ONTOGENIC TIME AND MORPHOMETRIC ANALYSIS OF *IN-VITRO* REARING OF STINGLESS BEE QUEEN, *Heterotrigona itama* (HYMENOPTERA: APIDAE: MELIPONINI)

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ABSTRACT

The incubation period and life cycle of the queen of stingless bee, *Heterotrigona itama* is poorly understood. Little is known about the growth and development time of *H. itama* queen during ontogenic development which is the vital key to understanding many aspects of their biology. This study examined the growth rate and ontogenic period of Indo-Malaya stingless bee queens under *in-vitro* conditions. The mean ontogenic time for queens of *H. itama*, was maintained at 25±2.5°C and relative humidity 75-100% approximately for 56 days, which is dissimilar to the *H. itama* workers. The immature stage showed an apparent variation in head capsule width measurement, with the early instar ranging from 0.49±0.07 mm and late instar ranging until 2.83±0.10 mm. Overall, there are five to six instar stages that could be found from this species. Ten morphological characters were measured and compared between *in-vitro* queens and wild workers of *H. itama*. The body and abdominal length of *in-vitro* queens of *H. itama* were significantly greater than wild workers. However, the wings and legs of *H. itama* workers were significantly lengthier compared to the *in-vitro* queens. It is hoped that the outcomes of this study will contribute to conservation efforts in Indo-Malaya stingless bees.

Keywords: *Heterotrigona itama*, stingless bee, incubation, life cycle, morphometrics

ABSTRAK

Tempoh inkubasi dan kitar hidup ratu lebah kelulut, *Heterotrigona itama* kurang difahami. Tidak banyak yang diketahui mengenai kadar pertumbuhan dan tempoh perkembangan ratu *H.*

itama semasa perkembangan ontogenik yang merupakan kunci penting untuk memahami banyak aspek biologi serangga tersebut. Kajian ini mengkaji kadar pertumbuhan dan tempoh ontogenik ratu lebah Indo-Malaya dalam keadaan in-vitro. Purata masa ontogenik untuk ratu *H. itama* yang dikekalkan pada suhu 25$\pm$2.5ºC dan kelembapan relatif 75-100% adalah sekitar 56 hari, berbeza dengan pekerja *H. itama*. Peringkat belum matang menunjukkan variasi yang jelas dalam pengukuran lebar kapsul kepala, dengan instar awal dari 0.49±0.07 mm dan instar akhir berjulat hingga 2.83±0.10 mm. Secara keseluruhan, terdapat lima hingga enam peringkat instar yang dapat dijumpai dari spesies ini. Sepuluh ciri morfologi diukur dan dibandingkan antara ratu *in-vitro* dan pekerja liar *H. itama*. Panjang badan dan abdomen ratu *in-vitro H. itama* jauh lebih besar daripada pekerja liar. Walau bagaimanapun, sayap dan kaki pekerja *H. itama* jauh lebih panjang berbanding dengan ratu *in-vitro.* Diharapkan hasil kajian ini dapat menyumbang kepada usaha pemuliharaan lebah kelulut Indo-Malaya.

Kata kunci: *Heterotrigona itama*, lebah kelulut, inkubasi, kitar hidup, morfometrik

INTRODUCTION

Stingless bees in Malaysia are highly diverse as a total of 45 species from 14 genera have been identified and documented so far (Rasmussen 2008; Samsudin et al. 2018). Out of that number, five domestic species capture a profitable endorsement: *H. itama*, *Geniotrigona thoracica*, *Tetragonula fuscobalteata*, *T. laeviceps* and *Lepidotrigona terminata* (Norowi et al. 2008).

Stingless bees play an important role in ecological and economic aspects as well as in their medicinal value. Due to that, stingless beekeeping or meliponiculture recently became one of the significant areas in the agricultural sector of Malaysia (Ismail & Ismail 2018). *Heterotrigona itama* is the most popular species for beekeeping and a good candidate for pollination services in the agricultural ecosystem in Malaysia (Azmi et al. 2016; 2017; 2019; Jaapar et al. 2021). Due to the high demand for stingless bee products (i.e., honey, bee bread and propolis), the meliponiculture activity is currently an emerging profitable venture for many Malaysian beekeepers. To fulfill the public requirements, the stingless bee colonies need to be multiplied in a short period of time. One of the bottlenecks in large-scale breeding for stingless bees is the limited number of queens that is usually found in most species' colonies.

Considerable studies have been conducted on the taxonomy, mellissopalynology, diversity, medicinal properties, foraging behaviour and pollination efficiency of the Indo-Malaya stingless bees. However, very little attention has been made to study the ontogenic time of the stingless bees specifically the queen. Understanding the overall life cycle or ontogenic period and the stingless bee queens' growth development are crucially important aspects of meliponiculture activity. According to Winston (1991), the ontogenic period in bees begins at oviposition and progresses through larval development and pupation to emergence as an immature adult (imago).

There are few studies on the ontogenic period of Indo-Malaya stingless bees such as *H. itama* and *T. minangkabau* by Salmah et al. (1991) and Salmah et al. (1996), respectively. To date, very few studies have been made on this aspect due to technical difficulties in continuously tracing individual life histories, particularly the queen. Little is known about the growth rate and development time of *H. itama* queen during ontogenic development which is the important key to understanding many aspects of their biology. Therefore, the objective of this study is to determine the rate of growth and ontogenic period of *H. itama* queen under *in-vitro* conditions. Then, ten selected morphological characters of *in-vitro* queens were measured and compared with the wild workers of *H. itama* to distinguish its morphometric differences. The outcome of this study is hoped to give an insight into the queen's biological aspect, which is essential for the sustainability of the meliponiculture industry and the conservation and management of this species.

MATERIALS AND METHODS

Study sites and samples collection

The samples collection was carried out at Big Bee Honey Farm, Marang, in Terengganu (5.03203° N, 103.29389° E). It is one of the largest bee farms in Terengganu state. For *in-vitro* queen rearing samples, at least four colonies of *H. itama* were used to extract the eggs from the brood combs. A few new layers of broods were gently taken out of the topping box using a knife and scalpel to avoid massive destruction to the colonies. The broods were opened and the eggs of *H. itama* were transferred to the laboratory to prevent contamination. For comparison of morphometrics measurement, at least 10 individuals of *H. itama* workers were collected randomly from different colonies at Big Bee Honey Farm. The samples were collected using an insect net and then preserved in 70 % ethanol. The samples were correctly labeled and brought back to the Ecology Laboratory at UMT for further analysis.

Procedures of in-vitro queen rearing and incubation period observation

The *in-vitro* rearing of *H. itama* queen was carried out according to the methods described by Nurul Izdihar et al. (2021). At least 100-200 eggs of *H. itama* were aseptically removed from the brood cells using a pointed dissecting needle, and the brood cells were then squeezed to obtain the larval food and the beneficial microbes from the brood combs. The procedure was handled in the biological safety cabinet and the handling area was sterilized with ethanol to prevent contamination. A 150µl of larval food was pipetted into the Elisa plate with 96 cavities which provided sufficient space for each larva of *H. itama*. Out of ~200 eggs, only 96 eggs were placed vertically on the larval food to prevent them die from asphyxiation. The incubation period inspection of 96 potentially queen individuals was done daily.

All the *in-vitro* rearing procedures were conducted in an incubator (Model: Rcom Reptile MAX 60) under 28°C. The relative humidity was adjusted to 100% for the first six days and 75% for the rest of larval incubation. Distilled water was used to maintain the humidity at 100%, whereas saturated sodium chloride (NaCl) solution was used in the incubator to achieve a relative humidity level of 60-75% for the larval incubation to undergo the pupation stage.

Measurement of head capsule width was taken every week until the emergence stage using digital calipers to record the larvae growth. Determination of larval instar stages in *H. itama* are based on the Dyar's ratio (Mohammadi et al. 2010). The observation from egg to adultery stages under *in-vitro* platform were recorded using a stereomicroscope (Model: Carl Zeiss (V12)). Considerately, the sample used for measurements was discarded as they died from high sensitivity towards the surrounding exposure. The data was recorded on the appearance and morphological changes between each period. As soon as queen was noticed to emerge, the queen was taken out from the incubator and the selected morphological characters were measured and recorded to be compared with the natural wild workers.

Morphological and morphometrics measurement

A total of 10 morphological characters of both *in-vitro* queen and wild workers of the stingless bee were measured for the morphometric analysis based on Samsudin et al. (2018). The parameters included body length (BL), head length (HL), head width (HW), forewing length

(FWL), forewing width (FWW), hindwing length (HWL), hindwing width (HWW), tibia width (TW), abdomen length (AL) and abdomen width (AW). A re-description of head, thorax, forewing and hind leg, and the abdomen for both *in-vitro* queens and workers were also carried out. All the morphological characters were observed and measured using a stereomicroscope (Model: Carl Zeiss (V12)).

Statistical Analysis

Means and standard deviation were summed up for the data differentiation between *in-vitro* queens and wild workers of *H. itama*. The samples were measured based on the parameters stated in the methodology section. One-way ANOVA was used to analyse the head capsule measurement outgrow weekly. T-test analysis were used to analyses morphometric measure between *in-vitro* queens and wild workers. All data analysis were conducted by Statistical Package for the Social Science (SPSS) version 25.0.

RESULTS AND DISCUSSION

Ontogenic time and developmental growth

The developmental period of *H. itama* queens from the day deposited in brood cells can be referred in Figure 2 (n = 10). The eggs usually took around 1-2 days to hatch into larvae. After hatched, the larvae started to feed on the food provisioning provided in the cells. The immature periods of the larval stage took a longer time duration which was 35 days than the pupal stage which took 14 days. The stages were considered critical because the highest mortality was before the pupation stage in *in-vitro* platform. In terms of developmental growth, the mean of head capsule width of early instar ranged from 0.49±0.07 mm and the late instar ranging until 2.83±0.10 mm (Figure 1). The head capsule of *in-vitro* queens was significantly different throughout the weeks of incubation ($p<0.05$). A huge difference in head capsule width from week 5 to week 6 could distinguish the penultimate (early) instar larvae to final instar larvae (Minakuchi et al. 2009).

Significant changes in head capsule measure mediated the regulation action from two essential hormones during post embryonic development. The two essential hormones are ecdysone and juvenile hormone (JH). The mechanism is primarily involved in the stimulation of corpus allata (endocrine gland) to secrete juvenile hormones that physiologically help in the larval-larval molting process. Once the corpus allata stopped secreting JH, the larvae suppressed to the final instar larvae. Immediately the ecdysone hormone took place in the progress of larval-pupal transition and pupal-adult molts (Minakuchi et al. 2009). The process is called metamorphosis. Physically, they started to resemble adult and were immobile after the final instar stage (pupal stage). There are probably five or six instar stages of *H. itama* queen in this study that could be observed under the *in-vitro* conditions. Overall, the mean ontogenic time for queens of *H. itama* was approximately 56 days.

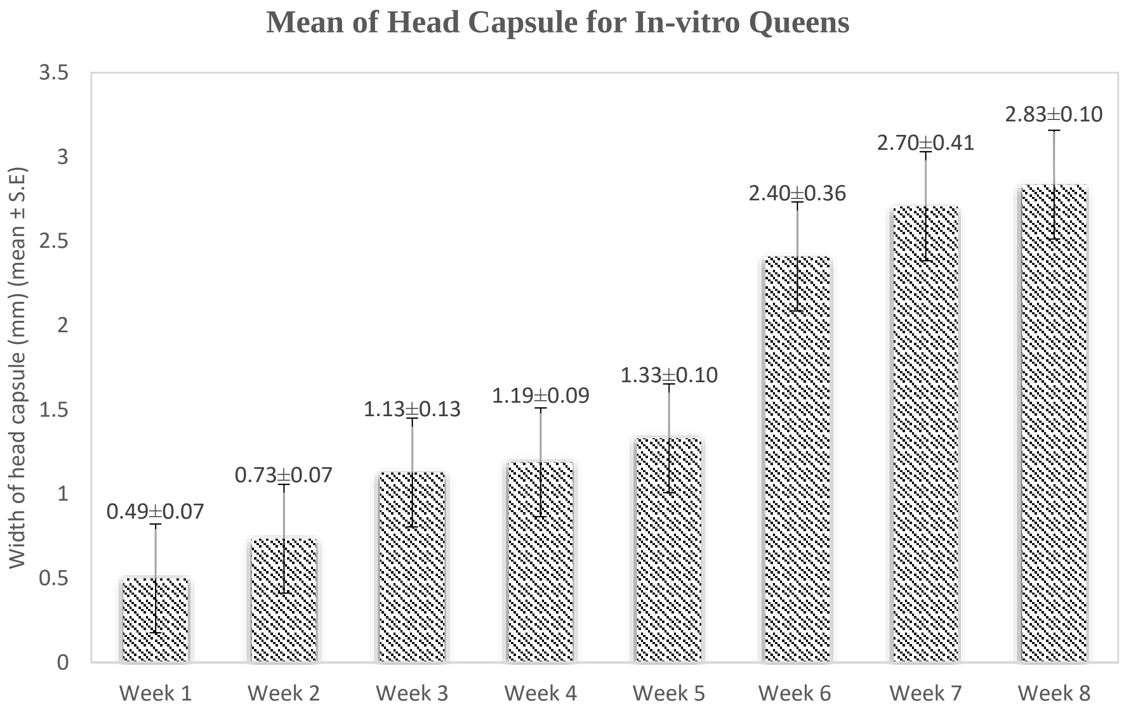

Figure 1. The mean of head capsules of *in-vitro* queen individuals (n=10). There is a constant increment in size from Week 1 to Week 8

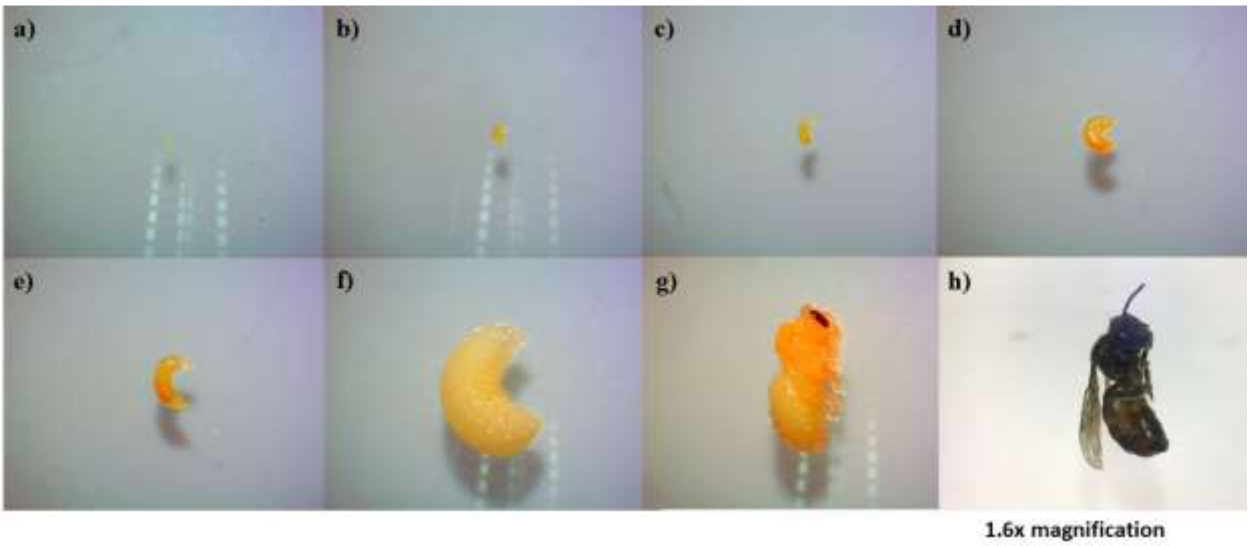

Figure 2. Life cycle of *in-vitro* queen of *H. itama*. a) An egg; b) 1-3 days old larvae; c) 3- 5 days old larvae; d) 5-6 days old larvae; e) 6-7 days old larvae; f) 35 days old larvae; g) 56 days old pupae; and h) *in-vitro* queen of *H. itama*

In this study, the ontogenic period for *H. itama* queens (56 days at 28^0C) is 2.6 times longer than that of *H. itama* workers (46.5 days) as reported by Salmah et al. (1996). The ontogenic period varies due to the consumption of larval food amount. Regarding the response to the food consumption, different species swallowed as much as regurgitated by workers in the brood cells. Different amounts of larval food consumption led to an inconstant ontogenic period of stingless bee species (Table 1). Somehow, the incubation period required from these species nearly similar as most of these species lived in a dense tropical region.

Table 1. A compilation of the incubation period of stingless bees and honeybee species (workers) in tolerating the food amount, relative humidity and temperature

Species	**Ontogenic period (day)**	**Incubation temperature (°C)**	**Source**
Heterotrigona itama	46.5	23 - 31	Salmah et al. (1996)
Tetragonula minangkabau	42	NA	Salmah et al. (1991)
Melipona beecheii	53	30	Moo-Valle et al. (2004)
Austroplebeia australis	55	27	Halcroft et al. (2013)
Apis mellifera	21	28	Winston (1991)

According to the previous report by Baptistella et al. (2012), the larvae of stingless bee have to consume larger quantity of larval food to increase the chance to become a queen, which may reflect the developmental growth as well as the ontogenic period of the queen larvae. Throughout the rearing experiment, the life cycle of *H. itama* larvae was observed from the egg to adultery phase (Figure 2). From the result, the incubation period of *H. itama* took longer during the larval phase (35 days). Based on Salmah et al. (1996), prepupal substage was employed longer (10 days) due to the process of caste differentiation in *H. itama* workers. In contrast to a situation in which queens took longer to adult emergence, due to the increased potential larval food amount to be consumed.

Roubik (1989) found that many stingless bee species do not thermoregulate their nests, but the internal temperatures inside the brood may fluctuate with the ambient temperatures at the optimum incubation temperature of 30–32 ^{0}C (Engels et al. 1995). These fluctuations may lead to the variations in the ontogenic times for stingless bees, particularly when the brood development occurs outside the nest. In this case, it was carried out under *in-vitro* conditions. Nevertheless, the information of queen differentiation mechanism in Malaysian stingless bees is scarce, but this study area is important to understand caste differentiation mechanism and follow the current research to complement the needs of colonies by mass-producing queens through *in-vitro* procedure (Wee et al. 2020).

Morphological characteristics and morphometric analysis

Five individuals of *in-vitro* queens of *H. itama* were selected for morphological characteristics study. Description of morphological characteristics such as head, thorax, wing, leg and abdomen with coloration and morphometric measurements were reported in this section.

In-vitro queens' heads appeared in brownish-black while the workers were dark black. The frons of both entirely covered with fine hair and slightly thick hairs approaching clypeus. The length and width of the workers head (2.19±0.33 mm) was significantly greater than *in-vitro* queens (2.08±0.03 mm) of *H. itama*. Both are composed of black compound eyes and ocelli (Figure 3). Both *in-vitro* queens and workers of *H. itama* possessed one tooth of brownish-black mandible. The antenna of both *in-vitro* queen and workers were geniculated bent like an elbowed shape and built of 11 flagellum.

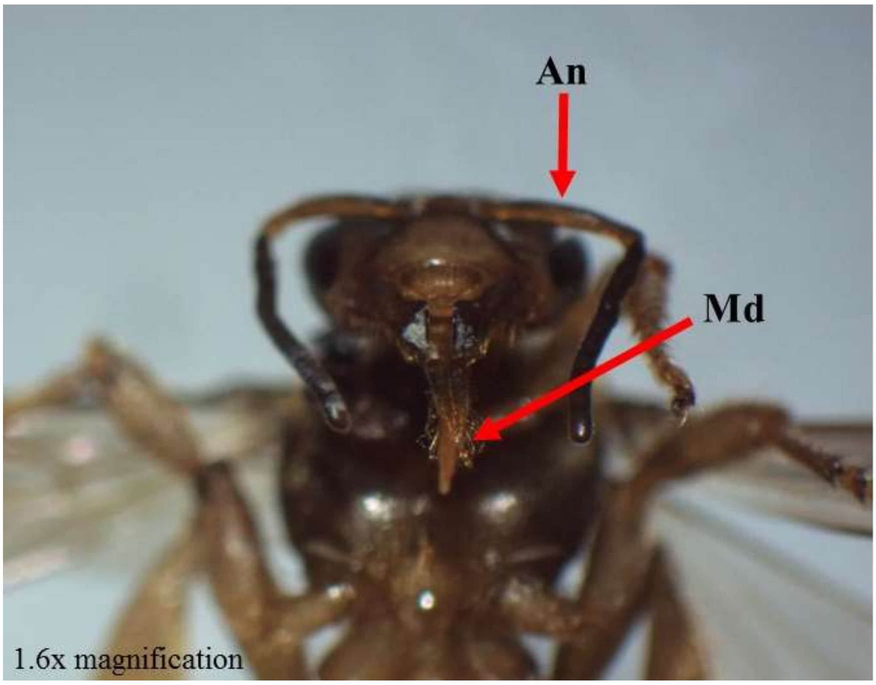

Figure 3. The head capsule of *in-vitro* queen of *H. itama*. Notes: An=Antenna; Md=Mandible. Scale attached in the figure

In-vitro queens' mesoscutum appeared in a complete black (Figure 4) and was covered with fine hair similar to the workers. Meanwhile, the *in-vitro* queens' tegula was slightly brownish than workers in full black colour.

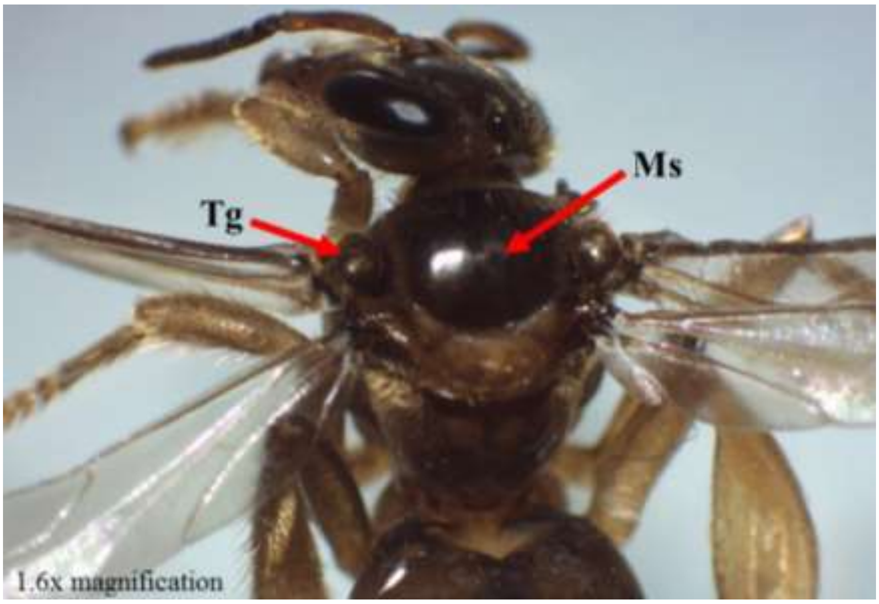

Figure 4. The dorso-anterior view of thorax of *H. itama in-vitro* queen. Notes: Ms = Mesoscutum; Tg = Tegula. Scale attached in the figure

In-vitro queens' hind tibia were longer and broader than workers, and the basitarsus appeared brownish-black in colour for the queen (Figure 5) but entirely black in workers. Both *in-vitro* queens and workers were covered with long setae at apical and short setae at the basal of hind tibia. Tibia width of workers (0.71±0.20 mm) was significantly larger than the *in-vitro* queens (0.63±0.14 mm) of *H. itama*.

Figure 5. The anterior view of hind legs of *H. itama in-vitro* queen. Notes: Ht = Hind tibia; Bs = Basitarsus. Scale attached in the figure

In this study, the *in-vitro* queens' bodies were predominantly brownish black (Figure 6) while the workers were darker. The abdominal part of *in-vitro* queens was brownish-black differed from the workers which were darker in colour. The abdominal length of *in-vitro* queens was significantly lengthier (4.47±0.47 mm) than the workers (1.96±0.29 mm) (Figure 7).

Figure 6. The anterior view of abdomen part of *H. itama in-vitro* queen. Scale attached in the figure

Figure 7. The lateral view of abdominal segment of *H. itama in-vitro* queen. Scale attached in the figure

The *in-vitro* queen and workers of *H. itama* have two pairs of wings which are forewings and hindwings. The forewing width of workers (2.35±0.54 mm) was significantly greater than the *in-vitro* queen of *H. itama* (1.52±0.28 mm). Wing venation for both appeared in dark brown and semi-transparent (Figure 8). *In-vitro* queens are composed of seven hamuli per hindwing (Figure 9), similar to the worker.

Figure 8. The two pairs of wings of *H. itama in-vitro* queen. Scale attached in the figure

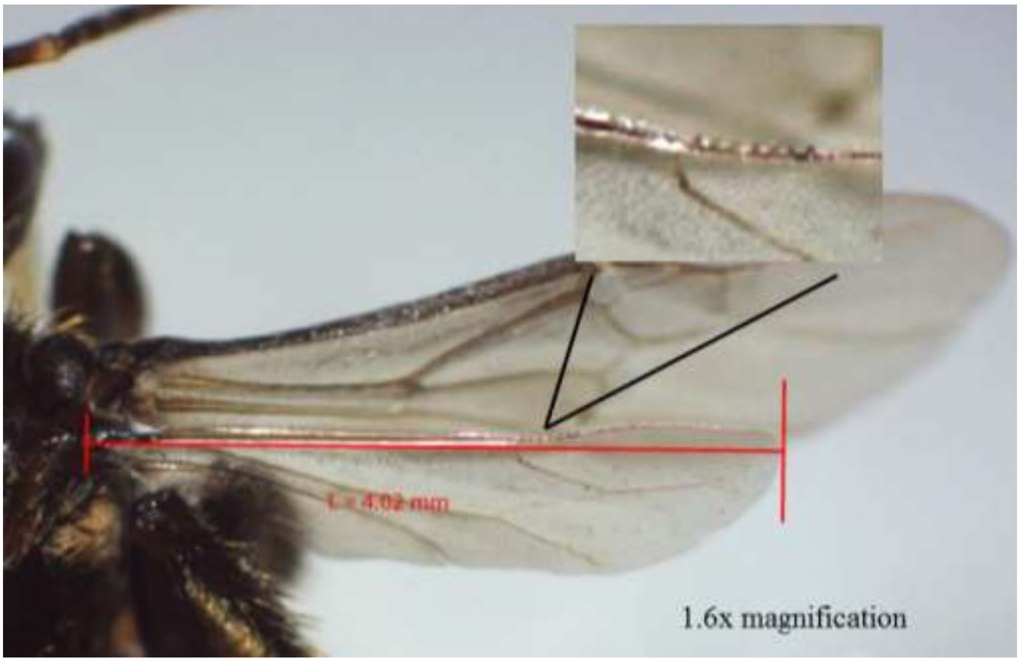

Figure 9. The hamuli of *H. itama in-vitro* queen. Notes: Hm = Hamuli. Scale attached in the figure

The comparison of morphometric measurement for 10 individuals of *in-vitro* queens and workers of *H. itama* is shown in Figure 10. All of the morphometric measurements were significantly different between *in-vitro* queens and workers of *H. itama* ($P<0.05$) except for tibia width ($P>0.05$).

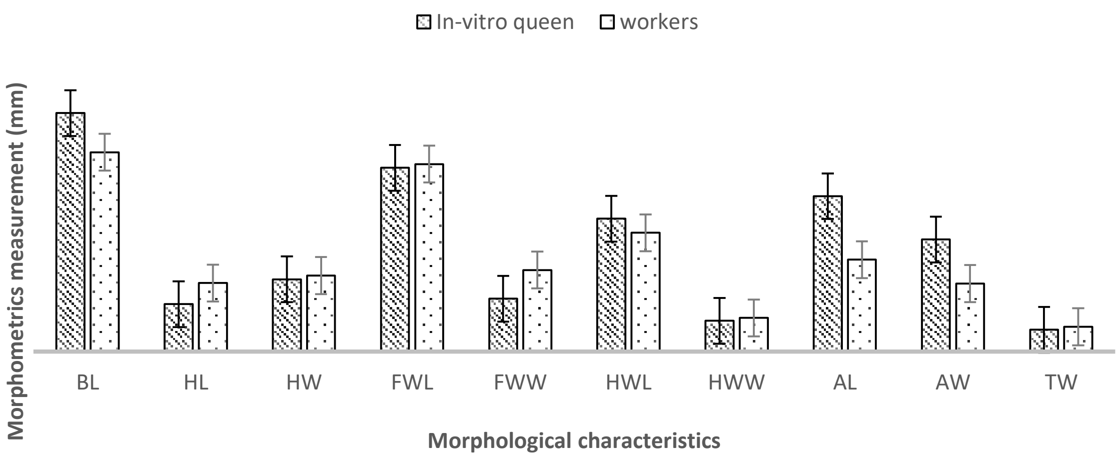

Figure 10. Mean and standard deviation of morphometric measurements for *in-vitro* queens and workers of *H. itama* (N=10). Notes: BL=body length, HL=head length, HW= head width, FWL=forewing length, FWW=forewing width, HWL=hindwing length, HWW=hindwing width, AL= abdomen length, AW= abdomen width, TW= tibia width.

The queen's role in insect caste is favoured as a specialised female with different morphology mainly to accelerate colony reproduction by laying eggs (Koedam et al. 2007).

Throughout the lifespan, the queen only helps to develop the colony and regulates the colony unity through the pheromone release. The queen acts in 'queen policing' by being dismissed behind any worker-laid eggs. Colony regulation by eating worker-laid eggs from the queen increases the quality of egg laid. This can be seen for the capability of the queen to lay fertilized eggs that are much different to workers that lay unfertilized eggs issued from an inability to mate (Alves et al. 2009). Morphologically, the body appearance of the queen is an indicator of segregated mating, presumably to attract male drones. This study showed that the queens produced through *in-vitro* and workers of *H. itama* had considerable differences in morphological characteristics and morphometric measurements. This was due to the amount of larval food consumption during post-embryonic development responsible for regulating caste determination in stingless bee (Baptistella et al. 2012). The amount of larval provision consumption can significantly affect the individual body mass (Helm et al. 2017).

The first morphological characteristics for differentiation between both *in-vitro* queens and workers derived from integument colour. In traditional bee taxonomy, body colour features were widely utilized to recognize the taxa (Ruttner 1988; Waldschmidt et al. 2000). A similar result was found when analysing the taxonomic of the stingless bee workers in Peninsular Malaysia (Samsudin et al. 2018) where the body colour was predominantly black. Interestingly, this study showed that the *in-vitro* queens were brownish black in colour which was different from workers.

From the result, the abdominal length of *the in-vitro* queen (4.47±0.47 mm) was generally greater than the size of other morphometric characters. This can be supported by a study of Fahimee et al. (2020) where the abdominal part of *in-vitro* queens from their research was larger than workers. The abdominal part of *in-vitro* queens built in with the reproductive system which operated copulating and laying eggs (Menezes et al. 2018). According to De Souza et al. (2018), queens were larger (150-250 mg) with large abdomen embodied with well-developed reproductive organs, including ovaries with 200-400 ovarian filaments (ovarioles) and a spermatheca (Snodgrass 1956).

However, the tibia width of workers (0.71±0.20 mm), and wings of workers (2.35±0.54 mm) were significantly greater than the *in-vitro* queens. These body characters were found to play prominent roles in the activities of stingless bees such as the hind leg shifted to a modified structure called corbiculae or pollen basket derived for pollen collection adaptation and transportation efficiency (Oladimeji 2014). Somehow, the size of tibia was slightly longer (0.71±0.20 mm) than previous research conducted by Trianto et al. (2020) for Indonesian specimens (0.53±0.79 mm). According to Kwapong et al. (2010), workers of stingless bees functionally have a direct role for corbiculae utilized in collecting and transporting pollen and other material to their colony. Furthermore, wing size was found different between *H. itama* and *in-vitro* queen due to the role of workers specialized for food searching accelerated more energy with huge wing size (Saufi et al. 2015). Morphological characteristics of *H. itama* worker wings are necessary for foraging activity (Azizi et al. 2020; Trianto et al. 2020). Some species may reach up to 2 km far from the mother nest (Bak-Badowska et al. 2019). It has been reported that the wing size might be influenced from flight activities during thermal regulation of nest and foraging activity (Oladimeji 2014).

CONCLUSION

This study reveals that the mean ontogenic time for queen of *H. itama*, maintained at $28\pm1^{o}C$ and relative humidity 90% was approximately 56 days, which is dissimilar for other stingless bees species and previous study. This study could be the first report of the ontogenic period of the queen of the Indo-Malaya of stingless bees that have been conducted under *in-vitro* conditions. In terms of morphological characteristics, certain body parts such as abdominal length, sizes of wings and legs showed huge differences between the two castes, *H. itama* workers and queens. Findings from the study will provide crucial information on the life cycle and external morphological characteristics of *H. itama in-vitro* queens before pre-release assessment in the field. It is hoped that the outcomes of this study will contribute to the conservation efforts in Indo-Malaya stingless bees as well as for the meliponiculture industry in Malaysia.

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