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Morphological descriptions on the larvae of *Hypopygiopsis fumipennis* (Walker, 1856) (Diptera: Calliphoridae)

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Abstract. The study on biodiversity of forensically important Diptera in the tropical rain forest in Malaysia is scarce. Thus, a preliminary survey was conducted at a jungle fringe near Kampung Bahagia Bukit Lagong, Sungai Buloh, Selangor. A rat carcass was offered to attract carrion flies and we collected an adult female calliphorid, *Hypopygiopsis fumipennis* (Walker, 1856) during the fresh stage of carcass decomposition. The female fly was allowed to oviposit on chicken liver in a container and the resulting larvae were reared to the adult stage. Along the developmental process, several individuals from each instar were collected and preserved in 70% ethanol and then processed on the slides. We recorded the duration of development for each instar and described its larval features for the first time. The third instar larvae of *H. fumipennis* showed accessory oral sclerite present, anterior spiracle with 13-15 papillae, intersegmental spines mostly unicuspid with pointed end, and posterior spiracles heavily sclerotized with inter-slit projections. Some larval differences between *H. fumipennis* and *Hypopygiopsis violacea* were noted.

INTRODUCTION

Five species of *Hypopygiopsis* (Diptera: Calliphoridae) have been catalogued in the Oriental region (Kurahashi, 1977; Verves, 2005). However, there are only two species of *Hypopygiopsis* recorded in Malaysia, namely *Hypopygiopsis fumipennis* (Walker, 1856) and *Hypopygiopsis violacea* (Macquart, 1835). Other species including *Hypopygiopsis infumata* (Bigot, 1877), *Hypopygiopsis tumrasvini* (Kurahashi, 1977) and *Hypopygiopsis robusta* (Malloch, 1926) are endemic in other Asian countries such as Indonesia, Thailand, Vietnam, Laos, Cambodia, India and Southern China (Kurahashi, 1977). They are generally large flies measuring more than 15 mm in length, and some of them are either larviparous or viviparous (Kurahashi, *et al.*, 1997).

Hypopygiopsis is considered one of the least studied forensically important blow flies in this region. Chen *et al.* (2008) studied the life cycle of *H. violacea* and made a comparison with the blow fly, *Chrysomya (=Achoetandrus) rufifacies* (Macquart, 1842). Ahmad Firdaus *et al.* (2010) reported that second and third instar larvae of *H. violacea* have been collected from a deceased in Malaysia. In Thailand, Moophayak *et al.* (2011) described the larval morphology of *H. tumrasvini* which could be forensically important. A detailed morphological study by electron microscopy on immature stages

of *H. tumrasvini* has been demonstrated by Sanit *et al.* (2012). While *H. fumipennis* adults are known to be attracted to excrement and decaying organic matter in the tropical rain forest (Kurahashi, 1977), however, its larvae have not been recovered from human or other vertebrate carcasses in Malaysia (Lee *et al.*, 2004; Syamsa *et al.*, 2010).

Hypopygiopsis fumipennis is distributed throughout Malaysia (Peninsular and Borneo) and other Southeast Asian countries including southern Thailand, Singapore and Indonesia (Sumatra) (Kurahashi, 1977; Kurahashi *et al.*, 1997; Kurahashi & Leh, 2009). The adults of *H. fumipennis* can be differentiated from *H. violacea.* They have orangish antennae, a golden yellow facial tomentum and the tarsi in male without fringe. In contrast, *H. violacea* has black antenna, silver facial tomentum and the tarsi in males have long fringes. *Hypopygiopsis fumipennis* adults are larger than *H. violacea*, where their body size ranged from 12.0 – 20.0 mm and 9.0 – 16.0 mm, respectively (Kurahashi, 1977; Kurahashi *et al.*, 1997).

The correct identification of *Hypopygiopsis* larvae is of paramount important in the context of forensic entomology services in Malaysia as there are many closely related species such as *Hemipyrellia* and *Lucilia* (both genera are in tribe Luciliini) co-existing on decomposing dead bodies. Due to the similarity in the characteristics among the species, misidentification may occur. As a consequence, this may contribute to biases when estimating the minimum postmortem intervals (mPMI) as different species of flies may have different durations of life cycle. Furthermore, fly larvae are the most frequent encountered specimens in forensic entomology cases (Lee *et al.*, 2004). Hence, species differentiation among the fly larvae is critical in ensuring the reliability of the profession. In this paper, we described the larval morphology of *H. fumipennis* for all larval stages as well as the duration to complete its life cycle and highlighted the taxonomical differences between *H. fumipennis* and *H. violacea* larvae.

MATERIALS AND METHODS

An entomological study was conducted in October 2011 at a jungle fringe near Kampung Bahagia Bukit Lagong, Sungai Buluh, Selangor (3°13'14"N 101°35'58"E, 55 meters above sea level). The objective of the study was to observe the decomposition process and to collect fauna of forensic importance in the tropical rain forest. A frozen rat carcass, *Rattus norvegicus* (Berkenhout, 1769), weight approximately 300 gm was obtained and placed on the ground at the study site on 25th October 2011. The rat carcass was observed daily and adult flies were collected by using a sweep net. A *H. fumipennis* female was captured and brought to the Parasitology Research Laboratory, Institute for Medical Molecular Biotechnology (IMMB), Faculty of Medicine, Universiti Teknologi MARA for rearing purposes. The fly was transferred from the net to a transparent container (height 6 cm; diameter 4 cm) which contained chicken liver (~25 gm) that served as oviposition medium. Chicken liver was added *ad-libitum* to maintain sufficient food supply to the resulting larvae and few drops of water were added into the container to avoid dehydration. Several tiny openings (~1 mm) were made on the cap of the container to allow airflow. When the captured fly died, we confirmed the species identification by using the keys in Kurahashi (1977) and Kurahashi *et al.* (1997).

The female fly oviposited thereafter and the deposited eggs were allowed to develop on the chicken liver in the cup. The mean temperature in the rearing room was 26.5 ± 3.1°C, mean humidity at 81.8 ± 2.4% and photo period at 12 h dark: 12 h light. During the larvae developmental process, six individuals (n=6) from each instar were collected and preserved in 70% ethanol (hence total n=18 as there are three instars altogether) while the remaining larvae (n=7) were allowed to pupate. Larval and pupal lengths were measured by an ordinary ruler and three readings were taken for each stage. Mouthparts and other morphological features were prepared by using slide mounting methods modified from Lee *et al.* (2004)

and Ahmad Firdaus *et al.* (2010). The sub-posterior ends of the preserved larvae were cut partially and soaked in 10% KOH for 24 hours. Internal organs of the larvae were then removed carefully to avoid damaging the external part by using a modified applicator stick. The larvae were transferred into 10% acetic acid for 5 minutes. Larvae were then dehydrated by a series of 70%, 90% and absolute alcohol for 30 minutes each. The dehydrated larvae were transferred into clove oil for 30 minutes and cleared in xylene for an additional 30 minutes. Lastly, they were placed on glass slides with a few drops of Canada Balsam or DPX for mounting purposes. Slides were then kept in an incubator to dry at 38ºC for three days. The larval morphology and associated characteristics were then studied by using digital stereomicroscope (Olympus SZX7, Japan) and a compound microscope (Olympus BX53, Japan) under different magnifications with a standardize measurement bar accordingly.

Resulting adults (F1 progeny) were kept in plastic containers (height 6.5 cm; diameter 9.0 cm) with supply of water and sugar under the laboratory condition. Observations were made on daily basis to determine longevity; however, only two adults were studied for longevity purposes. At the end of experiment, all dead adults were pinned, labeled and kept as voucher specimens in the laboratory.

RESULTS

A gravid female of *H. fumipennis* was brought to the laboratory on Day 1 (considered as the initial day of experiment) and the fly oviposited one hour after being transferred to the rearing container. The first instar larvae hatched from the eggs on the same day and second instar larvae were first noticed nine hours after egg hatched (Day 2). Third instar larvae were observed on Day 3 and the post-feeding larvae were seen on Day 17. The formation of pupae began on Day 49 and the first adult fly emerged on Day 62. Under the laboratory condition (26.5 ± 3.1°C, 81.8 ± 2.4% RH, dark: light 12:12), the complete life cycle of *H. fumipennis* ranged from 62 days to 70 days (n=7). The summary of the life cycle and the length of each larval stage are given in Table 1.

The body lengths among the instars of *H. fumipennis* were compared in Table 2. The mean length for the first instar was 1.96 mm (taken during Day 1), 5.3 mm for second instar (measured on Day 2) and 10.5 mm for early third instar on Day 3. The fully grown third instar (third instar that were a week old) was measured up to 17.3 mm and the longest larva ever recorded was 19.0 mm. The mean length for post feeding stage (which began on Day 17 and onwards) was reduced to 14.8 mm while for the pupae stage, the mean length was 10.67 mm.

Table 1. The stages of *H. fumipennis* at 26.5 ± 3.1ºC, 81.8 ± 2.4% RH and photoperiod 12 h dark: 12 h light

Stage	Day of transition / eclosion	Mean length ± sd (mm)
Egg	Oviposited on Day 1 (initial day of experiment)	
1st instar	Day 1	1.96 ± 0.06
2nd instar	Day 2	5.3 ± 0.29
3rd instar	Day 3 to Day 10	10.5 ± 1.3 to 17.3 ± 0.29
Post-feeding	Day 17	14.8 ± 0.29
Pupa	Pupation between Day 49 to Day 54	10.67 ± 0.58
Adult*	Eclosion between Day 62 to Day 70	

* = Developmental data based on seven pupae that were allowed for emergence.

Table 2. The differences among third instar larva of *H. fumipennis*, *H. violacea* and a Thai species, *H. tumrasvini*

Features	*H. fumipennis*	*H. violacea* (Ahmad Firdaus *et al.*, 2010)	*H. tumrasvini* (Thai species) (Moophayak *et al.*, 2011)
Length (3rd instar)	17.3 mm[a]	15 mm	13.7 mm
Accessory oral sclerite	Present[b]	Present	Absent
Anterior spiracle	13-15 papillae[b]	8-9 papillae	8-11 papillae
Intersegmental spines	Mostly unicuspid, some bicuspid, pointed end[b]	Unicuspid, dome-shaped, pointed end	Irregular rows, arranged in sets of posteriorly projecting acuminate spines with darkly pigmented tips

[a] = sample size (n) = 3
[b] = sample size (n) = 6

Under gross observations, *H. fumipennis* larvae are smooth and creamy white in color, with three thoracic segments and eight abdominal segments. Microscopic examination of first instar larvae reveals cephalopharyngeal skeleton lacks an accessory oral sclerite, and the anterior spiracles cannot be located. The intersegmental spines are unicuspid with the tips darkened apically (Figure 1A). The posterior spiracle is heavily sclerotized. The spiracular slit is robust, short and of "v-shaped". The peritreme is thin, partially complete without encircling the spiracular slit and the button is indistinct (Figure 1B). For the second instar, the tentorial phragm is thickened, pharyngeal sclerite is shorter and the mouth hook became more solid and bending downward. Anterior spiracles can be seen at this stage and consisted of 13 papillae (n=6) arranged in a single row (Figure 1C). The posterior spiracles of second instar possess a light brown peritreme that is incomplete and encircled the two spiracular slits inconspicuously. The spiracular slits were thin and long. However, the spiracular button is still absent at this stage (Figure 1D).

The gross specimen of third instar larva of *H. fumipennis* is shown in Figure 2. The structures of larva are microscopically discernible after being processed on a slide (Figure 3). The cephalopharyngeal is darker in color (Figure 3A) with a comma-shaped accessory oral sclerite found below the mouth hook. The dorsal cornuae and ventral cornuae are heavily pigmented. There are six dorsal tubercles and six ventral tubercles located at the last segment. The anterior spiracle consists of 13-15 marginal papillae (n=6) and are arranged in a single row (Figure 3B). The posterior spiracle is encircled completely by a heavily sclerotized peritreme and is well developed with a button slightly projecting outward from the posterior end of the peritreme. The posterior spiracle contains three cigar-shaped slits, which are blunt at both ends (Figure 3C). The integument of the third instar larva is covered by numerous intersegmental spines. Majority of the intersegmental spines are unicuspid in characteristic, however, some of them are bicuspids. The spines are usually darkly pigmented apically and sharply pointed (Figure 3D). The habitus of an emerged adult fly was also provided here (Figure 4). The body sizes of emerged adult flies ranged from 11 mm to 19 mm. Out of seven emerged adults, two were used to study longevity. Their life span ranged from 8 to 9 days under the laboratory condition.

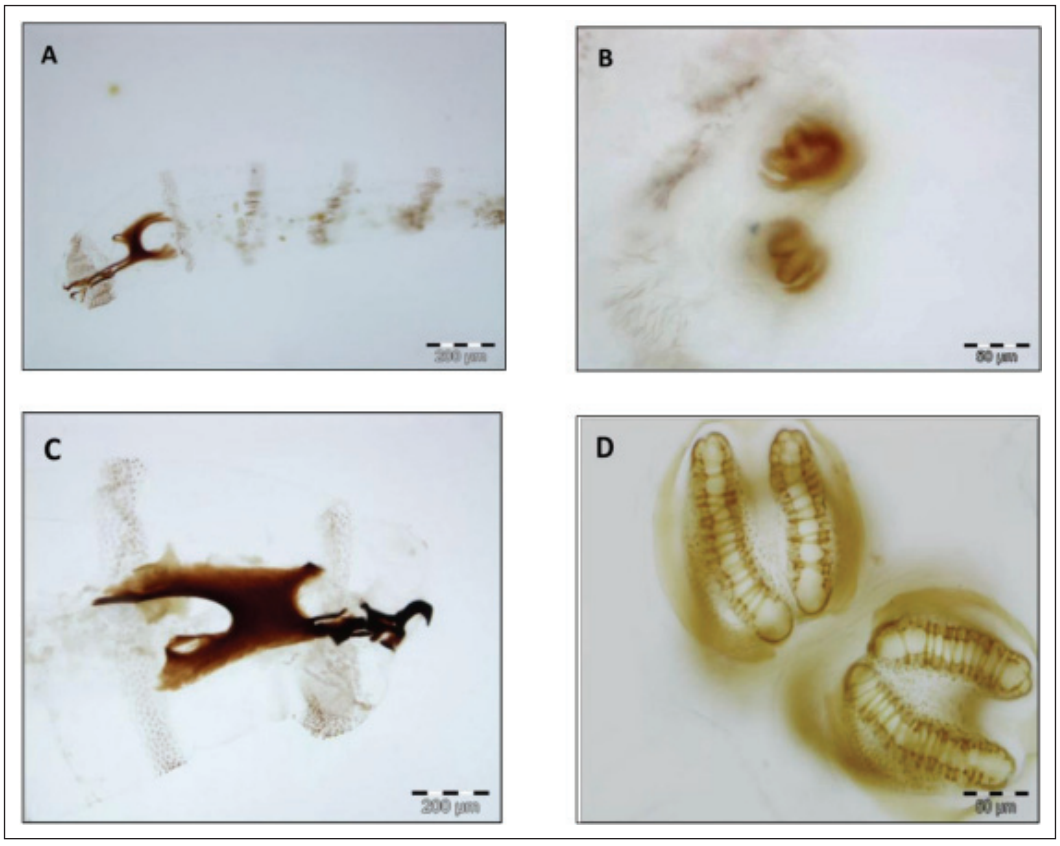

Figure 1. First and second instar larva of *H. fumipennis*. A. Cephalopharyngeal skeleton of first instar x10. B. Posterior spiracles of first instar x40. C. Cephalopharyngeal skeleton of second instar x10. D. Posterior spiracles of second instar x40.

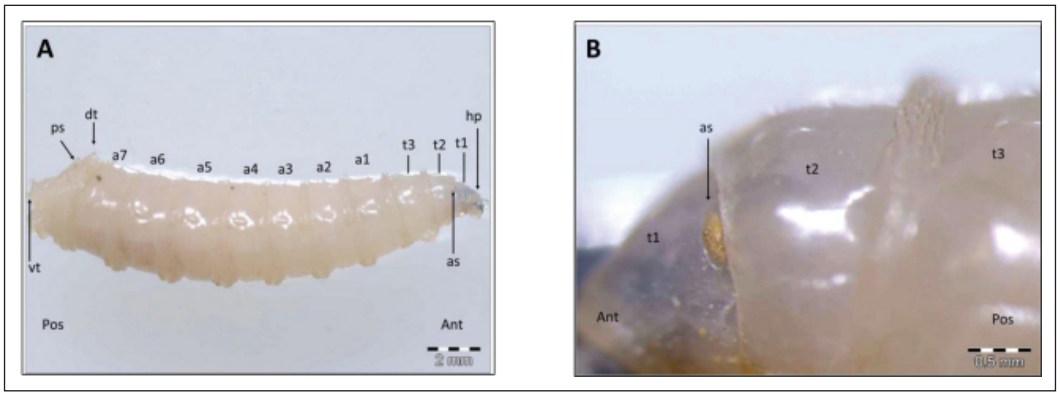

Figure 2. A. Gross specimen of third instar larva of *H. fumipennis* (x0.8). Head part (hp), anterior spiracle (as), posterior spiracle (ps), thoracic segments (t1-3), abdominal segment (a1-7), dorsal tubercle (dt), ventral tubercle (vt). B. Anterior spiracle (as) is located between t1 and t2 (x4).

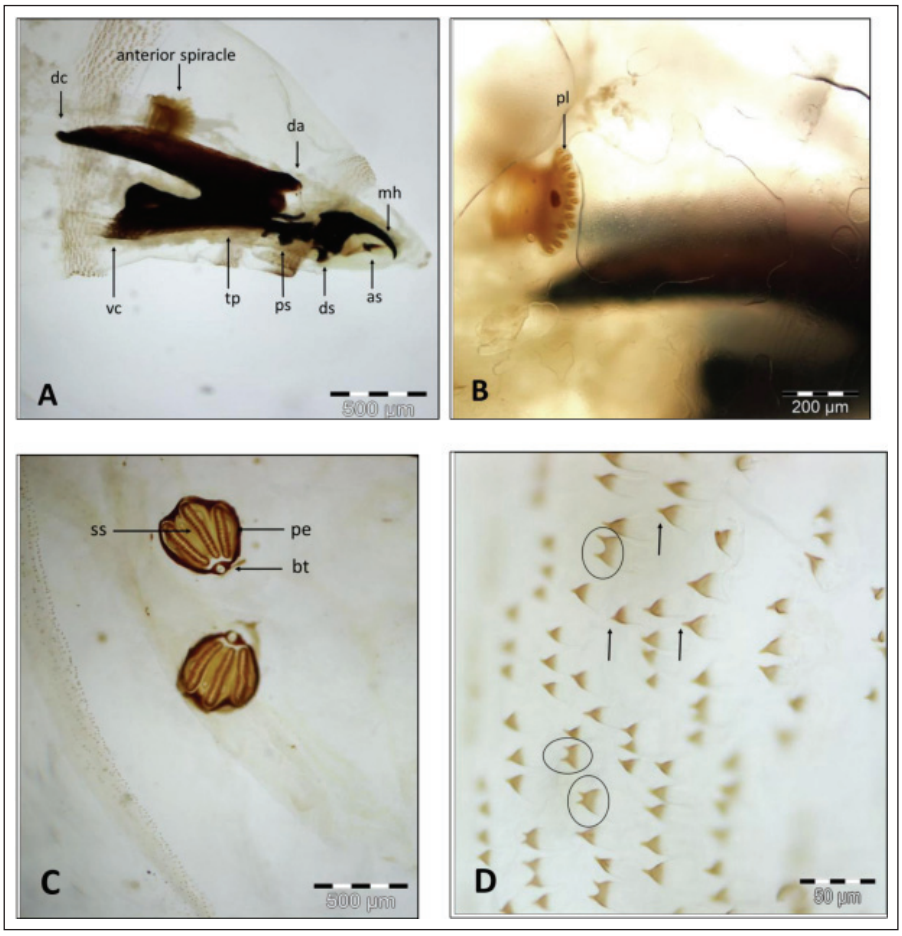

Figure 3. Light microscopy structures of third instar larva of *H. fumipennis*. A. Cephalopharyngeal skeleton x4, mouth hook (mh), accessory oral sclerite (as), tentorial phragma (tp), dorsal cornua (dc), dental sclerite (ds), ventral cornua (vc), pharyngeal sclerite (ps), dorsal arch (da). B. Anterior spiracle with 14 papillae x10, papillae (pl). C. Posterior spiracle x4, peritreme (pe), spiracular slit (ss), button (bt). D. Intersegmental spine x40, unicuspid spine (thin arrow), bicuspid spine (in circle).

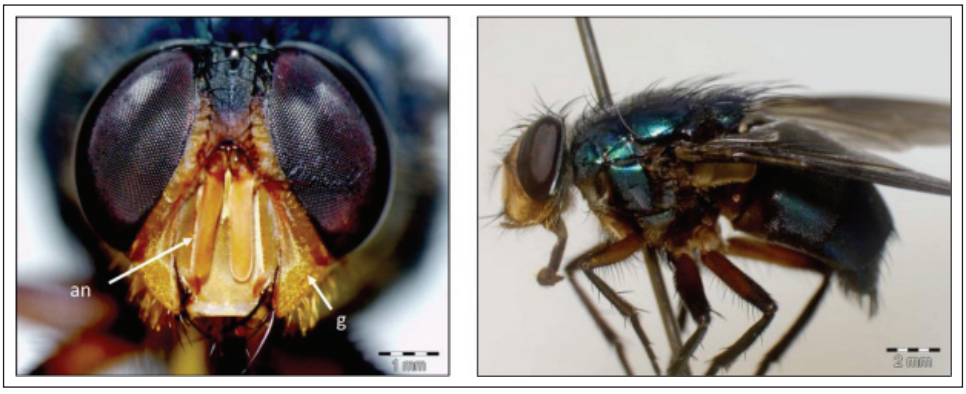

Figure 4. Habitus of *H. fumipennis* adult. A. frontal view (x2), note the golden gena (g) and yellowish orange antennae (an). B. Lateral view (x0.8). Body appears metallic bluish green and the wing slightly infuscated.

DISCUSSION

The third instar larvae of *H. violacea* share some morphological similarities with *H. fumipennis*. However, the number of marginal papillae at the anterior spiracle is the key to distinguish between the two species. Ahmad Firdaus *et al.* (2010) stated that the total number of marginal papillae of anterior spiracle for *H. violacea* is 8-9 while in the present study, the number of papillae for *H. fumipennis* ranged from 13-15. On the other hand, the body size of a fully grown third instar larva of *H. fumipennis* is larger than *H. violacea*. For instance, *H. fumipennis* achieves the mean body length of 17 mm (maximum length up to 19 mm) while the length for *H. violacea* was reported as 15 mm (Ahmad Firdaus *et al.*, 2010). Due to the fact that there could be various possibilities of measurement errors, we recommend that the measurements of larval length should be taken rigorously as many factors contribute to variations during the process of killing and methods of preservation (Amendt *et al.*, 2007; Amendt *et al.*, 2011). Furthermore, the size of larva largely depends on its nutritional status and food supply during the development process. The insect's hormonal regulation also play an important part in larval development (Higley & Haskell, 2001).

Moophayak *et al.* (2011) found that there was no accessory oral sclerite present in *H. tumrasvini* as compared to *H. violacea* and for the anterior spiracle, there were only 8-11 papillae seen in each spiracle. In the present study, we observed both *H. fumipennis* and *H. violacea* presented with an accessory oral sclerite during third instar stage. Hence, we suggest herein two major features to differentiate the larvae among *H. fumipennis*, *H. violacea* and *H. tumrasvini*, that is, the number of papillae of anterior spiracle and the presence of accessory oral sclerite (Table 2).

Chen *et al.* (2008) reported that the duration of life cycle of *H. violacea* required 308.25±8.25 hours (equivalent to 12 or 13 days) under the temperature 28 ± 2ºC with relative humidity 70±5%. However, the colonies of *H. violacea* which are currently maintained in the insectariums in the Institute for Medical Research (IMR), Kuala Lumpur have been observed to range from 19 to 23 days (Heah SK- personal communication). As for *H. fumipennis*, our results showed it ranged from 62-70 days, which is much longer than *H. violacea*. Despite the disparities observed between species, several factors need to be considered such as the effect of environmental temperatures on the growth of larvae which may lead to variations in life cycle (Wells & Lamotte, 2001; Greenberg & Kunich, 2002; Kumara *et al.*, 2010).

Hypopygiopsis violacea was previously recorded as the first Diptera to arrive on monkey carcasses in rubber plantation and tropical rain forest (Omar *et al.*, 1994; Chen *et al.*, 2008). Similarly, our study demonstrated that *H. fumipennis* was the first calliphorid to reach the rat carcass and breed on carrion, indicating that this species could be one of the potential candidates in the application of forensic entomology in the tropical rain forest ecoregion. Besides, we collected adults of *H. violacea* and *Catapicephala* sp. (Diptera: Calliphoridae) on the same carcass as both species arrived slightly after *H. fumipennis*. It is interesting to note that these calliphorids are co-habiting in the same ecological niches and were attracted to carcass during the early stage of decomposition. Thus far, *H. violacea* was the only member in *Hypopygiopsis* that had its larvae collected from a human cadaver found in an oil palm estate in Johor Bharu, Malaysia (Ahmad Firdaus *et al.*, 2010). We expect that the present study could be useful for the investigators to differentiate *H. fumipennis* from the other closely resembling species such as *H. violacea*, *Hemipyrellia ligurriens* (Wiedemann) and *Lucilia cuprina* (Wiedemann). Because of its highly specific distribution and preference to feed on fresh carcass, there is possibility that carrion found near jungle fringes or rural areas are likely to be infested with *Hypopygiopsis*. Despite of its forensic interest, *Hypopygiopsis* could be the main necrophagous species and a nutrient recycler in carrion ecosystem in the tropical rain forest. We recommend more ecological and

behavioral studies on *Hypopygiopsis* as its biology and ecology are largely unknown to the scientific communities.

As supplement to this study, we provide a modified key to differentiate the larvae of tribe Luciliini associated with carrion in Malaysia (key was partly adopted from Omar (2002):

1. – Accessory oral sclerite absent; anterior spiracles with 5-7 papillae; inter-segmental spines unicuspid and pointed end; posterior spiracle small, peritreme lightly sclerotized, without inter-slit projection, button present and the lining around button projected inward, posterior spiracular slits wide and stout .....*Lucilia cuprina* (Wiedemann)
   – Accessory oral sclerite present 2

2. – Body size 13 mm or less; anterior spiracle with 6-9 papillae; sharp unicuspid spines found on segment 1-7, with some bicuspids; posterior spiracle big, heavily sclerotized with inter-slit projection; button present and distinct; posterior spiracular long and thin .....*Hemipyrellia ligurriens* (Wiedemann)
   – Body size 15 mm or more, anterior spiracle with 8-15 papillae, inter-segmental spines mostly unicuspid, with or without bicuspid, dome-shaped, pointed end, posterior spiracle big, heavily sclerotized with inter-slit projection; button present and distinct; posterior spiracular long and thin 3

3. – Anterior spiracle with 8-9 papillae, body size 15 mm.....*Hypopygiopsis violacea* (Macquart)
   – Anterior spiracle with 13-15 papillae, body size 17-19 mm.....*Hypopygiopsis fumipennis* (Walker)

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