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Ontogenensis and developmental rate of the blow fly, *Hypopygiopsis tumrasvini* Kurahashi (Diptera: Calliphoridae)

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Abstract. Blow flies of the genus *Hypopygiopsis* are considered forensically important. In Thailand, four *Hypopygiopsis* species coexist, i.e., *Hypopygiopsis fumipennis*, *Hypopygiopsis infumata*, *Hypopygiopsis violacea* and *Hypopygiopsis tumrasvini*. In this study, the ontogeny and developmental rate of *H. tumrasvini* eggs, larvae and pupae were determined in the laboratory chamber reared at 25.0±2.0°C and 80.0±5.0% RH. Larvae emerged from eggs 10-12 h after deposition. Mean length of the first, second, third (feeding phase), third (post-feeding phase) instars and puparia were 3.5±1.1, 7.2±1.1, 13.5±1.8, 12.5±0.5 and 9.0±0.7 mm, respectively. The median development time for first, second, third instar (feeding phase), third instar (post-feeding phase) and pupariation period was 8 h, 10 h, 34 h, 22 d and 9-10 d, respectively. Developmental curve of the larval length indicated the rapid progression from 0 until 40 h from the first instar until the feeding third instar. Video recording of pupariation revealed the development of pupal respiratory horn beneath the larval integument at 27.0 h; whereas it protruded through the orifice of the integument at 27.5 h.

INTRODUCTION

Blow flies (Diptera: Calliphoridae) are among the first arthropod colonizers of human remains (Greenberg & Kunich, 2002; Williams, 2008). Thus, their larvae can be used in forensic investigations to estimate the minimum postmortem interval (mPMI) of the decedent (Goff & Odom, 1987), determine the presence of toxins and potentially the manner of death (Gunatilake & Goff, 1989; Byrd & Castner, 2001). However, to utilize entomological evidence, it is important to identify the specimens correctly and then apply development data to determine their age. Such information can then be used to estimate the age of the larvae, and mPMI.

In Southeast Asia, blow flies in the genus *Chrysomya* are most often associated with human remains (Lee *et al.*, 2004; Sukontason *et al.*, 2007). However, cases have also been found with the genus *Hypopygiopsis* present (Ahmad Firdaus *et al.*, 2010). In Thailand, four species of *Hypopygiopsis* have been identified: *Hypopygiopsis fumipennis* Walker, *Hypopygiopsis infumata* Bigot, *Hypopygiopsis violacea* Macquart and *Hypopygiopsis tumrasvini* Kurahashi (Kurahashi & Bunchu, 2011). In Malaysia, Ahmad Firdaus *et al.* (2010) provided morphological descriptions of second and third instars of *H. violacea*; while Chen *et al.* (2011) investigated the immature growth rate of this species.

The *H. tumrasvini* species is distributed throughout Bangladesh, Cambodia, China (Hainan Is., Yunnan), India (Assam, Uttar Pradesh), Laos, Thailand and Vietnam (Verves, 2005). This species is mainly found in high elevations (349 – 1,700 m) in Asia,

and dense forested areas where human remains associated with criminal activity are often discovered (Moophayak *et al.*, 2014).

In Thailand, morphology of the *H. tumrasvini* larvae and puparia have been described using both light and scanning electron microscope (Moophayak *et al.*, 2011; Sanit *et al.*, 2012). The purpose of this study was to determine the ontogeny and developmental rate of *H. tumrasvini*.

MATERIALS AND METHODS

Fly colony

A colony of *H. tumrasvini* was obtained from the mixed deciduous forest area higher than 500 m above sea level, high relative humidity and low temperature at Suthep-Pui Mt. (18°48'20"N, 98°54'34"E, 952 m a.s.l.), Mueang Chiang Mai district, Chiang Mai province, northern Thailand. Bait consisted of 1-day-old, tainted beef. A sweep net was used to capture female flies arriving at the bait. Adult females of *H. tumrasvini* were identified according to identification key (Tumrasvin *et al.*, 1979). In the field, all gravid females were transferred into a transparent glass tube (8 cm height, 2.3 cm diameter) containing ~3 g of fresh beef and a small one piece of leaf as oviposition medium to imitate a species specific natural breeding site. Any kind of leaf around that collected area could be used for oviposition. The glass tube was covered with a layer of gauze for air ventilation, and transported to the laboratory at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, northern Thailand.

The laboratory rearing environment was designed to resemble the natural habitat. The adult rearing cage was built using a glass fish tank (25x50x30 cm) and covered the entrance with white muslin cloth (Fig. 1). The cage contained 6 plastic trays (20x40x6 cm) which were filled with water and pebble to maintain temperature and relative humidity. These glass tubes were observed every 2 h for oviposition on both beef and leaf settings. When females laid eggs, the tubes were not disturbed until the newly emerged larvae hatched from the eggs. These larvae were transferred using a wet fine brush and maintained in the transparent rearing plastic box (13x17x7 cm) of which ¾ of the lid covering with a fine muslin cloth to prevent entering of parasitoid and ventilation, containing fresh beef *ad libitum* and sawdust soaked with water (2:1) to increase relative humidity and simulate the natural breeding site. The larvae were reared under ambient temperature (25±2°C), relative humidity of 80±5% and a light/dark regime of 12:12 h in a rearing chamber (Fig. 1). Adults were fed with honey (serve as carbohydrate to provide energy), water and fresh beef (provided every 3 days) as a protein source for reproductive development and oviposition.

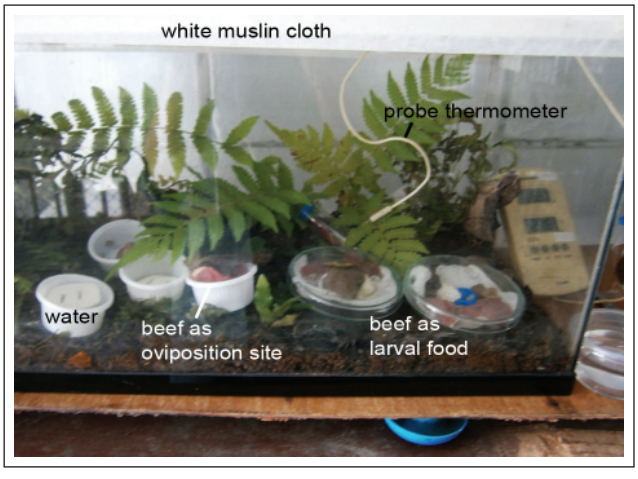

Figure 1. Rearing cage of *H. tumrasvini*, maintained at 25.0 ± 2.0°C and 80.0 ± 5.0% RH.

Developmental rate of egg

Eggs were divided into five groups of 24 and placed on a small piece of gauze (4.5x3.5 cm) located on a plastic tray (5.5x2.5 cm). A second piece of gauze soaked with water was placed over the eggs. These trays were then transferred into a plastic cage (13x17x7 cm) at 25.0±2.0°C, 80.0±5.0% RH and light/dark regime of 12:12 h. Eggs were observed every 2 h under a light microscope (Olympus®; CX31, Japan) until hatching. The experiment was replicated twice. Photographs were taken with a digital camera (Olympus®; VG-130, Japan). A video recorder function of a digital camera (Olympus®; VG-130, Japan) was also used to observe the hatching process.

Developmental rate of larvae

Larvae of *H. tumrasvini* were reared in a plastic container (13x17x7 cm) containing saw dust with water (2:1) at 25.0±2.0°C and 80.0±5.0% RH. Larvae were provided fresh beef *ad libitum*. Three larvae were sampled every 2 h from each egg batch, until the post-feeding stage when the third instar moves away from the beef toward the sawdust. Afterwards, two larvae (post feeding stage) were sampled until they entered the pupal stage.

Collected larva was sacrificed in 90°C water for 1 min. The length and width of each larva was measured using a digital caliper (Pittsburgh® 6 inch Digital calipers, model Soya, Taiwan). Larvae were then stored in 70% alcohol for preservation. As for puparia, each pupa was measured using digital caliper and then stored in 70% alcohol. Larval and pupal duration as well as time spent from newly hatched first instar to post-feeding stage was recorded.

Statistical analysis

Data were analyzed using the Mann-Whitney *U* test (SPSS version 17). A *P* value of less than 0.05 was considered significant.

RESULTS

Embryo development of *H. tumrasvini* is presented in Fig. 2. No obvious features of the embryos were observed during 0-6 h (Fig. 2A). One hundred fifty first instars occurred in each experiment, and two replications were conducted. Circular spinulation along the body was first observed at 8 h (Fig. 2B). Intense spinulation occurred during the 10 h period (Fig. 2C).

Each step of the hatching process was observed using a video recorder. During 10-12 h, the embryo was continuously active, as shown by changing in position of cuticular spines (Figs. 3A, 3B). The embryo shrunk vigorously prior to hatching, causing collapse of the latero-anterior margin of the eggshell (Fig. 3C), and later pushed out the most anterior region (Fig. 3D). Rupturing along the anterior hatching line allowed the first instar to eclose (Figs. 3E-G).

Morphometric measurement of larvae and puparia of *H. tumrasvini* was examined. The average length of the first, second, third (feeding phase), third (post-feeding phase) and puparia were measured: 3.5±1.1, 7.2±1.1, 13.5±1.8, 12.5±0.5 and 9.0±0.7 mm, respectively. Developmental period of immature *H. tumrasvini* reared in the laboratory at 25.0±2.0°C and 80.0±5.0% RH is shown in Table 2. The embryonation time (time for development of embryo inside the egg) was 10-12 h, while most of the first and second instars were rapid in development, taking 8 and 10 h, respectively. In contrast, the third instar development was

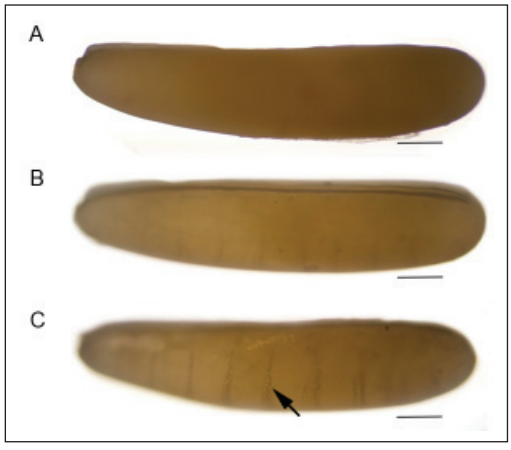

Figure 2. Embryo development of *H. tumrasvini*. A: 0-6 hr. B: 8 hr, showing circular spinulation of embryo. C: 10 hr, showing intense spinulation of embryo (arrow). Bar = 0.2 mm.

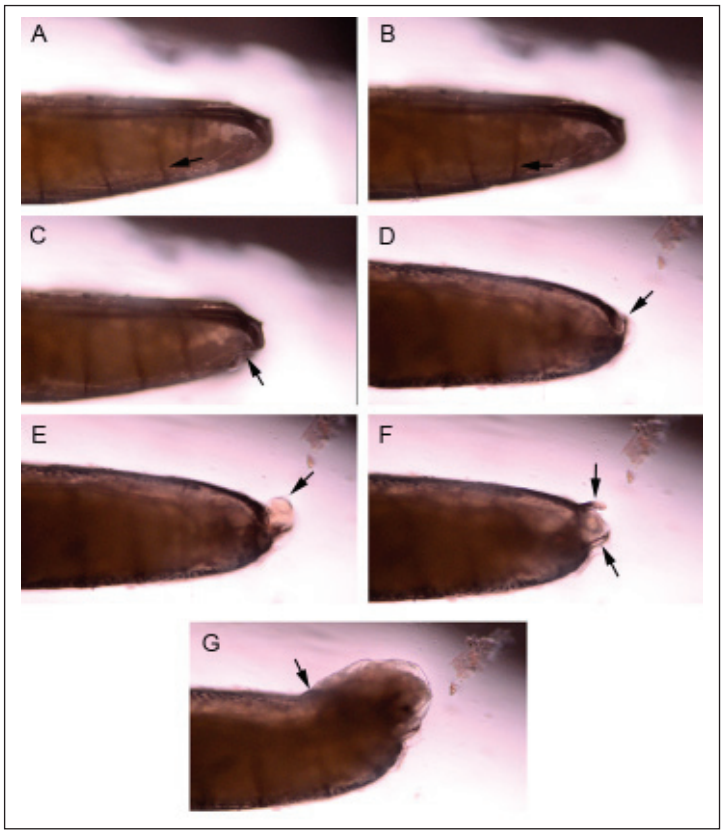

Figure 3. Hatching process of *H. tumrasvini* as demonstrated by a video recorder. A,B: Active movement of 10-12 hr embryo, as shown by changing in position of cuticular spines (arrows). C: Collapse of the latero-anterior margin of the eggshell due to shrunken embryo prior to hatching. D: Anterior straining by the larva. E,F,G: Rupturing along the anterior hatching line allowed the eclosion of first instar larva (arrows).

Table 1. Average length and width of immature *H. tumrasvini*[*]

Stage	*n*	Size (mean ± SD; mm)	
		Length	Width
First instar (L1)	30	3.5 ± 1.1	0.5 ± 0.2
Second instar (L2)	30	7.2 ± 1.1	1.1 ± 0.2
Third instar (L3, feeding phase)	102	13.5 ± 1.8	2.2 ± 0.3
Third instar (L3, post- feeding phase)	88	12.5 ± 0.5	2.3 ± 0.1
Puparia	40	9.0 ± 0.7	3.5 ± 0.1

[*]At 25±2°C and 80±5% relative humidity in laboratory.

considerably longer at 34 h in feeding phase; while 22 d in post-feeding period. Pupariation time took a longer period, being 9-10 d before emergence. The total developmental time of this species was 34-35 days. Figure 4 represents third instar length and width growth curves from the first until feeding phase. Both length and width median larval

Table 2. Developmental time of immature *H. tumarasvini*, with comparison to *H. violacea*

Stages	*H. tumrasvini*[*]		*H. violacea*[**]
	n	Duration (median)	
Egg	120	10-12 h	~6 h
First instar (L1)	30	8 h	12 h
Second instar (L2)	30	10 h	22 h
Third instar (L3, feeding phase)	102	34 h	16 h
Third instar (L3, post- feeding)	88	22 d	4 d 18 h
Puparia	40	9-10 d	5 d 18 h
First instar – pupariation		25 d	–
Total period (egg – emergence)		34.5-35.5 d	12 d 20 h

[*]At 25±2°C and 80±5% relative humidity in rearing chamber (present study)
[**]At 28±2°C and 70±5% relative humidity; 12 dark: 12 light (Chen *et al.*, 2011)

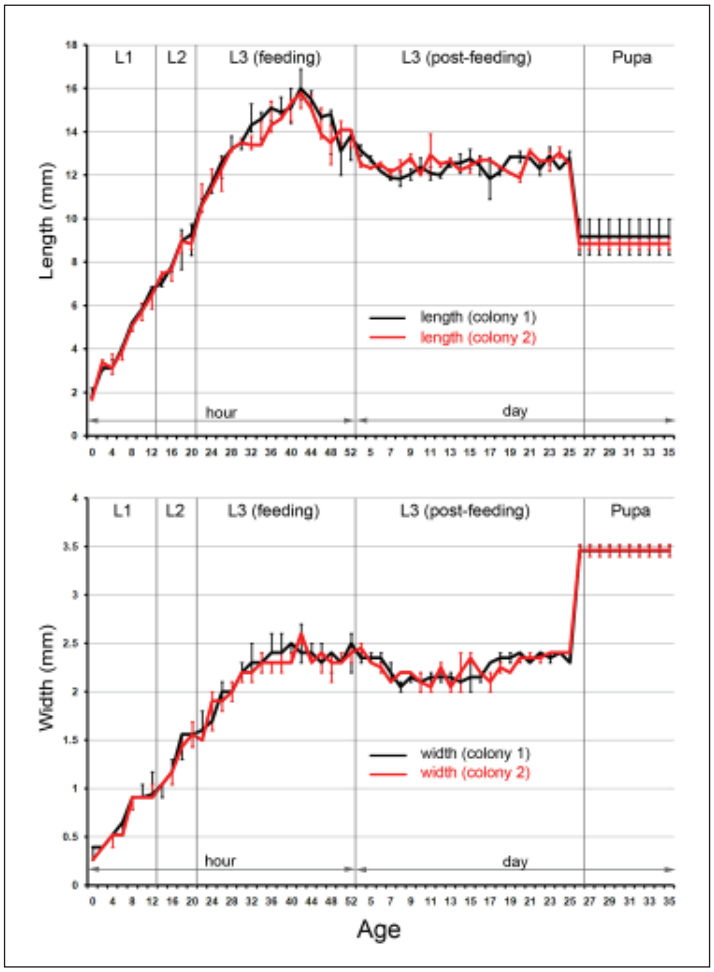

Figure 4. Developmental growth of *H. tumrasvini* showing median of larval length (upper) and width (lower). No significant differences between median of both colonies of their length and width (Mann-Whitney *U* test; $P>0.05$).

sizes were similar between colonies (Mann-Whitney *U* test; $P>0.05$).

Observation of the gradual coloration of *H. tumrasvini* puparia was displayed under stereomicroscope (Olympus SD30, Japan) (Fig. 5). At the first day of pupariation, the integument rapidly changed colors; from creamy-white (h 0) to orange, orange-brown, then light brown. Coloration of dark red-brown to almost black was observed from days 2-9. During this time, observation was conducted to examine development of the pupal respiratory horn, located at the dorso-lateral margin of the fifth segment (Fig. 6A-H). At h 0, a globular orifice appeared along the bubble membrane (Fig. 6A). The pupal respiratory horn began to emerge underneath the position of bubble membrane at 27 h thereafter. Rapidly the horn protruded through the orifice, at the center of bubble membrane with complete protrusion observed at 27.5 h (Figs. 6B-H). Microscopic observation also demonstrated that the position of the pupal respiratory horn is connected with the pharate adult (Fig. 7).

DISCUSSIONS

Species specific rates of development are the key information for estimating minimum PMI. Despite this potential utility in forensic investigations, ontogeny and developmental rate of *Hypopygiopsis* has not yet been established. Morphometric measurement in this study indicated that larvae of *H. tumrasvini* were relatively large, especially the third instar with lengths up to ~15.3 mm (range 11.7 – 15.3 mm). This size is comparable to the third instar stage of *H. violacea* (Ahmad Firdaus *et al.*, 2010).

Moreover, it is difficult to compare the developmental rate of immature stages of blow flies with that of other forensically important species (e.g., *Chrysomya megacephala* Fabricius, *Chrysomya rufifacies* Macquart, *Lucilia cuprina* Wiedemann, *Chrysomya nigripes* Aubertin, *Hemipyrellia ligurriens* (Wiedemann); or flesh flies, e.g., *Boettcherisca nathani* Lopes, *Lioproctia pattoni* (Senior-White), *Liopygia ruficornis* (Fabricius) and

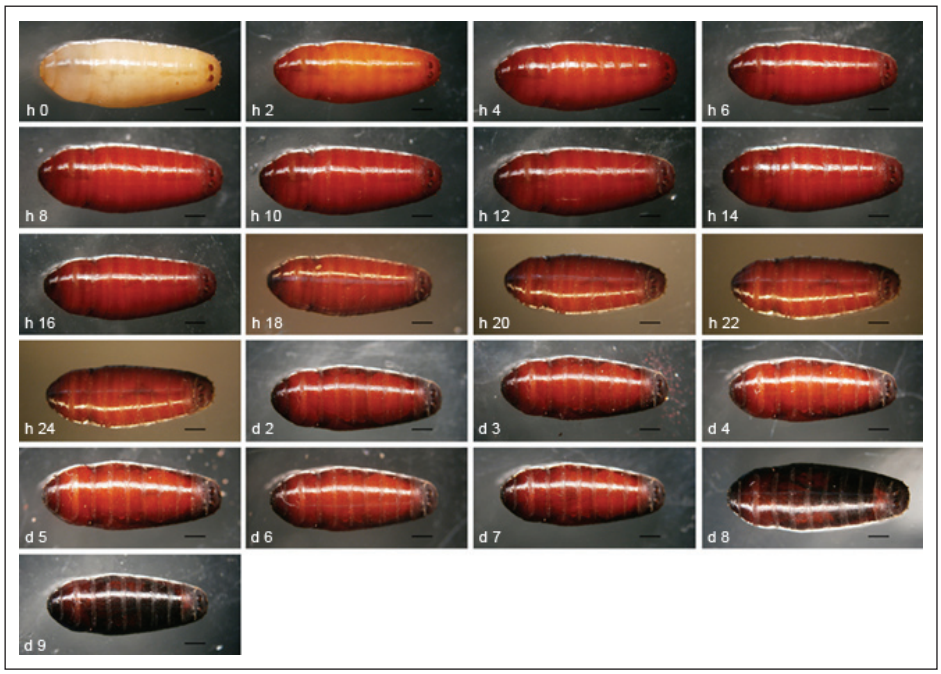

Figure 5. Gradual coloration change of puparia *H. tumrasvini* from h 0 to d 9. Bar = 1 mm for all figures.

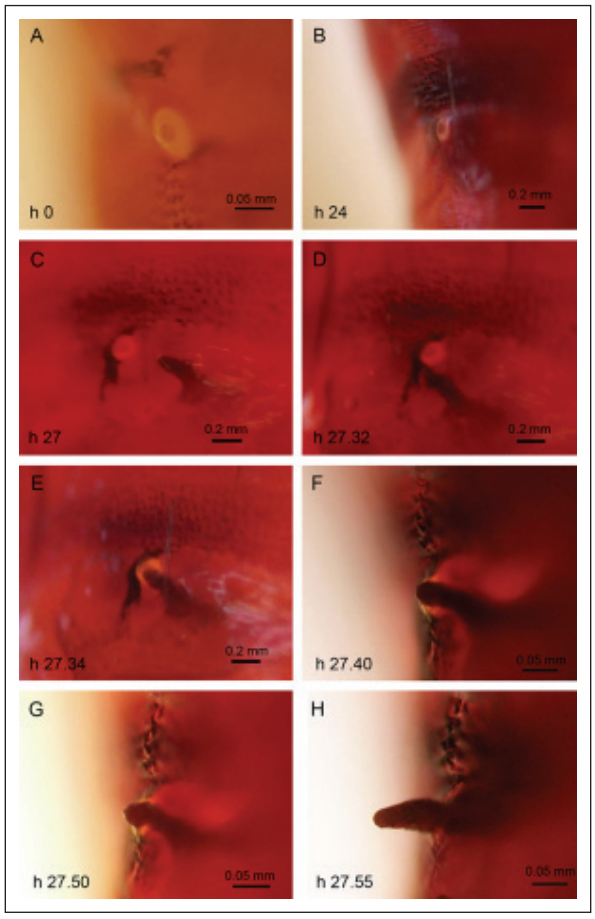

Figure 6. Development of pupal respiratory horn of *H. tumrasvini* as recorded via a video recorder. A-H: A remnant of the bubble membrane at h 0, and complete protrusion at h 27.55.

Figure 7. The connection between the pupal respiratory horn and anterior spiracle as shown in the pharate adult *H. tumrasvini*.

Parasarcophaga (*Liosarcophaga*) *dux* (Thomson) (Sukontason *et al*., 2008a; 2008b; 2010) due to a notably different biology. Results obtained from this study indicate the embryonation time of *H. tumrasvini*, reared at 25.0±2.0°C and 80.0±5.0% RH, was 10-12 h, which is consistent with embryonation of *C. rufifacies* (Sritavanich *et al*., 2009) but slower than that of *H. violacea* reared in ~6 h at 28.0±2.0°C and 70.0±5.0% RH (Chen *et al*., 2011). Also, the lengthy post feeding (~22 days), larval, and puparial periods of *H. tumrasvini* were longer than those of *H. violacea* (see Table 1), it is likely due to the lower temperature conditions (Chen *et al*., 2011).

First instar hatching from the eggshell was elucidated with the aid of a video recorder. In addition, the protrusion of pupal respiratory horns through puparial skin, at the position of bubble membrane was visualized to be completed at 27.5 h post-pupation. This protrusion of the pupal respiratory horn suggests oxygen supply requirements for the internal pharate adult as the structure observed in Fig. 7.

Furthermore, unique laboratory conditions are necessary for the blow fly development. This species exists in high altitude forests with low temperature and high humidity. Initially, our attempt to rear *H. tumrasvini* in ambient temperature (29-34°C and 42-74%RH) was ended up in failure. Rearing success occurred when we customized the cage at 25±2°C and 80±5% RH simulating a moist forest environment (see Fig. 1).

Mating conditions of *H. tumrasvini* were also distinct from that of other blow flies. Whereas other species of blow fly can reproduce freely while coexisting in a same cage, however, it was essential to separate newly emerged male and female *H. tumrasvini* and then reintroduce the both sexes for mating purposes in the ratio of 1:2, respectively. After successful mating, females produced eggs within the ovary (n = 107-312 eggs in single female), of which 80-218 eggs (75.3%; n = 845, hatching = 636) are able to hatch into first instar. This low fecundity limits the sampling in each interval and prohibits rearing experiments across multiple temperatures.

In conclusion, the present study expands the previous biological information of *H. tumrasvini* in describing the developmental rate of egg, larva and puparia. Such species-specific developmental data is imperative for future use in forensic investigations.

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REFERENCES

Ahmad Firdaus, M.S., Marwi, M.A., Syamsa, R.A., Zuha, R.M., Ikhwan, Z. & Omar, B. (2010). Morphological descriptions of second and third instar larvae of *Hypopygiopsis violacea* Macquart (Diptera: Calliphoridae), a forensically important fly in Malaysia. *Tropical Biomedicine* **27**(1): 134-137.

Byrd, J.H. & Castner, J.L. (2001). Insects of forensic importance. In: *Forensic entomology: The utility of arthropods in legal investigations*. (Editors: J.H. Byrd & Castner, J.L.). pp. 43-79. CRC Press, Florida.

Chen, C.D., Nazni, W.A., Ramli, R., Chia, K.H.M. & Sofian-Azirun, M. (2011). First study on the larval growth parameter and growth rate of a forensically important blow fly, *Hypopygiopsis violacea* (Macquart, 1835) (Diptera: Calliphoridae). *Proceeding of the ASEAN congress of Tropical Medicine and Parasitology* **11**: 159-163.

Goff, M.L. & Odom, C.B. (1987). Forensic entomology in the Hawaiian Islands. Three case studies. *American Journal of Forensic Medicine and Pathology* **8**(1): 45-50.

Greenberg, B. & Kunich, J.C. (2002). *Entomology and the law. Flies as forensic indicators*. UK: Cambridge University Press.

Gunatilake, K. & Goff, M.L. (1989). Detection of organophosphate poisoning in a putrefying body by analyzing arthropod larvae. *Journal of Forensic Sciences* **34**(3): 714-716.

Kurahashi, H. & Bunchu, N. (2011). The blow flies recorded from Thailand, with the description of a new species of *Isomyia* Walker (Diptera: Calliphoridae). *Japanese Journal of Systematic Entomology* **17**(2): 237-278.

Lee, H.L., Krishnasamy, M., Abdullah, A.G. & Jeffery, J. (2004). Review of forensically important entomological specimens in the period of 1972-2002. *Tropical Biomedicine* **21**(2): 69-75.

Moophayak, K., Klong-klaew, T., Sukontason, K., Kurahashi, H., Tomberlin, J.K. & Sukontason, K.L. (2014). Species composition of carrion blow flies in northern Thailand: altitude appraisal. *Revista do Instituto de Medicina Tropical de São Paulo* **56**(2): 179-182.

Moophayak, K., Sanit, S., Sukontason, K., Vogtsberger, R.C. & Sukontason, K.L. (2011). Morphological descriptions for the identification of *Hypopygiopsis tumrasvini* Kurahashi (Diptera: Calliphoridae). *Parasitology Research* **109** (5): 1323-1328.

Sanit, S., Sukontason, K.L., Sribanditmongkol, P., Klong-klaew, T., Samerjai, C., Sontigun, N., Limsopatham, K. & Sukontason, K. (2012). Surface ultrastructure of larva and puparia of blow fly *Hypopygiopsis tumrasvini* Kurahashi (Diptera: Calliphoridae). *Parasitology Research* **111**(6): 2235-2240.

Sritavanich, N., Jamjanya, T., Chamsuwan, A. & Hanboonsong, Y. (2009). Biology of hairy maggot blow fly, *Chrysomya rufifacies* and its applicaiton in forensic medicine. *Khon Kaen University Research Journal* **9**(2): 10-15. (in Thai with English abstract)

Sukontason, K., Bunchu, N., Chaiwong, T., Moophayak, K. & Sukontason, K.L. (2010). Forensically important flesh fly species in Thailand: morphology and developmental rate. *Parasitology Research* **106**(5): 1055-1064.

Sukontason, K., Narongchai, P., Kanchai, C., Vichairat, K., Sribanditmongkol, P. & Bhoopat, T. *et al*. (2007). Forensic entomology cases in Thailand: a review of cases from 2000 to 2006. *Parasitology Research* **101**(5): 1417-1423.

Sukontason, K., Piangjai, S., Siriwattanarungsee, S. & Sukontason, K.L. (2008a). Morphology and developmental rate of blowflies *Chrysomya megacephala* and *Chrysomya rufifacies* in Thailand: application in forensic entomology. *Parasitology Research* **102**(6): 1207-1216.

Sukontason, K.L., Sribanditmongkol, P., Chaiwong, T., Vogtsberger, R.C., Piangjai, S. & Sukontason, K. (2008b). Morphology of immature stages of *Hemipyrellia ligurriens* (Wiedemann) (Diptera: Calliphoridae) for use in forensic entomology applications. *Parasitology Research* **103**(4): 877-887.

Tumrasvin, W., Kurahashi, H. & Kano, R. (1979). Studies on medically important flies in Thailand VII. Report on 42 species of calliphorid flies, including the taxonomic keys (Diptera: Calliphoridae). *Bulletins of Tokyo Medical and Dental University* **26**(4): 243-272.

Verves, Y.G. (2005). A catalogue of Oriental Calliphoridae (Diptera). *International Journal of Dipterology Research* **16**(4): 233-310.

Williams, R.E. (2008). Case histories of the use of insects in investigations. In: *Entomology and Death: A Procedural Guide.* (Editors: N.H. Haskell & Williams, R.E.). pp. 10-39. East Park Printing, Clemson, S.C.