สัญญาเลขที่ RSA5580010

รายงานวิจัยฉบับสมบูรณ์

โครงการ ชีววิทยาของแมลงวันหัวเขียว *Achoetandrus rufifacies*: การประยุกต์ใช้ในนิติเวชกีฏวิทยาและการควบคุม

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มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย,
มหาวิทยาลัยเชียงใหม่ และคณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

กิตติกรรมประกาศ

ขอขอบพระคุณ สำนักงานกองทุนสนับสนุนการวิจัย, คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่และมหาวิทยาลัยเชียงใหม่ ซึ่งเป็นผู้สนับสนุนการวิจัยในโครงการนี้ ขอขอบคุณ รองศาสตราจารย์ นายแพทย์นิเวศน์ นันทจิต อธิการบดีมหาวิทยาลัยเชียงใหม่, รองศาสตราจารย์ นายแพทย์วัฒนา นาวาเจริญ คณบดีคณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่, รองศาสตราจารย์ ดร. ปรัชญา สมบูรณ์ หัวหน้าภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ที่อนุญาตและสนับสนุนการทำวิจัยครั้งนี้

ขอขอบคุณ ศาสตราจารย์ ดร. นายแพทย์ คม สุคนธสรรพ์ ที่เป็นผู้สนับสนุนการวิจัย ทำให้งานวิจัยครั้งนี้สำเร็จลุล่วงไปด้วยดี และคณะผู้ร่วมงานวิจัย Dr. Hiromu Kurahashi (Department of Medical Entomology, National Institute of Infectious Diseases, Japan), Dr. Jeffery Tomberlin (Department of Entomology, Texas A&M University, USA), คุณธันวดี คล่องแคล่ว, คุณนารินทร์ สนธิกันย์, คุณสงบ สนิท, คุณขวัญกมล ลิ้มโสภาธรรม, คุณจุฑารัตน์ เสมอใจ, คุณสุดธิดา สุวรรณยศ และเจ้าหน้าที่ทุกท่านของภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

บทคัดย่อ

รหัสโครงการ RSA5580010

ชื่อโครงการ ชีววิทยาของแมลงวันหัวเขียว *Achoetandrus rufifacies*: การประยุกต์ใช้ในนิติเวชกีฏวิทยาและการควบคุม

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ระยะเวลาโครงการ 3 ปี (16 กรกฎาคม 2555 ถึง 16 กรกฎาคม 2558)

เนื้อหางานวิจัย

แมลงวันหัวเขียว *Achoetandrus rufifacies* เป็นแมลงวันที่มีความสำคัญทางการแพทย์ และพบมากในประเทศไทย ตัวเต็มวัยเป็นพาหะนำเชื้อโรคต่างๆมาสู่มนุษย์ ส่วนตัวอ่อนก่อให้เกิดโรคหนอนแมลงวันทั้งในมนุษย์และสัตว์ การหาวิธีควบคุมจำนวนประชากรแมลงวันจึงนับว่าเป็นสิ่งที่จำเป็น ซึ่งต้องอาศัยองค์ความรู้พื้นฐานทางชีววิทยาในด้านต่างๆของแมลงวันหัวเขียวชนิดนี้ การศึกษาครั้งนี้มีจุดประสงค์เพื่อ 1) ศึกษาการกระจายตัวเชิงพื้นที่ของแมลงวันหัวเขียวชนิดนี้ และหาความสัมพันธ์ระหว่างการแพร่กระจายตัวกับปัจจัยทางกายภาพ (เช่นอุณหภูมิ ความชื้น) ในเขตเมืองเชียงใหม่และศึกษาช่วงการบินในแต่ละฤดูกาลและในช่วงเวลาของวัน ตั้งแต่เดือนกรกฎาคม 2556-มิถุนายน 2557 โดยจับแมลงวัน 2 ครั้งต่อเดือน จับแมลงวันตัวเต็มวัยโดยใช้กรงดักแมลงวันอัตโนมัติ ณ ศูนย์วิจัยสาธิตและฝึกอบรมการเกษตรแม่เหียะ จังหวัดเชียงใหม่ โดยตั้งกรงในจุดศึกษา 3 จุด คือ สวนปาล์ม, พื้นที่ป่า, สวนลำไย ใช้เครื่องในวัวเน่า 1 วัน (300 กรัม) เป็นเหยื่อล่อ พบว่าจับแมลงวันชนิดนี้ได้ 55,988 ตัว พบมากที่สุดในป่า (53%) รองลงมาคือสวนปาล์ม (27%) และสวนลำไย (20%) จำนวนแมลงวันที่จับได้มีความสัมพันธ์เชิงบวกกับอุณหภูมิ แต่มีความสัมพันธ์เชิงลบกับความชื้น พบแมลงวันจำนวนมากในฤดูร้อนรองลงมาคือฤดูฝนและฤดูหนาว ช่วงที่จับแมลงวันได้มากที่สุดคือ 15.00 - 18.00 น. ส่วนการศึกษาชีววิทยาด้านการสืบพันธุ์ของแมลงวันชนิดนี้ในห้องปฏิบัติการ ทำการผ่าแมลงวันเพศเมียเพื่อศึกษาการเจริญของเซลล์ไข่ ตั้งแต่อายุ 1 วันถึง 9 วัน

จากที่ได้เสนอไว้ในโครงร่างทุนว่า หากมีตัวอย่างแมลงวันที่จับได้จากภาคสนามจากจุดประสงค์ที่ 1 จะทำการศึกษาชีววิทยาของแมลงวันดังกล่าวด้วย จึงได้ศึกษาช่วงการบินในแต่ละฤดูกาลและในช่วงเวลา ของแมลงวันหัวเขียว *Chrysomya megacephala* ในช่วงเวลาเดียวกับที่ศึกษาใน *A. rufifacies* พบแมลงวันจำนวนมากในฤดูร้อนรองลงมาคือฤดูฝนและฤดูหนาว ช่วงที่จับแมลงวันได้มากที่สุดคือ 15.00 - 18.00 น. ส่วนการศึกษาชีววิทยาด้านการสืบพันธุ์ของแมลงวันชนิดนี้ในห้องปฏิบัติการ พบว่าตัวเต็มวัยเพศเมียสามารถออกไข่ได้ถึง 7 ครั้งในช่วงชีวิต นอกจากนี้ยังจำแนกชนิดของแมลงวันหัวเขียวชนิดต่างๆที่มีความสำคัญทางการแพทย์ โดยวิธีอณูชีววิทยา โดยใช้ COI gene ผลจากการศึกษานี้ นับเป็นข้อมูลพื้นฐานสำคัญที่จะนำไปสู่การพัฒนาวิธีการควบคุมประชากรแมลงวันหัวเขียวต่อไปในอนาคต

คำหลัก แมลงวันหัวเขียว *Achoetandrus rufifacies*, *Chrysomya megacephala*, การควบคุม, การกระจายตัว, ชีววิทยา

Abstract

Project Code: RSA5580010

Project Title: Biology of the blow fly, *Achoetandrus rufifacies* (Macquart): Application in forensic entomology and control

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Project Period: 3 years (16 July 2013-16 July 2015)

Content

Achoetandrus rufifacies (Macquart) (Diptera: Calliphoridae) is the important blow fly species of medical concern in Thailand. The adults are mechanical vectors of various pathogens, while the larvae can cause myiasis. Regarding this, a method to control fly population is mandatory, with all aspects of the basic research of this fly species being needed. In this study, we investigated the response of this fly species to climatic and physio-environmental factors in Chiang Mai, northern Thailand. Adult fly surveys were carried out every two weeks from July 2013- June 2014 at study sites in Mae Hia Agricultural Research, Demonstrative and Training Center using automatic baited trap with 1-day tainted beef offal as bait. A total 55,988 adult *A. rufifacies* were captured, with peak densities being observed in summer, followed by rainy season and winter. Population density had positive correlation with temperature (r = 0.461), but negative correlation with relative humidity (r = 0.621). The daily activity showed major peak during 15.00 – 18.00 pm. Changes in the ovariole during egg development of this species were conducted from day 1 to day 9.

As has been proposed in the grant proposal, the extra researches were conducted for biological information of other medically important flies, if species were available from the field collections. In this regard, seasonal and daily activity of blow fly, *Chrysomya megacephala* were conducted, of which the experimental design was the same as that has been described in *A. rufifacies*. Peak population was found in summer, followed by rainy season and winter. The daily activity showed major peak during 15.00 – 18.00 pm. Biological investigation in the laboratory revealed that females of this species can oviposit upto 7 times in her lifetime. Moreover, molecular identification of blow flies of medical importance in Thailand was performed using COI gene. Information gained from this thorough study in both alimentary and reproductive systems established a basic database, which was useful in understanding their functional role and serving as knowledge to address a strategy to control this fly species in the future.

Keywords: *Achoetandrus rufifacies*, *Chrysomya megacephala*, control, distribution, biology

ชีววิทยาของแมลงวันหัวเขียว *Achoetandrus rufifacies*: การประยุกต์ใช้ในนิติเวชกีฏวิทยาและการควบคุม

PROBLEMS AND RESEARCH RATIONALE

The hairy maggot blow fly, *Achoetandrus rufifacies* (Macquart) is a medically important species worldwide. From a medical point of view, it has a positive aspect by acting as a forensically important species. Based on reports from many countries, e.g., Hawaii of the U.S.A., Malaysia and Thailand, the larvae of this fly have been collected from many human corpses, and thus used as entomological evidence in forensic investigations, particularly in estimating postmortem interval (PMI). In northern Thailand, this is one of the two most common fly larva species collected in corpses, along with *Chrysomya megacephala,* the Oriental latrine blow fly. Conversely, in the negative sense, the adult of this species is not only a mechanical carrier of various pathogens to humans (e.g., bacteria, virus and parasites), but its larva also carries the myiasis-producing agent as previously reported (Baumgartner 1993).

Ecologically, adult surveys in northern Thailand revealed that *A. rufifacies* presents in varied habitats, e.g., urban, suburban and forested areas, at altitudes from 25 to 1,000 metres above sea level. Flies are also collected as urban land use types, suggesting that their food source is associated with the human environment. In Thailand, this fly was recorded as the second most collected blow fly species in the fly survey while *C. megacephala* was the most common species (Sukontason et al. 2008). Although the biology, distribution and control of *C. megacephala* have been widely studied, but information for *A. rufifacies* is limited. Sukontason et al (2008) reported the morphology of the immature stage (egg, larva and puparium) and developmental rate of *A. rufifacies* larvae; while Byrd and Butler (1997) found that environmental temperature was as an important environmental abiotic factor for larval growth and development. In *C. megacephala*, other abiotic factors, such as relative humidity, solar radiation, wind velocity and rainfall, had influence on adult fly distribution and activities. However, the influence of these abiotic factors on the distribution of *A. rufifacies* has not been clarified. Therefore, fly distribution and the factors that affect fly population should be clarified in *A. rufifacies*. Hence, the first objective of this study was to determine the relationship between climatic factors (temperature, relative humidity, light intensity) and physical factors (land use types) that influence the distribution of *A. rufifacies* in central Chiang Mai province, northern Thailand. Using the field survey and Geographic Information System (GIS) methodology, the spatial and temporal distribution of *A. rufifacies* in urban and suburban areas of Chiang Mai province will be clarified. Association between population density of *A. rufifacies* and climatic factors was evaluated.

Information of fly behavior, especially activity patterns in nature, is important in each insect species for use in controlling its population. Daily and seasonal activity patterns have been investigated in many insects, but not for *A. rufifacies*. Therefore, the second objective of this study was to determine the daily and seasonal activity patterns of this fly under natural conditions in Chiang Mai using electronic timer control fly entrance traps. The last objective was to investigate the biology and behavior of *A. rufifacies* in the laboratory, which includes reproductive system of adult males and females, developmental rate of the egg, larva and pupa, etc. Data gaining from these experiments

were beneficial in understanding the biology of *A. rufifacies* in both laboratory and natural conditions. The inclusive knowledge of spatial and seasonal trends, activity patterns and biological laboratory data provide information that will be useful in applying where and when to implement effective control programs and for forensic entomology purpose.

OBJECTIVES

1. To determine the relationship between climatic factors (temperature, relative humidity, light intensity) and physical factors (land use types) that influent the distribution of *A. rufifacies* in central Chiang Mai province, northern Thailand.

2. To determine the daily and seasonal activity patterns of this fly under natural conditions in Chiang Mai using electronic timer control fly entrance traps.

3. To investigate the biology and behavior of *A. rufifacies* in the laboratory.

METHOD

Part I: Spatial and temporal distribution of *A. rufifacies* in urban and suburban areas of Chiang Mai province

Part II: Determination of the daily and seasonal activity patterns of *A. rufifacies* in Chiang Mai province

Experimental design (Parts I, II)

Study site: This study was conducted at the Mae Hia Agricultural Research, Demonstrative and Training Center, which is located on Canal road in Mueang Chiang Mai district. This training center is located in the area as wide as 2,068,800 sq.m. (1,293 Rai) which used to be a part of Doi Suthep-Pui National Park, then later Royal Forest Department gave permission to Chiang Mai University for use this area as a study and research center (http://web.agri.cmu.ac.th /maehia/index.php/background/information). The general description of this area, it is consisted of foothill slope, hill, and lowland. Furthermore, nine of water reservoirs were found within this area. It is surrounded by natural scenery. The elevation of this area is around 300 meter above sea level. Slope surface is about 0-15% of total area aligned from North to South.

Three different microenvironments were chosen as study plots included forested area, palm garden, and longan orchard.

- Forested area (N 18°46’01.08”, E 98°56’08.3”) is located at the altitude of 344 m above sea level. Fly traps were set at about 150 m far from the road at the foothill of mixed deciduous forest. This area was covered with Teak (*Tectona grandis*) and bushes. The study site was located close to rubber plantation. The grass-fed cows were also found in the pasture land located on the other side of the road opposite to the study site.

• Palm garden (N 18°45’27.841”, E 98°55’48.515”) is located at the altitude of 330 m above sea level. It is consisted with Tenera plam tree (*Elaeis guineensis* Jacq**.)** which is the oil palm with small nut and thin shell. The flower is produced in a dense cluster. The palm fruit is reddish and grow in large bunches. As the palm tree leaves are large, compound and arranged cluster at the top of the tree. When palm tree grow up, the leaves form in the dense cluster until the canopy closes. Palm garden is supplied with a large amount of water. It placed about 120 m far from the main research station road and opposite to the storage building.

• Longan orchard (N 18° 45’56.66”, E 98°55’40.13”) is located at the altitude of 347 m above sea level. The dominant species in this orchard is *Dimocarpus longan* Lour. Longan trees were planted in row. The flowering season of Longan in Thailand is during late December to late February and the harvesting season starts at late June to late August. The Longan orchard is 220 m far from the main road and placed at the foothill of the mountain.

Adult fly collection: By using automatic baited trap, invented by Kom Sukontason, fly trapping was performed every 2 weeks for 1 year (July 2013-June 2014). The traps consist of four basic parts: (1) an external metal case (40 x 40 x 60 cm), (2) a fly entrance module, which is made of transparent plastic board and controlled by an electronic timer mechanism, (3) a black fly net, and (4) an attractive bait (~300 g of tainted beef offal) put in a plastic container and placed at the base of the trap. To prevent the scavengers and rain, the automatic trap was placed inside a wire cage that half-length covered with transparent plastic sheet.

The operation of the automatic trap is controlled by timer which was adapted from the fish feeder that originated to feed fish at regular intervals. The timer was set to open and closed at the specific period of time. The timer was connected with electric relay, which is an electrically operated switch that used to control a circuit by a low-power signal. At the beginning of trap operation, timer rotated the food hopper axle at the setting intervals and triggered the relay. Then, electric power, from 12 voltage battery, was released to supply the CD-player tray and let it slided from the slot loading drive. As the tray was connected with fly entrance module by fishing net, therefore, the movement of fly entrance module was depending on the movement of the tray. When, the tray slide out of the slot pulled the fishing net, then fly entrance module was lifted up and let the flies to enter into the trap. By using one-day tainted beef offal (300 g) as bait, adult flies were lured to the attractive bait. As flies are phototaxis insects so when they enter inside the fly net, they generally fly upward to the light at the top of the trap and hold back within the fly capture net. When time reached, timer triggered the relay again and let power supplied CD-player tray. Then, the tray was slide back into the loading slot. The fishing net was released, let the entrance module slided down and closed fly entrance. Therefore, all captured flies were retained inside the fly net.

The adult fly collection was performed twice a month for data collection throughout the year. The traps were set to operate at the time intervals as showed in Table 1.

Table 1. The automatic trap operation time

	Open	Closed	Duration (h)
DAY TRAP	6.00	9.00	3
	9.00	12.00	3
	12.00	15.00	3
	15.00	18.00	3
NIGHT TRAP	18.00	6.00	12

Traps were emptied at 3 hours for day trap and 12 hours for night trap by removed fly net from the trap and installed the new one for the next loop of experiments. During the experimental time, hourly temperature ($^{\circ}$C) and relative humidity (%) were recorded using Temperature and Humidity loggers (Ebro EBI 20-TH1; ebro Electronic GmbH & Co. KG, Germany).

Fly identification: The collected flies were transported to the laboratory of the Department of Parasitology, Faculty of Medicine, Chiang Mai University. They were sacrificed by placing a fly net in a refrigerator set at 4°C for 2 hours. Then, all flies were identified individually into species under dissecting microscope using the taxonomic keys (Tumrasvin et al. 1979), sexed and counted.

RESULTS

A total of 55,988 *A. rififacies* was collected during July 2013 – June 2014. Among three microhabitats, the largest number of *A. rufifacies* was found in forested area (53%) followed by palm garden (27%) and longan orchard (20%) (Fig. 1).

In northern Thailand, three seasons are defined as summer, rainy season, and winter. Summer is the time during mid-February to the beginning of May, rainy season occurs from mid-May to the beginning of October, and winter starts from mid-October to the beginning of February. During July 2013 – June 2014: in summer, the weather was hot and dry. The temperature was high and relative humidity was low. Furthermore, the lowest relative humidity (41.14%) was recorded in summer (mid-March 2014). The rainy season was dominated by the southwest monsoon, during that time; rainfall was at its heaviest. Therefore, highest relative humidity (85.99 %) was recorded in this season (mid-June 2014) but temperature was lower when comparing with summer time. Interestingly, relative humidity was constant at the beginning of winter, and then it started to decrease in mid-December 2013 and continued drop through winter to the beginning of summer. From this study, temperature in winter was not low and the highest average temperature (34.87°C) was record in this season also (mid-October 2013). In contrast, the lowest average temperature (23.31°C) was recorded in rainy season (the beginning of September 2013) (Fig. 2). Pearson's correlation coefficient showed a significant relationship between the number of *A. rufifacies*, temperature (P =0.000) and relative

humidity. As the number of *A. rufifacies* was positively correlated with temperature(r=0.461), while negative correlation was found between the number of fly and relative humidity (r =0.621).

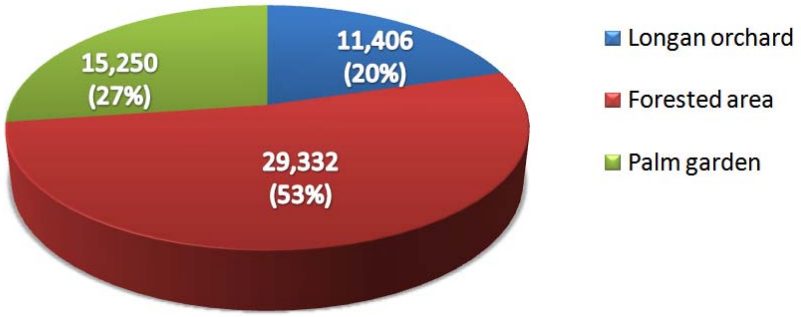

Figure 1. Total number of *A. rufifacies* captured from each microhabitat during July 2013 – June 2014.

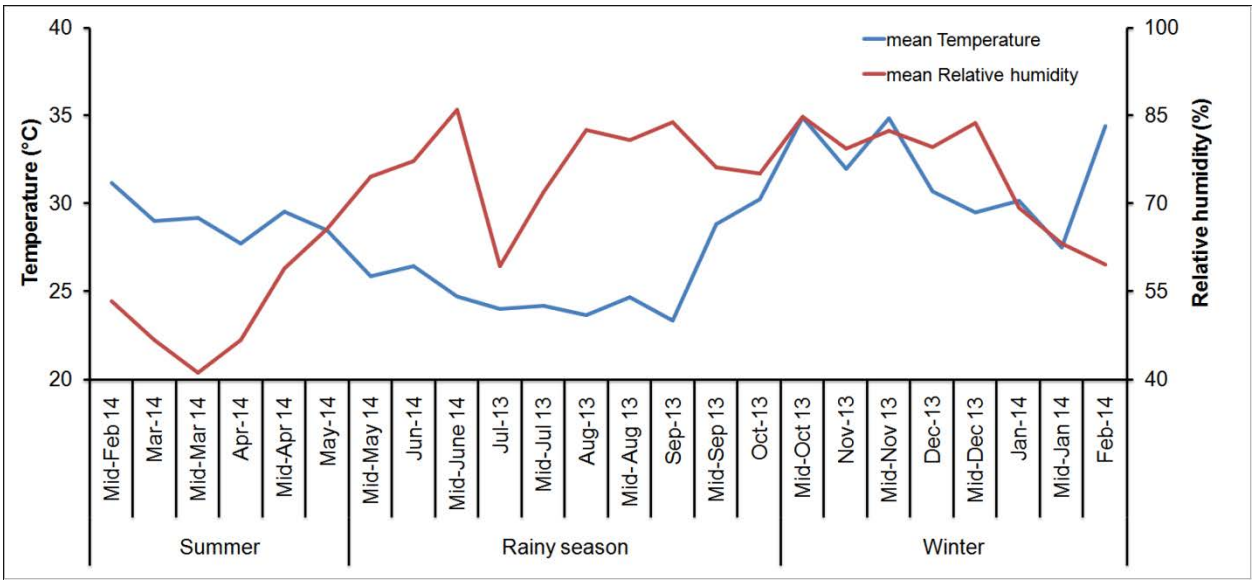

Figure 2. The fluctuation of average temperature (°C) and average relative humidity (%) recorded during July 2013 – June 2014.

According to this study, *A. rufifacies* was established throughout the year. High number of *A. rufifacies* was collected in summer (63.50% of collected *A. rufifacies*) and then the population was decreased during rainy season (25.71%) through the whole winter (10.79%). Peak population (8,669) was recorded in summer (the beginning of April 2014) and the lowest population density (132) was found in winter (the beginning of January 2014). Although high number of *A. rufifacies* was collected mainly in forested area, seasonal distribution patterns among those three microhabitats (forested area, palm garden, and longan orchard) were not different (Fig. 3).

High number of female (43,834 flies) was captured more than male. About eighty percents of them (35,247 flies) were non-gravid female. The highest ratio of male flies per one female (0.48) was

recorded at the end of summer (during mid-April 2014) suggested that high number of flies was emerged at that time, however the fluctuation of sex ratio did not show a clear pattern (Fig. 4). The average ratio between male per female was 0.26:1.

The daily activity of *A. rufifacies*, the major peak population was collected during 15.00 – 18.00, however; there was no significant different between the number of fly captured during 9.00-12.00, 12.00-15.00, and 15.00-18.00 ($P \geq 0.05$; One-way ANOVA, Dunnett's T3 post Hoc test). In summer, high number of *A. rufifacies* was capture during 15.00 -18.00. Interestingly, in rainy season, *A. rufifacies* showed peak population at 12.00-15.00 and similar pattern was also observed in winter (Fig. 5). Only small number of fly was captured during night time (18.00 -6.00). In summer and rainy season, the day length was longer than in winter, it was longer than 12 hours. High number of *A. rufifacies* was collected by the night time trap. On the contrary, when the day length was short (less than 12 hours) as in winter, only a few number of fly was collected at the night time (Fig. 6).

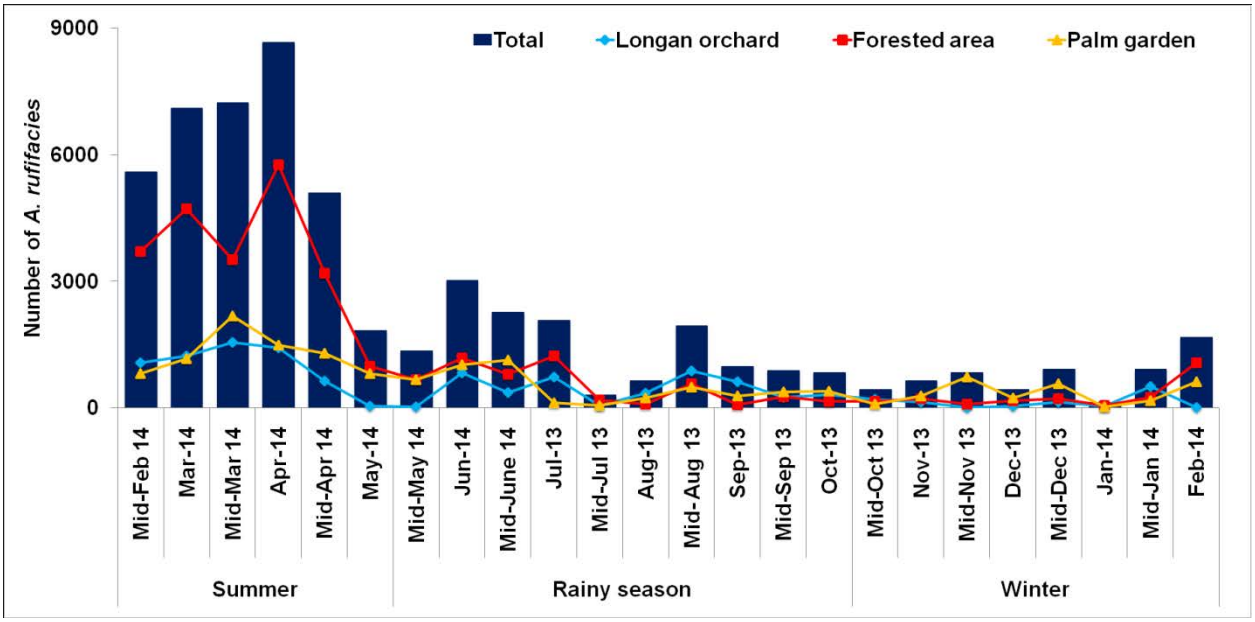

Figure 3. Seasonal fluctuation of population density of *A. rufifacies* collected during July 2013 – June 2014.

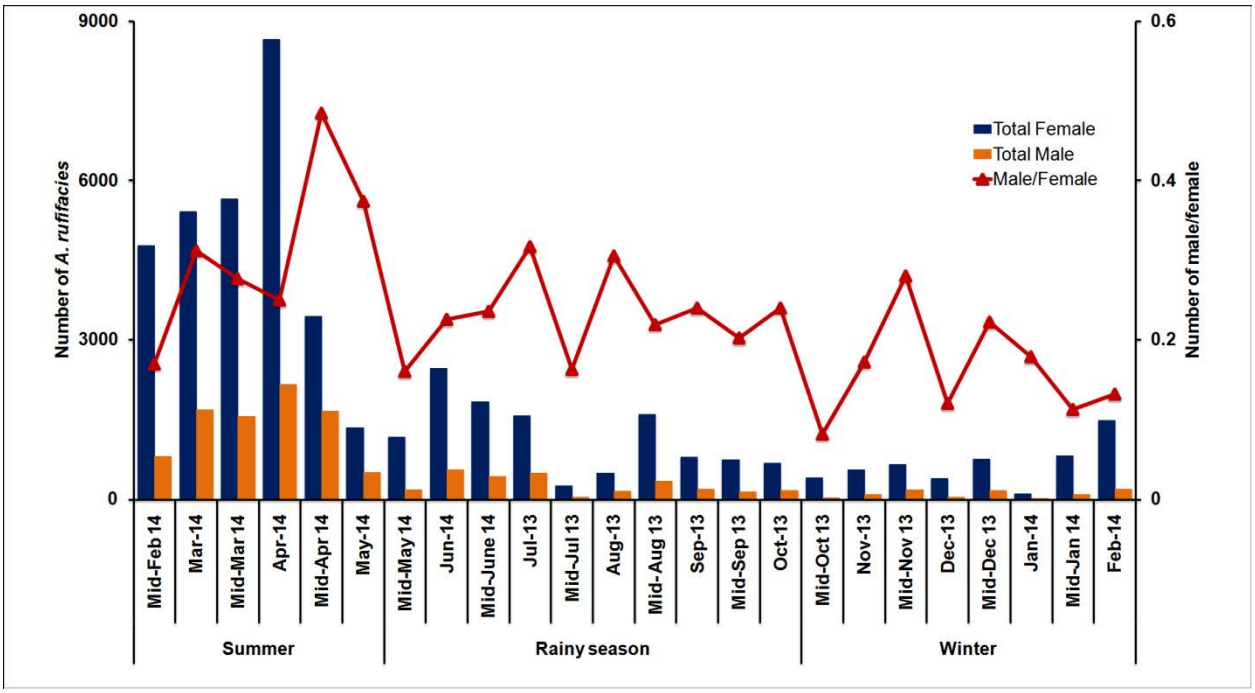

Figure 4. Sex ratio of *A. rufifacies* capture by the automatic trap during Jyly 2013 – June 2014.

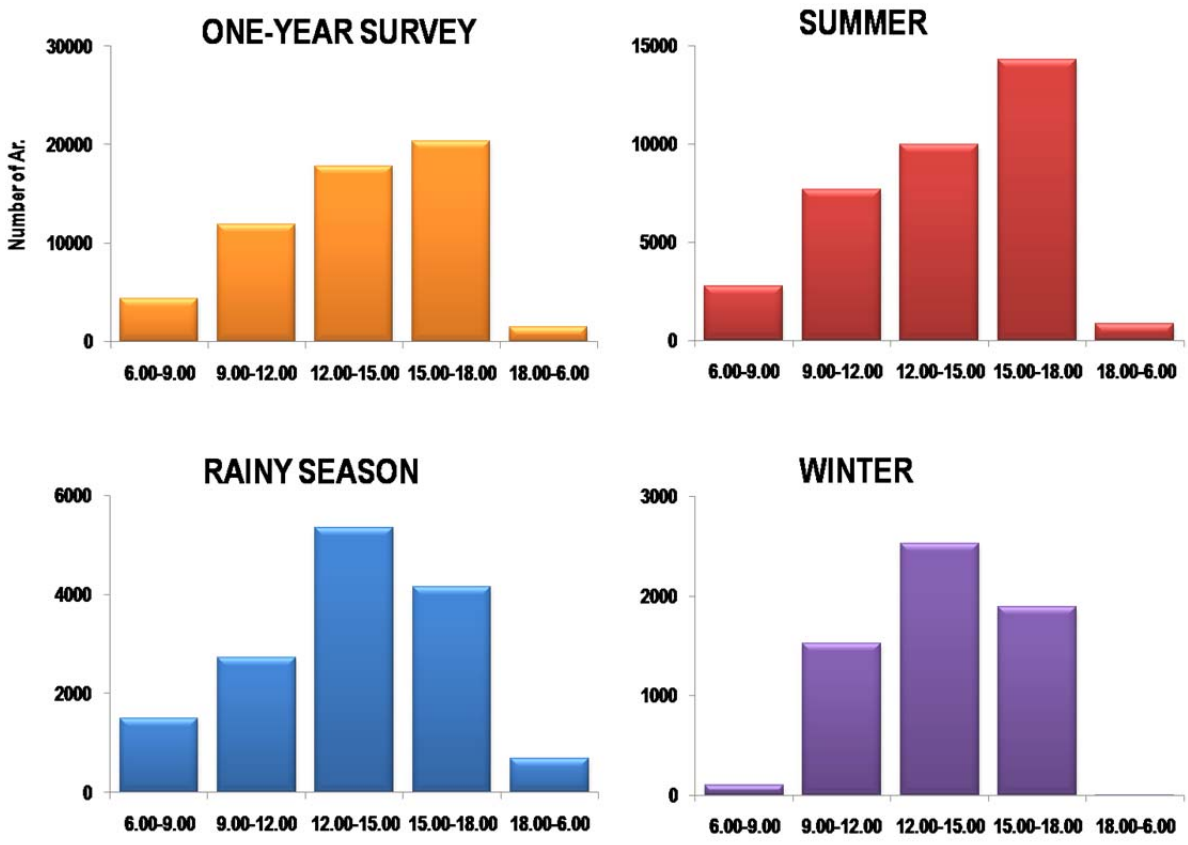

Figure 5. Daily activity pattern of *A. rufifacies* collected during July 2013 – June 2014)

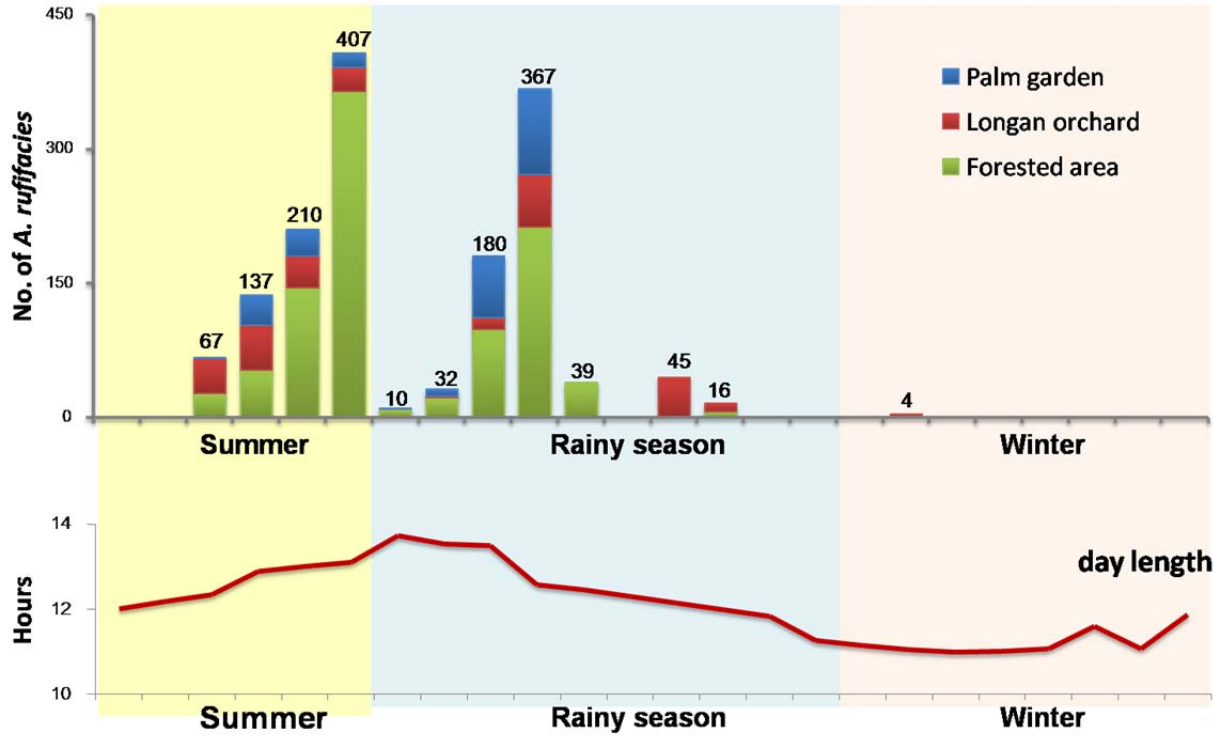

Figure 6. The number of *A. rufifacies* captured during the night time (6.00 p.m. – 6.00 a.m.) (above) and day length (below)

Part III: Determination of the biology of *A. rufifacies* in the laboratory

Dissection of female genitalia

The female internal reproductive organs of *A. rufifacies* consist of 2 ovaries, 2 lateral oviducts, a common oviduct, 3 spermathecae, vagina and 2 accessory glands.

Changes in the ovariole during egg development:

The stages described here for *A. rufifacies* form the basis of an aging technique that could be used to differentiate the ages of field collected female blowflies. In this experiment the ovarioles of *A. rufifacies* were observed for changes from day 0 (just after emergence) until 9-days-old under ambient temperature conditions of 18-27 $^{\circ}$C during the study period. The observations of the ovarian stages were conducted using fresh preparations in which the ovarioles were placed on a slide and covered with a cover slip. They were then examined and measured under an ordinary light microscope. The ovarioles of *A. rufifacies* are of the polytrophic ovariole type**.**

Day 1: The ovariole has a piriform germarium, which will develop to be oocyte. The follicles are not yet well differentiated from the germarium.

Day 2: The ovariole is enlarged. The follicle is nearly spherical and distinct from the germarium. Stalk cell is clearly observed. The cystocytes are clearly visible and situated in the basal region of the follicle which is surrounded by the follicular cells.

Day 3: The ovariole expanded into the globular shape. Nurse cells are more distinct. Oocyte is more developed, and the development of the 2nd follicle from the germarium cells was observed.

Day 4: The follicles are now oval in shape with yolk (oocyte) occupying up to one-third to one-half of the total follicle length. The epithelium around the oocyte has become columnar. Separation of the 2nd follicle from the germarium cell was apparent.

Day 5: The follicles are more develop, and have greatly increased in length and width. The yolk (oocyte) now accounts >two-third of the total follicle. The 2nd follicle showed enlargement, compared with the germarium cells.

Day 6-9: The eggs are full size with the oocyte occupying the entire follicle and the nurse cells have disappeared. Initiation of the 3rd follicle was apparent.

Part IV: Conduct biological experiments of other medically important flies (if specimens are available from field collections, e.g., investigation morphology using scanning electron microscope, study biology, etc.)

This part has been proposed in the grant proposal. In this regard, the extra researches were conducted for biological information of other medically important flies. Our team investigated the seasonal and daily activity of blow fly, *Chrysomya megacephala*, of which the experimental design was the same as that has been described in *A. rufifacies*. Result of seasonal activity was demonstrated in Fig. 7, while the daily activity was shown in Fig. 8. More female *C. megacephala* were collected than male (Fig. 9). Dissection of the reproductive status of female *C. megacephala* from the field collected revealed more non-gravid status than those gravid (Fig. 10).

The biological investigation of *C. megacephala* was conducted in the laboratory, focusing on the longevitiy of adult flies. Moreover, molecular identification of blow flies of medical importance in Thailand was performed.

Seasonal and daily activity of *C. megacephala*

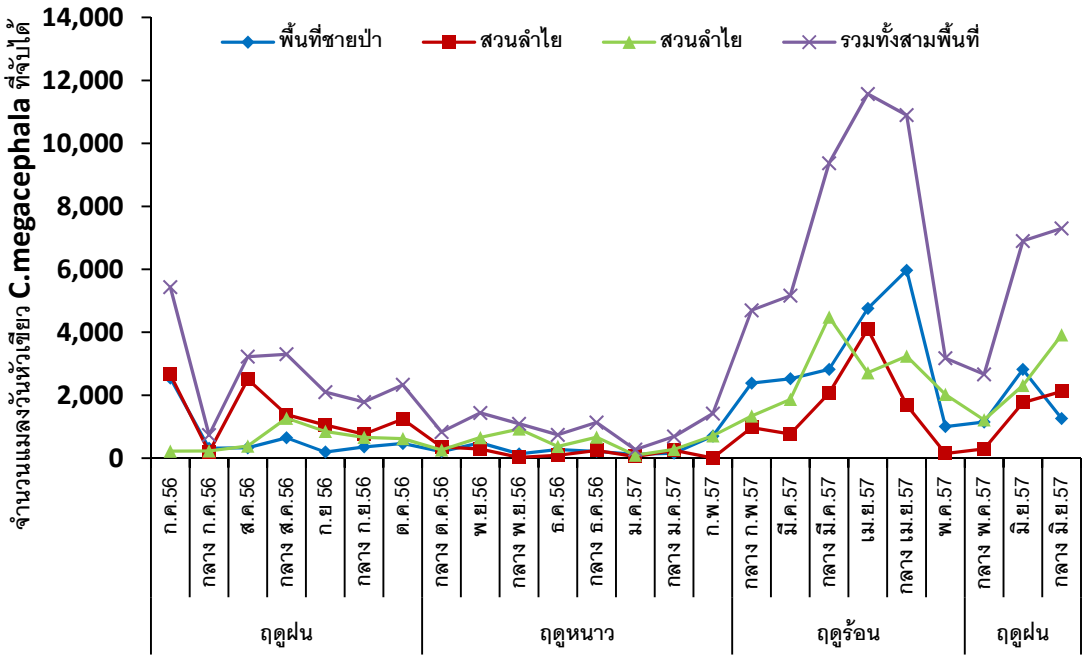

Figure 7. Seasonal activity of *C. megacephala* collected from 3 study areas. January was the month that collects the minimal number of this species, while the most collected flies was in April.

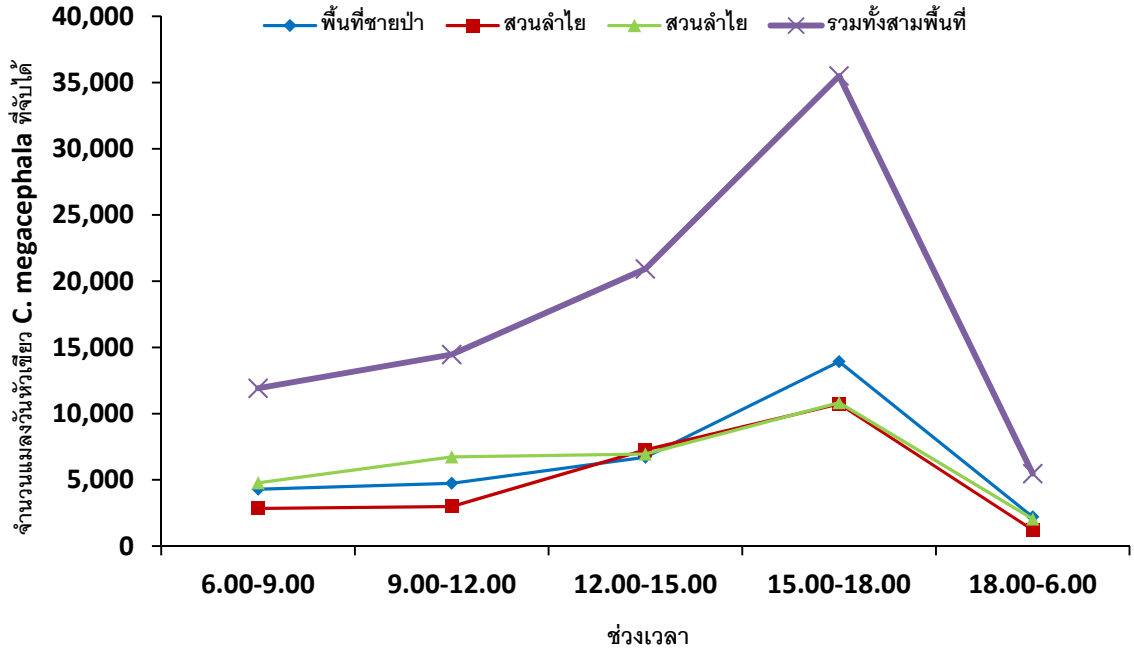

Figure 8. Dialy activity of *C. megacephala* in three study areas.

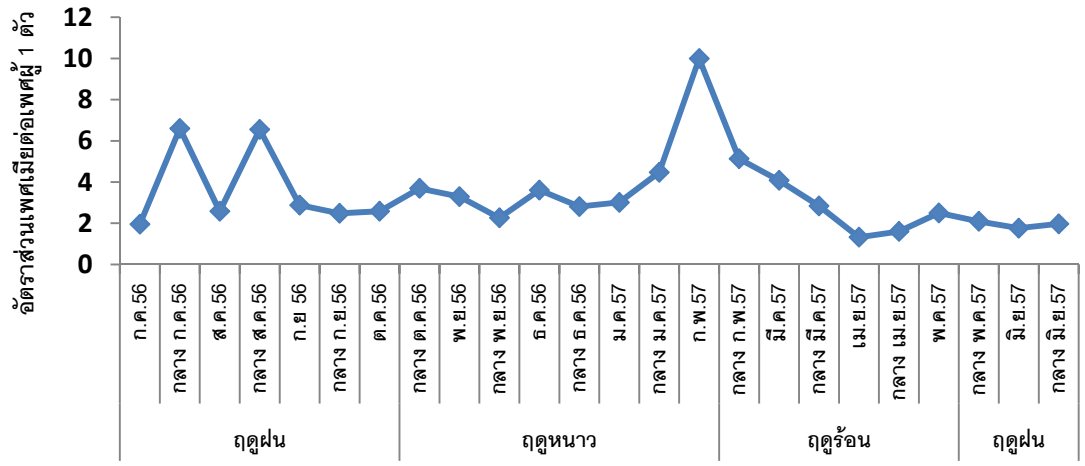

Figure 9. More female *C. megacephala* were collected than males during study period.

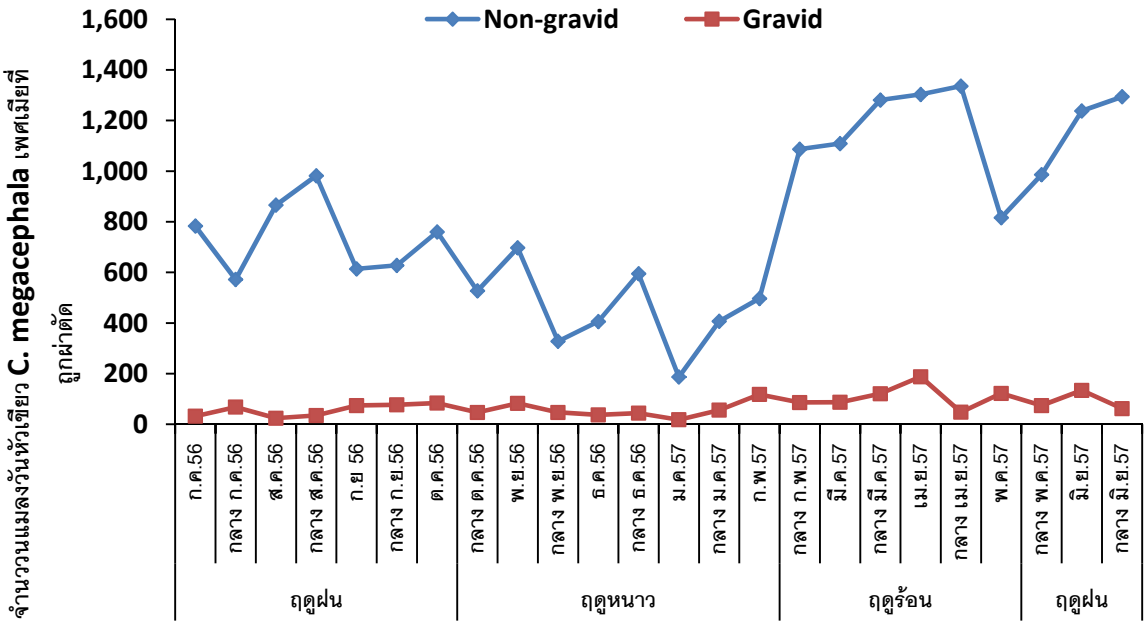

Figure 10. Reproductive status of female *C. megacephala* collected showing more non-gravid status than those gravid.

Longevity of adults *C. megacephala*

Adults were evaluated for their longevity in the laboratory condition. They were reared in a standard rearing cage and provided with a food source. One hundred males and one hundred females emerged from the same batch of egg were reared in the cage (30×30×30 cm) and fed with sugar and water. Fresh pork liver was provided as the protein source and oviposition site. Dead flies were counted daily. This experiment was replicated 3 times. Results indicated that in winter, males averaged 55.6 days in longevity, while 51.6 days in females.

Experiment of gonotrophic cycle in female *C. megacephala* was performed in the laboratory. After mating, gravid females were individually transferred into a small cage, and fed with sugar, water

and fresh pork liver. Once oviposit, the number of egg was counted. The number of oviposition was determined until death. The fresh pork liver was changed every 2 days. The experiment was conducted in 3 seasons. The result indicated that in winter, female can oviposit upto 7 times in her lifetime, and number of eggs in each batch was shown in Table 2.

Table 2. Gonotrophic cycle of *C. megacephala* reared in the labortory

No. oviposit	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Average of ovariole development until oviposit (day)	13 (7-27)	6.8 (2-21)	5.4 (3-15)	4.7 (3-8)	9.0 (6-17)	8.3 (4-16)	6.0
Average of number of eggs in each oviposition	222.7 (121-370)	196.1 (78-330)	210.2 (60-339)	196.9 (100-300)	212.2 (99-305)	226.7 (165-284)	259.0
No. flies examined	63	51	28	15	6	3	1

Molecular identification of forensically important blow flies in Thailand

Specimen collection

Adults of blow fly species (*C. megacephala*, *Chrysomya chani, Chrysomya pinguis, Chrysomya thanomthini, Ceylonomyia nigripes, A. rufifacies, A. villeneuvi, Hemipyrellia ligurriens, Hemipyrellia pulchra, Hypopygiopsis infumata, Lucilia cuprina, Lucilia porphyrina, Lucilia papuensis* and *Lucilia sinensis*) were included in this study. All specimens were identified by the morphological identification method. The identified flies were pinned and some preserved in 70% ethanol, and stored at room temperature. All specimens used for molecular identification have been confirmed by a specialist (Dr. Hiromu Kurahashi). Besides, adults of *C. megacephala*, *A. rufifacies*, and *L. cuprina* from a laboratory colony will be used to compare with field captured specimens.

DNA extraction, amplification, and sequencing

DNA extraction and amplification was performed. Briefly, genomic DNA was extracted from the thorax of individual fly specimens using the NucleoSpin® Tissue Kit (MACHEREY-NAGEL, Germany) according to the manufacturer's instructions, while remaining parts of the flies was maintained as a voucher specimen. The extracted DNA was eluted in 200 µl of elution buffer, measured for concentration by the NanoDrop 8000 Spectrophotometer (Thermo Scientific, USA), and diluted to 50 ng/µl prior to the PCR amplification step. The mitochondrial COI regions were amplified using three pairs of primer sets. PCR reactions was performed in total volumes of 50 µl containing 100 ng of template DNA, 1 unit of Tag DNA polymerase (Promega®, USA), 1x PCR buffer (Promega), 1.5 mM of $MgCl_2$ (Promega), 200 µM of each dNTPs (Promega), and 0.4 µM of each forward and reverse primer (1st Base, Singapore). The thermal cycling conditions was carried out in a TPersonal Combi Thermo Cycler (Biometra, Göttingen, Germany) consisting of a pre-denaturation step at 94ºC for 5

min, followed by 35 cycles of (1) denaturation step at 94ºC for 1 min, (2) annealing step at 46ºC for TY-J-1460&C1-N-2800 primers, 58ºC for C1-J-2495&TK-N-3775 primers or 45ºC for C1-J-2495& C1-N-2800 primers for 1 min 30 sec, and (3) extension step at 72ºC for 2 min followed by a final extension period at 72ºC for 5 min. The amplified products were subjected to electrophoresis in 1% agarose gel and stained with ethidium bromide. The PCR products were purified by using the NucleoSpin® Extract II Kit (MACHEREY-NAGEL, Germany) and subsequently sent to Macrogen DNA Sequencing Services (Macrogen Inc., Seoul, Korea) for automatic sequencing. All specimens were sequenced in both directions using the same forward and reverse primers as those used in the PCR amplification.

Phylogenetic analysis

The complete sequence of COI gene was obtained by aligning the overlapping DNA segments using the CLUSTALX version 1.83 (Thompson et al. 1997). All sequences obtained from this study was compared with those on the NCBI website via BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for species identification, and submitted to the GenBank database for assigning the accession numbers. A phylogenetic tree was constructed by the Neighbour-joining method using Kimura's 2-parameter model (Kimura 1980) implemented in MEGA© version 4.0 (Tamura et al. 2007), with 1,000 bootstrap replications. The result was demonstrated in Fig. 11, Table 3.

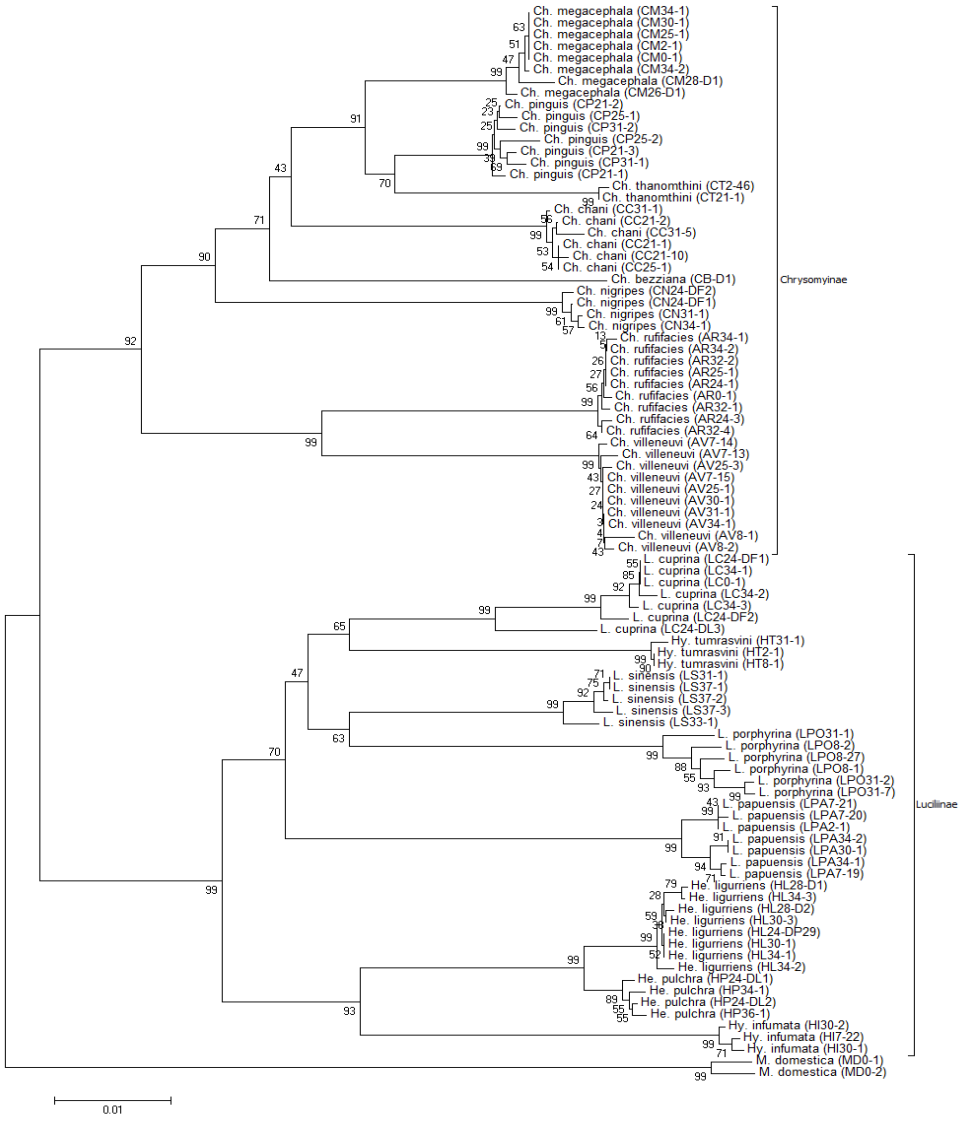

Figure 11. Neighbour-joining tree of Tamura-Nei substitution model for 92 COI sequences from all 16 species of forensically important blow flies and 1 species muscid outgroup. Number above branches refers to bootstrap proportions among 1000 replicates.

Table 3. Average of percentage of intraspecific divergences and interspecific divergences of COI gene (1247 bp) analyzed with neighbor-joining with Tamura-Nei model of substitubion

No.	Species	n	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*Ch. bezziana*	1	**n/c**															
2	*Ch. megacephala*	8	5.0	**0.2**														
3	*Ch. chani*	6	5.7	4.6	**0.2**													
4	*Ch. pinguis*	7	5.4	2.7	4.4	**0.4**												
5	*Ch. thanomthini*	2	5.7	3.5	4.5	2.9	**0.1**											
6	*Ch. nigripes*	4	6.7	5.5	6.0	6.3	6.3	**0.2**										
7	*Ch. rufifacies*	9	8.3	7.1	6.9	8.0	8.1	8.1	**0.1**									
8	*Ch. villeneuvi*	10	7.8	6.8	7.6	8.0	7.6	7.8	4.9	**0.1**								
9	*L. cuprina*	7	9.8	8.9	8.0	9.8	9.1	9.8	10.5	10.3	**0.8**							
10	*L. papuensis*	7	10.8	10.9	8.5	10.9	10.3	10.1	11.0	10.2	6.8	**0.5**						
11	*L. porphyrina*	6	11.4	10.4	9.4	10.6	10.0	11.3	10.9	10.6	6.2	8.0	**0.7**					
12	*L. sinensis*	5	8.9	8.8	8.8	9.7	9.3	9.6	10.4	9.3	5.4	6.1	5.5	**0.4**				
13	*He. ligurriens*	8	9.9	9.2	9.1	10.1	9.8	10.1	10.8	10.3	6.2	8.8	7.6	6.2	**0.2**			
14	*He. pulchra*	4	9.6	9.1	8.6	10.1	9.6	9.9	10.4	9.9	6.2	8.5	7.4	6.0	1.2	**0.3**		
15	*Hy. infumata*	3	9.6	10.3	9.6	10.2	10.5	10.2	11.3	10.5	7.7	9.2	9.2	7.3	5.7	5.7	**0.2**	
16	*Hy. tumrasvini*	3	10.0	9.6	8.6	10.6	10.3	9.8	9.8	10.2	5.0	6.9	7.1	5.2	6.1	6.0	7.8	**0.1**

Number in bold indicate intraspecific divergens. The intraspecific divergence for *Ch. bezziana* is not available because there is only one sequence

Discussion

This study was the first to determine the population dynamics, seasonal and daily activity of *C. megacephala* and *A. rufifacies*, the two most common blow fly species in Thailand, over a full year period using automatic fly trap. Result of this study indicated that fly abundance was peaked in the summer, thus significantly influenced by temperature. Similar findings were reported in the previous investigations in Thailand (Ngoen-klan et al. 2011;Klong-klaew et al. 2014) and Australia, with fly density peak being in the summer months from December to February (Urech et al. 2012). Greater fly density coincided with the summer period, which was characterized by higher temperature and this would favor a rapid developmental rate (Zuha et al. 2012). Although peak population was collected recorded in the summer months (the beginning of April 2014), population was also collected in the winter time, but with the lowest population density (the beginning of January 2014). Experiment of Cammack and Nelder (2010) in the South Carolina, USA, showed that *A. rufifacies*is capable of developing below the lower threshold of 10.0 °C and adults are active as low as 9.0°C. To conclude, the total number of males and females of both blow fly species differed significantly among seasons.

As for other climatic factor such as relativel humidity, result of this study showed that *A. rufifacies* density was negatively correlated with relative humidity. Such phenomenon was correlated with previous investigations in India (Das et al. 1979), *C. megacephala* and *A. rufifacies* northern in

Thailand (Ngoen-klan et al. 2011), with *Achoetandrus albiceps*, and *C. megacephala* in Brazil (Azevedo and Kruger 2013).

More female *A. rufifacies* and *C. megacephala* were collected than males in this study. This information was consistent with other reports (e.g. Ngoen-klan et al. 2011; Klong-klaew et al. 2014). The explanation of the lower number of the male to female collected by our automatic traps is not easy. The following possible explanation may be that male need protein source as food, while beyond these two vital functions, females also need for food and oviposit. The potential for finding protein was from natural sources used for oviposition, food source and/or breeding places (Spradbery 1979).

Understanding the behavior and daily activity of flies of medical importance may help in their control management. This study was the first to determine the daily activity of *A. rufifacies* throughout the one-day period using automatic trap. The trapping number of this fly species in the course of a day had a major peak around 15.00 – 18.00 PM. Only small number of fly was captured during night time (18.00 - 6.00), correlated with other previous reports. Greenberg (1990) reported that three common and forensically important flies --*Calliphora vicina, P. sericata* and *Phormia regina*--oviposited during the dark hours of the night during the summers of 1988 and 1989. Moreover, the blow flies, *Phaenicia eximia* and *Cochliomyia macellaria*, oviposited nocturnally on ground beef under artificial lighting of at least 1,500 footcandles at temperatures 26°C or higher during onsets of low-atmospheric pressure at study sites near Snook, Texas, during 2003 (Kirkpatrick and Olson 2007). Work conducted in the US suggested that nocturnal oviposition of blow fly *Phormia regina* is rare in the natural environment (Berg and Benbow 2013). In a study reported from the USA, the probability of nocturnal oviposition of blow fly *Lucilia sericata* on pig carcasses in Michigan was extremely low to nonexistent (Zurawski et al. 2009). The findings from this study may help to improve the control strategy of both *A. rufifacies* and *C. megacephala* by identifying the best time for sampling and application of insecticides or other management strategies. Additional studies are needed to better understanding the ecological mechanisms governing blow fly oviposition important to forensic entomology.

While observing the reproductive potential performed in *C. megacephala*, the present study indicated the highly fecundity in this species. Females can lay egg upto 7 batches, much more time than other blow flies species. In *Calliphora vicina*, females laid a mean of 3 ± 0.3 batches of eggs (George et al. 2015). In addition, this work found that female has 51.6 days in their longevity, much longer than *C. vicina* that most females did not survive until day 38 (mean survival = 33 ± 1 day) (George et al. 2015). Our results presented here confirm that *C. megacephala* has a high reproductive potential in the environment of northern Thailand. Understanding reproductive potential in calliphorids and other Diptera provides important insights to future control strategy.

Identification of blow fly species is mandatory in the medical application in forensic investigation. As the conventional identification methods using external morphology has limitations for similarity, sibling and closely related species of blow fly. In this regard, molecular identification has

offered in the field of forensic entomology is species determination. Documents have been found for molecular identification in many groups of flies of forensic importance. Examples of these were provided in Malaysia of which mitochondrial cytochrome oxidase I (COI) and cytochrome oxidase II (COII) have been used for phylogenetic analyses of *C. megacephala, A. rufifacies* and *Chrysomya nigripes* (Kavitha et al. 2012). In China, forensically important blow flies were genetically analyzed using a 278-bp segment of the cytochrome oxidase subunit I gene. These species were *C. megacephala, A. rufifacies, Protophormia terraenovae, Lucilia caesar, Lucilia bazini, Lucilia porphyrina, Hemipyrellia ligurriens, Lucilia sericata* and *Calliphora vicina* (Liu et al. 2011). In the US, Wells and Williams (2007) identified blow fly of the subfamily Chrysomyinae (Diptera: Calliphoridae), which are often found to be associated with a human corpse in Canada or the USA. They used phylogenetic analysis of DNA sequence from a short segment of the mitochondrial gene for cytochrome oxidase one (COI) for *P. terraenovae, C. megacephala, Chrysomya bezziana, Chrysomya semimetallica, A. rufifacies, A. albiceps, Chrysomya putoria, Chrysomya norrisi, Chrysomya varipes, L. sericata, L. illustris, Eucalliphora latifrons, Protocalliphora sialia, Protophormia atriceps, P. regina, Cochliomyia macellaria* and *Cochliomyia hominivorax*. in Southeastern Nebraska, eight species of blow flies of forensic importance were genetically analysed in *Cochliomyia macellaria*, *Lucilia illustris*, *Lucilia sericata*, *Cynomya cadaverina*, *Lucilia silvarum*, *P. regina*, *C. vicina* and *A. rufifacies* using the utility of RFLP of the mitochondrial COI region (Samarakoon et al. 2013). In Taiwan, eight species have been genetically identified (*C. megacephala, Chrysomya pinguis, A. rufifacies, H. ligurriens, L. bazini, L. cuprina, Lucilia hainanensis* and *L. prophyrina*) (Chen et al. 2004). These authors used mitochondrial cytochrome oxidase subunit I (COI) DNA of the preceding blow fly species to study its application value for their differentiation, of which the cloning and sequencing of the COI gene was ≈1,588 base pairs. In Thailand, little information was found for investigation of such topic. Preativatanyou et al. (2010) documented that COI–COII sequence was useful for identification of *C. megacephala, A. rufifacies* and *L. cuprina*. Based on the literatures, result of the present study provided the strength molecular information in analyzing upto 14 blow fly species of forensically important in Thailand. Although *A. rufifacies* and *A. villeneuvi* are very closely related species with larvae that act as predator to other fly species, there is a detected difference in their genetic, as shown in the phylogenetic profile (see Fig. 11). Similarly, although the male genitalia of *C. megacephala, C. pinguis, C. chani* and *C. thanomthini* are morphologically similar, their genetic analysis usind COI is markedly divergence (see Fig. 11). Therefore, result of this molecular study provides a strong evidence for species identification and would augment the accurate identification of blow flies of forensical importance in Thailand.

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1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 Suwannayod S, Sanit S, Sukontason K, **Sukontason KL.** *Parasarcophaga (Liopygia) ruficornis* (Diptera: Sarcophagidae): A flesh fly species of medical importance. Tropical Biomedicine 2013;30:174-80. (Impact Factor = 0.85)

1.2 Sanit S, Sukontason K, Klong-klaew T, Tomberlin JK, Limsopatham K, Samerjai C, Sontigun N, **Sukontason KL**. Ontogenesis and developmental rate of the blow fly, *Hypopygiopsis tumrasvini* Kurahashi (Diptera: Calliphoridae). Tropical Biomedicine 2014;31:760-8. (Impact Factor = 0.85)

1.3 Klong-klaew T, Sukontason K, Ngoen-klan R, Moophayak K, Irvine KN, Kurahashi H, Prangkio C, Sanit S, **Sukontason KL**. Impact of abiotic factor changes in blow fly, *Achoetandrus rufifacies* (Diptera: Calliphoridae), in northern Thailand. Parasitology Research 2014;113:1353-60. (Impact Factor = 2.098)

1.4 **Sukontason KL**, Sanit S, Klong-klaew T, Tomberlin JK, Sukontason K. *Sarcophaga (Liosarcophaga) dux* (Diptera: Sarcophagidae): A flesh fly species of medical importance. Biological Research 2014, 47, 14. (Impact Factor = 1.48)

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1.8 Chaiwong T, Kurahashi H, Sanit S, Moophayak K, Sukontason K, **Sukontason KL**. Three sarcophagid species (Diptera: Sarcophagidae) newly recorded in Thailand. Tropical Biomedicine 2015 (In press). (Impact Factor = 0.85)

หมายเหตุ Reprint ผลงานวิจัยเรื่องที่ 1-8 อยู่ในภาคผนวก

ผลงานวิจัยที่คาดว่าจะตีพิมพ์เพิ่มเติม

- Daily and seasonal activity of blow flies, *Chrysomya megacephala* and *Achoetandrus rufifacies* (Diptera: Calliphordae) in Chiang Mai, northern Thailand
- Molecular identification of medically important blow flies in Thailand
- Bionomic of of blow flies *Chrysomya megacephala* and *Achoetandrus rufifacies* (Diptera: Calliphordae) in Thailand
- Survey of medically important flies in Nan and Phitsanulok provinces, northern Thailand

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงสาธารณะ

มีการสร้างเครือข่ายความร่วมมือกับหน่วยงานอื่นคือ

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- Institute of Forensic Medicine, Goethe-Universität, Germany

- เชิงวิชาการ

มีการผลงานวิจัยไปพัฒนาการเรียนการสอน ในกระบวนวิชากีฏวิทยาการแพทย์ในระดับบัณฑิตศึกษา ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ และมีการสร้างนักวิจัยใหม่ จากโครงการนี้ โดยร่วมมือกับโครงการปริญญาเอกกาญจนาภิเษก คือ นส. ธันวดี คล่องแคล่ว, นส. นารินทร์ สนธิกันย์

3. การเสนอผลงานในที่ประชุมวิชาการ

- ได้รับเชิญให้เสนอผลงานวิจัยเรื่อง Forensic Entomology in Thailand ในการประชุมวิชาการนานาชาติ International Conference on Earth, Environment and Life Sciences เมืองดูไบ ประเทศสาธารณรัฐอาหรับเอมิเรตส์ วันที่ 23-24 ธันวาคม 2557

Tropical Biomedicine 30(2): 174–180 (2013)

Review Paper

Parasarcophaga (Liopygia) ruficornis (Diptera: Sarcophagidae): A flesh fly species of medical importance

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Abstract. *Parasarcophaga (Liopygia) ruficornis* is a well-known flesh fly species of medical importance, both as a myiasis-producing agent and fly seen in a forensic entomology context. This study performed a comprehensive literature review of this fly species, dealing with morphology, bionomics and medical involvement. Important characteristics used to identify *P. ruficornis* have been provided for both its third instar and adult for identification purpose in the future.

INTRODUCTION

The medical importance of the flesh fly species, *Parasarcophaga (Liopygia) ruficornis*, is of particular interest in many parts of the world, either as a myiasis-producing agent or fly seen in a forensic entomology context. Geographically, this species has been recorded as an Old World fly although it has invaded the New World. In the USA, it has been recorded from Hawaii (Davis & Goff, 2000), California, Florida, Massachusetts, New York, North Carolina, Pennsylvania and Washington D.C. (Alfred, 2011). In the Oriental region, *P. ruficornis* has been found in many countries such as Thailand (Sucharit *et al*., 1976), Malaysia (Kumara *et al*., 2012), Singapore (Sugiyama *et al*., 1990) and India (Sreevatsa *et al*., 1990).

The genetic characterization of *P. ruficornis* has been compared with that of *Parasarcophaga dux* and *Parasarcophaga argyrostoma* using allozyme and RAPD-PCR markers, which indicated a very close relationship between these species (Bajpai *et al*., 2011). However, based on the sequences of mitochondrial cytochrome oxidase gene *subunits I* and *II* (*COI* and *COII)*, the *ruficornis*-group was one of the six major clades of forensically important sarcophagids in Malaysia, with the other five being the *peregrina*-group, *albiceps*-group, *dux*-group, *pattoni*-group and *princeps*-group (Tan *et al*., 2010).

Morphology

In the forensic entomology aspect, identification of larval specimens that are found to be associated with a corpse is essential before they are used further in investigation [e.g., estimation of post-mortem interval (PMI_{min}) from the larvae collected]. Although flesh flies, including *P. ruficornis*, are commonly larviparous (larviposit the first instar), they also have been documented as oviparous in a laboratory colony. Sukhapanth *et al*. (1988) provided information of *P. ruficornis* eggs measuring 1.6±0.33 mm in length. Morphologically, the *P. ruficornis* larva, which is similar to other flesh fly larvae, exhibits a distinct morphological feature in having its posterior spiracle

situated within a terminal concavity of the last abdominal segment. The larva typically has a vermiform robust body, comprising a head region, three thoracic segments and eight abdominal segments (Figure 1A). The length of the first, second and third instar is 6.8±0.45 mm, 11.8±0.07 mm and 16.9±0.08 mm, respectively (Sukhapanth *et al.*, 1988). With the aid of light microscopy, the third instar of *P. ruficornis* has been documented by focusing on the main features used to differentiate from the other forensically important flesh fly species. On each lateral side of the prothorax, a pair of anterior spiracles having 11-15 papillae arranged in a single row (Figure 1B). The posterior spiracle is located in a terminal concavity of the last abdominal segment and is characterized by (1) the distinct inner projections between the spiracular slits (Figure 1C), (2) the prominent tail of the upper end of the peritreme, and (3) position of the peritreme at the base of the middle slit (Sukontason *et al.*, 2010). All larval instars of this fly species have been described by scanning electron micrograph (SEM) (Singh *et al.*, 2012), highlighting the sensory organs (dorsal, terminal and ventral organs) located on the cephalic segment as well as strong, slightly curved mouth hooks. In this review, the sharp bladed mouth hooks of the first instar are clearly demonstrated in Figure 2. Features of the internal cephalopharyngeal skeleton of all instars were also illustrated by Singh *et al.* (2012).

Puparia of *P. ruficornis* were measured as 11.7±0.14 mm in length (Sukhapanth *et al.*, 1988). A very short pupal respiratory horn was observed dorsolaterally in the first abdominal segment (Singh *et al.*, 2012). Regarding adults, males are slightly larger than females, measuring 13.8±0.16 mm and 13.2±0.01 mm, respectively (Sukhapanth *et al.*, 1988). The prime characteristics of *P. ruficornis* differ from two other forensically important species; *P. dux* and *P. peregrina*, which have yellowish orange third antenna and palpus (Figure 3). In adult flesh flies, only males could be identified, of which each species was endowed with distinct characteristics of terminalia (Figure 4). Regarding flesh flies of forensic importance, the key to identify adult males of the South American genera was published (de Carvalho & de Mello-Patiu, 2008), while that for identifying species including *P. ruficornis* in Thailand has been updated (Chaiwong *et al.*, 2009). Informative characteristics of male genitalia of this species, particularly the distiphallus, have been displayed using SEM (Giroux *et al.*, 2010).

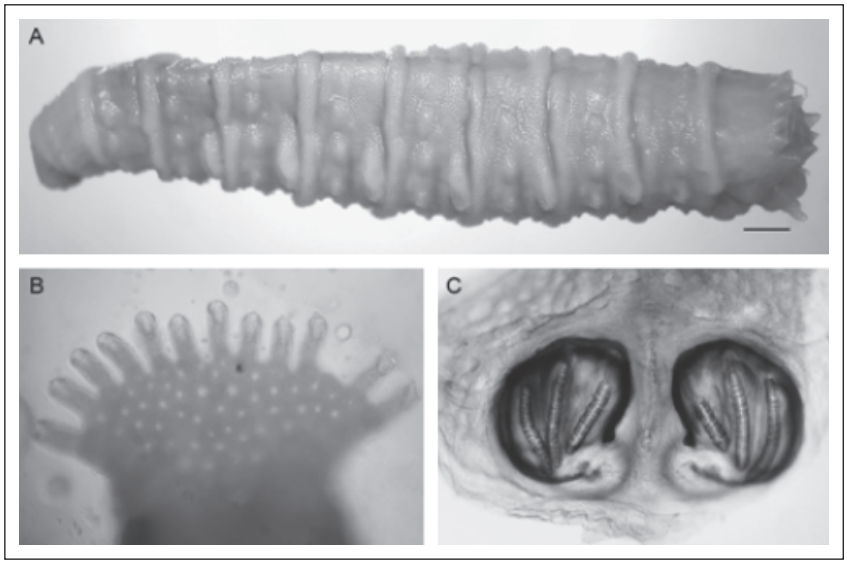

Figure 1. Third instar of *P. ruficornis*. (A) Lateral view. Bar = 1 mm. (B) Anterior spiracle. (C). Posterior spiracle. Online figure in color

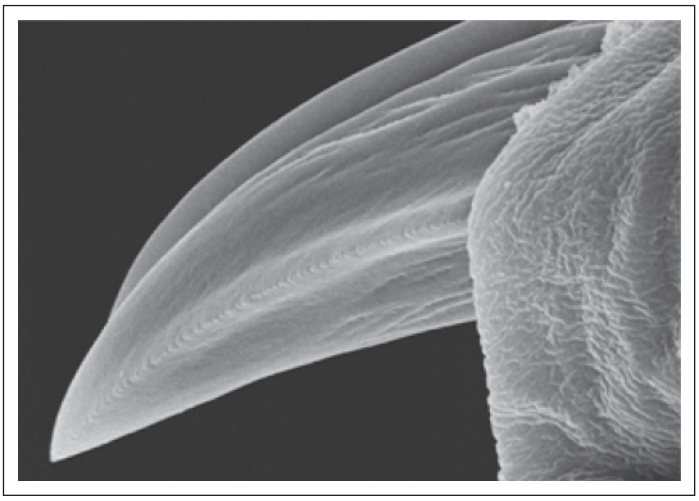

Figure 2. Scanning electron micrograph showing the sharp bladed mouth hook of the first instar of *P. ruficornis* (original picture of KL Sukontason)

Figure 3. Adult male of *P. ruficornis*. Inset displays important features of this species, yellowish orange of the third antenna (arrowhead) and palpus (arrow). Online figure in color

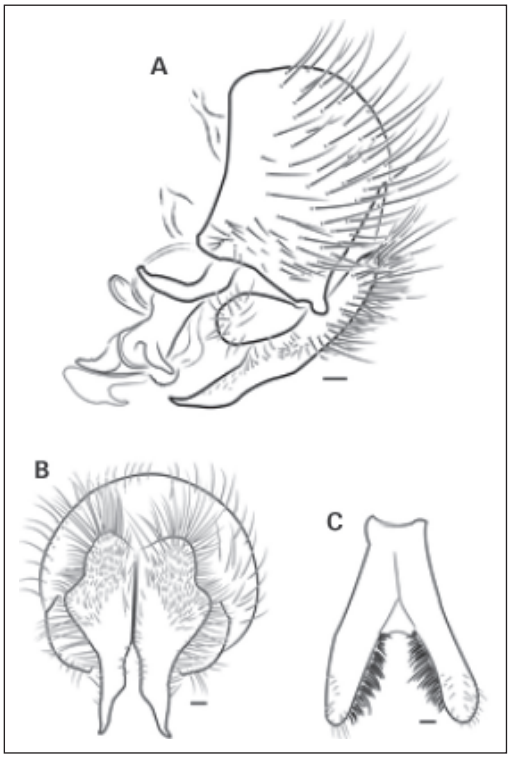

Figure 4. Male terminalia of *P. ruficornis*. (A) Lateral view. (B) Cercus, caudal view. (C) Sternite 5. Bar of all figures = 0.02 mm

Table 1. Documentation of human cases associated with larvae of *P. ruficornis*

Year	Country	Death scene	PMI_{min}	State of decay	Reference
2002	Thailand	indoor	ca. 3-4 d	bloat	(Sukontason *et al.*, 2007)
2006	Thailand	indoor, constant 25°C	unknown	mummified	(Sukontason *et al.*, 2007)
2011	Kuwait	indoor, 2nd floor (in air condition)	ca. 7.5-8.5 d	decomposed	(Al-Mesbah *et al.*, 2011)
2012	Malaysia	indoor	ND	ND	(Kumara *et al.*, 2012)

ND, no available data

Bionomics

Based on extensive study on the bionomics of sarcophagids in Thailand by Bänziger & Pape(2004), female *P. ruficornis* occasionally laid eggs on the mesh of boxes containing the breeding medium. Its laying habit is of an amphibiodotic type (larviposit on both faeces and carrion). Females typically behave in a larviparous manner by depositing 40-80 first instars (Verma & Ishikawa, 1984), but in special circumstance such as laboratory rearing, oviparous behavior also has been documented. This phenomenon was similar to that which occurred in *P. dux* in laboratory conditions (Sukontason *et al.*, 2005). Laboratory rearing conditions set at 27±4ºC and 78±4% RH demonstrated that female *P. ruficornis* laid eggs 5-7 days after emergence, with the number of eggs per batch being 19.1±11.1 (Sukhapanth *et al.*, 1988). Larval growth development (larviposition until pupariation) varied according to records. In Guam it took 7 days at 29.5ºC (Bohart & Gressitt, 1951); in Thailand 8.0±1.8 days at 27±4ºC (Sukhapanth *et al.*, 1988); and in Hawaii 7.5-10.8 days at 26ºC during experiments (Goff *et al.*, 1997). In Saudi Arabia, Amoudi *et al.* (1994) reared larvae at the constant temperatures of 13, 16, 19, 22, 25, 28, 31, 34 and 37ºC, and the larval development time took 31.5, 17.5, 10.5, 9.7, 8.5, 6.8, 6.0, 5.5 and 6.3 days, respectively. In Thailand, larvae were reared at 27±4ºC, and the pupal period was 11.7±0.14 days. Longevity of males and females ranged from 3.5 to 39 days and 2.0 to 31.8 days, respectively (Sukhapanth *et al.*, 1988).

This fly species is synanthropic, that is, it lives closely associated with the human environment. In Pakistan, adult *P. ruficornis* was trapped in residential areas, where flies fed and larviposited on rabbit, fish and chicken carcasses (Shazia *et al.*, 2006). In Thailand, adults were captured from the flowers of *Bulbophyllum putidum* and the fruit of *Dimocarpus longan* (Bänziger & Pape, 2004), as well as animal waste such as putrid fish (Sucharit *et al.*, 1976). Similarly, in northeast Thailand adult *P. ruficornis* was collected from restaurants and school cafeterias, but not from fresh-food markets, garbage piles or paddy fields (Chaiwong *et al.*, 2012). In southern India, this species was collected in several villages that were hit by the tsunami on 26th December, 2004 (Srinivasan *et al.*, 2006).

Myiasis

There are limited records of myiasis caused by *P. ruficornis* and it is either reported as the sole species responsible or co-infesting with other fly larvae. In Thailand, Sucharit *et al.* (1981) reported on cases in the vagina and in India patients suffering from leprosy have been documented as being infected (Sreevatsa *et al.*, 1990). A wound co-infested with the blow fly, *Chrysomya megacephala*, and house fly, *Musca domestica*, was recorded from the scalp of a man in Brazil (Ferraz *et al.*, 2010). Adult *P. ruficornis* were caught in the intensive care unit (ICU) of a hospital in Malaysia providing further evidence of the potential for the nosocomial infestation(Nazni *et al.*, 2011).

Forensic entomology

Although blow flies (Diptera: Calliphoridae) are often used in forensic investigations, several documented cases report the presence of flesh fly larvae, suggesting that there are other fly groups of forensic importance. Of these flesh fly species, specimens of *P. ruficornis* has been recorded to associate with human death scenes, as well as from pig carcasses (*Sus scrofa*); an animal experimental model in forensic entomology in Brazil (de Souza & Linhares, 1997; Barbosa *et al*., 2009), the USA (Oahu island of Hawaii) (Davis & Goff, 2000), and Thailand (Vitta *et al*., 2007; Sukjit, 2011). Investigation on the island of Oahu, Hawaii, U.S.A. indicated that this species was an early invader and insect colonizer of the death scene (Nolte *et al*., 1992). A document reporting from Kuwait demonstrated the significance of post feeding third instar *P. ruficornis*, which was collected from the blanket which the body remain was wrapped. Based on the age of *P. ruficornis* collected and the location of the body, ~7.5–8.5 days PMI_{min} was estimated (Al-Mesbah *et al*., 2011). Based on the literature, few number of human death scenes were found to be associated with this species, mostly indoor cadavers (Table 1). Difficulty in identification of flesh fly larvae and/or incorrect identification may lead to have lower number of cases infesting with *P. ruficornis*, than the actual cases. According to the indoor death scenes associated with *P. ruficornis*, this fly species might have special features in its biology that allow them to be colonized in houses or other dwelling. This phenomenon was similarly documented in other species, *Sarcophaga caerulescens* Zetterstedt, the sarcosaprophagous flesh fly species found in indoor death scene in southern Finland (Pohjoismaki *et al*., 2010). Such phenomenon is worthy of note in forensic investigation.

In addition, study pertaining to more precise estimation and/or analysis of death related to drugs has used specimens of *P. ruficornis*. Goff *et al*. (1994) reported the larval developmental rate of this fly species when fed on phencyclidine; a common drug abused in many country and legitimate veterinary tranquilizer, from the decomposing tissue of rabbit. No significant differences in larval growth rate were observed among rabbits administered with 3.66, 7.31, and 14.62 mg of phencyclidine via ear vein infusion. Pupariation period of *P. ruficornis* were longer for animals fed on tissues containing the drug.

In conclusion, despite information on all aspects of *P. ruficornis* being relatively limited; the significance of this fly species is increasing, particularly from a forensic entomology viewpoint. Based on the literature review, the indoor environment preferred by *P. ruficornis* is worthy of note in forensic investigation. Therefore, to increase worldwide reviews of human cases involving this fly species is mandatory, and their clarification would benefit forensic investigations.

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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.

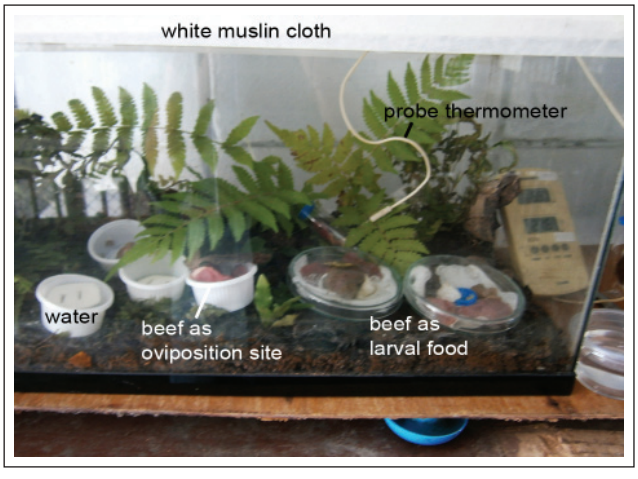

Figure 1. Rearing cage of *H. tumrasvini*, maintained at 25.0 ± 2.0°C and 80.0 ± 5.0% RH.

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

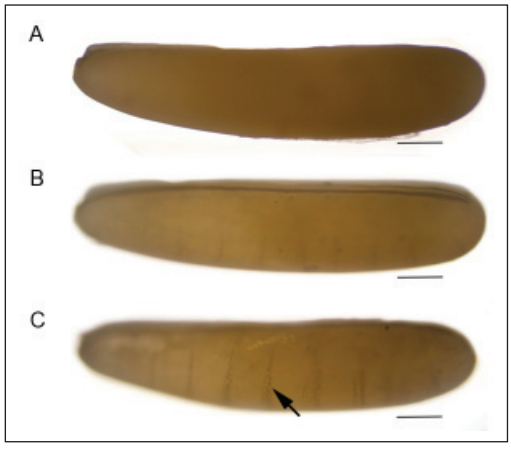

Figure 2. Embryo development of *H. tumrasvini*. A: 0-6 hr. B: 8 hr, showing circular spinulation of embryo. C: 10 hr, showing intense spinulation of embryo (arrow). Bar = 0.2 mm.

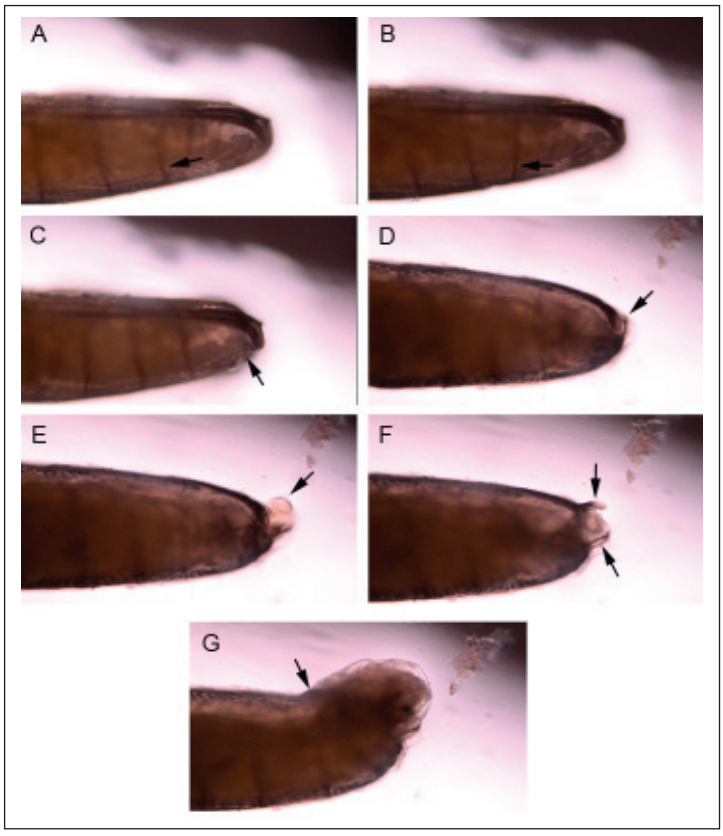

Figure 3. Hatching process of *H. tumrasvini* as demonstrated by a video recorder. A,B: Active movement of 10-12 hr embryo, as shown by changing in position of cuticular spines (arrows). C: Collapse of the latero-anterior margin of the eggshell due to shrunken embryo prior to hatching. D: Anterior straining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

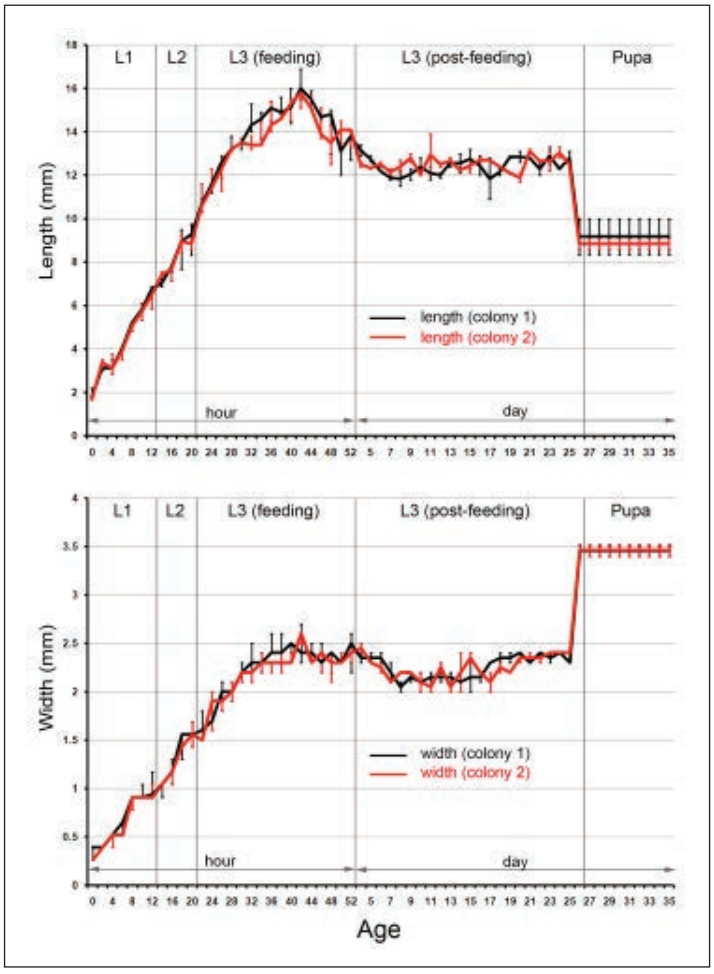

Figure 4. Developmental growth of *H. tumrasvini* showing median of larval length (upper) and width (lower). No significant differences between median of both colonies of their length and width (Mann-Whitney *U* test; $P>0.05$).

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

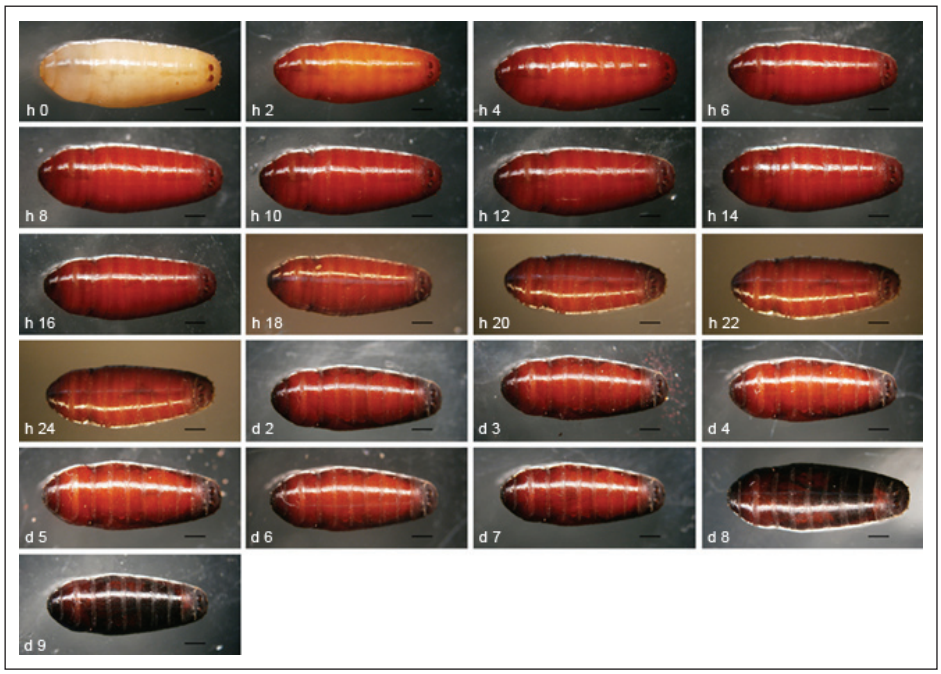

Figure 5. Gradual coloration change of puparia *H. tumrasvini* from h 0 to d 9. Bar = 1 mm for all figures.

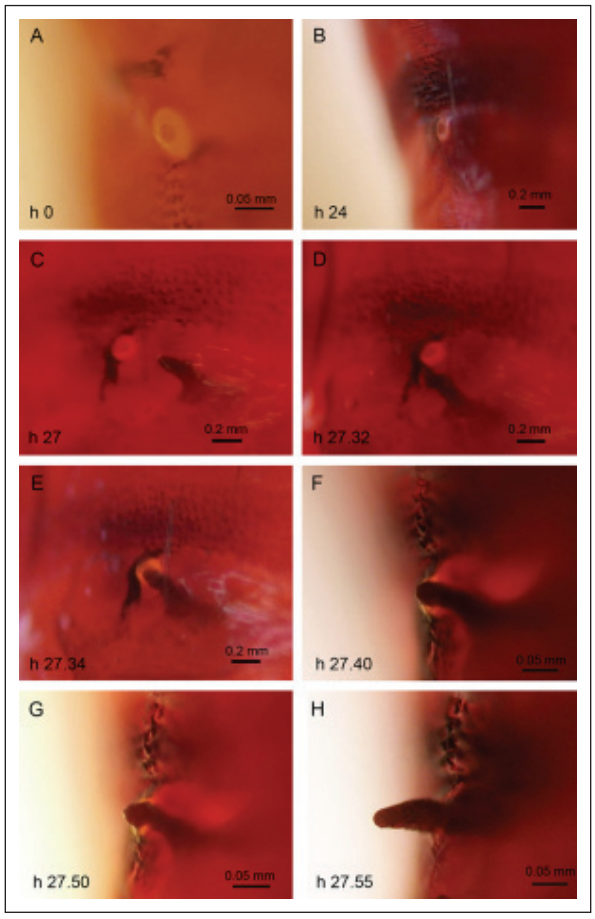

Figure 6. Development of pupal respiratory horn of *H. tumrasvini* as recorded via a video recorder. A-H: A remnant of the bubble membrane at h 0, and complete protrusion at h 27.55.

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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ORIGINAL PAPER

Impact of abiotic factor changes in blowfly, *Achoetandrus rufifacies* (Diptera: Calliphoridae), in northern Thailand

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Abstract Understanding how medically important flies respond to abiotic factor changes is necessary for predicting their population dynamics. In this study, we investigated the geographical distribution of the medically important blowfly, *Achoetandrus rufifacies* (Macquart) (Diptera: Calliphoridae), and ascertained the response to climatic and physio-environmental factors in Chiang Mai, northern Thailand. Adult fly surveys were carried out every 2 weeks from May 2009 to May 2010 at 18 systematically randomized study sites in three districts of Chiang Mai province (Mueang Chiang Mai, Mae Rim, and Hang Dong), using reconstructable funnel traps with 1-day tainted beef offal as bait. During the study period, 8,861 adult *A. rufifacies* were captured, with peak densities being observed at the end of winter (i.e., late February) and throughout most of the summer (May to March). Population density had a weak but significant (α=0.05) positive correlation with temperature (r=0.329) and light intensity (r=0.231), and a weak but significant (α=0.05) negative correlation with relative humidity (r=−0.236). From the six ecological land use types (disturbed mixed deciduous forest, mixed deciduous forest, mixed orchard, lowland village, city town, and paddy field), greater fly densities were observed generally in the disturbed mixed deciduous forest and lowland village, but not in the paddy fields. In conclusion, *A. rufifacies* are abundant from the end of winter and throughout most of the summer in northern Thailand, with population density being weakly positively correlated with temperature and light intensity, but weakly negatively correlated with relative humidity. The greatest densities of this fly species were collected in disturbed mixed deciduous forest and lowland village land uses. The prediction of annual and season specific distributions of *A. rufifacies* were provided in each season and all-year patterns using a co-kriging approach (ArcGIS9.2).

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Introduction

The hairy maggot blowfly, *Achoetandrus rufifacies* (Macquart) (Diptera: Calliphoridae) is a medically important species commonly associated with anthropogenic activities. The adult fly acts as a mechanical carrier of pathogens that may cause diseases. For example, several species of bacteria were isolated from flies captured in Malaysia, e.g., *Escherichia coli*, *Klebsiella ozaenae*, *Proteus vulgaris*, *Pseudomonas fluorescens*, and *Burkholderia pseudomallei* (Sulaiman et al. 2000). Also, *A. rufifacies* were documented as a predominant mechanical carrier of helminth eggs (e.g., *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm, *Taenia* spp., *Hymenolepis nana*) and protozoan cysts (e.g., *Entamoeba histolytica*/*dispar*, *Entamoeba coli*, *Cryptosporidium*, and *Giardia lamblia*) in Ethiopia (Fetene and Worku 2009). In Malaysia, the presence of *A. lumbricoides* and *T. trichiura*

eggs were detected in the gut lumen (Sulaiman et al. 1988). In addition to the fly larvae being a myiasis-producing agent in both humans and animals (Zumpt 1965), it plays another medically important role, since the larvae found in human corpses and/or death scenes can be used as entomological evidence in forensic investigations. Examples of such cases have been recorded in Europe (e.g., Benecke 2001), Thailand (Sukontason et al. 2007), Malaysia (Kavitha et al. 2013), India (Suri Babu et al. 2013), and Hawaii (USA) (Goff and Odom 1987). The presence of larvae in human corpses was reported at both outdoor and indoor death scenes (Sukontason et al. 2007; Kumara et al. 2012). In Australia, *A. rufifacies* was the second most abundant nuisance fly species collected in cattle feedlots, and it was noted that not only can such flies cause complaints from people living in the vicinity, but they can also contribute to production losses and animal welfare issues (Urech et al. 2012).

The geographical distribution of *A. rufifacies* has been expanding globally from Australasia/Oceania, Palaearctic, and Nearctic to Neotropical regions (Verves 2005). In the USA, flies are now found in Texas, Louisiana, Florida, California, Alabama, Arizona, Arkansas, Colorado, Georgia, Nebraska, Tennessee (Verves 2005), South Carolina (Cammack and Nelder 2010), and as far north as Wisconsin (Marche 2013). The species has also recently been identified in Canada (Rosati and VanLaerhoven 2008). It was the most abundant fly species collected from and near pig carcasses in Guam and Mexico (Jenson and Miller 2001; Valdes-Perezgasga et al. 2010) and the second most abundant species found in a regional survey of northern Thailand (Bunchu 2012).

A. rufifacies has a complete metamorphosis life cycle, comprising four distinct stages (egg, larvae or maggot, pupa, and adult) and their developmental rate is temperature dependent (Byrd and Butler 1997; Sukontason et al. 2008). The biology of this species has been reviewed in detail, i.e., taxonomy, ecological role, control strategy, and medical importance (myiasis, medico-legal importance) (Baumgartner 1993). Using applicability of the 304-bp cytochrome oxidase I gene fragment in molecular identification, *A. rufifacies* population displayed 0–0.8 % intraspecific variations in individuals collected from different locations in China (Aly and Wen 2013). This phenomenon was similar with work conducted in Australia (Harvey et al. 2003). Ecologically, although the seasonal distribution of this fly has been described in Australia (Vogt 1988; Urech et al. 2012), limited information exists for Thailand (Lertthamnongtham et al. 2003), especially with respect to abiotic factors (temperature, rainfall, and relative humidity). Hence, the objective of this study was to employ geographical information system (GIS) analytical techniques to investigate temporal population dynamics of this fly in relation to climatic factors and land use types in urban and suburban areas of Chiang Mai, northern Thailand.

Materials and methods

Study area

Three districts of Chiang Mai province were selected for this study: urban Mueang Chiang Mai (MU), suburban Mae Rim

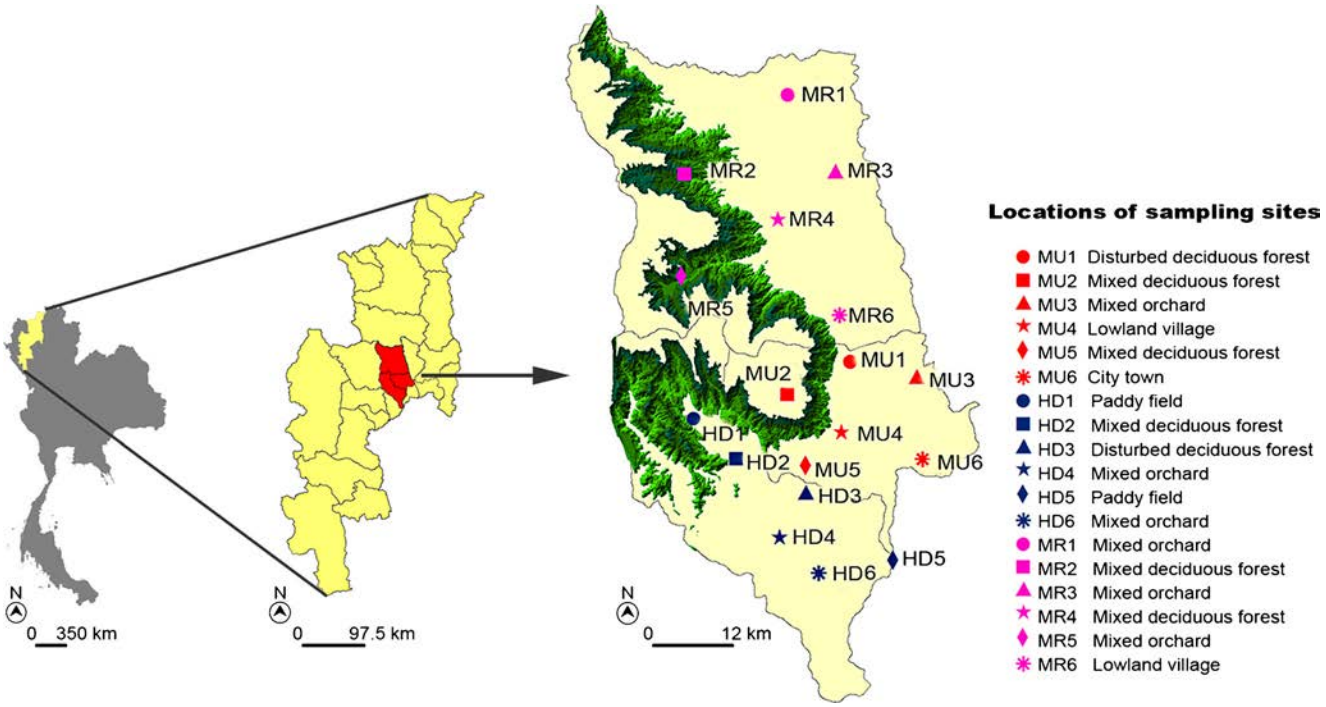

Fig. 1 Map of Thailand showing the three sample districts (Mueang Chiang Mai, *MU*; Mae Rim, *MR*; and Hang Dong, *HD*) of Chiang Mai province and the *A. rufifacies* sampling locations. *Green shade* represents mountainous area

Fig. 2 A reconstructable funnel trap used for adult fly collection

(MR), and Hang Dong (HD) to the north and south of MU, respectively (Fig. 1). Following a systematic random sampling method (Rogers and Williams 1993), the study area was plotted using the topographical maps of Chiang Mai (MapMagic™ scale 1: 150,000 with a UTM projection type, Everest Spheroid and the Indian 1975 Datum). A more detailed discussion of the sample site selection procedure is provided (Ngoen-klan et al. 2011). The land use categories were obtained from the Geo-Informatics and Space Technology Development Agency (Public Organization), Northern Region, Thailand.

Fly collection

Fly collection was performed every 2 weeks from May 2009 to May 2010 using an in-house prototype reconstructable funnel trap kit (Fig. 2). The collection procedure was described previously (Ngoen-klan et al. 2011). Two hundred and fifty grams of 1-day tainted beef offal (Bunchu et al. 2008) was used as bait positioned under the trap. The traps were exposed for a 1-h period between 9.30 a.m. and 12.00 noon. The following physical data was recorded for each study site: the GPS coordinates [Garmin™ eTrex Handheld GPS (China)], temperature (degree Celsius) and relative humidity (percentage) [Digital Hygro-Thermo Meter (DHT-1); Daeyoon Scale Industrial Co., Ltd (China)] and light intensity (lux) [LUX/FC light meter TM-204 Tenmars (Taiwan)].

Fig. 3 Ecological land use types sampled in this study. **a** Disturbed mixed deciduous forest. **b** Mixed deciduous forest. **c** Mixed orchard. **d** Lowland village. **e** City town. **f** Paddy field

The collected flies were transported to the laboratory of the Department of Parasitology, Faculty of Medicine, Chiang Mai University within 1 h. Trapped specimens were sacrificed by placing them in a freezer set at 0 °C for 2 h, then counted individually, sexed, and identified.

Statistical analysis

For the statistical and geospatial analyses, all raw data were transformed using a logarithm base 10 ($\log_{10}$) transformation to improve approximation of normality. The relationship of climatic factors with fly populations was determined using bivariate correlation analysis and Pearson Product Moment Correlation (r), while the data for all land uses of these three regions were combined before analysis. One-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test were employed to investigate the relationship between land use types and mean total number of *A. rufifacies* using SPSS 12.0 for Windows (α=0.05). A co-kriging approach (using the Geostatistical Analyst tool of ArcGIS 9.2) was utilized to explore the spatial relationship between fly population and climate data (Ngoen-klan et al. 2011).

Results

A total of 18 study sites were selected for fly collection. Figure 3 shows the six ecological land use types sampled (disturbed mixed deciduous forest, mixed deciduous forest, mixed orchard, lowland village, city town, and paddy field). Collectively, a total of 8,861 adult *A. rufifacies* were captured during the 1-year study period. Trapped fly numbers varied seasonally with peak numbers occurring around late February coinciding with end of winter/early summer followed by the summer (April to June) (Fig. 4, upper). Fly numbers decreased during the rainy season, rapidly declining at the end of rainy season, and remained low throughout winter (September to January). A similar trend was observed in all three districts. There were more *A. rufifacies* females than males in all sample collections; the average ratio was 3 female:1 male (Fig. 4, lower). Neither nongravid nor gravid was examined in the captured *A. rufifacies*.

Pearson product moment correlation results indicated that fly population density had a weak positive correlation with temperature (r=0.329, P=0.000) (Fig. 5a) and light intensity (r=0.231, P=0.000) (Fig. 5c). However, a negative correlation was found between fly population and relative humidity (r=−0.236, P=0.000) (Fig. 5b). Based on land use types in central Chiang Mai (Table 1), it was observed that flies exhibited a preference for disturbed mixed deciduous forest and lowland village, followed by mixed deciduous forest and mixed orchard. A significantly lower abundance of flies was captured in the paddy field area (Bonferroni post hoc test, P<0.05).

To assess the spatial distribution patterns of *A. rufifacies*, co-krigged seasonal maps were produced using fly population

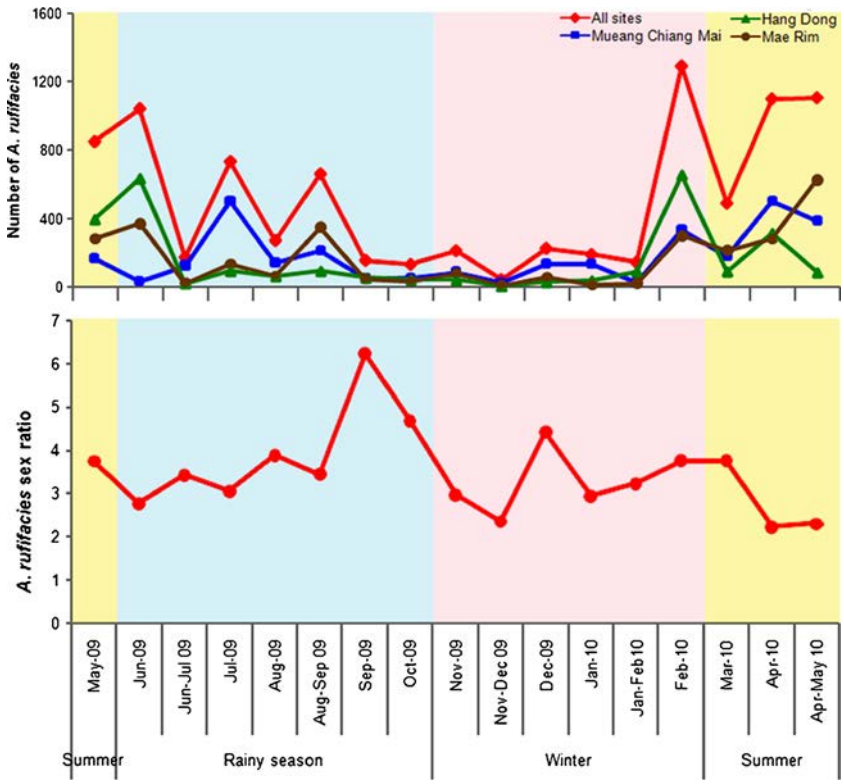

Fig. 4 Monthly fluctuations in population density of *A. rufifacies* determined using a reconstructable funnel trap baited with 1-day tainted beef offal in Chiang Mai, northern Thailand, May 2009 to May 2010

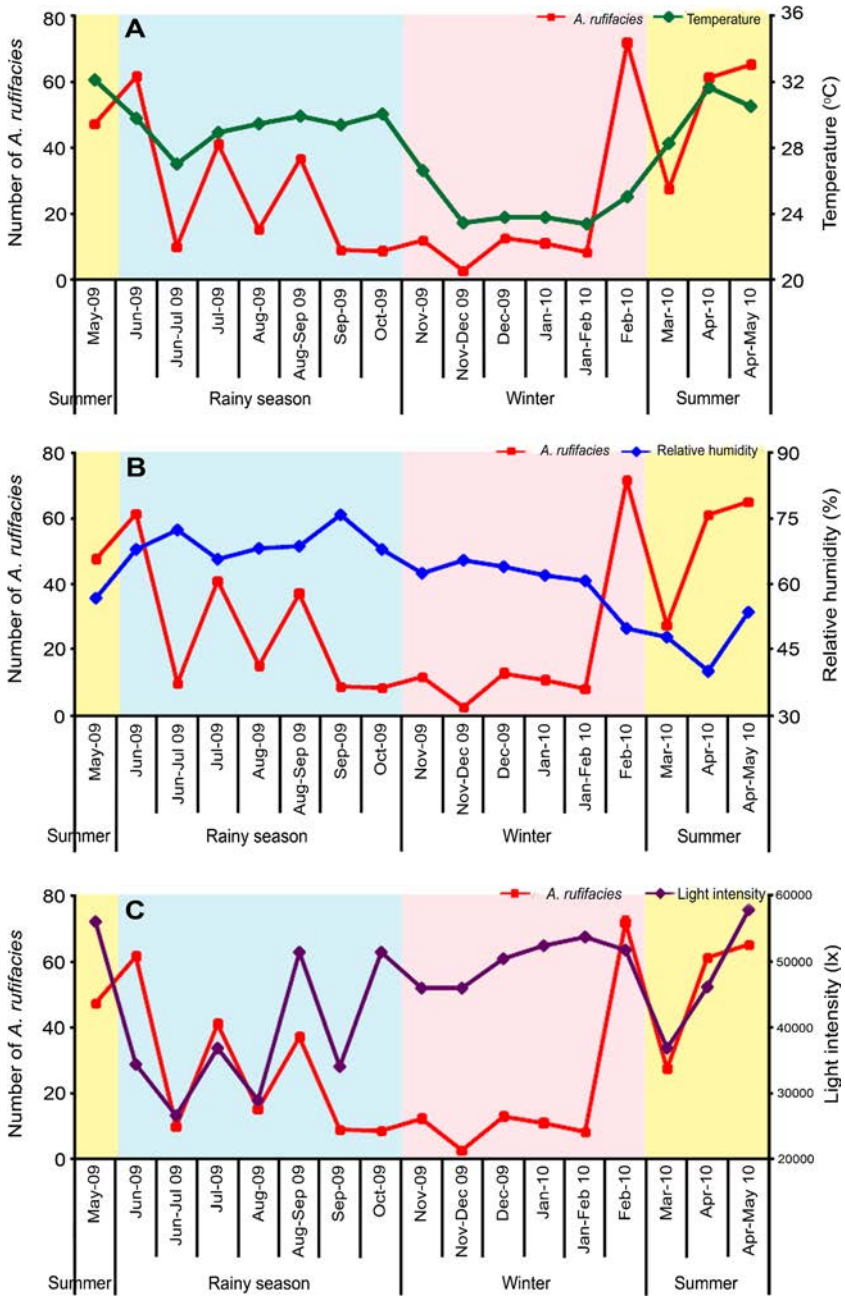

Fig. 5 Correlation of the overall *A. rufifacies* population density with climatic factors. **a** Weakly positive with temperature (r=0.329, P=0.000). **b** Negative correlation with relative humidity (r=−0.236, P=0.000). **c** Weakly positive with light intensity (r=0.231, P=0.000)

data, land use types, temperature, relative humidity, and light intensity. The total predicted seasonal abundance across the three districts is displayed in Fig. 6a–c, while the all-year pattern is shown in Fig. 6d. The red areas represent the highest population of *A. rufifacies* predicted by the analysis, while the blue areas represent the lowest population density predicted.

Discussion

Although the impact of *A. rufifacies* on humans and animals has been well documented, information pertaining to the fly's biology and ecology is scarce. Local surveys of flies of forensic importance, which include *A. rufifacies*, are critical in identifying what species are present in the various areas (Brundage et al. 2011). This study was the first to determine the population dynamics over a full year and to predict population density of *A. rufifacies* in Thailand using systematic random sampling and GIS.

Blowfly abundance was significantly influenced by climatic factors. Our results further indicate that although found year-round, *A. rufifacies* exhibited a bimodal peak with one maximum at the end of winter or early summer and another through the summer and shading into the

Table 1 The population density of *A. rufifacies* based on land use types in Chiang Mai, northern Thailand, using a reconstructable funnel trap

Land use types	Number	Log_{10} no. of AR[a]
Disturbed mixed deciduous forest	34	1.26±0.64a
Lowland village	34	1.26±0.79a
City town	16	1.09±0.54ab
Mixed orchard	101	0.81±0.67b
Mixed deciduous forest	84	0.79±0.78b
Paddy field	34	0.61±0.68b

Different lowercase letters indicate significant difference (Bonferroni post hoc test, $P<0.05$)

[a] Mean±SD of flies captured

rainy season and winter. Similar findings were reported in Australia, with fly density peak being in the summer months from December to February (Urech et al. 2012). Greater fly density coincided with the summer period which was characterized by higher temperature, and this would favor a rapid developmental rate (Zuha et al. 2012). In this context, the increasing temperature during early summer probably generates flight activity from breeding places. In Australia, it was found that flies did not enter the trap at temperatures <13 °C (Vogt 1988). Results from our study agree with this observation as only a few *A. rufifacies* were captured in November to December (see Fig. 4), when temperatures were lowest, around 18 °C. Also, *A. rufifacies* require a temperature >15.0 °C to pupate normally (O'Flynn 1983) which may further explain the temperature related to fly density results. Interestingly, adult *A. rufifacies* were still observed flying around deer carrion when the ambient temperature was ~9 °C in South Carolina of USA (Cammack and Nelder 2010).

In addition to temperature, our results clearly indicated that *A. rufifacies* density was negatively correlated with relative humidity, which is consistent with the findings in India (Das et al. 1979), *Chrysomya megacephala* (F.) in Thailand (Ngoen-klan et al. 2011), with *Achoetandrus albiceps* (Wiedemann), and *C. megacephala* in Brazil (Azevedo and Kruger 2013).

The weak positive correlation observed between *A. rufifacies* density and light intensity is in contrast with studies on field-captured *C. megacephala* that suggest no relationship ($P=0.420$) (Ngoen-klan et al. 2011). Similarly, density-related experiments using an I-box wind tunnel were also negatively associated with light intensity (Moophayak et al. 2013b). A greater attractive index to bait occurred in the afternoon (1–5 p.m.) as compared to the morning hours (9–12 a.m.). Perhaps, the increased attraction of *C. megacephala* to bait in higher light intensity is due to greater visibility for landing (Burkett and Butler 2005). More research is needed to confirm whether light intensity impacts *A. rufifacies* behavior similarly to *C. megacephala*. Currently, automatic trap-based field experiments are underway to capture *A. rufifacies* and *C. megacephala* behavior data at 3 h increments.

In this study, we found more *A. rufifacies* females than males in traps baited by tainted beef, a finding also noted by other studies (Mulieri et al. 2006; Bunchu et al. 2008; Ngoen-klan et al. 2011). The greater proportion of captured females may result from proteinaceous food requirements for oviposition, similar with *C. megacephala* (Bunchu et al. 2008). Recent experiments conducted in 2013 revealed that 80 % of total *A. rufifacies* collected were nongravid females ($n\sim6{,}000$ specimens) (KLS, unpublished data). Dissection of *A. rufifacies* revealed compact cells of ovary and densely supplied tracheoles, suggesting a newly emerged young female fly population.

In this study, *A. rufifacies* were densely localized in disturbed mixed deciduous forest and lowland village land use types. Similarly, earlier research conducted in urban Chiang Mai City identified *A. rufifacies* larvae frequently in human corpses from both environments (Sukontason et al. 2007). A survey in northern Thailand also found *A. rufifacies* in varying elevations from lowlands to 1,050 m above sea level, indicating an extensive geographical range for this species (Moophayak et al. 2013a). As for urban settings per se, although we found that lowland villages had higher fly densities, we did not target sampling to specific anthropogenic activities. In Ethiopia, high densities of this species were found at butchery sites, moderate numbers at defecating ground sites, and low amounts in rubbish bins at market places (Fetene and Worku 2009). Further studies of localized fly densities at specific anthropogenic activity sites could also be beneficial in Thailand.

In this study, the estimated annual distribution of *A. rufifacies* was similar to each of the three seasonal periods, with high populations consistently identified in disturbed mixed deciduous forest and lowland village. The mapping facilitates an understanding of the geographical distribution of this species in the study area, and offers the potential to identify approaches for management.

In conclusion, this study provides two important findings. First, population density of *A. rufifacies* correlates with several abiotic factors; positive with both temperature and light intensity, and negatively with relative humidity. Secondly, GIS co-kriging methods effectively visualized the relationship between fly population density and land use. Taken together, this data can contribute to baseline information for local control strategies and add value to forensic entomology approaches.

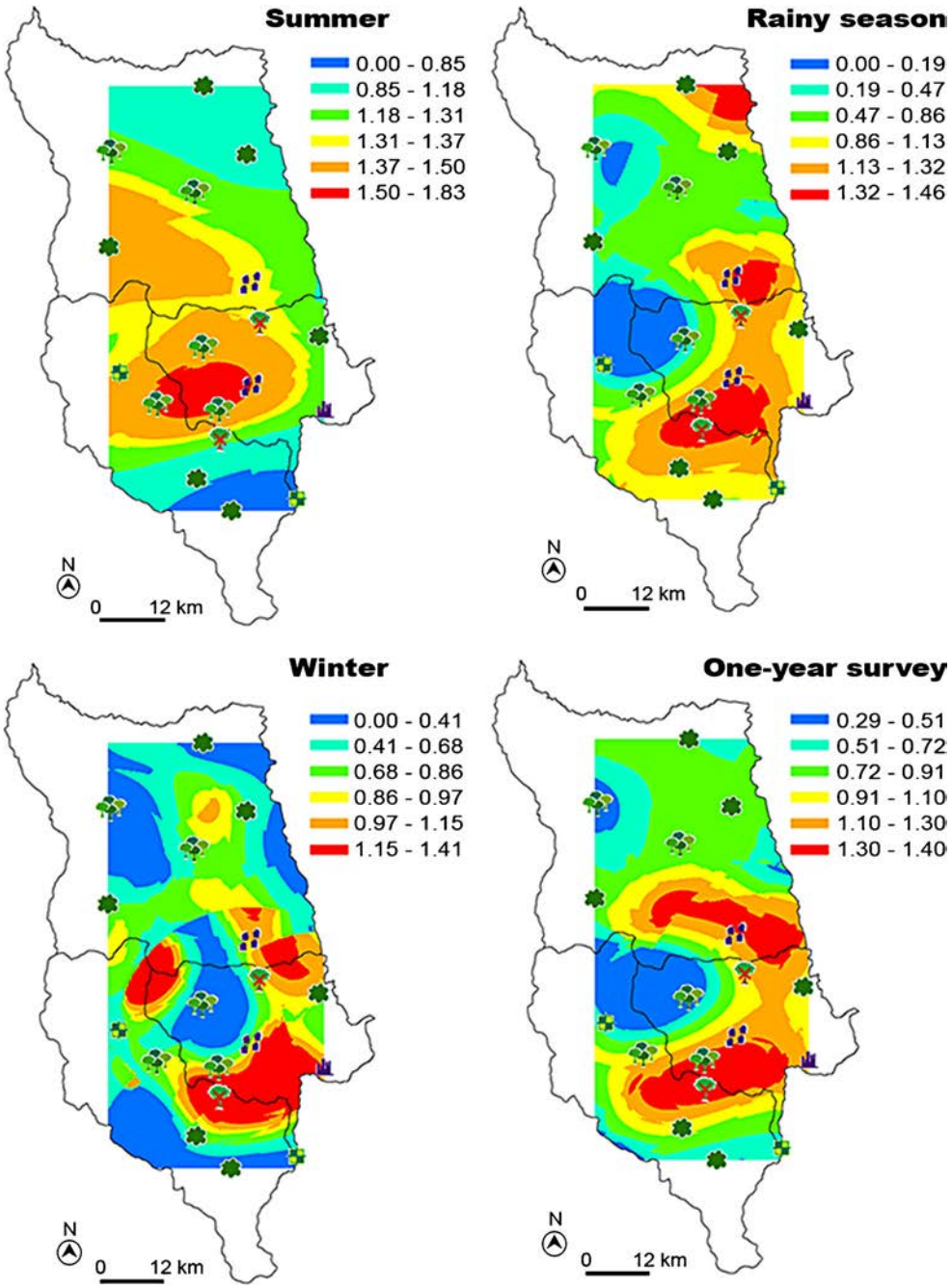

Fig. 6 Co-kriging to predict spatial distribution of *A. rufifacies* in three districts of Chiang Mai, northern Thailand. The *red areas* represent the highest population of *A. rufifacies* predicted by the analysis, while the *blue areas* represent the lowest population density predicted

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