Songklanakarin J. Sci. Technol.
42 (6), 1215-1220, Nov. - Dec. 2020

http://www.sjst.psu.ac.th

Original Article

Species identification of forensically important fly larvae by integrating morphological characteristics and protein profiles

Pluemkamon Phuwanatsarunya[1], Nuttanan Hongsrichan[2], Tarinee Chaiwong[3], Marutpong Panya[3], and Nophawan Bunchu[1, 4*]

[1] *Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Mueang, Phitsanulok, 65000 Thailand*

[2] *Department of Parasitology, Faculty of Medicine, Khon Kaen University, Mueang, Khon Kaen, 40002 Thailand*

[3] *College of Medicine and Public Health, Ubon Ratchathani University, Warin Chamrap, Ubon Ratchathani, 34190 Thailand*

[4] *Centre of Excellence in Medical Biotechnology, Faculty of Medical Science, Naresuan University, Mueang, Phitsanulok, 65000 Thailand*

Received: 1 October 2018; Revised: 14 March 2019; Accepted: 20 August 2019

Abstract

The correct identification of fly species has a crucial role in accurately performed forensic investigations. Species identification of fly larvae by observing only morphological characteristics is difficult and less accurate. Therefore, the objective of this study was to develop an alternative tool for identifying fly larvae by integrating morphological characteristics and protein expression profiles. Excretory-secretory (ES) protein profiles from third stage larvae of five fly species were evaluated to differentiate these species, including *Chrysomya megacephala*, *Achoetandrus rufifacies*, *Lucilia cuprina*, *Musca domestica*, and *Boettcherisca nathani*, based on the SDS-PAGE technique. They were also assessed for morphological characteristics. The results showed that different protein patterns in ES products and morphological characteristics were observed among these fly species. A novel dichotomous key for identification of fly larvae was developed by combining unique patterns of ES protein profiles with morphological characteristics. However, studies including other fly species should be pursued.

Keywords: excretory and secretory (ES), protein profile, third stage larva, identification, flies

1. Introduction

The fly larvae of *Chrysomya megacephala* (Diptera: Calliphoridae), *Achoetandrus rufifacies* (Diptera: Calliphoridae), *Lucilia cuprina* (Diptera: Calliphoridae), *Musca domestica* (Diptera: Muscidae), and *Boettcherisca nathani* (Diptera: Sarcophagidae) have long been recognized as crucial clues in medicolegal forensic entomology, especially in Thailand (Bunchu, 2012; Sukontason, Bunchu, Chaiwong, Moophayak, & Sukontason, 2010). The determination of the minimum time since death (MTD) from fly larvae plays a primary role in estimating the post mortem interval (Harvey, Gasz, & Voss, 2016). In addition, human myiasis (Bunchu *et al.*, 2012), the infestation of fly larvae in a living person, may be found in neglected people, in which case the fly larvae can be used to estimate the minimum abandonment duration. The

*Corresponding author
Email address: nophawanb@nu.ac.th, bunchu_n@hotmail.com

accuracy of MTD or abandonment duration mainly relies on correct species identification. Species identification based on morphological characteristics is more difficult at the larval stage than at the adult stage. Moreover, the taxonomic key for identification is inappropriate for forensic investigators or technicians who are not familiar with the technical terms of entomology. Although molecular techniques have been accepted for fly species identification, both equipment and reagents required are expensive and fit for the laboratory, not for field use. In addition, with all current methods the larvae must be destroyed after processing, and hence further confirmation or other study of live specimens is impossible. Therefore, our goal was to develop a simpler live specimen identification method. Excretory-secretory (ES) products from the fly larvae have been widely studied to discover antimicrobial agents and for application in larvae debridement therapy (Valachova *et al*., 2014). Previous studies have reported differences in protein profiles of the ES products from different fly species (Pinilla, Moreno-Pérez, Patarroyo, & Bello, 2013; Suriyakan *et al*., 2016). However, the ES protein profile has not been used for species identification. Therefore, the objectives of this study were primarily to determine the protein profiles in ES products of forensically important fly species of Thailand, and to develop a simple identification key based on both protein profiles and morphological characteristics. This key may be useful for non-taxonomic experts, and could be subjected to further studies including other fly species.

2. Materials and Methods

2.1 Rearing of flies in the laboratory

The five fly species used in this study were *C. megacephala*, *A. rufifacies*, *L. cuprina*, *M. domestica* and *B. nathani*. All are laboratory strains and have been maintained in the laboratory for more than 20 generations. The larvae used in this study were reared with fresh pork liver in plastic containers, as described previously (Bunchu *et al.*, 2012). All flies were maintained in the laboratory at the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, under light- and temperature-controlled conditions (light:dark 12 h:12 h, temperature 25.0 ± 0.5 °C).

2.2. Preparation of excretory/secretory (ES) products from larvae

The ES products were prepared as described elsewhere, with slightly modifications (Van der Plas *et al*., 2007). Briefly, one hundred third-stage larvae were collected from a rearing box and washed three times with 70% alcohol and sterile distilled water. They were kept in a sterile centrifuge tube (15 ml) with 100 µl of sterile ultra-pure water and then incubated for 60 min at room temperature in the dark. Next, the ES products were collected and transferred into a new sterile microcentrifuge tube. Protease inhibitor cocktail (AMRESCO, USA) was added to inhibit the degradation of proteins (protease inhibitor: ES products, 1: 100). The ES products were then centrifuged at 1,300× g for 5 min at 4 °C to remove particulate materials. The supernatant was transferred into a new sterile microcentrifuge tube and kept at -80 °C. All larvae were transferred from the centrifuge tube to the rearing box to determine the survival rate. The protein concentration of each ES sample was determined with the Bradford protein assay (BioRad Protein assay kit, USA), according to the manufacturer's instructions. Cold acetone was used to precipitate proteins in the ES products. Briefly, 50 µl of ES product was mixed with 200 µl of cold acetone and incubated at -20 °C for 1 h. During the incubation period, the ES products were mixed every 15 min. The ES products were then centrifuged at 13,000-15,000× g at 4 °C for 30 min. Subsequently, the supernatant was removed and the protein precipitate was air-dried at 25 °C for 5 min. The precipitate was dissolved in 1 ml of acetone and centrifuged at 13,000× g for 10 min at 4 °C. The supernatant was then removed and the precipitate was air dried at 25 °C for 30 min. Finally, the precipitate was dissolved in 20 µl of phosphate-buffered saline (PBS), and 1 µl of 1x protease inhibitor cocktail. The protein concentration in the ES products after precipitation was then re-determined with the Bradford assay, as previously described.

2.3 Separating ES proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The ES products of each fly species were run on 4-12% bis-tris gradient gel (Invitrogen, Germany) in reducing conditions. Briefly, 60 µg of ES product from third-stage larvae in 4× loading of SDS reducing buffer (0.5M Tris-HCl pH 6.8, 2% glycerol, 10% SDS, 0.5% bromphenol blue, and 5% β-mercaptoethanol) was heated at 95 °C for 5 min and separated on SDS-PAGE at 180 V for 45 min. A protein marker (Vivantis, Malaysia) was used as the protein reference. The gels were stained with Coomassie blue (0.1% Coomassie blue G-250, 40% methanol, and 10% acetic acid) or Simply Blue Safe Stain (Invitrogen, USA) for 1 h at room temperature and then destained with a solution of 40% methanol and 10% acetic acid overnight to enable visualization of the protein profile. The relative molecular weight of each protein band in the gel of each fly strain was calculated by measuring the molecular mobility of proteins in the gel, compared with the standard curve of a standard marker protein. The retention factor (Rf) was calculated using the formula

$$Rf = \frac{\text{Distance moved by protein band from a starting place}}{\text{Distance moved by tracker color from a starting place}}$$

The standard curve of the reference protein of known molecular weight was made using the retention factor (X-axis) and the logarithm of the logarithm of the molecular weight (Y-axis). The resulting linear regression fit was used to estimate the molecular weight of each protein band. All ES products were run and analyzed in triplicates.

2.4 Determination of the morphological characteristics of fly larvae using 10% potassium hydroxide (KOH) preparation

Determination of the morphological characteristics of fly larvae was done after the clearing method as described previously (Bunchu *et al.*, 2012). Each fly larva was cut as shown in Figure 1 to remove the anterior and posterior parts. Only the anterior and posterior parts were cleared in KOH.

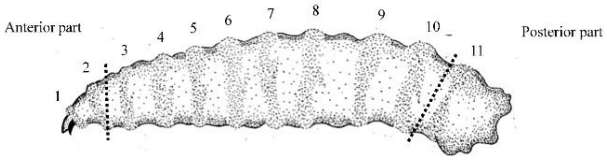

Figure 1. Dissection of larva to remove the anterior and posterior parts.

Important morphological characteristics were observed and illustrated.

3. Results

3.1 Protein profiles of ES products

The analysis of the ES proteins from the larvae of five fly species revealed a difference by species in the number of proteins bands, corresponding to a molecular weight range from 8 to 240 kDa. The ES protein profiles of the five species of forensically important fly larvae as visualized by SDS-PAGE and representative diagrams are shown in Figures 2 and 3, respectively. The numbers of protein bands seen for ES products derived from the larvae of *C. megacephala*, *L. cuprina*, *B. nathani*, *A. rufifacies*, and *M. domestica* were 10, 9, 9, 8, and 7 bands, respectively. The estimated molecular weight of each protein band is shown in Table 1.

A unique protein pattern was observed for each species. The 21 kDa protein band was found only for *M. domestica*, whereas the weak specific bands at 37 kDa and 83 kDa were found for *C. megacephala.* The 146 kDa protein band was observed for *A. rufifacies* and the 115 kDa protein band was seen only for *L. cuprina.* Therefore, these specific bands may be used to discriminate between the fly species. Common protein molecular weights that were found in the ES products of all species included 41 and 71 kDa. There were six protein sizes shared by four species (6, 14, 17, 28, 56, and 72 kDa) that were expressed as weak or faint bands, as shown in Figure 2. The expression of a 19 kDa protein was predominant in *L. cuprina* and *B. nathani*, whereas low expression of a 67 kDa protein was found in the ES products of *C. megacephala*, and *L. cuprina*, and a 72 kDa protein band was seen for *C. megacephala*, *A. rufifacies*, *M. domestica*, and *B. nathani.* The 146 kDa and 115 kDa protein bands were respectively seen only for *A. rufifacies* and *L. cuprina.*

3.2 Survival rate of fly larvae after the collection of ES products

The survival rate of fly larvae after the collection of ES products was determined to assess whether the ES collection method affects the viability of larvae. The larvae were allowed to develop into adults and it was shown that the lowest survival rate was observed in *C. megacephala* (64.79%), while the highest survival rate was observed in *B. nathani* (88.70%), see Table 2.

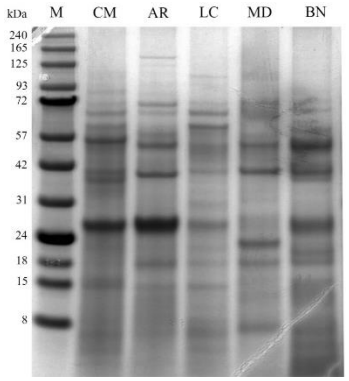

Figure 2. Excretory-secretory (ES) protein profiles of the larvae representing five species using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). M: molecular weight marker proteins; CM: *Chrysomya megacephala*; AR: *Achoetandrus rufifacies*; BN: *Boettcherisca nathani*; MD: *Musca domestica*; and LC: *Lucilia cuprina*.

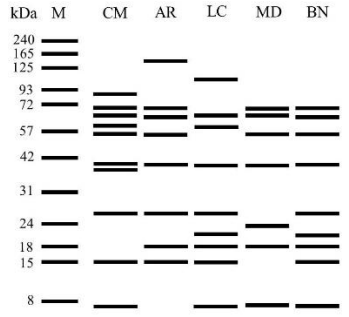

Figure 3. A diagram of the total protein SDS-PAGE results for larvae representing five species M: molecular weight marker proteins; CM: *Chrysomya megacephala*; AR: *Achoetandrus rufifacies*; BN: *Boettcherisca nathani*; MD: *Musca domes-tica*; and LC: *Lucilia cuprina*.

Table 1. Protein bands allowing to discriminate the five fly species from ES proteins secreted by the larvae.

Estimated molecular weight of protein band (kDa)				
C. megacephala	*A. rufifacies*	*L. cuprina*	*M. domestica*	*B. nathani*
	146			
		115		
83				
72	72		72	72
71	71	71	71	71
67		67		
56	56		56	56
41	41	41	41	41
37				
28	28	28		28
			21	
		19		19
	17	17	17	17
14	14	14		14
6		6	6	6

The values are presented in kDa and represent approximate protein band positions.

Table 2. Survival rates of the larvae of five fly species after the collection of ES products.

Species	Number of tested larvae	% Survival rate
Chrysomya megacephala	5600	64.79 ± 26.31
Achoetandrus rufifacies	3072	83.55 ± 9.07
Lucilia cuprina	4060	73.97 ± 29.6
Musca domestica	9270	70.40 ± 21.5
Boettcherisca nathani	3590	88.70 ± 9.45

3.3 Dichotomous key for the identification of third-stage larvae of five forensically important fly species in Thailand

In summary, a dichotomous key for the identification of five fly species from their third-stage larvae in Thailand is as follows.

1.	–	Posterior spiracle with straight slit, point to button (Figure 4A-4C) and ES product protein bands at ~14 and ~28 kDa.	2.
	–	Posterior spiracle with straight slits, not point to button (Figure 4D), or coil slits (Figure 4E) and ES product protein bands at ~6, ~17, ~56, and ~72 kDa .	3.
2.	–	Body of larva without tubercle)Figure 5A(, ES product protein bands at ~6 and ~67 kDa.	4.
	–	Body of larva with tubercle (Figure 5B), ES product protein bands at ~14, ~17, ~28, ~41, ~56, ~71, ~72, and ~146 KDa.	*Achoetandrus rufifacies*
3.	–	Posterior spiracle with straight slits, non-point to button (Figure 4D), ES product protein bands at ~6, ~14, ~17, ~19, ~28, ~41, ~56, ~71, and ~72 kDa.	*Boettcherisca nathani*
	–	Posterior spiracle with peritreme like D letter, coil slit like M letter (Figure 4E), ES product protein bands at ~6, ~17, ~21, ~41, ~56, ~71, and ~72 kDa.	*Musca domestica*
4.	–	Posterior spiracle with incomplete peritreme, fat slits, straight slits and point to button (Figure 4A), ES product protein bands at ~6, ~14, ~28, ~37, ~41, ~56, ~67, ~71, ~72, and ~83 kDa.	*Chrysomya megacephala*
	–	Posterior spiracle with complete peritreme, straight slits and clear button (Figure 4C), ES product protein bands at ~6, ~14, ~17, ~19, ~28, ~41, ~67, ~71, and ~115 kDa.	*Lucilia cuprina*

4. Discussion

To the best of our knowledge, this is the first experimental study on identifying (distinguishing between) five species of medically and forensically important flies in Thailand (*C. megacephala*, *A. rufifacies*, *L. cuprina*, *M. domestica*, and *B. nathani*) by comparing their larval secreted protein profiles using the SDS-PAGE method. This study demonstrated that the protein profile of the ES products produced by the third instar larvae (3 days old) can be used to distinguish between the five fly species. The protein patterns of these five species were clearly different. Similarly, previous studies have demonstrated that different fly species in the same stage provide different protein profiles of ES products (Pinilla *et al*., 2013). The protein profile showed a band at 14 kDa for three species: *C. megacephala*, *A. rufifacies*, and *L. cuprina*. This observation is similar to previous studies in other blow fly species, such as *Chrysomya bezziana* (Sukarsih *et al*., 2000) and *Cochliomyia hominivorax* (Giglioti *et al*., 2016). Therefore, this may be a common blow fly protein band. Moreover, this study demonstrated that the 14 kDa protein band can be used to separate the blow fly from the house fly. A protein band with 28 kDa was found in four species and similarly found in *Sarconesiopsis magellanica* (Pinilla *et al*., 2013) and in *C. hominivorax* (Giglioti *et al*., 2016). Therefore, this protein band may be common for fly species. This study found specific bands that may be used to discriminate fly species. The protein band at 21 kDa was observed only for *M. domestica*. Weak specific bands at 14 kDA, 37 kDa, and 56 kDa were found for *C. megacephala*, and were related to the ranging band of *C. megacephala* in previous studies (El-Ebiarie & Taha, 2012; Suriyakan *et al*., 2016; Taha, Abdel-Meguid, & El-ebiarie, 2010). Moreover, different stages of fly larvae may provide different protein profiles (Alborzi, Jolodar, Seyfi Abad Shapouri, & Bagherian Pour, 2014; Brant, Guimarães, Souza-Neto, Ribolla, & Oliveira-Sequeira, 2010; Giglioti *et al*., 2016; Pinilla *et al*., 2013; Pires, Moya-Borja, Barreira, Pinho, & Alves, 2007). Therefore, studies on the same fly species at different larval stages should be carried out in the future. Recently, a study on the species identification of fly eggs based on amino acid profiles revealed differences by direct analysis in real-time, high-resolution mass spectrometry (Giffen, Rosati, Longo, & Musah, 2017). However, the data on the fly species used in that study have not been reported.

As regards the survival rates of fly larvae after collecting the ES products, the results showed a high survival rate of 65-88%, agreeing with previous studies that have also found a high emergence rate (>60%) in normal rearing conditions (Begum, hasan, Saifullah, & Howlader, 2013; Gabre, Adham, & Chi, 2005; Sukontason, Bunchu, Sukontason, & Choochote, 2004). Therefore, this method provided sufficient live larvae for further study if needed. In addition, the morphological characteristics of the third-stage fly larvae of the five species were similar to those observed in previous studies (Mathison & Pritt, 2014; Samerjai *et al*., 2016; Sukontason, Piangjai, Siriwattanarungsee, & Sukontason, 2008).

5. Conclusions

The protein profiles of ES products in combination with morphological characteristics greatly increased the

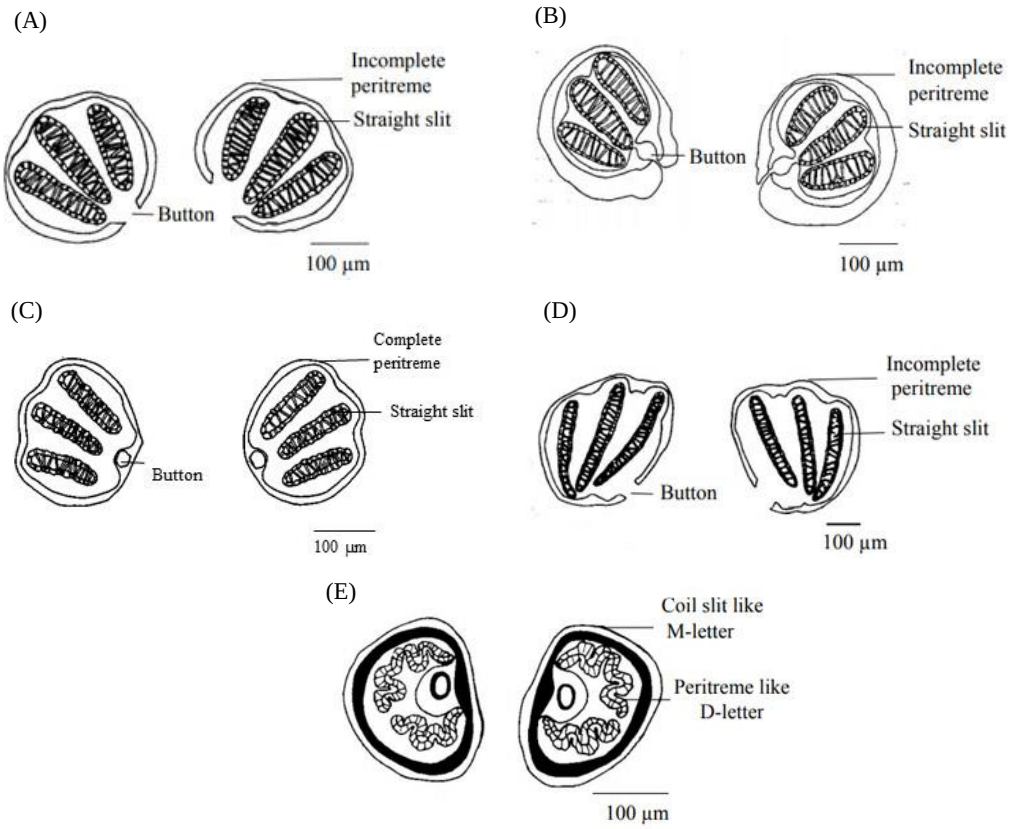

Figure 4. Posterior spiracle of third-stage larvae: (A) *Chrysomya megacephala*, (B) *Achoetandrus rufifacies*, (C) *Lucilia cuprina*, (D) *Boettcherisca nathani*, and (E) *Musca domestica*.

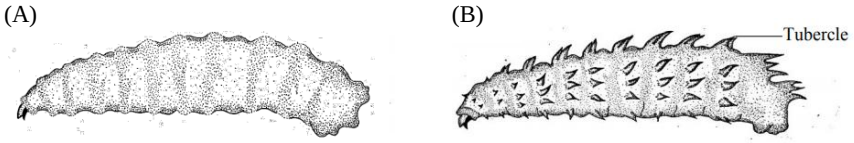

Figure 5. Third-stage larvae: (A) larva without tubercles, and (B) larva with tubercles.

accuracy of species identification of fly larvae. The novel dichotomous key reported in this study is an alternative way to identify fly species at the larval stage, and could prove very useful to non-taxonomic experts. However, studies including further fly species should be carried out in the future.

Acknowledgements

We are grateful for the financial support of Naresuan University research fund (R2558C003 to NB). The authors gratefully acknowledge Wirawan Wannacha for technical assistance, and Pakinai Junhortone and Wannacha Nakhonkam for maintaining the fly colonies in the laboratory.

References

Alborzi, A., Jolodar, A., Seyfi Abad Shapouri, M., & Bagherian Pour, E. (2014). Isolation and identification of excretory-secretory and somatic antigens from the *Oestrus ovis* larvae by SDS-PAGE and immunoblotting. *Veterinary Research Forum*, *5*(4), 307-311.

Begum, M., hasan, M., Saifullah, A. S. M., & Howlader, M. A. (2013). Effects of sodium chloride on oviposition and development of blow fly, *Lucilia cuprina*, (wiedmann) (Diptera: Calliphoridae). *Dhaka University Journal of Biological Sciences*, *23*(1), 47-51.

Brant, M. P. R., Guimarães, S., Souza-Neto, J. A., Ribolla, P. E. M., & Oliveira-Sequeira, T. C. G. (2010). Characterization of the excretory/secretory products of *Dermatobia hominis* larvae, the human bot fly. *Veterinary Parasitology*, *168*(3-4), 304-311.

Bunchu, N. (2012). Blow fly (Diptera: Calliphoridae) in Thailand: Distribution, morphological identification and medical importance appraisals. *International Journal of Parasitology Research*, *4*(1), 57-64.

Bunchu, N., Thaipakdee, C., Vitta, A., Sanit, S., Sukontason, K., & Sukontason, K. L. (2012). Morphology and developmental rate of the Blow Fly, *Hemipyrellia ligurriens* (Diptera: Calliphoridae): Forensic ento-

mology applications. *Journal of Parasitology Research*, *2012*, 1-10.

El -Ebiarie, A. S., & Taha, N. (2012). Molecular characterization of serine proteases from both first and third larval instars of *Chrysomya megacephala*. *Life Science Journal*, *9*(3), 2086-2093.

Gabre, R. M., Adham, F. K., & Chi, H. (2005). Life table of *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae). *Acta Oecologica*, *27*(3), 179-183.

Giffen, J. E., Rosati, J. Y., Longo, C. M., & Musah, R. A. (2017). Species identification of necrophagous insect eggs based on amino acid profile differences revealed by direct analysis in real time-high resolution mass spectrometry. *Analytical Chemistry*, *89*(14), 7719-7726.

Giglioti, R., Guimarães, S., Oliveira-Sequeira, T. C. G., David, E. B., Brito, L. G., Huacca, M. E. F., . . . Oliveira, M. (2016). Proteolytic activity of excretory/secretory products of *Cochliomyia hominivorax* larvae (Diptera: Calliphoridae). *Pesquisa Veterinaria Brasileira*, *36*(8), 711-718.

Harvey, M. L., Gasz, N. E., & Voss, S. C. (2016). Entomology-based methods for estimation of postmortem interval. *Research and Reports in Forensic Medical Science*, *6*, 1-9.

Mathison, B. A., & Pritt, B. S. (2014). Laboratory identification of arthropod ectoparasites. *Clinical Microbiology Reviews*, *27*(1), 58-60.

Pinilla, Y. T., Moreno-Pérez, D. A., Patarroyo, M. A., & Bello, F. J. (2013). Proteolytic activity regarding *Sarconesiopsis magellanica* (Diptera: Calliphoridae) larval excretions and secretions. *Acta Tropica*, *128*(3), 686-691.

Pires, F. A., Moya-Borja, G. E., Barreira, J. D., Pinho, R. T., & Alves, C. R. (2007). The main proteinases in *Dermatobia hominis* second and third instars larvae are serine-proteinases. *Veterinary Parasitology*, *145* (3-4), 326-331.

Samerjai, C., Sanit, S., Sukontason, K., Morakote, N., Wannasan, A., Pereira, R. M., & Sukontason, K. L. (2016). Morphology of immature stages of flesh flies, *Boettcherisca nathani* and *Lioproctia pattoni* (Diptera: Sarcophagidae). *Acta Tropica*, *163*, 109-120.

Sukarsih, S. M., Riding, G., Partoutomo, S., Hamilton, S., Willadsen, P., & Wijffels, G. (2000). Identification and characterization of the excreted/secreted serine proteases of larvae of the old world screwworm fly. *Chrysomya bezziana*. *International Journal for Parasitology*, *30*(6), 705-714.

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

Sukontason, K. L., Bunchu, N., Sukontason, K., & Choochote, W. (2004). Effects of eucalyptol on house fly (Diptera: Muscidae) and blow fly (Diptera: Calliphoridae). *Revista do Instituto de Medicina Tropical de Sao Paulo*, *46*(2), 97-101.

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

Suriyakan, S., Kanthawong, S., Chaiwong, T., Lamlertthon, S., Thongwat, D., Panya, M., . . . Bunchu, N. (2016). Antimicrobial activity of excretory and secretory products from *Chrysomya megacephala* (Diptera: Calliphoridae) Larvae. *Journal of the Medical Association of Thailand*, *99*(9), 1-8.

Taha, N., Abdel-Meguid, A., & El-ebiarie, A. (2010). Application of native excretory/secretory products from third larval instar of *Chrysomya megacephala* (Diptera:Calliphoridae) on an artificial wound. *Journal of American Science*, *6*(7), 313-317.

Valachova, I., Prochazka, E., Bohova, J., Novak, P., Takac, P., & Majtan, J. (2014). Antibacterial properties of lucifensin in *Lucilia sericata* maggots after septic injury. *Asian Pacific Journal of Tropical Biomedicine*, *4*(5), 358-361.

Van der Plas, M. J., van der Does, A. M., Baldry, M., Dogterom-Ballering, H. C., van Gulpen, C., van Dissel, J. T., . . . Jukema, G. N. (2007). Maggot excretions/secretions inhibit multiple neutrophil pro-inflammatory responses. *Microbes and Infection*, *9*(4), 507-514.