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# Pteridine fluorescence in age-determination of immature *Chrysomya megacephala* (Fabricius) and *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae)

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**Abstract.** In the practice of forensic entomology, the chronological age of the maggots retrieved from the cadaver is used to determine the minimum post-mortem interval (mPMI) i.e. minimum time of death. The conventional method of aging the maggots is based on measuring the growth rate of these maggots. Although effective, the constraint associated with conventional method necessitates the development of new age determination method, such as pteridine determination. Pteridine, a by-product of protein metabolism in insects is known to correlate with the age of a variety of dipterans. A number of studies were conducted on aging the adults of forensically important flies. In this study, pteridine was extracted from *Chrysomya megacephala* and *Chrysomya rufifacies* maggots of known age using established methods and determined by measuring the fluorescence at excitation of 330nm and the emissions between 350nm and 600nm. Results exhibited significant positive linear relationships between the pteridine accumulations and age of the fly immature. Pteridine determination is a potential new age determination tool that can be used to determine mPMI.

## INTRODUCTION

In forensic entomology, the main application used is the estimation of minimum Post Mortem Interval (mPMI) by determining the time between death and corpse discovery (Catts, 1992). The method is normally conducted by observing succession pattern of the insects and estimating the age of the oldest specimens of forensic flies found on the corpse. The forensic flies are naturally attracted and oviposited on the corpse a few minutes after death and the maggots are associated with crime scene investigation (Nazni *et al.*, 2011; Heo *et al.*, 2007).

Conventional method used in age determination is by observing the anatomical and morphological changes of the fly larvae. Classical taxonomy is still considered as the gold standard to determine the species of the larvae. However, this conventional method required well-trained technicians with the expertise. Hence, a number of alternative techniques have been developed to facilitate this difficulty. Several new-age determination approaches using molecular and biochemical techniques had been conducted in determining the age of the larval stages of flies such as pteridine fluorescence, gene expression analysis and cuticular hydrocarbon analysis (Zhu *et al.*, 2003; Zhu *et al.*, 2006; Tarone *et al.*, 2007; Zehner *et al.*, 2009; Boehme *et al.*, 2012 & Zhu *et al.*, 2012).

One of the methods that have been developed by Mail *et al.* (1983) to age grading the adult flies of *Stomoxys calcitrans* L. (Diptera: Muscidae) is using pteridine measurement extracted from

the adult head. Pteridine is a group of fluorescent chemicals derived from a pyrimidine-pyrazine ring structure and their amount increases with chronological age in populations of various dipteran taxa. The compound is a chemically recognizable group of substances mainly responsible for pigmentation (Ziegler and Harmsen, 1969). Quantification of these particular chemical compounds make this technique a potentially useful method for age grading of insects with several advantages.

Pteridine compound has a widespread prevalent in insects and it appears to have higher concentrations in insects than most organisms (Ziegler and Harmsen, 1969; Mail and Lehane, 1988). In addition, the ability to detect and characterize the small quantities found in individuals by using fluorescence spectrometer with simple sample preparation makes this technique assayable. Furthermore, the method was found to be applicable to many insect species (Tomic-Carruthers *et al.*, 1995; Robson *et al.*, 2006), including the necrophagous flies, *Chrysomya bezziana* (Diptera: Calliphoridae) (Wall *et al.*, 1990), *Lucillia sericata* (Diptera: Calliphoridae) (Wall *et al.*, 1991), *Musca domestica* (Diptera: Muscidae) (McIntyre and Gooding, 1995), *Chrysomya megacephala* (Diptera: Calliphoridae) (Zhu *et al.*, 2003), *Boettcherisca peregrina* (Diptera: Sarcophagidae) (Zhu *et al.*, 2012) and *Calliphora vicina* (Diptera: Calliphoridae) (Bernhardt *et al.*, 2017). Nevertheless, none of the above study has conducted on immature stages of flies. In this study, we investigated the usefulness of pteridines from larvae of *Ch. megacephala* and *Chrysomya rufifacies* (Diptera: Calliphoridae) which are the common necrophagous flies of forensic importance . Both *Ch. megacephala* and *Ch. rufifacies* are the dominant forensic flies found on corpses in Malaysia (Lee *et al.*, 2004).

## MATERIALS AND METHODS

### Colonization and sample collection

A total of two species of forensically important flies were used in this study. *Ch. megacephala* and *Ch. rufifacies* were reared in different cages and placed in the same room at constant temperature (25 ± 1°C) and relative humidity (70%–80%) with a photoperiod of Light:Dark of 12H:12H. Fresh cow liver (50gm) was introduced into the cage of specific fly species as a medium for oviposition. The eggs were transferred to a new container (30cm L x 30cm W x 4cm H) with cow liver and reared to first instar (L1). In order to determine the pteridine level of all species with respect to age, thirty maggots (n = 30) were taken from the cage daily and placed in separate tubes. The samples were immediately killed in 60°C water for 30 seconds. This procedure was conducted daily until the surviving larvae developed into pupal stage.

### Sample preparation

Pteridine levels in flies were determined using methods modified from Mail & Lehane (1988). Age, length and weight of larvae of both species were measured individually and placed in the 2.0 mL tubes before extraction. Sample was homogenized for 60 seconds using sonication. Then 0.5 mL of mixture in a ratio of 2:1 of chloroform:methanol were added to the sample and further homogenized for another 30 seconds. Solution of 0.75 mL, 0.1N NaOH adjusted to pH 10 using glycine was added to optimize the pteridine extraction. All samples were mixed by vortexing followed by centrifugation for 5 minutes at 8000g in 4°C to remove the remaining particles from the samples of larvae and adults. For comparison, head of the adult was also processed accordingly to assay the pteridine fluorescence.

**Detection of pteridine compound**

Supernatants were transferred into new tubes and read immediately using a fluorescence spectrophotometer (Perkin Elmer LS-55, USA). Monochrometer of the excitation was set at 330nm and the wavelength of the emission was set between 350nm and 600nm. The greatest intensity of the spectra emission wavelength was recorded between 350nm and 600nm proportional to 6-biopterin concentration (Mail and Lehane, 1988). A stock solution of 6-biopterin (Sigma-Aldrich, Australia), a primary component of dipteran pteridines (Mail and Lehane, 1988) served as the standard and also used to confirm the detectable level of pteridine in adults and larvae.

**Statistical Analysis (Linear regression)**

All statistical analysis was carried out using SPSS 17.0 (SPSS Inc., USA). Simple linear regression was used to examine the relation of pteridine concentration with age of the fly larvae. A *P* value of 0.05 was considered statistically significant. The predictive equations for the relationship between age of the adults and larvae and the concentration of the pteridine were determined. Analysis was conducted based on the individual larvae and species.

## RESULTS

**Pteridine detection in larval stage of flies**

Figure 1 compares the emission spectra of 6-biopterin, adult head and third instar larvae of the fly. There is a significant similarity of the wavelength value of all samples ($p > 0.5$). Maximum emission wavelength for all these samples was detected at 450 nm. The data of pteridine concentration of larvae of *Ch. megacephala* and *Ch. rufifacies* were within the statistical normality (Shapiro-Wilk test for normality) and hence the analysis was continued with parametric correlation test. There was moderate and high relationship between pteridine concentration and age of the larvae of *Ch. megacephala* and *Ch. rufifacies* at $p$=0.01 level using Pearson's correlation test ($r^2$ = 0.556, 0.849, respectively). The value of pteridine concentration was plotted against the age of the larvae of *Ch. megacephala* and *Ch. rufifacies* and there were differences in increasing pattern of pteridine from these species (Figure 2).

A linear relationship was observed between pteridine concentration and chronological age for larvae of *Ch. megacephala* ($p < 0.001$, $r^2 = 0.556$). Similar result was observed for *Ch. rufifacies*

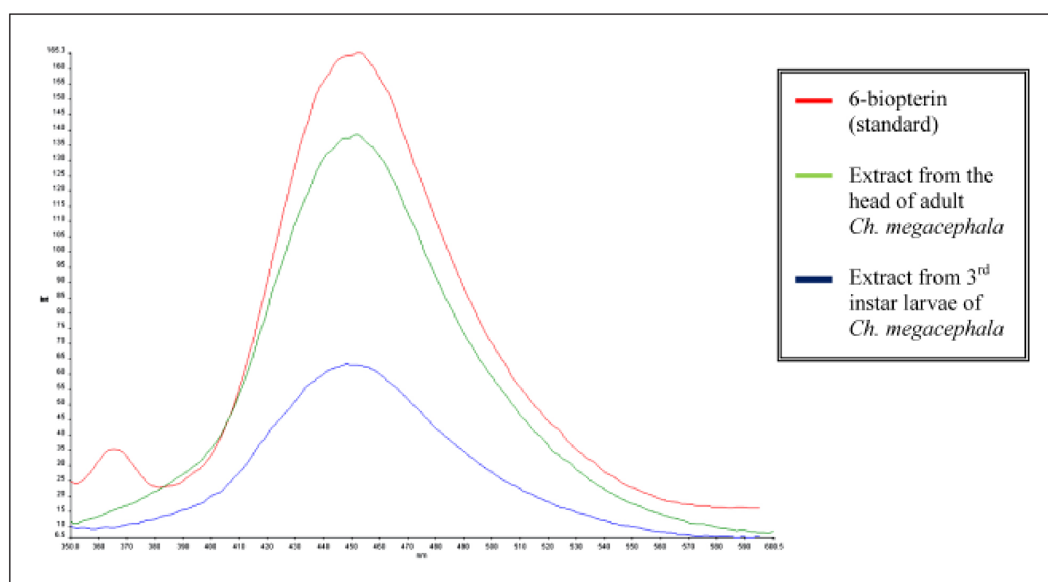


Figure 1. Emission spectra of 6-biopterin, adult head and larvae of *Ch. megacephala*.

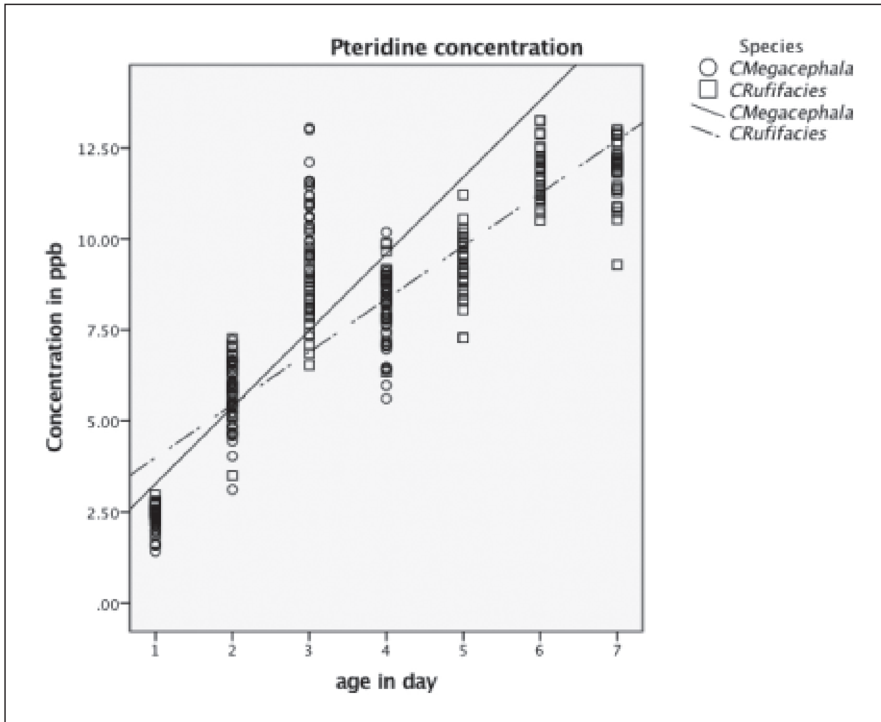


Figure 2. Pteridine concentrations plotted against age of the larvae of *Ch. megacephala* and *Ch. rufifacies*.

Table 1. Regression models between pteridine concentration and age of larvae for *Ch. megacephala* and *Ch. rufifacies*

| Species | Regression model | $r^2$ | n | *p* value |
|---|---|---|---|---|
| *Ch. megacephala* | y = 1.17 + 2.10 x | 0.556 | 209 | < 0.05 |
| *Ch. rufifacies* | y = 2.54 + 1.45 x | 0.849 | 122 | < 0.05 |

larvae where the relationship can be described using linear regression model ($p < 0.001$, $r^2 = 0.849$) (Table 1).

## DISCUSSIONS

This study has successfully detected the pteridine compound from larvae of *Ch. megacephala and Ch. rufifacies*. It supports previous studies suggesting that pteridine was discovered in most of the dipterans (Zhu *et al.*, 2012; Robson *et al.*, 2006; Wall *et al.*, 1990; Wall *et al.*, 1991). Pteridine is detected from a number of dipterans of both economical and forensic importance. Most of the previous studies emphasized the pteridines from adult flies as some of it provides useful information on pest control programme and indoor-death scene investigations (Catts & Goff, 1992). Nonetheless, in forensic entomology, the information provided from larvae found on corpses are more useful for mPMI estimation. Therefore, detection of pteridine from larvae is more promising in forensic entomology application.

It is crucial to confirm that pteridine concentrations can be measured from individual fly larva to assess the suitability of this compound as age predictor. However, pteridine concentrations could not be

measured individually in some insects. Lardeux *et al.* (2000) has confirmed that individual extract of *Aedes polynesiensis* and *Culex quinquefasciatus* (Diptera: Culicidae) showed low concentrations of pteridines or below detectable level, although the pteridine level can be increased by pooling the mosquitoes in minimum of five individuals. The study also proved that there was decreasing pattern of pteridine level against age of the small dipterans. Penilla *et al.* (2002) also successfully detected the pteridine level by pooling five individual *Anopheles albimanus* (Diptera: Culicidae) using HPLC analysis.

In this study, pteridine concentrations were found increasing in both *Ch. megacephala* and *Ch. rufifacies* larvae. Pattern of the pteridine concentration was different as *Ch. rufifacies* showed higher increment compared to *Ch. megacephala*. It has been reported that most dipterans showed different pattern of pteridine concentration albeit the samples were collected from the same family (Wall *et al.*, 1991; Wall *et al.*, 1990). In this study, there was no observation conducted in terms of sexual differentiations as this was impossible at larval stages. However, Wall *et al.* (1990) showed that pteridine accumulation was significantly higher in *Ch. bezziana* males compared to females. It is also agreed by Berndart *et al.* (2017) where they showed the sex of insect is a significant factor in prediction of adult fly age of *Ca. vicina*. This concurred that there might be a difference at pteridine level extracted from different species and sex of insects.

This present study demonstrated that pteridine is increasing in larval stages of *Ch. megacephala* and *Ch. rufifacies* throughout the development process. It also showed that there was a significant linear positive relationship between the pteridine concentration and age of larvae for both species. Other factors such as temperature or source of food consumed may affect pteridine accumulation. According to Zhu *et al.* (2012), different temperatures produced different rates of pteridine accumulation in *B. peregrina* (Diptera: Sarcophagidae). Higher temperature will cause slightly higher accumulation of pteridine in the adult fly. On the contrary, Bernhardt *et al.* (2017) indicated that even though there was a significant effect between three different temperatures against pteridine concentrations, it can be synchronised using accumulated degree-days (ADD) approach and makes it less important in age estimation. Nevertheless, the temperature data of the area where the samples were collected could provide important information to determine the pteridine concentration and intensity.

The present study also indicated that the larva samples showed a moderate and high relationship with age for both *Chrysomya rufifacies* and *Chrysomya megacephala*, respectively. The significant correlation could be due to the fact that adult insect from the previous studies (Robson *et al.*, 2006; Zhu *et al.*, 2012) were a fully developed form. Therefore, aging in the adults will produce large amount of pteridine which is linearly correlated with age. On the other hand, larvae are continuously undergoing growth development until pupation. Therefore, their aging may be different from aging in adults. Hence, a correlation with other confounding factors such as length and weight are essential for the larvae rather than the age only.

Detection of pteridine compound from larvae could be a potential method in determining the minimum PMI. It could be used to provide the nearest estimated age of the insect specimen especially when the larvae had reached the third instar stage. Most importantly, it should be noted that the presence of pteridine in the larvae was the first reported in this study because previous literatures only reported the pteridine from the compound eyes of flies and other dipterans.

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