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Technical Note

Study on the pupal morphogenesis of *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae) for postmortem interval estimation

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ABSTRACT

Chrysomya rufifacies (Macquart) is one of the most common species of blow flies at the scene of death in Southern China. Pupae are useful in postmortem interval (PMI) estimation due to their sedentary nature and longer duration of association with the corpse. However, to determine the age of a pupa is more difficult than that of a larva, due to the fact that morphological changes are rarely visible during pupal development. In this study, eggs of *C. rufifacies* were reared in climatic chambers under four different constant temperatures (20, 24, 28 and 32 °C each ± 1 °C, respectively) with same rearing conditions such as foodstuff, substrate, photoperiod and relative humidity. Ten duplicate pupae were sampled at 8-h intervals from prepupae to emergence under the different constant temperatures, respectively. The pupae were sampled, killed, fixed, dissected and with the puparium removed, the external morphological changes of the pupae were observed, recorded and photographed. The morphological characters of *C. rufifacies* pupae were described. Based on the visible external morphological characters during pupal morphogenesis at 28 °C ± 1 °C, the developmental period of *C. rufifacies* was divided into nine developmental stages and recorded in detailed description. Based on above-mentioned nine developmental stages, some visible external morphological characters were selected as indications for developmental stages. These indications mapped to 8-h sampling intervals at the four different constant temperatures were also described in this study. It is demonstrated that generally the duration of each developmental stage of *C. rufifacies* pupae is inversely correlated to appropriate developmental temperatures. This study provides relatively systematic pupal developmental data of *C. rufifacies* for the estimation of PMI. In addition, further work may improve by focus on other environmental factors, histological analysis, more thorough external examination by shortening sampling intervals, PAE (the Pupal Age Estimator) method and parasitic insects of *C. rufifacies*.

1. Introduction

The time elapsed from the moment of death is also known as the postmortem interval (PMI). Regardless whether in the past or present, and whether the case is simple or complicated, the determination of PMI is still the primary problem in crime scenes. PMI is extremely relevant to criminal activities, and in most cases, it is identical to the time of crime [1]. Developmental times of immature necrophagous insects that consume the body can be a superb indicator for the minimum PMI (min PMI), as insects are usually the first to arrive on a corpse after death [2]. Therefore, the min PMI is extremely significant in identifying or eliminate a suspect, and to mark out an investigative area.

Blow flies (Calliphoridae) larvae serve as carrion feeders which play a very important role in recycling organic materials in the ecosystem. There are more than 1450 blow flies species distributing all over the world [3]. Among which the major species include *Calliphora*, *Lucilia*, and *Protophormia* genera [4]. Studies on necrophagous insects have been carried out in many countries of the world [5–8], including China [9–11]. Among these necrophagous species, *Chrysomya rufifacies* (Macquart) is one of the most common species of blow flies involved in homicide cases in Southern China [12–14], and even in other countries beyond China in East Asia [15,16] and Southeast Asia [3,17].

In the forensic field, age estimation methods have been most frequently focused on the larval morphology and developmental data available from numerous published studies [5–8]. Age can be estimated from appropriately preserved pupae using analysis of

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external morphology, histology and molecular biology [18–24]. Pupae are useful in PMI estimation due to their sedentary nature and longer duration of association with the corpse [25]. However, determining the age of a pupa is much more difficult than that of a larva, since it is impossible to observe morphological differences such as length and weight from the outside directly [26]. In addition, the pupal stage can last up to more than 50% of the whole juvenile development before adults emerge, and low temperatures can prolong the duration up to several weeks for certain species [27].

The external morphological characters of *Phormia regina* during pupal morphogenesis at temperatures of 22 °C and 29 °C have been described by Greenberg and Kunich [28] in 2002. It is the first published study focused on the pupal development stages and duration of these stages in specific species. In 2002–2008, the pupal morphogenesis of *Aldrichina grahami*, *Chrysomya megacephala* and *Lucilia cuprina* at different constant temperatures were studied in China [29–31]. In the same year 2012, the pupal development stages of *Calliphora vicina* at 22 °C were studied by Brown [32]; the pupal development stages of *Lucilia sericata* at 25 °C were studied by Zajac and Amendt [33], and similar studies were conducted on *Calliphora vomitoria* and *Chrysomya albiceps* by Ergil [34]. Feng and Liu [35,36] focused on the key developmental process during the pupal morphogenesis of *Megaselia spiracularis* and *Megaselia scalaris* at multiple different constant temperatures. In 2014, Karabey and Sert [37] studied the stages and duration of the pupal stage of *L. sericata* at temperatures of 20, 25 and 30 °C, whereas Brown et al. [20] developed a timeline of metamorphosis on development of the external morphology of *C. vicinia*, and with an observation that 23 external metamorphic developments were correlated to age in accumulated degree hours (ADH) after puparium removal [20].

In forensic applications, pupae of blow flies collected from corpse are usually brought back to laboratory, reared to emergence and calculated the min PMI reversely. This method requires a lot of time, therefore, leads to missing the best opportunity to solve a case. Reliable age estimations of forensically relevant pupae are necessary, especially when pupae represent the oldest specimens present at a crime scene or even the only source of entomological evidence available [35,36]. As a forensic potential species, the pupal morphogenesis of *C. rufifacies* at different constant temperatures may provide more accurate estimates of the min PMI.

2. Materials and methods

2.1. Colonies establishment

C. rufifacies colonies were established from wild adults and larvae caught using carcass of a pig as bait in Panyu, Guangzhou, Guangdong, China (Lat. 22°57′12.54″N, Long. 113°17′30.25″E) during the years 2012–2013. Adults were identified using the morphological characters described by Whitworth [38]. Five duplicate colonies were taken as F1, each with approximately 100 larvae. The larvae were reared to adults first by supplying them with pig liver in a climatic chamber (GCZ-160B, Ningbo Jiangnan Instrument Factory, China) under constant conditions of photoperiod 12:12 L:D cycle, 40–50% relative humidity and constant temperature of 28 ± 1 °C in the laboratory. The larvae were supplied with pig liver upon sand substrate for pupation, emergence to adults, ovary maturation and oviposition. The following initial oviposition was removed. F2 colonies were formed with approximately 300–600 newly laid eggs each.

Eggs of the F2 colonies were collected from above-mentioned F1 colonies. Then those were immediately transferred and reared in another similar climatic chamber, supplied with pig liver upon sand substrate and were kept under constant conditions of photoperiod 12:12 L:D cycle, 40–50% relative humidity, and four different constant temperatures (20, 24, 28 and 32 °C each ±1 °C, respectively).

2.2. Sampling of pupae

First sampling of pupae began with the formation of white puparium after postfeeding larvae pupated. Ten duplicate pupae were sampled from above-mentioned F2 colonies at 8-h intervals until emergence under four different constant temperatures, respectively. Samples were hot-water-killed (HWK) and fixed in the solution of acetic acid and ethanol (acetic acid:ethanol = 1:1) in a culture dish, and were then preserved in 80% ethanol. HWK 80% ethanol preservation facilitates puparium removal and kills the pupa immediately avoiding further development [22].

2.3. Observation of pupal morphogenesis

The samples were dissected for puparium removal by surgical knife blade and forceps under stereomicroscope (Zeiss Stime 2000). The external morphological characters of the pupae were observed, recorded, and photographed by digital camera (Nikon D700). The samples were subsequently preserved in 80% ethanol.

3. Results

3.1. Morphological description of pupae

Pupae were 7.2–11.5 mm in length based on account of approximately 100 measured samples. Puparium slightly curved (Fig. 1); anterior part slightly tapering than posterior part; dorsal part slightly darker than ventral part; mouth hook is visible through puparium in ventral view; each abdominal segment with 8 obvious pairs of conical processes, each process consists of dozens slightly recurvate spinules; posterior spiracles rounded, the interval between each posterior spiracle is less than their transverse diameter.

Fig. 1. Pupal stage of *C. rufifacies* in dorsal view.