Development time, body mass and length of immatures of *Paralucilia fulvinota* (Bigot, 1877) (Diptera: Calliphoridae) reared under natural conditions in a Central Amazon forest

Tempo de desenvolvimento, massa e comprimento corporal de imaturos de *Paralucilia fulvinota* (Bigot, 1877) (Diptera: Calliphoridae) criados sob condições naturais em uma floresta da Amazônia central

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Abstract: Blow flies (Calliphoridae) are used in forensic entomology studies, because their life cycle help to determine the minimum post-mortem interval (PMI_{min}). *Paralucilia fulvinota* (Bigot, 1877) is a forensic indicator and is reported from human corpses in the Amazonian region. Our objectives were to describe the development time of *P. fulvinota* under natural conditions at Ducke Reserve (Amazonas), provide the accumulated degree-days (ADD) and accumulated degree-hour (ADH), body length and mass of larvae, and the sex ratio of adults. Pregnant females were sampled on a swine carcass. From hatched eggs, we reared stock colonies and observed the immature development under uncontrolled conditions of temperature (24.5 ± 1.3 °C), rainfall (188 ± 11.5 mm^3) and humidity (84 ± 3.9%). Development time was 11 days. Adult sex ratio was 0.86. Larvae gained mass at constant rates through the instars but grew less in length from the second to the third instar. Accordingly, *P. fulvinota* grows more in mass than in length when in larval stage. The period from first stadium to adults lasted 159.5 degree-days and 3828 degree-hours. Our results may be helpful in providing a more accurate estimate of PMI_{min} on corpses encountered in forested areas of the Amazon region.

Keywords: Blow flies. Bionomy. Forensic entomology. Post-mortem interval.

Resumo: Moscas varejeiras (Calliphoridae) são usadas na entomologia forense, pois seu ciclo de vida ajuda a determinar o intervalo pós-morte mínimo (IPM_{min}). *Paralucilia fulvinota* (Bigot, 1877) é considerada indicadora forense e é relatada em cadáveres humanos na região amazônica. Nossos objetivos foram descrever o tempo de desenvolvimento dessa espécie em condições naturais na Reserva Ducke (Amazonas), informar sobre grau-dia acumulado (GDA) e grau-hora acumulado (GHA), comprimento e massa corporal das larvas e a razão sexual de adultos. Fêmeas grávidas foram criadas em carcaça suína. A partir de ovos eclodidos, criamos colônias de estoque e estudamos o desenvolvimento dos imaturos sob condições não controladas de temperatura (24,5 ± 1,3 °C), precipitação (188 ± 11,5 mm^3) e umidade (84 ± 3,9%). O tempo de desenvolvimento foi de 11 dias. A razão sexual foi de 0,86. As larvas ganharam massa a taxas constantes através dos ínstares, mas cresceram menos do segundo para o terceiro ínstar. Consequentemente, *P. fulvinota* cresce mais em massa do que em comprimento durante o estágio larval. O período de primeiro estádio a adulto durou 159,5 graus-dia e 3.828 graus-hora. Nossos resultados podem ser úteis na estimativa mais precisa do IPM_{min} em cadáveres encontrados em áreas florestais da região amazônica.

Palavras-chave: Mosca varejeira. Bionomia. Entomologia forense. Intervalo pós-morte.

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INTRODUCTION

Necrophagous insects are crucial in the decomposition process of cadavers (Feddern *et al*., 2019). Among the insects that visit corpses, Diptera and Coleoptera may represent up to 60% of the fauna found throughout the decomposition process (Charabidze *et al*., 2014; Mariani *et al*., 2014). Blow flies (Diptera: Calliphoridae) are among the first organisms to arrive in newly deceased corpses (Archer, 2003), because they are highly attracted by the odors released even at long distances (Anderson, 2009). Thus, Calliphoridae is a good forensic indicator and is often used for the determination of the minimum post-mortem interval (PMI_{min}) (Vairo *et al*., 2015; Faris *et al*., 2016; Abd-Algalil *et al*., 2017).

The PMI_{min} is usually based on the minimal age of insects associated with the corpses (Nuorteva, 1977), and can be estimated using fly larval body mass and length data (Greenberg & Kunich, 2002). However, the main method to obtain the PMI is using accumulated degree-day (ADD), which is based on an estimated linear relationship between development time and rearing temperature of insects (Amendt *et al*., 2007). This relationship can only be linear under controlled conditions of temperature (Higley & Peterson, 1994); under uncontrolled conditions, the temperature varies in a hyperbolic manner (Ikemoto & Takai, 2000).

Most studies on the life cycle of Calliphoridae have been conducted under controlled temperature (Lecheta *et al*., 2015; Bambaradeniya *et al*., 2019); however, small variations in temperature may result in alteration of the development time (Grassberger & Reiter, 2002). For example, species of Calliphoridae from different geographic regions may present divergent responses to temperature because they are intrinsically adapted to different conditions (Grassberger & Reiter, 2002).

The first study on the development time of the larval stages of *Paralucilia fulvinota* (Bigot, 1877) (Diptera: Calliphoridae) under uncontrolled temperature was performed by Greenberg & Szyska (1984) in the Peruvian Amazonia. The first application of forensic entomology in the Brazilian Amazonia was performed by Pujol-Luz *et al.* (2006). In this study, larvae of *Paralucilia fulvinota* were used to estimate the minimum post-mortem interval (PMI_{min}) of the corpses. The difficulty highlighted by the application of forensic entomology in the Amazon is related to the lack of knowledge about the biology of necrophagous insects (Pujol-Luz *et al*., 2006). Considering the importance of *P. fulvinota* as a forensic indicator, we conducted a study of this species as a way to provide the first information on ADD and accumulated degree-hour (ADH), body length and mass of immature stages, and the sex ratio of adults. Additionally, we provide a table with the development time in our study and those reported in previous papers. Our results may support PMI_{min} calculation in criminal cases that involves entomological evidence in a Central Amazon forest.

MATERIAL AND METHODS

This study was conducted at Ducke Reserve (02º 55' 56.7" S, 59º 58' 30.2" W), a federal protected area of 10,000 ha located on the km-26 of the AM-10 road in Manaus, Amazonas, Brazil. The region has an equatorial climate, with a mean annual temperature of ~26 ºC (variation: 23.3-31.4 ºC), a mean annual rainfall of ~2,286 mm^3 and a mean relative humidity of ~80% (Costa *et al*., 2013).

This experiment was performed between October and December 2014. The eggs were obtained from mature females of *Paralucilia fulvinota* collected from a carcass of *Sus scrofa* (Linnaeus, 1758) (ethical committee protocol: nº 007/2014-CEUA/UFAM). The females were placed inside rearing cages under uncontrolled temperature and humidity with a photoperiod of 12L:12D and were offered beef to stimulate the oviposition. The laid eggs were transferred to Petri plates containing 1 g of beef, with moistened filtering paper on the bottom.

Six replicate units were used, each containing 60 larvae and 60 g of beef for feeding. The larvae food was not replaced throughout the experiment. We used the individuals (sample unit) of one replicate to assess larval body mass and length throughout the development. The immatures were kept under environmental

temperature, which was recorded using a digital thermo-hygrometer (TH50, Incoterm).

To determine the development time, we made hourly observations from the oviposition period until the hatch of the eggs. Afterwards, observations were restricted to 12-hour intervals, and continued until the larvae ceased feeding (Queiroz, 1996). Then, we counted, weighted, and individualized each third-instar larvae in separate 15-mL Falcon vials. Observation continued until all adults had emerged. Each day, from each larval instar, we separated six of the total number of reared larvae from one replicate unit. Posteriorly, the larvae were killed with hot distilled water and preserved in 96% ethylic alcohol (Bugelli *et al*., 2017). We identified the instars based on their spiracle openings (Queiroz *et al*., 1997). The sex ratio of adults was calculated following Silveira-Neto *et al.* (1976). The mature females were identified using the key proposed by Amat (2009).

To evaluate the growth of *Paralucilia fulvinota*, we measured body mass and length. These measurements were taken under a Leica M165C stereomicroscope with calibrated lens (accuracy = 0.001 mm). Larval body length was measured as the distance from the cephalic segment to the distal margin of the final abdominal segment, considering any flexion of the larval body. The weighing of wet mass was performed for all three instars using a scale (ML, Mettler-Toledo, Columbus, EUA, accuracy = 0.01 mg). Larval growth was estimated according to the difference of weight and size between subsequent instars. We performed analysis of variance (ANOVA) in order to verify whether body mass differed among instars. This analysis is too used to test difference of length among instars. Statistically significant test was $p < 0.05$. All analysis was made in statistical software R (R Core Team, 2016).

To calculate accumulated degree-hour (ADH) and accumulated degree-day (ADH), we established a minimum temperature of 10 °C. We did so because there is no existing data on the thermal demands of *P. fulvinota*, and 10 °C is the bottom limit suggested for Neotropical species (Higley & Peterson, 1994). The temperature records were obtained from the Ducke Reserve Meteorological Station. We used 24.5 °C as the mean temperature, with 27.6 °C and 21.1 °C as the maximum and minimum values, respectively. We followed Higley & Haskell (2003) to calculate ADD and ADH from the formulas below.

$$ADD = (\text{rearing temperature - minimum threshold}) \times \text{development time}$$

$$ADH = \frac{(\text{rearing temperature- minimum threshold}) \times \text{development time}}{24}$$

RESULTS

We observed a larval survival rate of 14.72%, meaning that only 53 out of the 360 initial larvae developed into adults. Overall development time (immature + imago) lasted around 11 days (264 h). On average, the first-larval instar (L1) lasted 12 hours, the second-larval instar (L2) lasted 36 h, and the final instar (L3) lasted 72 h. Overall, larval stages L1–L3 lasted on average 120 h, while the pupal stage lasted around 144 h (Table 1). The sexual ratio was 0.86, with 46 females and seven males.

During development, the body length at egg hatching stayed around 2.72 ± 0.17 mm. Sixty hours after hatching, the third instar larvae were an average of 11.45 ± 1 mm long, thus significantly larger than L1 (3.05 ± 0.4 mm) and L2 (6.35 ± 1.38) (ANOVA: $F_{2, 33} = 119.66$; $p < 0.001$). Larvae grew more in length from the first to the second instar (4.48 mm) than from the second to the third instar (3.58 mm; Figure 1).

The larval mass increased approximately 420%. Larval mass at egg hatching was 0.09 mg, and after 60 h (L3), recorded weight was 31.67 ± 5.09 mg (Figure 2). Third instar larvae were significantly heavier than first (0.32 ± 0.38 mm) and second instar ones (6.59 ± 4.6) (ANOVA: $F_{2, 33} = 134.36$; $p < 0.001$). Contrary to the trend observed in body length, larvae gained more mass from the second to the third instar (19.22 mg) than from the first to the second instar (11.89 mg).

The estimates of ADD and ADH for development time of *Paralucilia fulvinota* were 159.5 and 3828, respectively. In both indexes, the values for L1 were lower than other stages (Table 2).

DISCUSSION

We obtained a low emergence rate (14.72%). Pérez *et al.* (2016) also observed a low percentage of emergence in the first generation of *Calliphora vicina* Robineau-

Table 1. Comparison of our results with other studies regarding the development time of *Paralucilia* Brauer & Bergenstamm, 1891 species under natural conditions of temperature (temp; °C). We demonstrate that development time differs even among closely related species. Legends: [1] = Greenberg & Szyska (1984); [2] = Pujol-Luz *et al*. (2006); [3] = Barros-Souza *et al.* (2012); [4] = Sales *et al.* (2013); [5] = our study.

Species	Temp	Average development time (hours)				
		L1	L2	L3	Pupa	Total
Paralucilia fulvinota[1]	21.7-26.0	18	20.5	99	120	257.5
		26	12	183	120	341
Paralucilia fulvinota[2]	26.0	30.3	16	118	122.3	286.6
Paralucilia paraensis[3]	25.8	11.4	13	81.1	144.14	249.64
Paralucilia paraensis[4]	26.0	13	18	46	96	173
Paralucilia fulvinota[5]	24.5	12	36	72	144	264

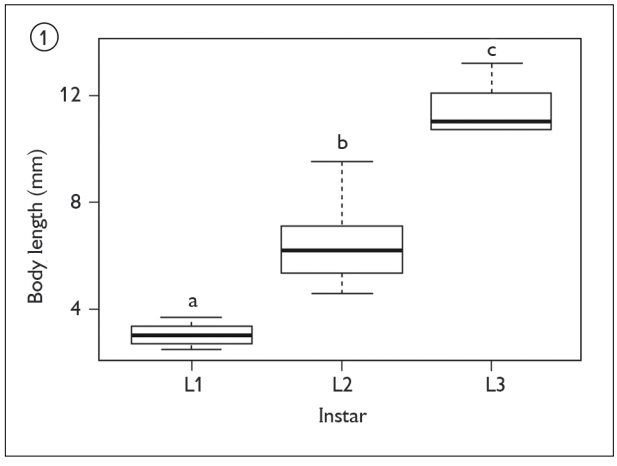

Figure 1. Body length (mm) of larvae of *Paralucilia fulvinota* (Bigot, 1877) reared under uncontrolled conditions of temperature with average value of 24.5 °C in Ducke Reserve, Manaus, AM. Legends: a (L1) = larvae of 1st instar; b (L2) = larvae of 2nd instar; and c (L3) = larvae of 3rd instar.

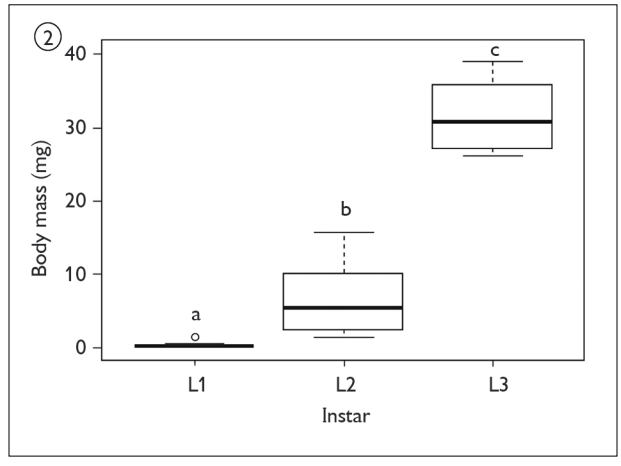

Figure 2. Body mass (mg) of larvae of *Paralucilia fulvinota* (Bigot, 1877) reared under uncontrolled conditions of temperature with average value of 24.5°C in Ducke Reserve, Manaus, AM. Legends: a (L1) = larvae of 1st instar; b (L2) = larvae of 2nd instar; and c (L3) = larvae of 3rd instar.

Table 2. Accumulated degree-hour (ADH) and Accumulated degree-day (ADD) for the larval and pupal development of *Paralucilia fulvinota* (Bigot, 1877) in Ducke Reserve, Manaus, AM, Brazil. The values were calculated using 24.5°C as temperature and 10°C as bottom limit (Higley & Peterson, 1994).

Index	Stages				
	L1	L2	L3	Pupa	Total
ADH	174.00	522.00	1044.00	2088.00	3828.00
ADD	7.25	21.75	43.5	87	159.5

Desvoidy, 1830. One of the factors that may have influenced our results is diet. We opted to use beef to feed the larvae. This resource is used in the rearing of flies for forensic study (Souza & Kirst, 2010). Nevertheless, the low emergence rate of adults may indicate that the amount of food offered may not have been sufficient for the larvae to complete their energy reserve. During development, the larvae compete for the food resource (personal observation) and some may not assimilate the nutrients necessary to achieve pupation (Gobbi *et al*., 2013). Furthermore, survive rate is higher for wild immatures than for those reared in the laboratory, which is likely due to wild larvae having access to additional nutrients (Dicke *et al*., 1989). It is also possible that the laboratory larvae experience stress caused by the inability to migrate to bury themselves in a space that is relatively small compared to the natural environment. This situation was also observed during a study of the development of *Lucilia eximia* (Wiedemann, 1819) (Calliphoridae) in Colombia (Vélez & Wolff, 2008).

Paralucilia fulvinota was recorded in Peru and Brazil (see Table 2) under a temperature range of 21.7 °C to 26 °C. In Peru, the development from L1 to pupa was approximate (~12.5 days; 299.3 h) to our study (Greenberg & Szyska, 1984). In Brazil, Pujol-Luz *et al*. (2006) used a development time of ~287 h, 23 h longer than our study. This difference of ~1 day is relevant, mainly because both studies were performed in Amazonas state. This difference can be related to the diet used during the rearing of the larvae. Pujol-Luz *et al*. (2006) used pork in the rearing of *P. fulvinota*, while the present study used beef. For example, larvae of *Chrysomya putoria* (Wiedemann, 1830) (Calliphoridae) needed more time to develop when reared with swine liver compared to bovine liver (Salazar-Souza *et al*., 2019).

In urban (Barros-Souza *et al*., 2012) and forested (Sales *et al*., 2013) areas of Manaus (Amazonas, Brazil), the development time of *P. paraensis* (Mello, 1969) differ from what we observed with *P. fulvinota* regarding overall development time and duration of instars. This demonstrates that even morphologically similar species may display differences in life cycle.

We recorded a higher number of females compared to males. On the other hand, Barros-Souza *et al.* (2012) registered a 1:1 ratio in *P. paraensis*. Although we may expect a similar quantity of males and females (*e.g*. Boatright & Tomberlin, 2010; Li *et al*., 2014), some females of Calliphoridae species (*e.g. Chrysomya albiceps* (Wiedemann, 1819)) can reproduce in a monogenetic manner (Serra *et al*., 2007).

Through the values of body mass and length, we could observe that the juveniles gained more mass than length. Thus, length measurements may not be appropriate variables with which to determine instar and development time in blow flies. Furthermore, according to Day & Wallman (2006), the larvae develop a slight bend on the anterior edge of the ventral side, which may mislead measurements. To overcome this issue, it was suggested that studies should use the junction of the width of the fifth and sixth segments in relation to body length (Day & Walmann, 2006).

In an urban area of Manaus, Barros-Souza *et al.* (2012) registered 4841.06 degree-hours and 201.71 degree-days for *P. paraensis* in uncontrolled conditions of temperature. Such values were similar to those recorded by our study (ADH = 3828; ADD = 159.5) and the differences may be due to the variation between the study areas (urban *versus* primary forest), species-specific features, and the climatic conditions of the experiments. Furthermore, studies conducted under uncontrolled temperature conditions are fundamental for obtaining more accurate values of PMI (Barros-Souza *et al*., 2012), as forensic entomology practices are applied in dynamic environments.

Studies with blow flies of forensic value are rather common in several regions (Nabity *et al*., 2006; Badenhorst & Villet, 2018; Langer *et al*., 2019). However, bionomic data on necrophagous species should not be extrapolated to differences localities, as this may mislead the PMI estimations (Barros *et al.*, 2019). Therefore, the precise development of data for forensic indicator species is essential for accuracy in PMI estimates (Nabity *et al*., 2006).

CONCLUSION

This study presents bionomic data on *P. fulvinota* in Amazonas state under uncontrolled conditions, contributing information on development time. It is the first study with data on ADH, ADD, and growth rates for larval stages of this species. This information is important in order to minimize errors in the calculation of PMI_{min} for subsequent utilization in medico-legal procedures. Moreover, our data may provide useful bionomic information on *P. fulvinota* for solving crimes associated with forested areas of similar characteristics in which this species is an entomological vestige.

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