

# Intra-puparial development of *Lucilia eximia* (Diptera, Calliphoridae)

Yardany RAMOS-PASTRANA<sup>1,2\*</sup>, Carlos Andres LONDOÑO<sup>1</sup>, Marta WOLFF<sup>2</sup>

<sup>1</sup> Universidad de la Amazonia, Laboratorio de la Entomología, Grupo -GAIA-, Grupo de Investigación en Agroecosistemas y Conservación en Bosques Amazónicos. Florencia, Caquetá, Colombia.

<sup>2</sup> Universidad de Antioquia, Grupo de Entomología GEUA. Medellín, Colombia.

\* Corresponding author: ya.ramos@udla.edu.co

## ABSTRACT

There are few studies about the intra-puparial development in Diptera, nonetheless its importance has been increased because several dipteran species are of forensic interest. Studies on the life cycles of flies often disregard the changes that occur inside the pupae. The objectives of this research were to analyze the intra-puparial development of *Lucilia eximia*, and to describe chronological and morphological changes that occur during this stage. Around 1.600 specimens were laboratory-reared. The pre-pupae were identified by the reduction of their size and change in coloration, and 10 individuals were sampled every three hours (n=1.000) until adult emergence. The specimens were fixed in 96% alcohol, subsequently immersed in Canoy solution for 24 hours and in formic acid (5%) for 48 hours, to facilitate dissection and analysis of the morphological changes of the individuals. Four stages of the intra-pupal development of *L. eximia* were observed: 1) Larva-pupa apolysis, which lasted  $23 \pm 1.08$  h; 2) Cryptocephalic pupa,  $5 \pm 0.53$  h; 3) Phanerocephalic pupa,  $92 \pm 1.94$  h; and 4) Pharate adult: Transparent eyes,  $125 \pm 2.15$  h; Yellow eyes,  $23 \pm 0.89$  h; Pink eyes,  $14 \pm 0.73$  h; and Red eyes,  $20 \pm 0.60$  h. The pharate adult is completely formed after 296 hours and the emergence of the adult occurred after  $302 \pm 3.81$  h. In addition, there were included the stage of pre-pupa, pupariation and the beginning of the adult stage, Imago and adult emergence. Each stage is described and compared with those described for *Chrysomya albiceps* (Calliphoridae).

**KEYWORDS:** Immature insects, Metamorphosis, Forensic entomology, Postmortem interval.

## Desenvolvimento Intra-pupal de *Lucilia eximia* (Diptera: Calliphoridae)

### RESUMO

No mundo existem poucos estudos sobre o desenvolvimento intrapupal de Diptera. Comumente, os estudos dos ciclos de vida das espécies de moscas são feitos eliminando o desenvolvimento embrionário que é muito importante, principalmente nas espécies de interesse forense. O principal objetivo foi o de analisar o desenvolvimento intrapupal de *Lucilia eximia*, descrevendo as mudanças morfológicas que ocorrem durante o desenvolvimento intrapupal. Foram analisados cerca de 1.600 espécimes cultivados em laboratório. Foram analisadas as fases de pré-pupa, pupa, o início da fase adulta, a imago e a emergência. Pré-pupas foram identificadas de acordo com a redução no tamanho e na alteração da coloração. Dez espécimes foram coletados a cada três horas (n=1.000), sem interrupção, até a emergência dos adultos. As pupas foram fixadas em etanol a 96%. Em seguida foram imersas em solução Carnoy por 24 horas e ácido fórmico a 5% por 48 horas para facilitar a dissecação e análises das alterações morfológicas. Foram determinados 4 estágios de desenvolvimento intrapupal. 1) Apólise larva-pupa com duração de  $23 \pm 1,08$  h; 2) Pupa criptocefálica:  $5 \pm 0,53$  h; 3) Pupa fanerocefálica:  $92 \pm 1,94$  h e; 4) Adulto farado: olhos transparentes:  $125 \pm 2,15$  h; olhos amarelos:  $23 \pm 0,89$  h; olhos róseos:  $14 \pm 0,73$  h e olhos vermelhos  $20 \pm 0,60$  h. O adulto farado estava completo após 296 horas e a emergência dos adultos ocorreu com  $302 \pm 3,81$  h. Cada fase foi descrita e comparada com o descrito para *Chrysomya albiceps* (Calliphoridae).

**PALAVRAS-CHAVE:** Insetos imaturos, Metamorfose, Entomologia forense, Intervalo pós-morte.

## INTRODUCTION

The decomposition process begins soon after the death of an organism. When a corpse is exposed in the environment, it can be colonized by insects and other arthropods, mainly larvae of necrophagous Diptera (Amendt *et al.* 2004). The presence of an insect is often used as a tool for the postmortem interval estimation (PMI) (Disney and Manlove 2005; Pujol-Luz *et al.* 2008).

The age of the pupa has been used to estimate the postmortem interval, and has also been documented for a few dipteran species of forensic importance such as in the Calliphoridae: *Calliphora erythrocephala* (Macquart, 1834) (Wolfe 1954), *Chrysomya albiceps* (Wiedemann, 1819) (Pujol-Luz and Barros-Cordeiro 2012) and *Chrysomya rufifacies* (Macquart, 1842) (Ma *et al.* 2015)]; Muscidae: *Musca domestica* Linnaeus, 1758 (Fraenkel and Bhaskaran 1973); Sarcophagidae: *Sarcophaga bullata* (Parker, 1916) (Fraenkel and Bhaskaran 1973); and Stratiomyidae: *Hermetia illucens* (Linnaeus, 1758) (Barros-Cordeiro *et al.* 2014). This information has also been reported for some dipterans with medical and veterinary interest: *Cephenemya phobifera* (Clark, 1815) (Bennett 1962), *Cuterebra tenebrosa* Coquillet, 1898 (Baird 1972; Baird 1975), *Dermatobia hominis* (Linnaeus Jr., 1781) (Lello *et al.* 1985), *Hypoderma linaetum* (Viller, 1789), *Hypoderma bovis* (Linnaeus, 1761) (Scholl and Weintraub 1988), *Cuterebra fontinella* Clark, 1827 (Scholl 1991), and *Oestrus ovis* (Linnaeus, 1758) (Cepeda-Palacios and Scholl 2000).

*Lucilia eximia* (Wiedemann, 1891) (Diptera: Calliphoridae) is associated with animal remains that decay in urban environments, mainly the ones exposed to sunlight (Barros-Souza *et al.* 2012; Archer and Elgar 2003). This species is considered of medical and veterinary importance and it has been used in forensic entomology as a biological indicator of PMI (Moura *et al.* 1997).

*Lucilia eximia* is a cosmopolitan fly (Whitworth 2014). In the neotropics this species is found from the north of Mexico to south South America (Whitworth 2014), and had also been reported in forensic studies in Europe (Velásquez *et al.* 2010), in North America (Debry *et al.* 2013; Sanford *et al.* 2014), in Central America (Garcés *et al.* 2004; Calderón-Arguedas *et al.* 2005), and in South America (Rocha *et al.* 2010; Ramos-Pastrana and Wolff 2011; Barros-Souza *et al.* 2012; Urahy-Rodrigues *et al.* 2013; Ramos-Pastrana *et al.* 2014). The objectives of this research were to analyze the intra-puparial development of *Lucilia eximia*, and to describe chronological and morphological changes that occur during this stage.

## MATERIALS AND METHODS

This study was carried out at the Entomology Laboratory of the Universidad de la Amazonia, Florencia – Caquetá, Colombia.

About 1.600 mature larvae (L3) of *L. eximia* originated from the eggs laid by approximately 45 females specimens collected in the city of Florencia (Caquetá, Colombia) were reared and monitored into an incubation chamber (BOD) ( $25.46 \pm 1.21$  °C,  $93.31 \pm 2.41$  RH, 12:12 L:D) until they stopped feeding, became pink and began leaving the carcass. They were then placed into plastic containers with vermiculite from which 10 individuals were continuously sampled every three hours day-night (n=1000) until the emergence of adults. The sampled specimens were fixed in ethanol (96%), followed first by a treatment with Carnoy solution for 24 hours and then with formic acid (5%) for 48 hours. This chemical treatment, facilitated the removal of the puparium using the methodology proposed by Cepeda-Palacios and Scholl (2000), Pujol-Luz and Barros-Cordeiro (2012) and Barros-Cordeiro *et al.* (2014).

The pupae and adults obtained were killed with ethyl acetate in a killing jar. Some adults and all pupae were preserved in ethanol (96%). The remaining specimens were pinned and then used to confirm their species with the keys proposed by Vargas and Wood (2010) and Whitworth (2014), and the morphology of the pupae was described. Finally, the specimens were deposited in the Entomological Collection of the Universidad de la Amazonia (CEUAM).

### Intra-puparial development

To observe the development of the puparium and pupa, fixed puparia were dissected and the pupae extracted using a surgical scalpel under a binocular stereoscope (Leica EZ4, Leica Microsystems (Schweiz), Germany). All observations were documented and imaged using a photographic camera (Optika PRO 5 Digital Camera 4083.12, Italy) adapted to the stereoscope. In addition it is included the stage of pre-pupa, pupariation and the morphological description of the pupa and the beginning of the imago and emergence, according to Fraenkel and Bhaskaran (1973), Pujol-Luz and Barros-Cordeiro (2012) and Barros-Cordeiro *et al.* (2014)

The methodology used in this study was adapted from Cepeda-Palacios and Scholl (2000) and Pujol-Luz and Barros-Cordeiro (2012). To describe the morphology of the intra-puparial stages we used the methodology of Fraenkel and Bhaskaran (1973), Cepeda-Palacios and Scholl (2000) and Pujol-Luz & Barros-Cordeiro (2012).

This study was designed to analyze and describe the morphology of the intra-puparial development stages, because it is important to have the duration of each stage determined. To describe the period of time for each stage, the mean and

the standard error were obtained using the software Estimates version 8.0 for Windows (Colwell 2006).

## RESULTS

Intra-puparial development lasted  $302 \pm 3.81$  hours, and four stages were observed: Larvae-pupa apolysis, Cryptocephalic pupa, Phanerocephalic pupa and Pharate adult.

### Pre-pupa

Pre-pupa and Pupariation (lasted 80 hour). Before the pre-pupa stage the larvae stop feeding, became pink, and leave the carcass to find a substrate to pupate, being active for an additional period of six hours (Figure 1A). Subsequently, they buried into the substrate, and began the pre-pupae stage. In this stage the cephalic segment was retracted into the second segment, and the cephalopharyngeal skeleton remained immersed. The 12<sup>th</sup> segment undergoes invagination into the 11<sup>th</sup>, and this process continues up to the invagination of the 8<sup>th</sup> into the 7<sup>th</sup> segment, giving it a barrel shape, and the intestine is emptied (Figures 1B, 1C). The length is reduced to 25.53% of its starting length, and lasted  $80 \pm 1.5$  h (Table 1).

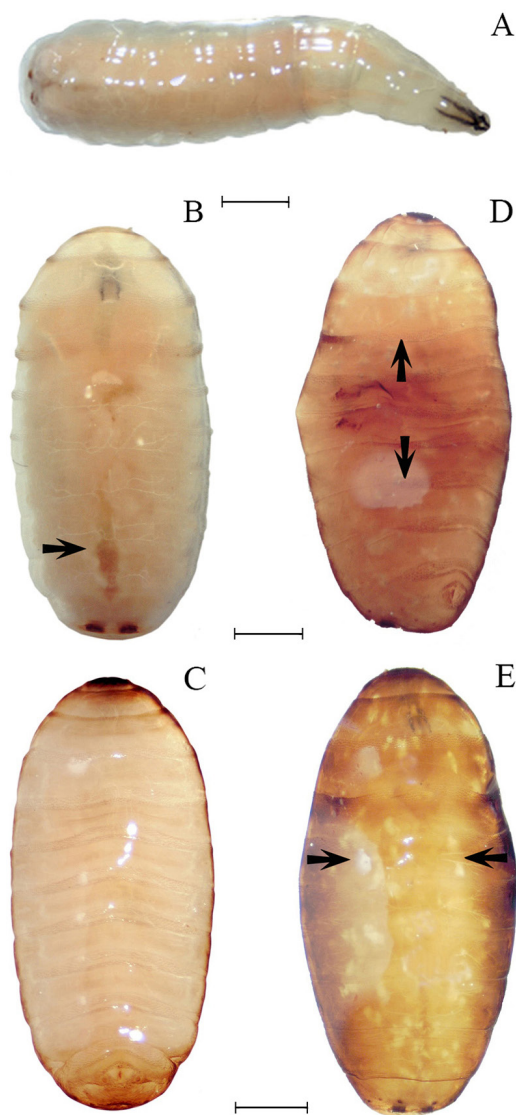
### Morphological description of the pupa

The pupa of *Lucilia eximia* possesses a typical coarctate form, between 6 mm long and 2.31 mm wide, with dark brown color. The fifth segment has a depression that marks the division of the head, and the mandible and maxilla are attached to the puparium. The top (ring 1) has a trapezoidal shape form (Figure 2A) and bears the anterior spiracles, with 6-8 buds each one (Figure 2B). The posterior ring has the spiracular plate, with two well pigmented spiracles, each one with three clefts, and a complete peritreme (Figure 2C).

### Stages of intra-puparial development

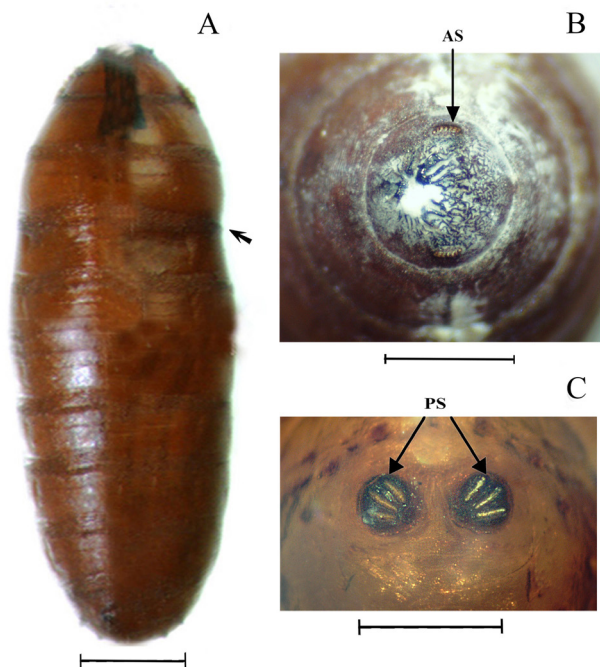
Larva-pupa apolysis – The darkening and hardening of the puparium begins from the lateral zone towards the middle area of the dorsal region, and from the middle zone of the ventral region towards the ends (Figure 1D), and then extending to the dorsal area (Figure 1E). During the first six hours, the epidermis was still attached to the puparium, which made it difficult its release. This phase lasts  $23 \pm 1.08$  h (Table 1).

Cryptocephalic pupa (observed at 23 to 28 h) – This stage is characterized by the secretion of the pupal skin and the complete separation of the epidermis from the puparium, which facilitates its extraction. Additionally, the head and the thoracic appendages are still invaginated (Figure 3A), the abdomen still has the aspect of the larva, and the head and thorax can not be differentiated in dorsal view (Figure 3B). All the structures of the cephalopharyngeal skeleton are found in the larva (Figures 3A and 3B), with the exception of the maxilla and mandible, which stay adhered to the puparium. This stage lasts  $5 \pm 0.53$  h (Table 1).



**Figure 1.** Morphological description of the pupariation process of *Lucilia eximia*. Post-feeding pink larva, in lateral view A. White puparium with empty intestine (arrow), in ventral view B. and dorsal view C. Larva pupa apolysis, darkening and hardening of the puparium (arrows) from the middle zone to the extremes, in ventral view D. Darkening and hardening of the puparium from the lateral zone of the dorsal region to the middle area (arrows), in dorsal view E. This stage lasts  $23 \pm 1.08$  h. PL: Pink Larvae. Scale bars: 1mm. This figure is in color in the electronic version.

Phanerocephalic pupa (observed at 28 to 120 h) – This period is characterized by the complete extroversion of the cephalic capsule, however the thoracic appendages continue invaginated (Figure 3C). Segmentation is visible and it is possible to identify the abdominal sternites and tergites, as well as the prothoracic spiracles; however, the specimen still retains a pupa aspect (Figure 3D). This stage lasts  $92 \pm 1.94$  h (Table 1).

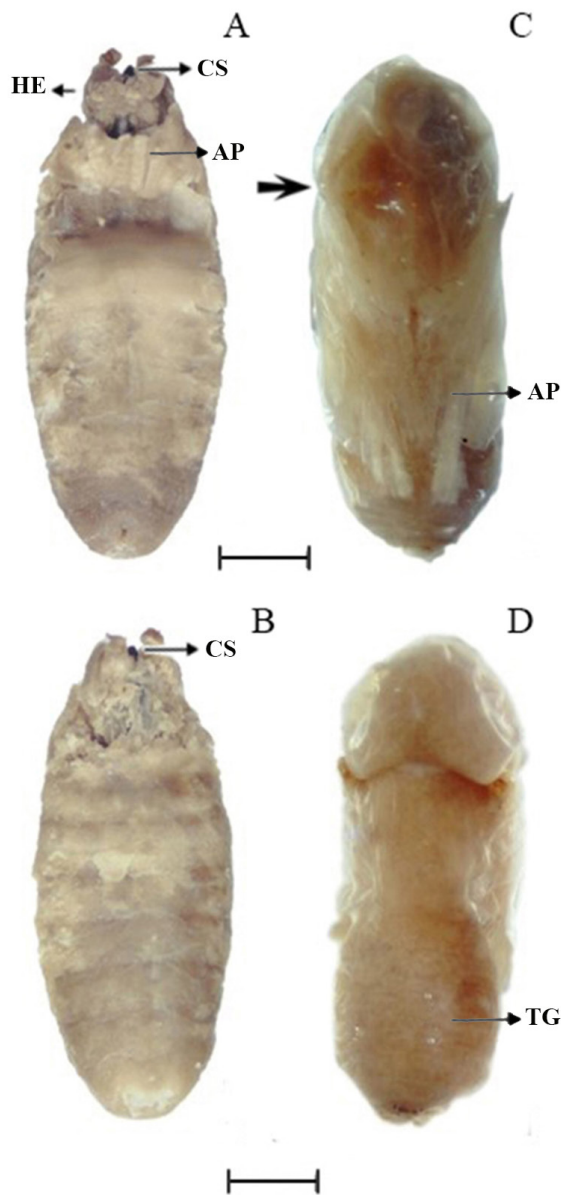


**Figure 2.** External morphology of the pupa of *Lucilia eximia*. Depression shows the division of the head (arrow) in a ventral view A. Anterior view of the ring two with anterior spiracles B. View of the posterior ring with spiracular plate C. AS: Anterior Spiracles, PS: Posterior Spiracles. Scale bars: A: 1mm; B and C: 0.5 mm. This figure is in color in the electronic version.

Pharate Adult – This stage is the longest in the intra-puparial development, which corresponds to the stage where adult maturation occurs and the individuals have an adult aspect. This stage is divided into four sub-stages, delimited according to the pigmentation of the compound eyes (Fraenkel and Bhaskaran 1973; Pujol-Luz and Barros-Cordeiro 2012):

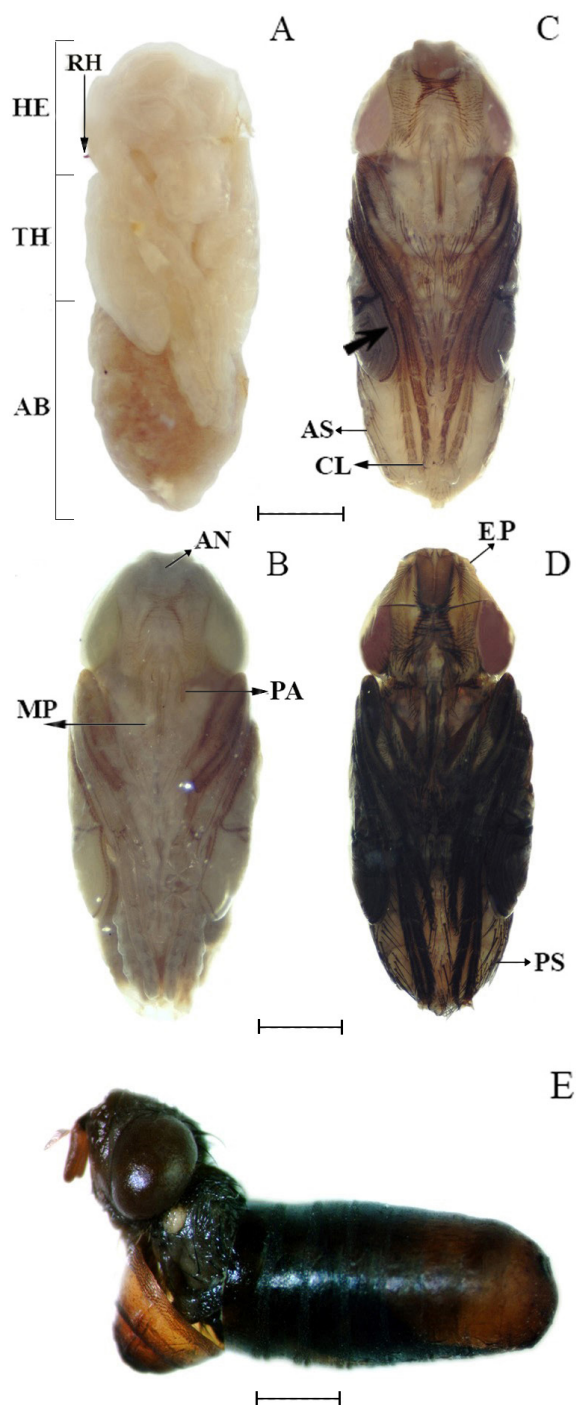
(1) Transparent eyes (observed at 120 to 245 h): The division of the body into its main tagma (head, thorax and abdomen) is evident. The respiratory horn with red pigmentation is visible, and at this phase starts the development of the mouthparts. Additionally, the legs and wings are differentiated, but the wings do not present their membranous condition (Figure 4A). This is the longest sub-stage lasting  $125 \pm 2.15$  h (Table 1).

(2) Yellow eyes (observed at 245 to 268 h): The terminalia is visible, and the antennae, vibrissae, palpi and mouthparts are fully developed. Bristles without pigmentation emerge on the edges, and bristles and setae start developing on the legs. The wings develop their primary venation and setae are visible on their margins. The thoracic and abdominal sutures are well-defined in this stage (Figure 4B). This stage last  $23 \pm 0.89$  h (Table 1).



**Figure 3.** Morphological description of the intra-puparial development of *Lucilia eximia*. Cryptocephalic pupa with head and the thoracic appendages are still invaginated, in ventral view A. The abdomen still with larval aspect, in dorsal view B. Phanerocephalic pupa with extroversion of the cephalic capsule (arrow) C. Differentiated thoracic segments and abdominal tergites D. CS: Cephalopharyngeal Skeleton, HE: Head, AP: Appendages, TG: Tergites. Cryptocephalic pupa:  $5 \pm 0.53$  h. Phanerocephalic pupa:  $92 \pm 1.94$  h. Scale bar: 1 mm. This figure is in color in the electronic version.

(3) Pink eyes (observed at 268 to 282 h): The development of the wings is completed and setae on the costal vein present black pigmentation. It is also possible to observe ocelli, spongy mouthparts, abdominal setae, head bristles, and well-developed claws (Figure 4C). It is the shortest sub-stage lasting  $14 \pm 0.73$  h (Table 1).



**Figure 4.** Morphological sequence of Pharate adult of *Lucilia eximia*, according to the pigmentation of the eyes. Transparent eyes A. Yellow eyes B. Pink eyes, with costal vein presenting black pigmentation (arrow) C. Red eyes D. Emergence of the adult E. HE: Head, TH: Thorax, AB: Abdomen, RH: Respiratory Horn, MP: Mouthparts, PA: Palpus, AN: Antennae, AS: Abdominal Setae, CL: Claws, AP: Arista Plumose, EP: Pigmented Sternites. Scale bar: 1 mm. Transparent eyes:  $125 \pm 2.15$  h. Yellow eyes:  $23 \pm 0.89$  h. Pink eyes:  $14 \pm 0.73$  h. Red eyes:  $20 \pm 0.60$  h. This figure is in color in the electronic version.

**Table 1.** Duration (in hours) of each stage of the intra-puparial developmental of the *Lucilia eximia*.

Stage	Phases	Beginning and ending of the phases (in hours)	Time Hours $\pm$ SE	Sample size (n)
Pre-pupa	Pupariation	80	$80 \pm 1.5$	260
	Larva-pupa apolysis	0 - 23	$23 \pm 1.08$	80
Pupa	Cryptocephalic pupa	23 - 28	$5 \pm 0.53$	20
	Phanerocephalic pupa	28 - 120	$92 \pm 1.94$	300
	Pharate adult (compound eyes)			
	Transparent eyes	120 - 245	$125 \pm 2.15$	420
	Yellow eyes	245 - 268	$23 \pm 0.89$	80
	Pink eyes	268 - 282	$14 \pm 0.73$	50
	Red eyes	282 - 302	$20 \pm 0.60$	50
Total time			302	1000

(4) Red eyes (observed at 282 to 302 h): The whole body is fully developed. The main feature is beginning of the development of the ptilinum, the wings with a membranous condition, the black mouthparts, and the plumose arista and visible genitalia. Furthermore, the setae, tergites and sternites are strongly pigmented and well defined (Figure 4D). This stage lasts  $20 \pm 0.60$  h (Table 1).

### Imago and emergence

After a period of  $299 \pm 1.5$  hours, it is possible to observe the imago inside of the puparium, with the ptilinum totally formed and expanded. Emergence of the adults ( $n=154$ ) (Figure 4E) started after  $302 \pm 3.81$  hours (Table 1).

### DISCUSSION

Four stages of intra-puparial development were identified, which is in agreement with previous reports (Pujol-Luz and Barros-Cordeiro 2012; Barros-Cordeiro *et al.* 2014; Cepeda-Palacios and Scholl 2000).

The larva-pupa apolysis stage in *L. eximia* lasts  $23 \pm 1.08$  h, which corresponds to 7.62% of the total time of intra-pupal development. This contrasts with the report of Pujol-Luz and Barros-Cordeiro (2012), where this same stage in *C. albiceps* lasted just 3 h when reared at  $26^\circ\text{C}$ , corresponding to 3% of the total development (Table 2).

Cryptocephalic pupa is the shortest stage ( $5 \pm 0.53$  h) in *L. eximia*, which corresponds to 1.66% of the total intra-puparial development time. Previously, Pujol-Luz and Barros-Cordeiro (2012) reported 3 h for this stage in *C. albiceps*.

**Table 2.** Duration (in hours) of each stage of the intra-puparial development and amount of time required up to the emergence of the adult (EAD) of eight species of Diptera. LPA larva-pupa apolysis, CPP Cryptocephalic pupa, PCP Phanerocephalic pupa, PHA Pharate adult. Adapted from Pujol-Luz and Barros-Cordeiro (2012).

Family	Species	T°C	LPA	CCP	PCP	PHA	EAD	References
Calliphoridae	<i>Lucilia eximia</i>	26,8	23	5	92	182	302	This study
Calliphoridae	<i>Calliphora erythrocephala</i>	24	–	–	24–25	–	80	Wolfe (1954)
Muscidae	<i>Musca domestica</i>	30	4	6–9	16–18	28	96	Frankel and Bhaskaran (1973)
Sarcophagidae	<i>Sarcophaga bullata</i>	24	20	20–28	46–48	168–192	–	Frankel and Bhaskaran (1973)
Oestridae	<i>Cuterebra tenebrosa</i>	24	120-144	–	–	–	1248	Baird (1975)
Oestridae	<i>Dermatobia hominis</i>	24	72–120	–	168–192	–	720	Lello <i>et al.</i> (1985)
Oestridae	<i>Oestrus ovis</i>	24	18	48	120	31	528	Cepeda-palacios and Sholl (2000)
Calliphoridae	<i>Chrysomya albiceps</i>	26	3	3–6	6–9	81	90	Pujol-Luz and Barros-Cordeiro (2012)

The phanerocephalic pupa stage lasted 92 hours, contrasting with the 3 hours reported by Pujol-Luz and Barros-Cordeiro (2012) for *C. albiceps*. At this stage the insect still has a pupal aspect, while Pujol-Luz and Barros-Cordeiro (2012) reported that, in *C. albiceps*, it is already possible to differentiate head, thorax and abdomen. At this stage in both species, it is possible to identify the prothoracic spiracles.

Pharate adult is the stage where *L. eximia* spends most of the intra-puparial development time (182 h). This data is in agreement with that observed by Pujol-Luz and Barros-Cordeiro (2012), who reported 81 h for *C. albiceps*. However, the starting point is different, being at 120 h for the current study, whereas it was at 9 h in the study by Pujol-Luz and Barros-Cordeiro (2012), which can probably be explained by the species belonging to different genera, beside the difference in relative humidity (RH) during their development, as the temperatures used in both studies were similar ( $25.46 \pm 1.21$  °C,  $93.31 \pm 2.41\%$  RH and  $26 \pm 1.0$  °C,  $60 \pm 10\%$  RH, respectively).

During the pharate adult stage, we were able to identify four sub-stages according to the pigmentation of the eyes. Previously, the same number of sub-stages were reported for *C. albiceps* (Pujol-Luz and Barros-Cordeiro 2012), but the starting times and length of each sub-stage differed from the current study: 1) transparent eyes 39.74-81.13% of the total time (against 10–23% in *C. albiceps*); 2) yellow eyes 81.14–88.85% (22–66% in *C. albiceps*); 3) pink eyes 88.93–93.57% (67–73% in *C. albiceps*) and 4) red eyes 93.67–100% (74–100% in *C. albiceps*). These discrepancies also may be due to the species belonging to different genera and to the different relative humidity conditions during their development.

*Chrysomya albiceps* is an Afrotropical species introduced in South America. It is considered the most abundant species in urban and rural zones (Barros-Souza *et al.* 2012). During its larval stage it is a facultative predator of eggs and larvae of other dipterans (Rosa *et al.* 2006). The features mentioned above can make *C. albiceps* responsible for the decrease of native species such as *L. eximia*, due to the interspecific competitions when these species are simultaneously present in a carcass. As a survival mechanism, *L. eximia* could have developed different colonization strategies, which would explain the different behavior when compared to *C. albiceps*.

Since the behavior and intra-puparial development time of *L. eximia* and *C. albiceps* are different, it is possible to state that when a person is trying to solve a cases involving death with long time of decomposition times, where the only entomological evidences are empty pupae and puparia of *L. eximia* and *C. albiceps*, the PMI estimation has to be calculated differently, as the PMI for each species is different.

## CONCLUSION

One of the most common species of blow flies at the crime scenes in Colombia is *L. eximia*. Thus, this species has great importance and potential in determining the postmortem intervals. This study provided pupa development data of *L. eximia* for determining PMI. This species presented a relatively longer period of intra-puparial development than *C. albiceps*, which is very relevant for an accurate assessment of the postmortem interval.

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