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A study of the pupal development of *Calliphora vomitoria* (L., 1758) (Calliphoridae: Diptera) at different temperatures

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Abstract: In this study, the pupal development process of the *Calliphora vomitoria* (L. 1758), widely used in the estimation of postmortem time in forensic entomology, was examined. In the last decade, studies of the development periods in the pupal stage and the development times of these periods have increased around the world and specifically in Turkey. In this study, the aim was to detect the pupal development periods of the *Calliphora vomitoria* via a process of hourly pupal dissection, a process that would be the only one of its kind in the literature for this species. This method was repeated under three different temperatures. Adult emergence from the pupae was observed at hours 501–504, 312–314, and 208–210 at 15 °C, 20 °C, and 25 °C, respectively. Nine new development periods, not specified in other studies, were detected with this study, thus producing new attained results for our country and the science world.

Key words: Calliphoridae, forensic entomology, pupal development, developmental biology, Turkey

1. Introduction

When death occurs, one of the first questions that immediately comes to mind is the answer to what the date of death is. For answering this question, forensic entomology has been of great benefit in many countries (Benecke, 2001). A minimum postmortem interval (PMImin) estimation can be conducted by examining the life cycles of some insect groups, which show holometabolous metamorphosis after they infest the corpse and by determining the development periods of these examined periods at certain temperatures. The use of insects in a forensic system is based on the fact that they lay their eggs in the corpse minutes after death and are present at every stage of decomposition. In addition, some species are peculiar to a certain region and season (Carvalho et al., 2000). Some species reach the corpse less than 5–10 min after death, some come during the active decomposition stage, and others arrive during the dry skin and bone stages. Insects continue to colonize until there is nothing to consume (Goff, 2001; Anderson, 2010; Şabanoğlu and Sert, 2010).

Flies, especially bottle flies, are generally the first and the most important forensic insect species that come to the cadaver (Arnaldos et al., 2005; Byrd and Castner, 2010). The development stages of the flies occur in a certain order and at certain time periods. This development time may change depending on nutrition conditions and the temperature of the environment. When the life span of a fly existent in any period of this development stage in the scene is detected, the death time will also have been detected (Hall, 1990). In particular, the larval stage provides concrete evidence for making an estimate of a time of death (Adams, 2003). The larval stage is a period in which morphological structures can be externally detected, and by considering these data, it becomes possible to estimate postmortem time. The pupal stage in carrion flies may take as much as the total of the previous stages or longer (Greenberg and Kunich, 2002). Although the pupal stage forms 50% of the immature development process (Zehner et al., 2009), since pupa is like a black box, forensic studies (Voris, 1939; Mandeville, 1988; Haskell, 1990; Tantawi and Greenberg, 1993; Carvalho et al., 2000; Grassberger and Reiter, 2002; Greenberg and Kunich, 2002; Amendt et al., 2004; Anderson, 2005; Gennard, 2007; Byrd and Castner, 2010; Goff, 2010) have been focused more heavily on larva (Greenberg and Kunich, 2002). Development in the pupal stage is a metamorphic change that occurs without any growth. The pupal time (age) may be assessed in a useful way for the first 10 h due to the fact that the color of the puparium changes from white to dark brown during this period. (Grenberg and Kunich, 2002). However, because the color no longer changes after 10 h, it has no impact on the determination of the pupal age after that time (Amendt et al., 2004). Convenient methods developed for

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age calculation in the pupal stage that forms most of the development may be important tools in entomologically determining the time that has passed since death (Zehner et al., 2006).

Although studies of the pupal development period have recently increased, there are still a limited number. Hitherto the pupal development of following species have been determined: *Phormia regina* (Meigen, 1826; Greenberg and Kunich, 2002), *Calliphora vicinia* (Robineau-Desvoidy, 1830; Brown, 2012; Brown et al., 2015; Zajak and Amendt, 2015; Pasquerault et al., 2013), *Lucilia sericata* (Meigen, 1826; Karabey and Sert, 2018; Zajak and Amendt, 2015; Pasquerault et al., 2013), *Chrysomya putoria* (Wiedemann, 1830; Proença et al., 2014), *Chrysomya rufifacies* (Macquart, 1843; Ma et al., 2015), *Chrysomya albiceps* (Wiedemann, 1819; Ergil, 2012; Pujol-Luz and Barros-Cordeiro, 2012), *Calliphora vomitoria* (Linnaeus, 1758; Ergil, 2012; Pasquerault et al., 2013), *Cochliomyia macellaria* (Fabricius, 1775), *Lucilia cuprina* (Wiedemann 1830; Barros-Cordeiro et al., 2016), *Lucilia eximia* (Wiedemann 1819; Ramos-Pastrana et al., 2017), *Chrysomya megacephala* (Fabricius, 1784; Sinha and Mahato, 2018), and *Sarcophaga argyrostoma* (Robineau-Desvoidy, 1830; Örsel, 2016; Sert et al., 2020).

In one study in the literature (Pasquerault et al., 2013), nine periods were specified for the pupa of the dissected *Calliphora vomitoria*. Pupal development was examined at 24 °C temperature and at 6-h intervals in the study, and the development periods were expressed as the percentage of the total pupal development period.

When the pupal studies were considered, it was seen that there were clear differences in terms of their methods as dissection intervals. Ames et al. (2006) and Zehner et al. (2009) conducted the molecular studies to determine the pupal age of the *Calliphora vicina*. For the purpose of developing postmortem time estimations, Richards et al. (2012) used three-dimensional microcomputerized tomography (micro-CT) on the pupae belonging to the *Calliphora vicina* in their study. However, applied gene expression and imaging methods are too expensive and difficult (Villet and Amendt, 2011). The study conducted by Agrel and Lundquist (1973) in which the pupal period was generally separated into periods without any specification of genera or species was the first study conducted on this subject. Sivasubramanian and Biagi (1983) examined the morphology of the pupal periods of the *Sarcophaga bullata* at a temperature of 25 °C and separated the pupal stage into nine periods. In the study conducted by Finell and Jarvilehto (1983) on *Calliphora erythrocephala* (=*C. vicina*), the pupal stage was separated into seven periods. Greenberg (1991) examined the pupal development of *Phormia regina* at 22 °C and 29 °C and detected 11 periods. There were few further studies until Zajac and Amendt (2009) examined the pupal development periods of *Calliphora vicina* and *Lucilia sericata* in terms of morphology and histology. While Pujol-Luz and Barros-Cordeiro (2012) examined the pupal development periods of *Chrysomya albiceps*, Richards et al. (2012) examined *Calliphora vicina* with the methods of micro-CT. Brown et al. (2015) examined *C. vicina* and isolated 23 periods. Afterwards, Ma et al. (2015) examined the pupal development periods and morphological changes of *Chrysomya rufifacies* at four different temperatures. Örsel (2016) examined the development of the larva and pupa stages of *Sarcophaga argyrostoma* at 28 °C and 32 °C, and the periods of the formation of the checkered pattern in the abdomen of the pupa and the formation of longitudinal bands in the thorax were detected for the first time. Karabey and Sert (2018) examined the pupal development of *Lucilia sericata* at three different temperatures and reported 18 periods. Sert et al. (2020) examined the pupal development of *Sarcophaga argyrostoma* at three different temperatures and reported 23 periods.

Perhaps the most important issue in the determination of pupal development periods is the pupal dissection hour interval. Being able to understand which morphological structure develops at what point in pupal development requires dissecting the pupa at very short time intervals and recording the development. As per the methodology of this study, conducting pupal dissection 24 times a day, once per hour, provided the opportunity for the precise detection of the pupal development and estimation of PMImin in a precise way. Regarding the dissection periods of various studies, Pujol-Luz and Barros-Cordeiro (2012) conducted dissection once per 3 h on the first day and once per 6 h on the remaining days, Brown et al. (2015) and Pasquerualt et al. (2013) once per 6 h, Ma et al. (2015) once per 8 h, Zajac and Amendt (2012) once per 24 h, and Richards et al. (2012) once per 72 h. The only studies in the literature in which dissection was conducted 24 times a day on an hourly basis were Ergil (2012), Örsel (2016), Karabey and Sert (2018) and Sert et al. (2020) (Table 1).

Although the development rate of an insect used as evidence for assigning minimum postmortem interval (PMImin) may depend on many factors (season, weather condition, drugs and poisons in the body, geographical area, etc.), temperature is seen as the most important. An insect's development generally accelerates within the optimum development range when the temperature increases and slows down when it decreases (Anderson, 2000; Byrd and Castner, 2010; Dourel et al., 2010; Damos et al., 2012). Within the frame of this linear relationship, the thermal requirements necessary for a species to reach certain development periods at a certain temperature are called a thermal constant. This constant, composed of units labelled degree day or degree hour, can be expressed as the total of the temperatures on the lowest

Table 1. Dissection periods and development temperatures of different studied species according to different researchers.

	Species	**Pupal sampling period**	**Temperature**
Present study	*Calliphora vomitoria*	1 h	15, 20, 25 °C
Zajac and **Amendt, 2012**	*Calliphora vicina* *Lucilia sericata*	24 h	25 °C
Pujol-Luz and **Barros-Cordeiro, 2012**	*Chrysomya albiceps*	3 h/6 h	26 ± 1 °C
Davies and **Harvey, 2013**	*Calliphora vicina* *Lucilia sericata*	24 h	22 °C
Pasquerault et al., 2013	*Calliphora vicina* *Calliphora vomitoria* *Lucilia sericata*	6 h	22–24 °C
Brown et al, 2015	*Calliphora vicina*	6 h	22 °C
Ma et al., 2015	*Chrysomya rufifacies*	8 h	20, 24, 28, 32 °C
Martín-Vega et al., 2017	*Calliphora vicina*	6 h	24 ± 0.8 °C
Salazar-Souza et al., 2018	*Chrysomya albiceps*	2 h (for the first 12 h) 12, 6, 18, 6, 18, 6, 18, 3 h (until adult emergence)	28°C day/26 °C night
Örsel, 2016	*Sarcophaga argyrostoma*	1 h	28, 32 °C
Karabey and **Sert, 2018**	*Lucilia sericata*	1 h	20, 25, 30 °C
Sert et al., 2020	*Sarcophaga argyrostoma*	1 h	20, 25, 30 °C

development threshold (basal temperature) of the insect and the multiplications of the development periods [ADD (accumulated degree days)/ADH (accumulated degree hours)] (Anderson, 2000; Reibe et al., 2010; Damos et al., 2012).

In this study, the pupal development periods, development times, and thermal values of the *Calliphora vomitoria* necessary for the development of the species were detected and photographed at different temperatures: 15 °C, 20 °C, and 25 °C (Table 2, Figures 1–25). Different from the 11 development periods determined by Greenberg and Kunich (2002) regarding this species, nine new development periods and times belonging to these periods were also detected in this study.

2. Materials and methods

Within the scope of this study, the *Calliphora vomitoria* collected from cattle livers left in various areas of Beytepe Campus at morning, midday, and evening hours were examined. Experimental studies were conducted in the Biocriminal Entomology Laboratory of the Biology Department at Hacettepe University between 2010 and 2012. A Leica MZ 16 A binocular stereoscopic microscope and imaging system were used for the identification of the species, pupa examination, and photographs; and the Sanyo MIR-253 cooling incubator was used for pupa development.

The samples were identified using the adult diagnosis keys in Greenberg and Kunich (2002) and the keys in Zumpt (1965), and, afterwards, they were placed in cages and were allowed to develop in the lab environment.

The optimum development temperature of the species is around 20–22 °C (Greenberg and Kunich, 2002). For this reason, one degree lower and one degree higher than this temperature were selected with the optimum temperature and human degree intervals (±5 °C) while determining the pupal development temperatures. The pupal development of the species was examined for all three temperatures every hour from the prepupa stage accepted as zero to the adult stage, and the hourly developmental differences were detected.

Adult flies were kept 60 per cages measuring 10 × 10 × 10 cm during the breeding experiments. Cotton saturated with sugar and milk powder mixture prepared with water and cattle liver were added to the cages as food source. The milk powder and sugar mixture were prepared at a ratio of 50%–50% (Byrd and Castner, 2001). Females laid approximately 180 eggs. After emerging, the first instar larvae were placed in groups of 50 in an 8 cm × 10 cm box filled with sawdust. One hundred grams of liver was provided for the 50 larvae (Ireland and Turner, 2005; Goff, 2010).

The collected pupae were randomly selected each hour and killed by boiling them in water for 30 s; they were

Table 2. Pupal development periods and range of the beginning times (minimum and maximum) of these periods of *Calliphora vomitoria* at 15 °C, 20 °C and 25 °C. ADH values were calculated using 3 °C as base temperature for *C. vomitoria* according to Marchenko, 2001. Newly identified stages indicated with asterisk (*).

Stage	15 °C	15 °C ADH	20 °C	20 °C ADH	25 °C	25 °C ADH
Prepupae	0th–1st h	0–12	0th–1st h	0–17	0th–1st h	0–22
Cryptocephalic pupae	40th–41st h	480–492	20th–21st h	340–357	8th–9th h	176–198
***Emergence of respiratory horns**	41st–45th h	492–540	21st–24th h	357–408	9th–11st h	198–242
***Divergence of respiratory horns**	54th–57th h	648–684	33rd–35th h	561–595	27th–28th h	594–616
Phanerocephalic pupae	66th–68th h	792–816	43rd–45th h	731–765	29th–30th h	638–660
***Stigma become evident**	70–73rd h	840–876	48th–49th h	816–833	32nd–33rd h	704–726
The beginning of pupal-adult ecdysis	77th–80th h	924–960	50th–52nd h	850–884	33rd–35th h	726–770
The completion of pupal-adult ecdysis	99th–103rd h	1188–1236	62nd–65th h	1054–1105	41st–43rd h	902–946
Segmented abdomen	110–114th h	1320–1368	71st–73rd h	1207–1241	49th–50th h	1078–1144
***Formation of a depression on the middle of the abdomen**	131st–134th h	1572–1608	88th–91st h	1496–1547	60th–62nd h	1320–1364
***Formation of a longitudinal band on the middle of the eye**	174th–179th h	2088–2148	112nd–116th h	1904–1972	82nd–85th h	1804–1870
Pigmentation of the eye's posterior	220–224th h	2640–2688	122nd–125th h	2074–2125	90th–92nd h	1980–2024
Pigmentation of the entire eye	231st–233rd h	2772–2796	131st–133rd h	2227–2261	99th–101st h	2178–2222
***Ocel eyes become evident**	257th–259th h	3084–3108	144th–145th h	2448–2465	119th–120th h	2618–2640
***Eyes become pinkish red color**	282nd–285th h	3384–3420	170th–173rd h	2890–2941	145th–147th h	3190–3234
Emergence of bristles on the head and thorax	347th–349th h	4164–4188	205th–207th h	3485–3519	146th–148th h	3212–3256
Emergence of bristles on the abdomen	376th–378th h	4512–4536	228th–232nd h	3876–3944	157th–159th h	3454–3498
***Tube like structure under respiratory horns**	424th–429th h	5088–5148	293rd–295th h	4981–5015	187th–188th h	4114–4136
***Shedding of pupal cuticle on pharate adult**	499th–500th h	5988–6000	310th–312nd h	5270–5304	206th–207th h	4532–4554
Adult emergence	501st–504th h	6012–6048	312nd–314th h	5304–5338	208th–210th h	4576–4620

Figure 1. Prepupae.

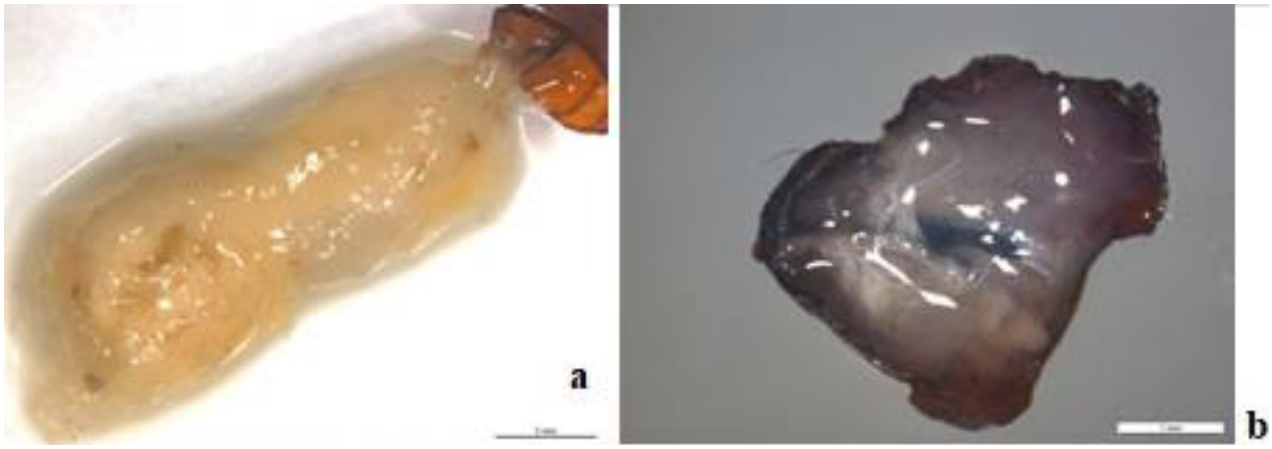

Figure 2. a) Pupae in viscous form, b) attached pharyngeal skeleton to the puparium.

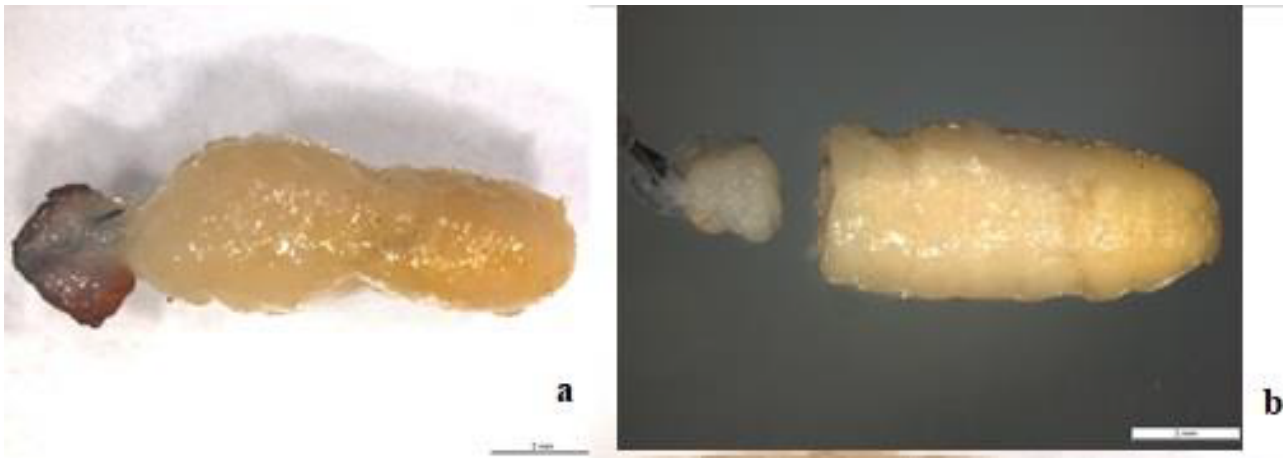

Figure 3. a) Ventral view of the nonviscous pupa attached to the pharyngeal skeleton at the anterior end, b) dorsal view of the dissected pupae in two pieces.

Figure 4. Dorsal view of the Cryptocephalic pupae.

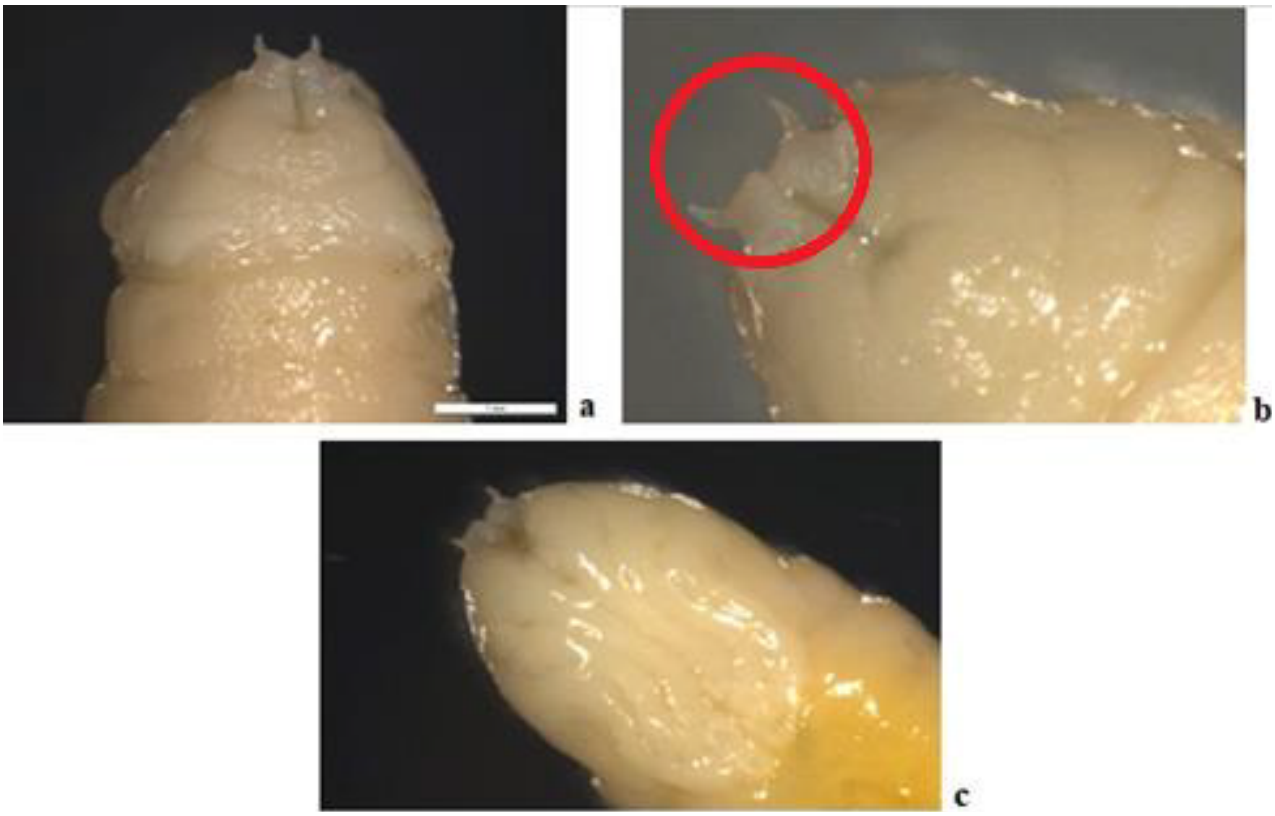

Figure 5. a and b) Dorsal view of the respiratory horns, c) ventral view of leg and wing buds.

dissected immediately after death (Brown et al., 2015). At least five individuals were dissected each hour. However, this number was increased to be able to correctly detect the transition periods. In total, 10,780 pupae were dissected during the study.

The puparia were carefully cut, the pupae were taken out, and the development periods were examined. Detailed photos of the pupae were taken in the dorsal, ventral, and lateral directions. Pupae were stored by placing them in 80% ethyl alcohol along with their puparia after these processes.

3. Results

The pupal development periods of *C. vomitoria* and the intervals of the beginning times of these periods (maximum and minimum) were separately given for 15

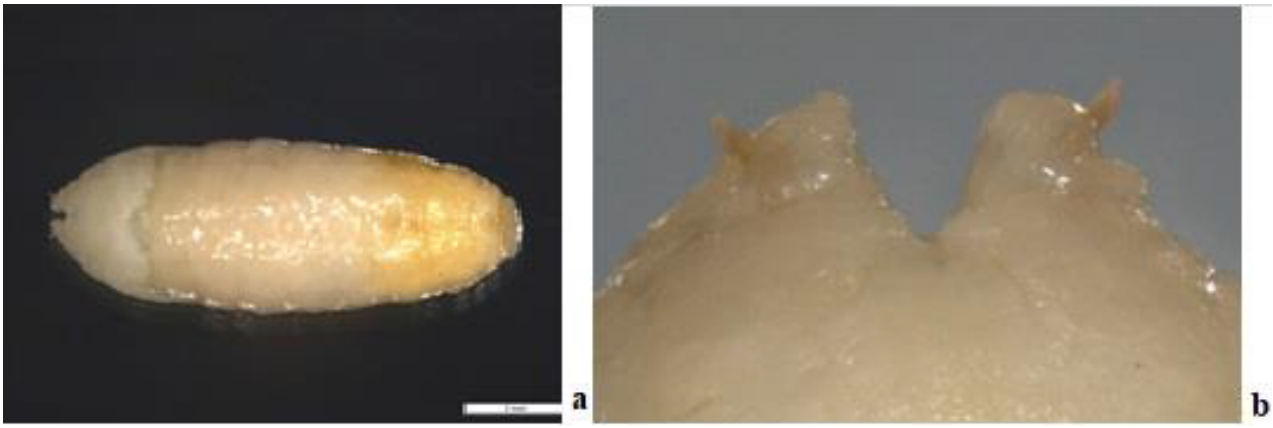

Figure 6. a) Nonadjacent respiratory horns, b) enlarged view of the respiratory horns.

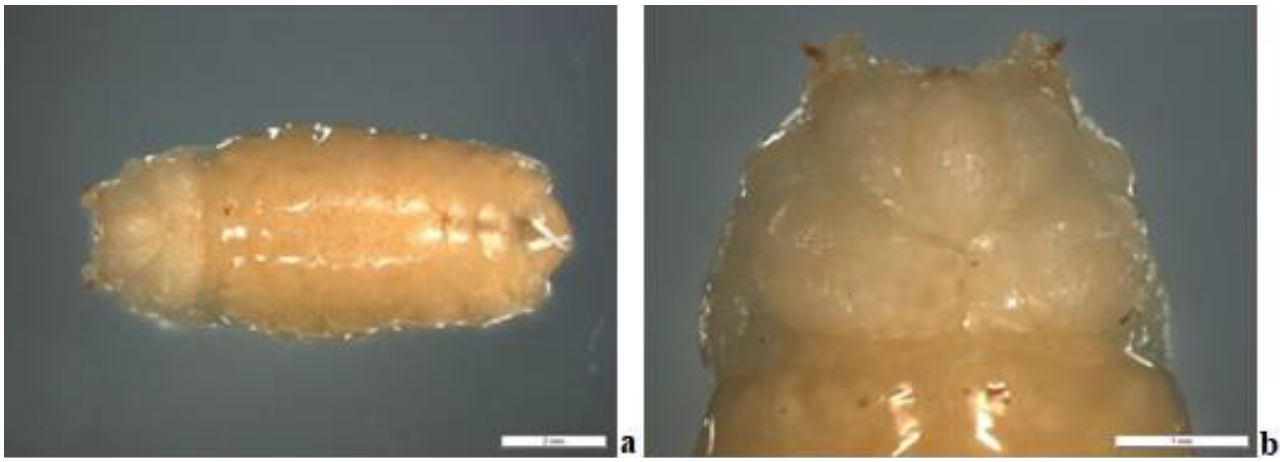

Figure 7. a) Straight shape of the area between respiratory horns, b) the protuberance behind the respiratory horns.

°C, 20 °C, and 25 °C (Table 2). The ADH values calculated by using the basal temperature taken from Marchenko (2001) are also given in Table 2. Because the morphological changes observed in the pupa were the same, photos were given only for 15 °C (Figures 1–24).

Pupal period results at 15 °C

Prepupae 0 h

Since this hour is the starting period of the transition to pupae, it is called the prepupa. Puparium was soft because it was white and had not been hardened completely (Figure 1).

Hours 1–4

When the puparium was cut, the pupae coming out of it was in viscous form. In the pupae, the part that will form the head at the anterior end was bound to the pharyngeal skeleton, and the posterior part was bound to the puparium from the place where there were posterior stigmas. The pharyngeal skeleton was visible in the anterior (Figure 2).

Hours 5–39

Pupa was not liquid and viscous at hour five and afterwards as in the first four hours. Because the part in the anterior was still tightly bound to the puparium, it broke when the puparium was pulled out. For this reason, the structure could not be taken out as a whole when the pupae was dissected, but it could be seen in two pieces. The pharyngeal skeleton of the pupae within these hours was less visible from the outside as time passed. Further, when the pupae was observed from the ventral side, yellow coloration was visible in some regions (Figure 3).

Hours 40–41

The pupae was no longer adjacent to the puparium at this hour. In other words, both the anterior and posterior ends were separated from the outer cover of the pupae. Therefore, the pupae could be completely taken out as a whole when dissected. This period is called the Cryptocephalic pupae. Adult structures including the head, thorax, and abdomen were not visible yet. The pharyngeal skeleton was not seen from the anterior end. The pupae had an appearance reminiscent of larval segments in a narrowing situation towards the posterior (Figure 4).

Hours 41–45

There were respiratory horns at the anterior end that were in the same color as the pupae. The wing and leg buds

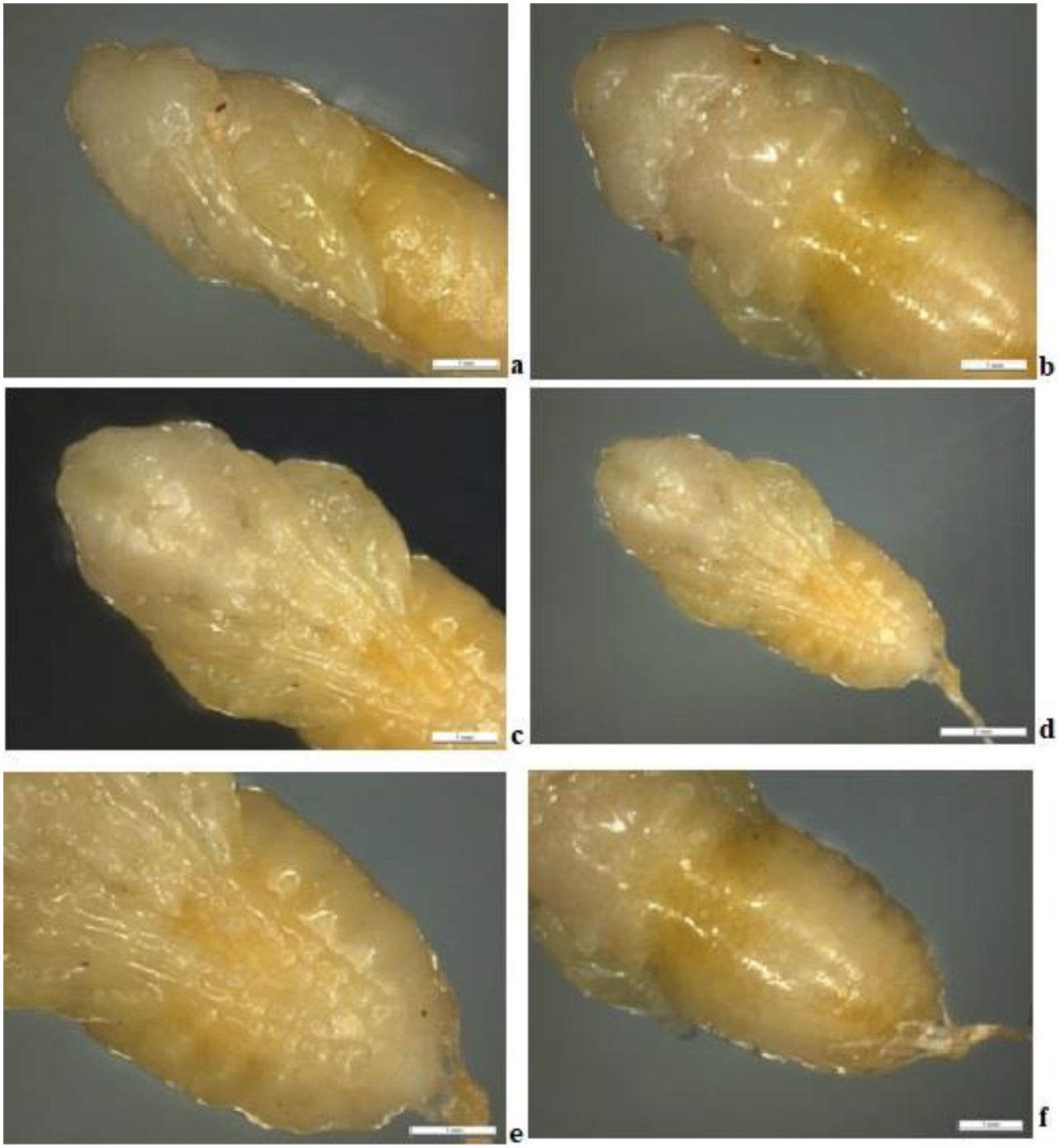

Figure 8. Formation of the head, thorax and abdomen and withdrawn of the respiratory horns anterolaterally to the back of the compound eyes a) lateral view, b) dorsal view, c) ventral view, wings, legs and segmented abdomen, d and e) ventral view, f) dorsal view.

became visible below the pupal skin. The legs reached the second and third segment border of the segment-like rings in the anterior (Figure 5).

Hours 54–57

Respiratory horns started to withdraw backwards, they were no longer adjacent, and there was space increasing between them over time (Figure 6).

Hour 65

Respiratory horns had withdrawn to the anterolateral sides. The area between the horns started to turn into a straight shape from an ovoid shape. There was a protuberance behind the horns in the middle. This image is an indicator that adumbrates that the head is close to everting (Figure 7).

Figure 9. The reduction of the lines on the abdomen and the stigmas a) dorsal view, b) ventral view.

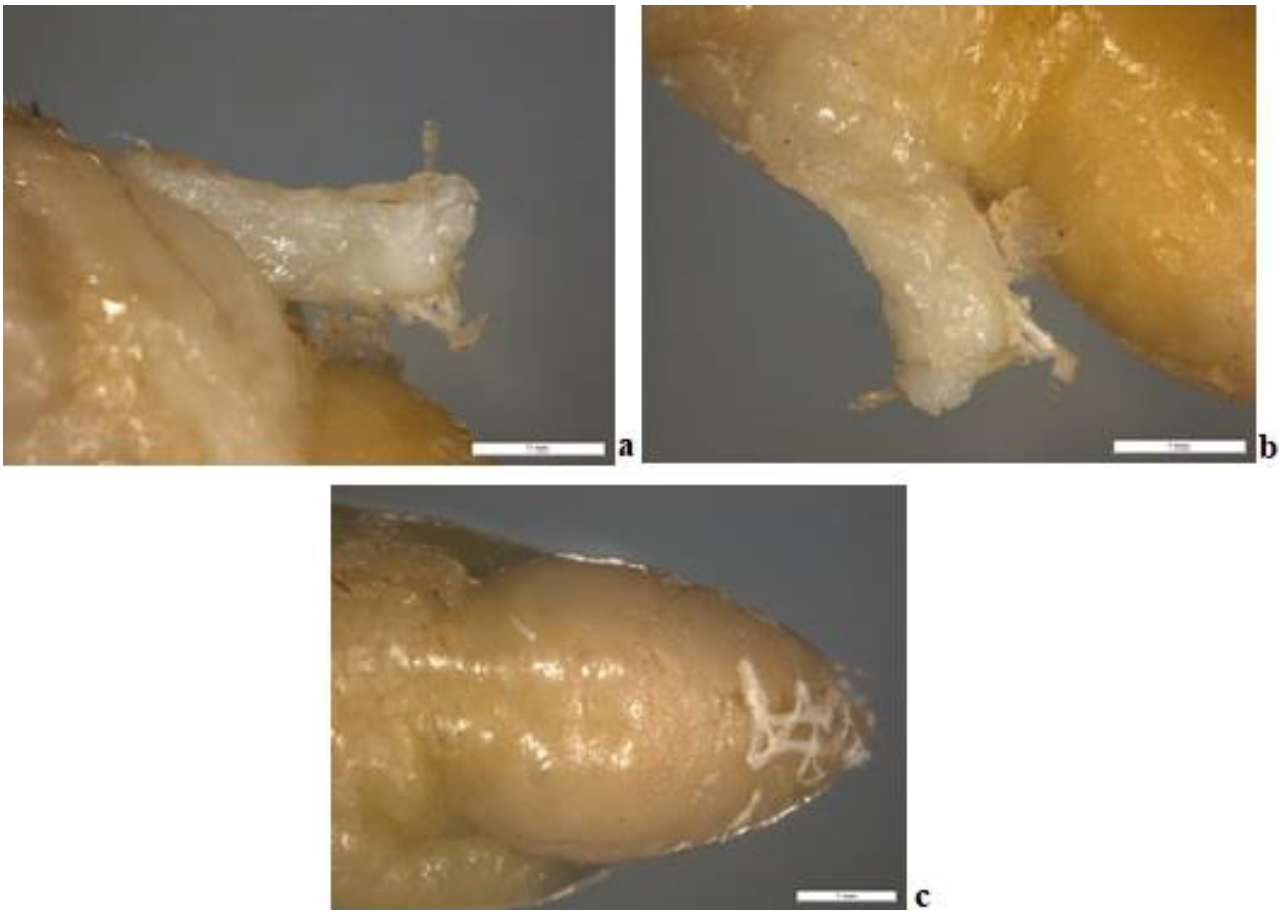

Figure 10. The view of the shed pupal skin a) ventral, b) dorsal, c) remnant of the shed skin at the end of the abdomen.

Hours 66–68

In the phanerocephalic pupal period, the respiratory horns had been withdrawn near the anterior stigmas behind the eyes. The eyes had emerged above the head. The head, thorax, and abdomen borders were clear. The wings and legs had extended towards the posterior end. There were stripes reminiscent of larva segments in the ring structure on the abdomen. Moreover, a yellowish coloration was visible on the abdomen and thorax border (Figure 8).

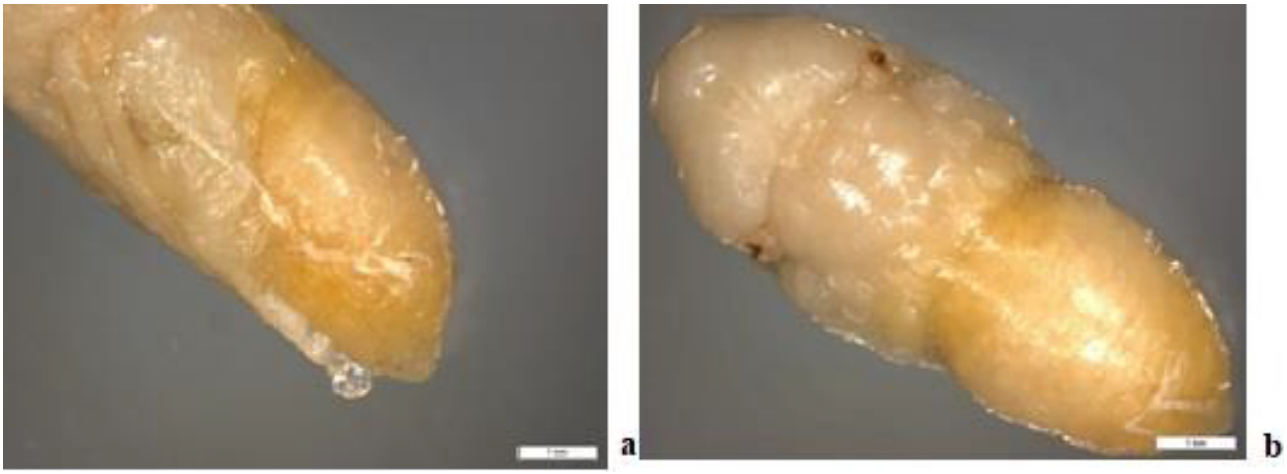

Figure 11. The view of pupal cuticle a) lateral view, b) dorsal view.

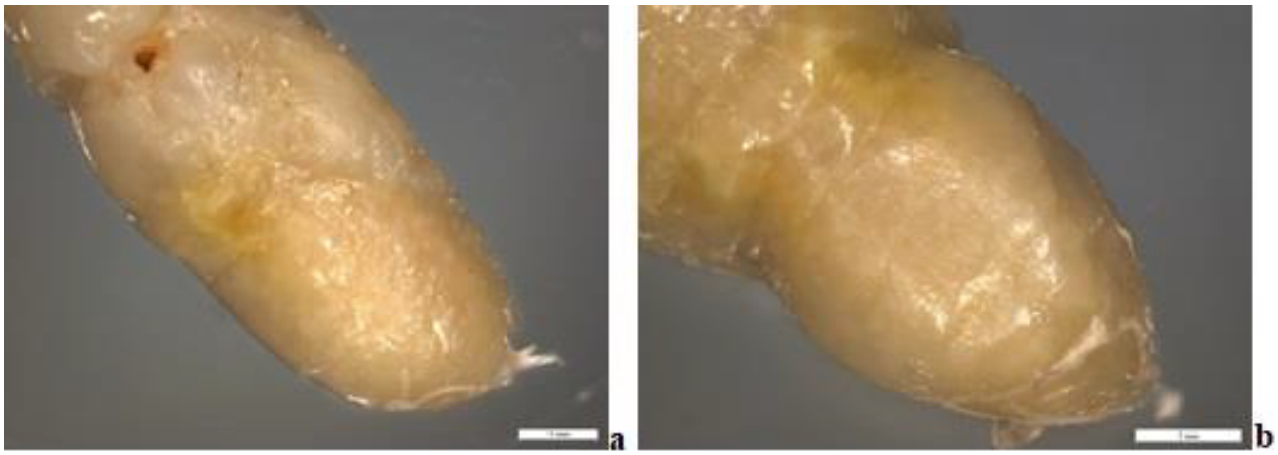

Figure 12. Borders of the abdominal segments a) lateral view, b) dorsal view.

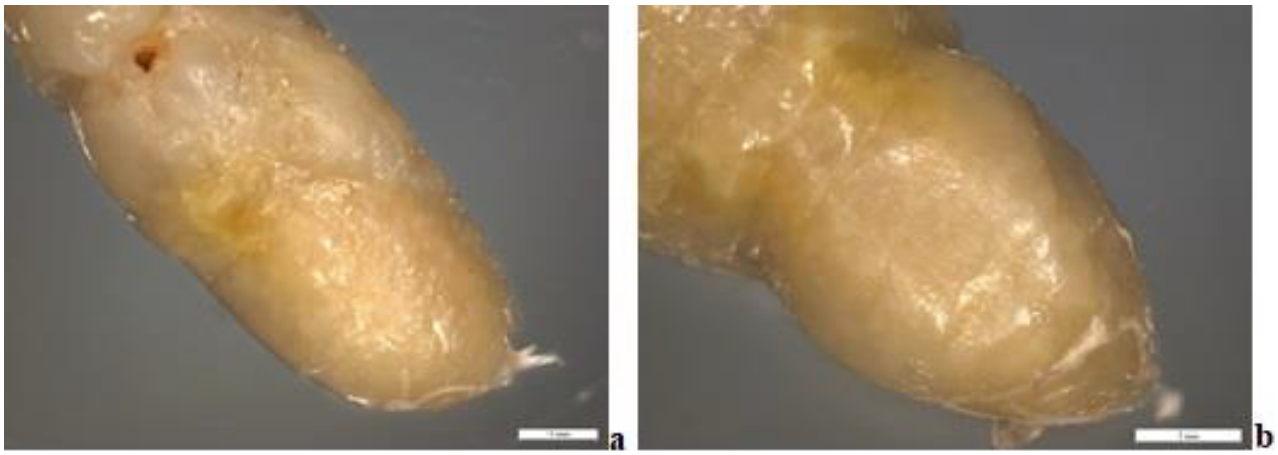

Figure 13. The dorsal view of depression formed in the middle of the abdomen.

Figure 14. Beginning of the pigmentation of eyes' posterior.

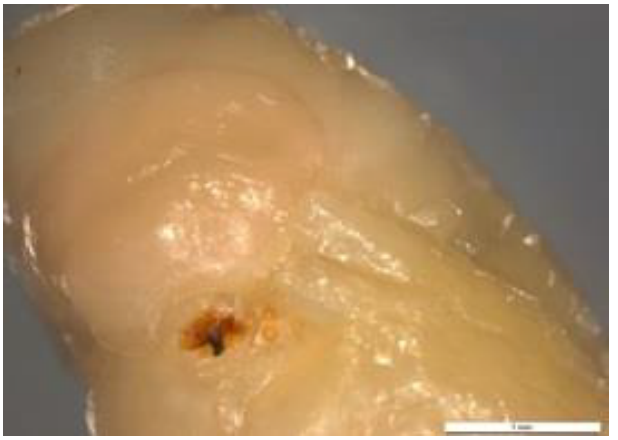

Figure 15. Pigmentation of the entire eye.

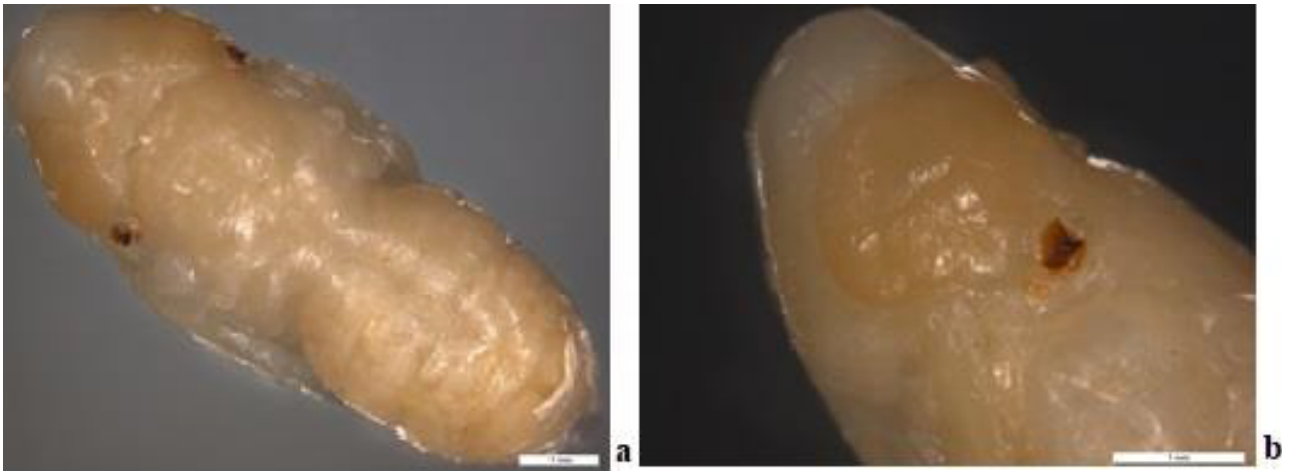

Figure 16. Pigmentation of the eyes a) dorsal view, b) lateral view.

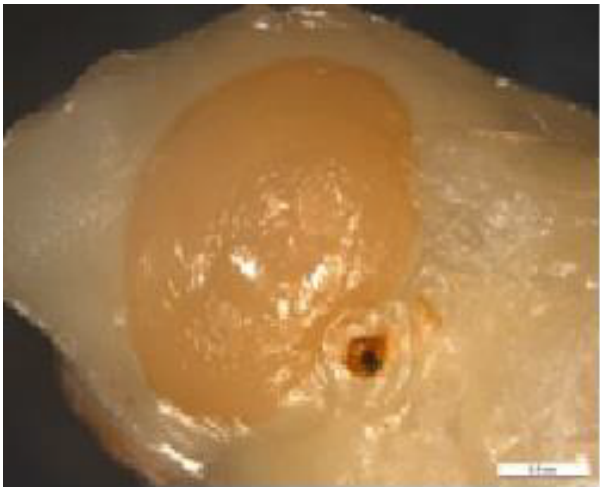

Figure 17. Darkness of the eye's border.

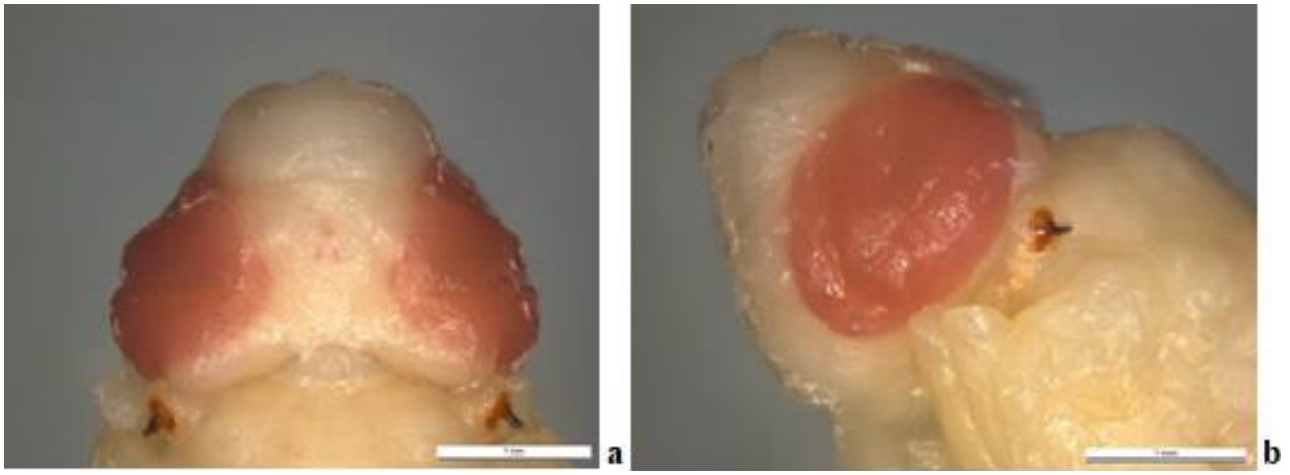

Figure 18. Color of the eyes a) dorsal view, b) lateral view.

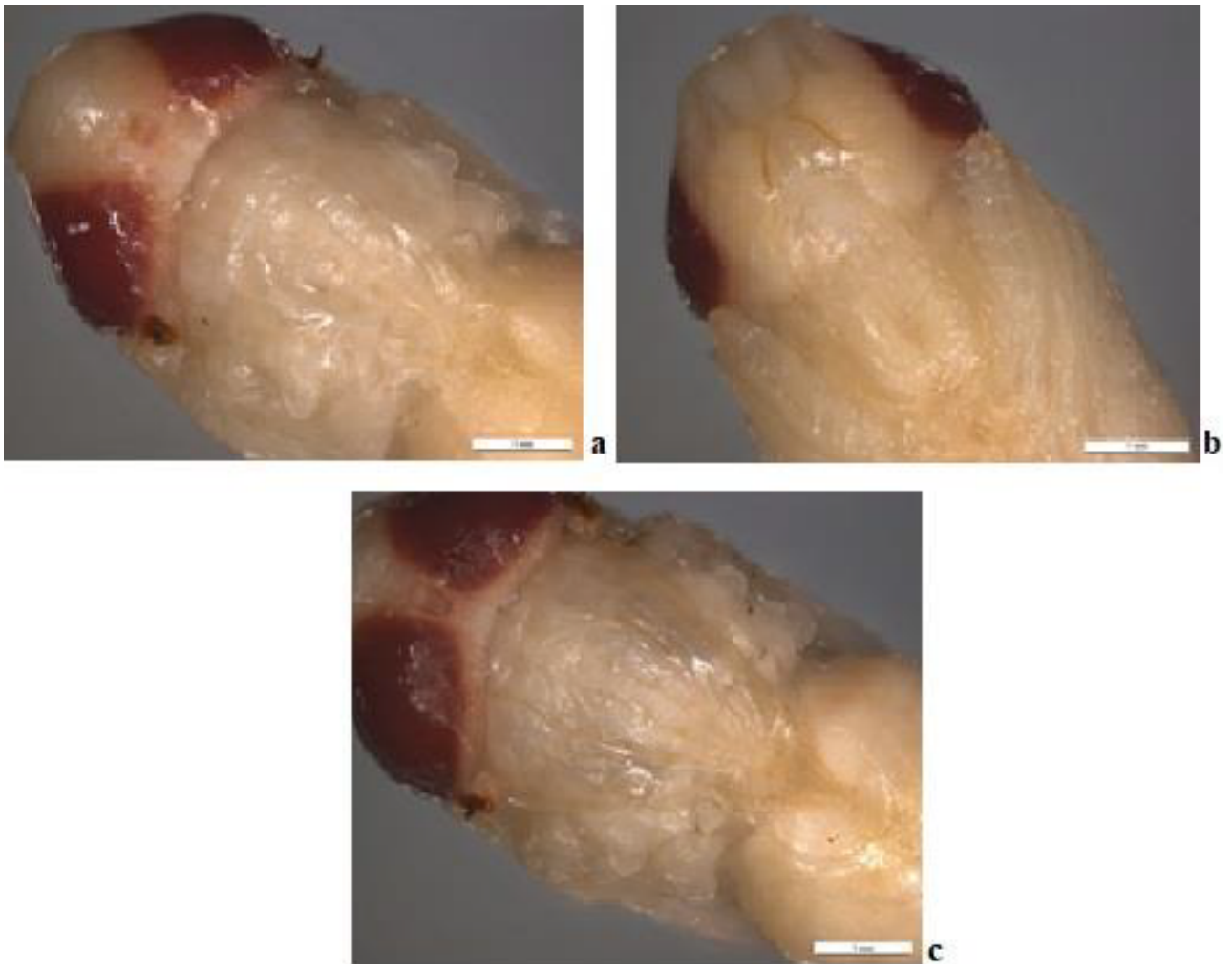

Figure 19. a) Bristles formation of head and thorax, b) genal bristles and pigmented frame around the mouth.

Figure 20. Pigmentation of the costal vein on the wing.

Figure 21. Bristle formation on the abdomen from dorsal view.

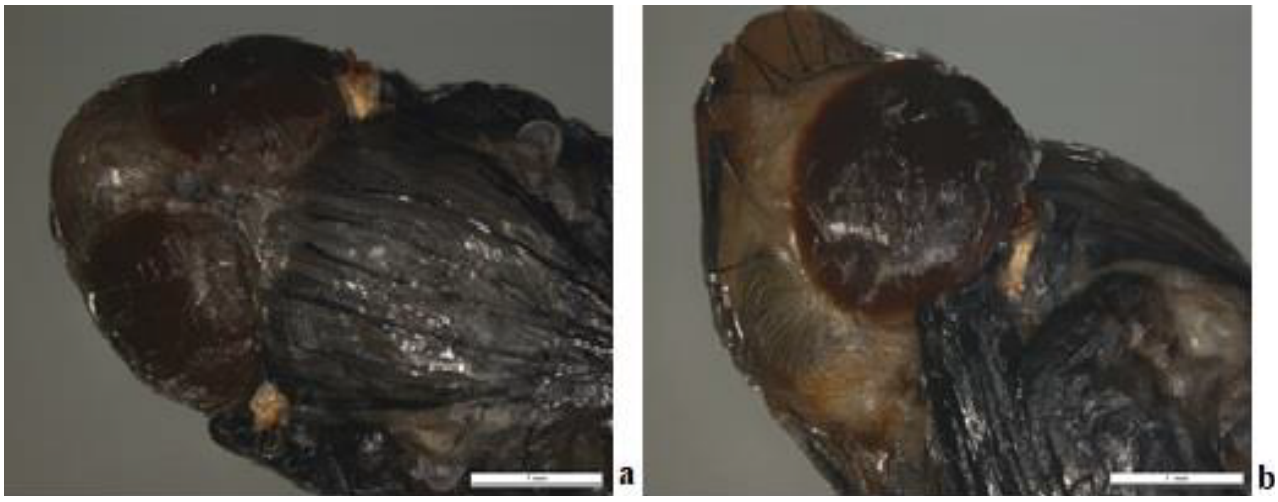

Figure 22. Tube like structure in the lower part of the respiratory horn a) dorsal view, b) lateral view.

Hours 70–73

The segmented appereance on the abdomen seen in the previous hours had decreased. Abdomen seemed smooth. Furthermore, the locations of the stigmas on the abdomen were visible. Pupae was a fairer color, and the yellow coloration in the thorax and the abdomen had decreased (Figure 9).

Hours 77–80

The pupal skin started to be shed in the wing buds and legs (Figure 10).

Hours 99–103

The skin apolysis was completed. The surface of the pharate adult was completely covered with membrane (pupal cuticule) (Figure 11).

Hours 110–114

The abdominal segments had started to be visible (Figure 12).

Hours 131–134

The formation of a depression started in the middle of the abdomen. The color of the pupae started to bleach, and the yellowish color at the border of thorax and abdomen was lost (Figure 13).

Hours 220–224

The posterior of the eye started to be slightly pigmented (Figure 14).

Hours 231–233

Pigmentation was visible in the whole eye. Pigmentation was completed in the border of the eye. The eyes were a light pink color (Figure 15).

Hour 252

The eyes continued to be gradually pigmented and darkened. The orange color of the eye was close to the color of the anterior stigmas (Figure 16).

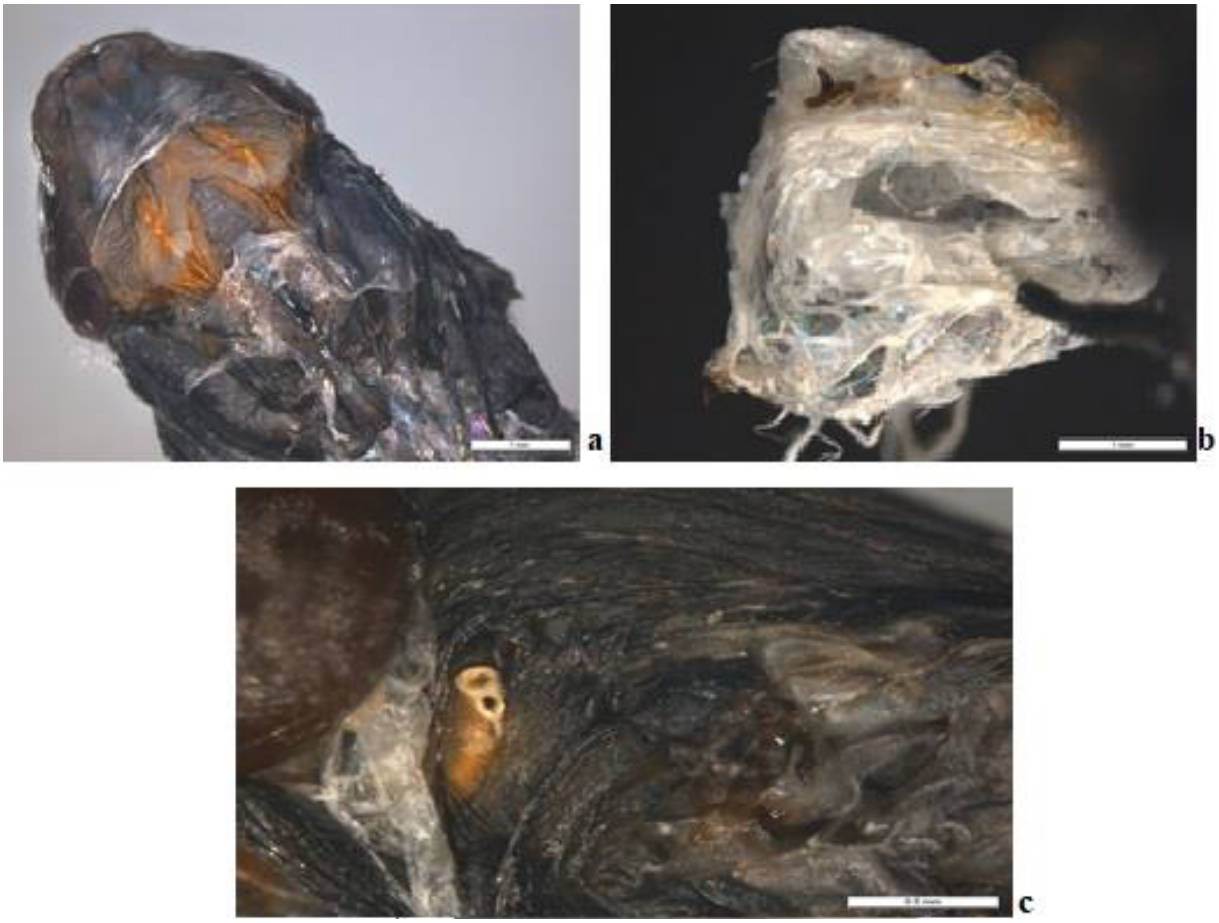

Figure 23. a) Shedding of the pupal cuticle around pharate adult, b) scraped respiratory horns with the shed pupal cuticle, c) open-ended tube with respiratory horns in place.

Hour 260

The compound eyes continued to be pigmented from posterior to anterior. The color of the border stripe of the eye had also become darker (Figure 17).

Hours 282–285

The eyes turned into a pinkish-red color. The eye color was no longer the same color as the anterior stigmas (Figure 18).

Hours 347–349

The sides of the eyes, forehead, and gena region and the bristles in the thorax had darkened and become visible. The frame around the mouth under the antennas had become pigmented (Figure 19).

Hour 378

The darkening of the costal vein on the wing was completed, and the blackness at the base had increased (Figure 20).

Hours 376–378

The formation of the bristles started on the abdomen. Bristles occurred part by part only in the first segment of the abdomen (Figure 21).

Hours 424–429

A tube like structure not seen before emerged in the lower part of the respiratory horn (Figure 22).

Hours 499–500

The adult was very close to emergence at this point. Extremities and structures were clearly visible after the shedding of the membrane covering the pharate adult above the adult. Respiratory horns were thrown together with the shedding membrane and, instead, an open-ended tube remained (Figure 23).

Hours 501– 504

Adult emergence occurred (Figure 24).

At the end of the study, nine different periods not existent in the study conducted by Greenberg (1991) were also observed along with the specified 11 periods. These nine periods determined for *Calliphora vomitoria* are the emergence of the respiratory horns, the withdrawn of the respiratory horns, the prominence of the stigmas, the formation of a depression on the middle of the abdomen, the formation of a longitudinal band on the middle of the eye, the prominence of the ocel eye, the turning of the eye

Figure 24. Adult emergence.

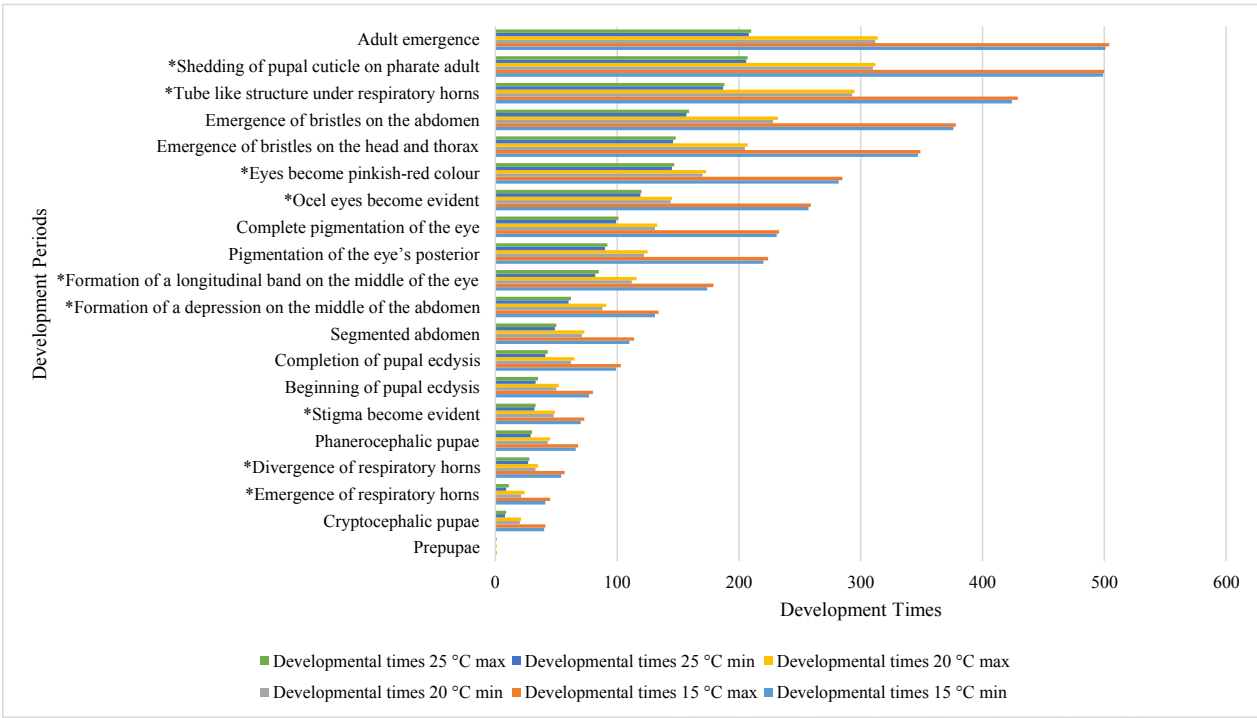

Figure 25. Pupal development periods and range of the beginning times (minimum and maximum) of these periods of *Calliphora vomitoria* at 15 °C, 20 °C and 25 °C.

color to pinkish-red, the detection of a tube-like structure under the respiratory horn, and the shedding of pupal cuticle on the pharate adult.

Calliphora vomitoria completed its development in a minimum of 501 h, namely 20 days 21 h at 15 °C, in 13 days at 20 °C, and in 8 days 16 h at 25 °C. According to the findings, this species reached Cryptocephalic pupa in a minimum of 40 h at 15 °C. It is also possible to divide this 40-h period according to different morphological developments. For instance, the pupa was in liquid form when the puparium was cut between zero and four h. It lost its liquid form and turned into a solid form between 5 and 39 h. The period between 40 and 66 h is the Chryptocephalic pupa period. This is the period when the bond between the pupa and the puparium was broken. In addition to this finding, it was seen that the respiratory horns emerged at hour 41 of this period, and the horns started to withdrawn backwards at hour 54. Phanerocephalic pupa occurred at hour 66, the stigma spaces on the abdomen occurred at hour 70, the beginning of pupal-adult apolysis occurred

at hour 77, and the completion of apolysis occurred at a minimum at hour 99. Abdomen segments started to occur at hour 110, and the formation of a depression on the middle of the abdomen segments started to occur at hour 131. A longitudinal band that could be determined by a microscope occurred in the middle of the eye at hour 174. The pigmentation of the posterior of the eye occurred at hour 220, and the pigmentation of the whole eye occurred at hour 231. During this time, the eyes seemed light pink in color. Ocel eyes were first visible at hour 257. The eyes had turned pinkish red from orange at hour 282. Bristles growth occurred at hour 347 in the head and thorax, and bristles growth occurred at hour 376 in the abdomen. A tube-like structure not seen before occurred at the lower part of the respiratory horns at hour 424. Adult emergence was very close at hours 499–500, and the extremities were clearly seen together with the shedding of the membrane covering the pharate adult. Adult emergence occurred at a minimum at hour 501, and pupal development was finalized (Table 1). The development of the *Calliphora vomitoria* at 20 °C and 25 °C is similar to that at 15 °C. However, as specified before, development periods shorten as temperature increases. As may be seen in Table 2, all of the development periods specified above at 20 °C decreased by 38.83% on average when compared to 15 °C. Similarly, the pupal development period at 25 °C decreased by 32.13% when compared to 20 °C.

4. Discussion

When the pupal morphological development studies becoming intense in the last decade are considered, it is seen that the studies conducted by Sivasubramanian and Biagi (1983), Finell and Jarvilehto (1983) and Greenberg (1991) are taken as the basis. Respectively 9, 7 and 11 pupal morphological development periods have been determined in these studies in which different temperature and pupal dissection intervals have been used. According to our findings in this study, there are 20 morphological development periods for the *Calliphora vomitoria* pupae. The number of developmental period to be higher in our study than the aforementioned studies stems from the hourly pupal dissection.

When pupal morphological development studies are considered, it is seen that the main difference between them is the pupal dissection intervals. The number of the detected periods changes also depending on these intervals. Besides the fact that the species are different from one another also causes the difference in pupal development periods and changes in the times of these periods. Among these studies; Zajac and Amendt (2009) have dissected the *Calliphora vicina* and *Lucilia sericata* once per 24 h and detected 11 pupal morphological periods, Pujol-Luz and Barros-Cordeiro (2012) have detected 8 pupal morphological development periods in *Chrysomya albiceps* by firstly dissecting once in 3 h and then in 6 h and Ma et al. (2015) have detected 9 pupal morphological development periods by dissecting *Chrysomya rufifacies* once in 8 h. The periods detected in the aforementioned studies also take place in our findings. We could count these periods as the emergence and coloration of the eyes, emergence of parts such as antenna, leg and wing, emergence of the thorax and abdomen parts and occurrence of segmentation, emergence and coloration of bristle growth in various parts of the body, which take place in general insect morphology. While the determination of these structures is seen as the common point of the previously mentioned studies, it is seen that our findings are more detailed in the issue of the time of these periods. For instance, Pujol-Luz and Barros-Cordeiro (2012) have specified that in *Chrysomya albiceps*, ocelli becomes visible, all hair and bristles get darker, wings become clear and veins thicken between the hours 66–90 at 26 ± 1 °C. According to our findings at 25 °C, it is seen that ocelli emerges at the 119th—120th h, bristles in head and thorax emerges at the 146th—148th h and the bristles in the abdomen emerges at the 157th—159th h.

Similarly, according to Ma et al., (2015), while respiratory horns, head, thorax and abdomen separation and wing and leg buds become visible at the 28th h, stigma becomes visible at the 42.4th hour and abdomen segmentation becomes visible at the 80th h at 24 °C in *Chrysomya rufifacies*, respiratory horn and wing and leg buds emerge at the 9th—11th h, head, thorax and abdomen separation emerges at the 29th—30th h, stigmas emerge at the 32nd—33rd h and abdomen segmentation emerges at the 49th—50th h according to our findings at 25 °C.

While it has been specified by Zajac and Amendt (2009) at 25 °C in *Calliphora vicina* that head, thorax, abdomen separation and eye, wing and legs become visible at the 48th h, abdomen segmentation and the coloration of eyes emerge at the 144th h, head, thorax and abdomen separation and the emergence of eyes occur at the 29th—30th h, wing and leg buds emerge at the 9th—11th h, abdomen segmentation emerges at the 49th—50th h and eye coloration emerges at the 90th—92nd h according to our findings at 25 °C in *Calliphora vomitoria*. Besides, the emergence of a longitudinal depression in the abdomen is a common period existent in both studies and it has emerged at the 72nd h in the study conducted by Zajac and Amendt (2009) and at the 60th—62nd h in our study. *Calliphora vomitoria* and *Calliphora vicina* to be two close species in forensic entomology and the performance of both studies at 25 °C make it important to compare the findings of the study conducted by Zajac and Amendt (2009) to our findings. It is clearly natural that there are some pupal development period differences among one another due to the fact that the specified species are two

different species. However, because pupal dissection is conducted once in 24 h in the study conducted by Zajac and Amendt (2009), it is thought that differences have been observed between the beginnings of the compared pupal periods.

Our findings show many similarities in terms of the development periods to the results of Karabey and Sert (2018) and Sert et al. (2020) who conducted studies on a similar topic, especially in our country, and used similar methodology. Karabey and Sert (2018) have stated at 20 °C in *Lucilia sericata* that the formation of the leg buds emerges at the 12th–14 h, head, thorax and abdomen separation emerges at the 41st—43rd h, stigma formation occurs at the 45th—48th hours and abdomen segmentation starts to be seen at the 132nd—136th h. According to Sert et al. (2020), while respiratory horns and the wing and leg buds emerge at the 29th—33rd h, head, thorax and abdomen separation emerges at the 60th—65th h, stigmas emerge at 68th—71st h and abdomen segmentation emerges at the 121st—132nd h at 20 °C in *Sarcophaga argyrostoma*, respiratory horn and the wing and leg buds emerge at the 21st—24th h, head, thorax and abdomen separation emerge at the 43rd—45th hours, stigmas emerge at the 48th—49th h and abdomen segmentation emerges at the 71st—73rd h according to our findings at 20 °C.

Actually, the existence of pupal period and time differences among the species compared above is based on conducting the studies on different species and using different methodologies. The existent comparisons conducted have been handled upon the similar characters detected. In addition, Pasquerault et al. (2013) have a study of pupal morphological development on the *Calliphora vomitoria* examined in this study. Nine periods in total have been given for the dissected pupal development in Pasquerault et al. (2013). They have expressed the developmental periods not in hourly data as we have given, but in the form of the percentage of the pupal development period. Due to being in the form of hourly data, our findings cannot be completely compared to the percentage data of Pasquerault et al. (2013). However, when it comes to conduct an assessment for some periods, the period "Tagmes not separated, mouth hooks attached to the pupa" specified to correspond to less than 10% of the development in Pasquerault et al. (2013) lasts up to respectively the 39th (7.7%), 19th (6.05%) and 7th (3.3%) h of the development at 15, 20 and 25 °C in our study and this corresponds to less than 10% of the total development. Similarly, the period "Red tube, deployment of ptiline, red ocelli" corresponding to 90%–100% of the development in Pasquerault et al. (2013) seems partly comparable to the period "Tube like structure under respiratory horns" in our study. This period is seen respectively at the 429th (85%), 295th (93%) and 188th (89.5%) h at 15, 20 and 25 °C in our study. Results are partly in accordance with each other and the differences in the comparison, though little, are thought to stem from the differences in study methods.

5. Conclusion

The developmental periods of the *Calliphora vomitoria* at the temperatures of 15, 20 and 25 °C regarding the pupal period used in the determination of minimum postmortem interval (PMImin) estimation have been examined every hour and revealed for the first time with this study. The data revealed in this study are considered to make very significant contributions to the science world and those conducting implementations in this subject in terms of both developmental biology and forensic entomology. Both the highness in the period number and clear determination of at which hour these morphological development periods emerge and how long it takes for them to complete their development both in our study and in similar studies are considered to provide opportunities for clearer determination of postmortem interval estimations conducted in murder or unexpected death cases.

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