Examining external morphological characteristics of *Lucilia sericata* pupae for age estimation in medico-legal investigations

by

Lisa Alberts (ALBLIS003)

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Name: Lisa Alberts

Student number: ALBLIS003

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Signature: Signed by candidate

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Abstract.

Insects play an essential role in the resolution of medico-legal investigations. For various insects, like necrophagous flies, it is vital for their survival to find and inhabit a food source such as a decomposing body. This makes it possible to use these insects as evidence in medico-legal investigations. A crucial part of any medico-legal investigation is estimating the time that has elapsed since death, otherwise known as the post-mortem interval (PMI). The age of the oldest insects can provide the most precise estimation of the PMI.

Flies undergo different stages of development. All the immature stages can be utilized as evidence at death scenes. Out of all the immature stages, pupae represent the oldest specimens, which makes them valuable in establishing a minimum time since death. Identification and aging of pupae is currently a challenging process since they all look similar in appearance. Few studies have been done on pupae for PMI estimation. This study aims to identify reliable morphological markers to aid in a more accurate age estimation of *Lucilia sericata* during the pupal stage.

A total of 145 pupae were collected and examined for external morphological changes over time. Five pupae were collected at each time point. A total of six external morphological characteristics of the puparium and twenty external morphological characteristics of the pupae were initially examined. These characteristics were linked to age in accumulated degree hours (ADH), with the aim of creating a timeline that can aid in the estimation of pupal age. The pupal ADH ranges from the youngest being 6550 ADH and the oldest being 11300 ADH. The timelines identified several characteristics that develop during the early or late stages of development. Multiple linear regression analysis was performed to assess characteristics which were useful for estimating the age of the pupae (ADH) and develop a regression equation based on the data collected. The regression analysis identified 10 characteristics that are the most significant in aging pupae. They were the colour and shape of the labellum, leg length, leg width at full length, thoracic setae, facial setae, abdominal macrosetae, palp shape, genal setae and labrum colour. Some of these characteristics like the leg length and width and abdominal macrosetae did provide important time-breaks on their respective timelines. However, the development and the pigmentation of the compound eye also provided valuable time-breaks it's timeline.

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First and foremost, I would like to thank my supervisor, Mr Calvin Mole. Without your guidance, support, and advice I don't think this study would have been possible. Thank you for always going the extra mile to help me when I was stuck. Thank you for always being patient while I was trying to figure out how to do statistics during our meetings.

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1. Forensic Entomology.

In terms of numbers and diversity, insects are the most diverse and largest group of animals on the earth. They have colonised almost every environment on earth and are associated with both life and death. Their economical and sanitary importance is well documented in history, but in recent years they have also been used in a forensic context (Williams and Villet, 2006; Vanin, 2018). Forensic entomology refers to the study of insects and other arthropods that populate the flesh of humans and animals (dead or alive) as well as the analysis of insect evidence for forensic and legal purposes (Amendt, 2018; Mona *et al.*, 2019).

Forensic entomology can provide evidence that is used in both civil and criminal cases, but is most commonly used in death enquiries (Williams and Villet, 2006; Amendt *et al.*, 2007). Forensic entomology can play an important role in providing essential information concerning the conditions surrounding a victim's death, including the season and location of death and even the use of drugs. However, its main application is the estimation of the minimum time since death (Amendt *et al.*, 2011). For various insects, like necrophagous flies, it is vital for their survival to find and colonise a food source such as a decomposing body (Sharma, Kumar Garg and Gaur, 2015). Thus, a minimum time since death is estimated by analysing the different species that make up the insect fauna found on the corpse and estimating the age of the oldest insects found on the deceased individual (Amendt *et al.*, 2011).

Forensic entomology has been categorized into three different categories; stored-product forensic entomology, urban forensic entomology, and medico-legal forensic entomology (Williams and Villet, 2006; Vanin, 2018). This literature review will focus on medico-legal forensic entomology and the estimation of the post-mortem interval (PMI) using larvae development and morphology. This will be followed by a critical review of the pupae and external puparium as a tool for PMI estimation.

1.2. Forensic Entomology in South Africa.

The first research focussing exclusively on forensic entomology in South Africa was conducted at least 40 years ago by André Prins and the South African Museum in the 1980's. Previously, entomological research focussed primarily on veterinary and agricultural entomology, medical entomology and ecological entomology (Williams and Villet, 2006). There have only been a couple of studies on the forensically important insects found in South Africa. These studies were on various species of flies and beetles, which aimed to generate entomological

data on developmental models, succession patterns and thermal summation model in a South African climate and environment. (Williams and Villet, 2006, 2019). As a result of the limited number of studies done in South Africa, the entomological data used in investigations is not suitable for the South African setting. This is due to the data being from other countries (Williams and Villet, 2006, 2019). This is problematic due to different species in different geographic regions. Each species in their respective geographic region will have a development rate that is specific to that region. These differences may be due to genetic variation between populations and the possibility that inherent biogeographical variation exists between populations of species in relation to development time is of considerable consequence toward generating an accurate minimum PMI estimation (Harvey, Gasz and Voss, 2016). Therefore, it is necessary to conduct further research which is directly applicable to the South African setting.

1.3. Estimation of the time since death.

A crucial part of any medico-legal investigation is estimating the time that has elapsed between the death of the individual and the discovery of the body, otherwise known as the post-mortem interval (PMI) (Amendt, 2018; Vanin, 2018; Mona *et al.*, 2019). This PMI estimation provides a starting point in time for the reconstruction of criminal events (Vanin, 2018). The forensic entomological estimation of the PMI assumes that insects that inhabit the flesh of humans and animals will arrive at a dead body within minutes of death. Most insects associated with forensic investigations are Diptera (flies) and then a small group of Coleoptera (beetles). The main dipteran species associated with forensic investigations are Sarcophagidae (flesh flies), Calliphoridae (blow flies), and Muscidae (house flies) (Joseph *et al.*, 2011). Research has shown that flies are attracted to the decomposing remains by various gasses released over the time of decomposition (Joseph *et al.*, 2011; Harvey, Gasz and Voss, 2016; Mona *et al.*, 2019). Thus, the time it takes for the insects to colonise, undergo their full developmental cycle, and leave the remains, is closely linked to the time since death (Harvey, Gasz and Voss, 2016).

Taking this into account, the species and the age of the oldest insects can provide the most precise estimation of a minimum time since death (Gennard, 2012). The oldest insects will provide the time when the first adult female insect arrived at that body after death, thus giving a minimum time since death (Amendt *et al.*, 2007).Thus, estimating the age of these

insects are vital for the medico legal investigation (Feng and Liu, 2014; Proença *et al.*, 2014; Ma, Huang and Wang, 2015). Current methods used for age estimation are based around the species-specific time required for an immature insect to progress through the different developmental stages. These stages include the eggs, first instar larvae, second instar larvae, third instar larvae and the pupae (Harvey, Gasz and Voss, 2016).

1.3.1 Succession patterns.

Should the discovery of the body be delayed for more than the first generation of insects, PMI estimations can be based on the succeeding colonisers (Greenberg and Kunich, 2002). This method of PMI estimation is based on the composition of the insect species present on the body at the time of discovery. This method is only recommended for PMI estimation of body that are in the later stages of decomposition (Amendt *et al.*, 2007). Research has shown that certain insect species are linked with different stages of decomposition and not all insects associated with decomposition will be present at the same time (Erzinçlioğlu, 1983; Amendt *et al.*, 2011). This makes it possible to estimate PMI by identifying and examining the composition of the species present on the corpse (Erzinçlioğlu, 1983; Díaz-Aranda *et al.*, 2018). This might seem like a quick and easy method for PMI estimation, but it is complicated by many factors that might influence succession (Erzinçlioğlu, 1983). These factors include season, location, weather conditions and whether a body has been buried or moved. The identification of the various insects is a procedure that requires expertise in insect taxonomy (Amendt *et al.*, 2011).

The insects found on the body can be divided into four main categories; predator species, necrophagous species, omnivorous species and adventive species (Smith, 1986; Amendt *et al.*, 2011). Necrophagous species feed on the decomposing tissue. These include various dipteran species like Calliphoridae and Sarcophagidae. Predators feed on other arthropods and might start out as necrophages and become predators in their later stages. Omnivorous species like ants and wasps aren't necessarily necrophagous in nature but will use the decomposing flesh as a food source when available. Adventive species don't feed on the decomposing flesh but will use the body as an extension of their natural habitat which it is found in (Smith, 1986). All of the insects in these various categories will arrive at different times and thus make it possible to estimate a PMI.

1.3.2 Developmental models.

Identifying the correct species is a critical step when using developmental models. Different species have different growth and maturation rates. To estimating the PMI, the age of the larvae must be determined (Joseph *et al.*, 2011; Feng and Liu, 2014; Brown, Thorne and Harvey, 2015; Ma, Huang and Wang, 2015). That is where developmental data of the dipteran species associated with decomposition can be useful for PMI estimation. The larvae of blow flies and flesh flies can aid in PMI estimation. However they are only useful during the first few weeks after the death occurred while the body is still in the earlier stages of decomposition (Introna *et al.*, 1989). Developmental data is information about the length of the development of the various immature stages recorded at different temperatures. This data then gets summarised into one or two developmental models, namely isomorphen and isomegalen diagrams. Developmental models rely on either size or stage of development to estimate PMI (Amendt *et al.*, 2011).

The most basic of these models is the isomorphen diagram. This is a contour plot that represents the time from an event, such as hatching or oviposition (X-axis) against the ambient temperature of the environment (Y-axis) (Amendt *et al.*, 2011). The second model, the isomegalen diagram, is a contour plot of age, or time since an event, such as hatching (X-axis) against temperature (Y-axis) (Harvey, Gasz and Voss, 2016). The age estimation of the specimens can be conducted with great accuracy when the scene temperature was consistent with the constant temperatures used in the diagram. However, the ambient temperatures in which specimens are developing on decomposing remains vary over time and this can cause age estimations to be less accurate. Other mathematical models have been developed to take these fluctuating ambient temperatures into account (Grassberger and Reiter, 2002; Amendt *et al.*, 2011; Harvey, Gasz and Voss, 2016; Mona *et al.*, 2019).

1.3.3 Thermal summation models.

Thermal summation models are more sophisticated than the above mentioned models (Amendt *et al.*, 2011). This model is also referred to as the accumulated degree hours (ADH) model (Amendt *et al.*, 2007, 2011; Mona *et al.*, 2019). ADH refers to how much thermal energy is needed per hour to develop from one stage of development to the next. This is dependent on the ambient temperature and the relative humidity of the environment the insect is in (Amendt *et al.*, 2007). Both the ambient temperature and relative humidity have

an effect on the physiology of the insects, thus it impacts their developmental rates (Gennard, 2012). The number of accumulated degree hours to complete development is specific to each species (Joseph *et al.*, 2011; Harvey, Gasz and Voss, 2016). The ADH model has the capability to incorporate larval size, developmental stage, as well as varying temperatures into one linear regression model. This capability to process more complex data sets makes it the preferred model to use when estimating PMI at an outdoor crime scene (Joseph *et al.*, 2011).

1.4. The Pupae.

The pupal stage is the final stage of the fly's immature life cycle. The pupal stage begins with the shrinking of the larvae and cessation of movement, closely followed by the formation of a pale puparium. The puparium refers to the hard external casing that encloses the soft pupa (Brink, 2009; Brown, Thorne and Harvey, 2015; Ma, Huang and Wang, 2015). Over the next few days, the pupa undergoes various physiological, anatomical, and structural changes (Zdarek and Fraenkel, 1972; Denlinger and Zdarek, 1994).

1.4.1 Morphology.

The puparium is generally oblong in shape and consist of two parts; the hard external puparium and the soft pupa on the inside (Brink, 2009; Brown, Thorne and Harvey, 2015; Ma, Huang and Wang, 2015). The general external morphology of the puparium remains universal across all species with slight variations in size and shape of some features. Due to the process of pupation, the puparium will retain some features characteristic of the third instar larva. These characteristics include (i) the cephalopharyngeal skeleton or CPS (the larval digestive system) which is merged with the inside of the ventral wall of the puparium, (ii) the anterior and posterior spiracles (respiratory structures of third instar larvae) and (iii) intersegmental spines (figure 1.1). Some features that are species specific include (i) constrictions in the anterior third of the puparium (figure 1.2), (ii) texture of the puparium surface (figure 1.3), (iii) the lateral ridge (figure 1.4), (iv) folds in the frontal field (figure 1.5) as well as (v) slight variation in puparium colour (Brink, 2009). It should be noted that some of these structures are only visible on Scanning Electron Micrograph. A full morphological discussion of species differences is beyond the scope of the current literature review, however for detailed descriptions of species differences the reader is referred to the following dissertation by Dr Sonja Brink (2009): Key Diagnostic Characteristics of the Developmental Stages of Forensically Important Calliphoridae and Sarcophagidae in Central South Africa.

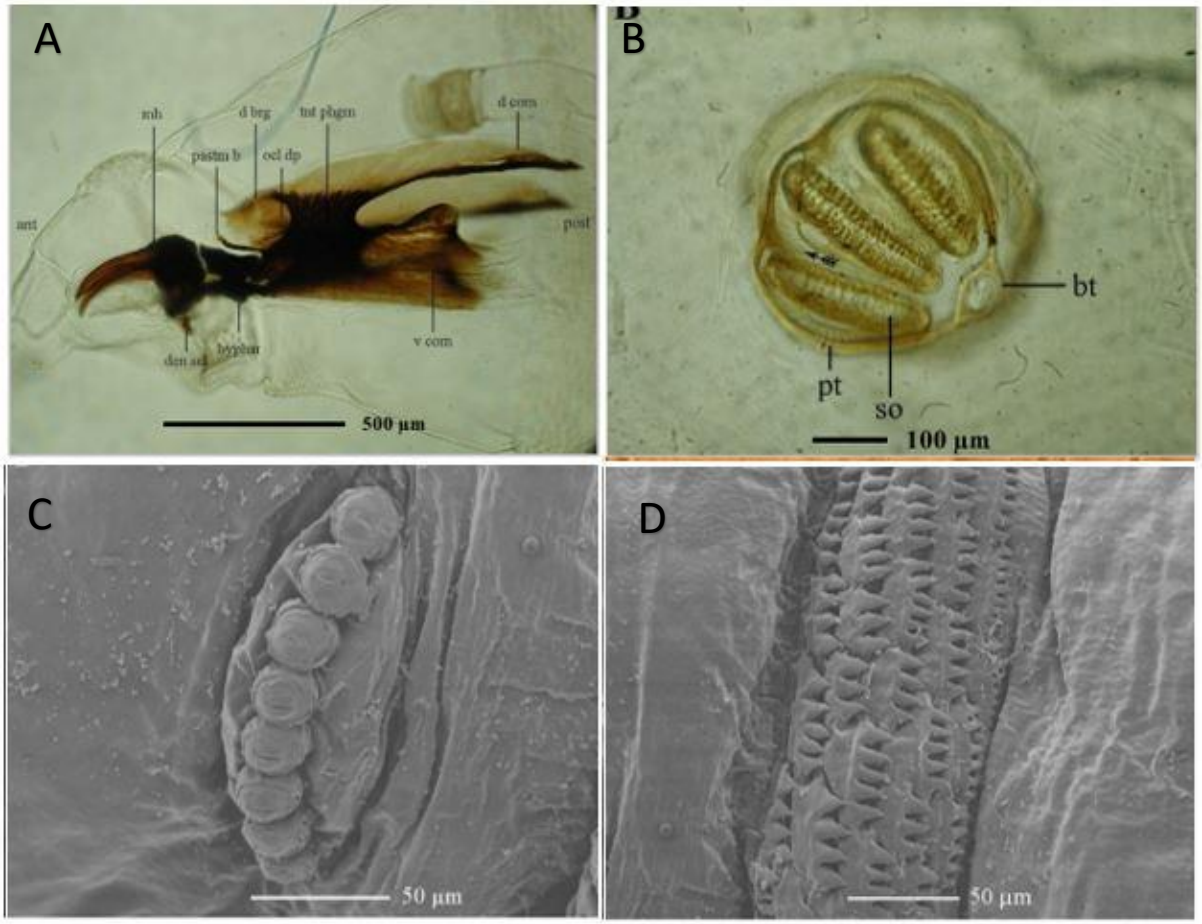

Figure 1.1: (A) Light micrograph of the lateral aspect of the cephalopharyngeal skeleton (CPS) of a third instar *Lucilia sericata* larva. (B) Light micrograph of the detail of posterior spiracles of third instar larvae of *Lucilia sericata*. (C) Scanning electron micrograph of the anterior spiracles of third instar larvae of *Lucilia sericata*. (D) Scanning electron micrograph of the spines of third instar larvae of *Lucilia sericata* (Brink, 2009).

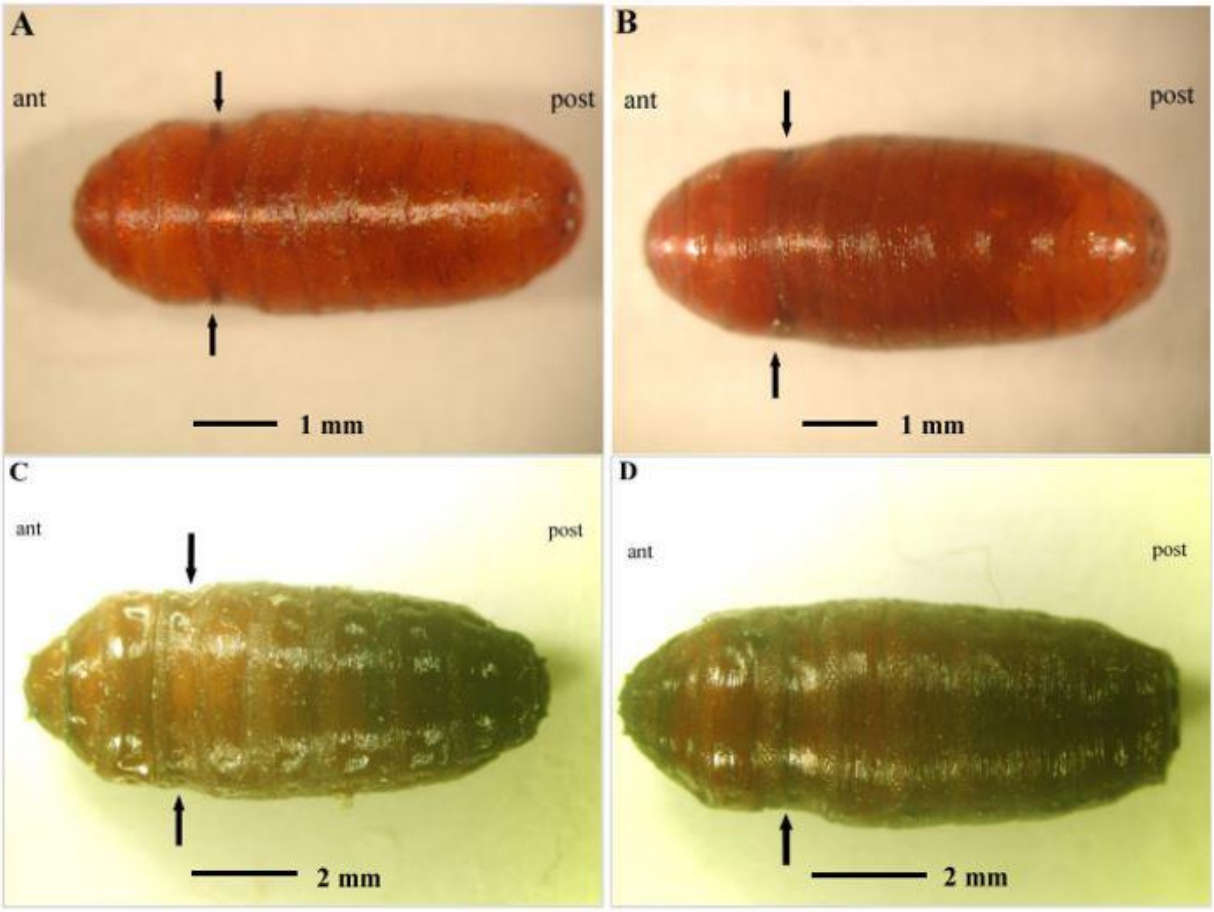

Figure 1.2: Light micrographs of the puparia of (A) *Lucilia cuprina*, (B) *Lucilia sericata*, (C) *Chrysomya chloropyga*, (D) *Chrysomya marginalis* (Brink, 2009).

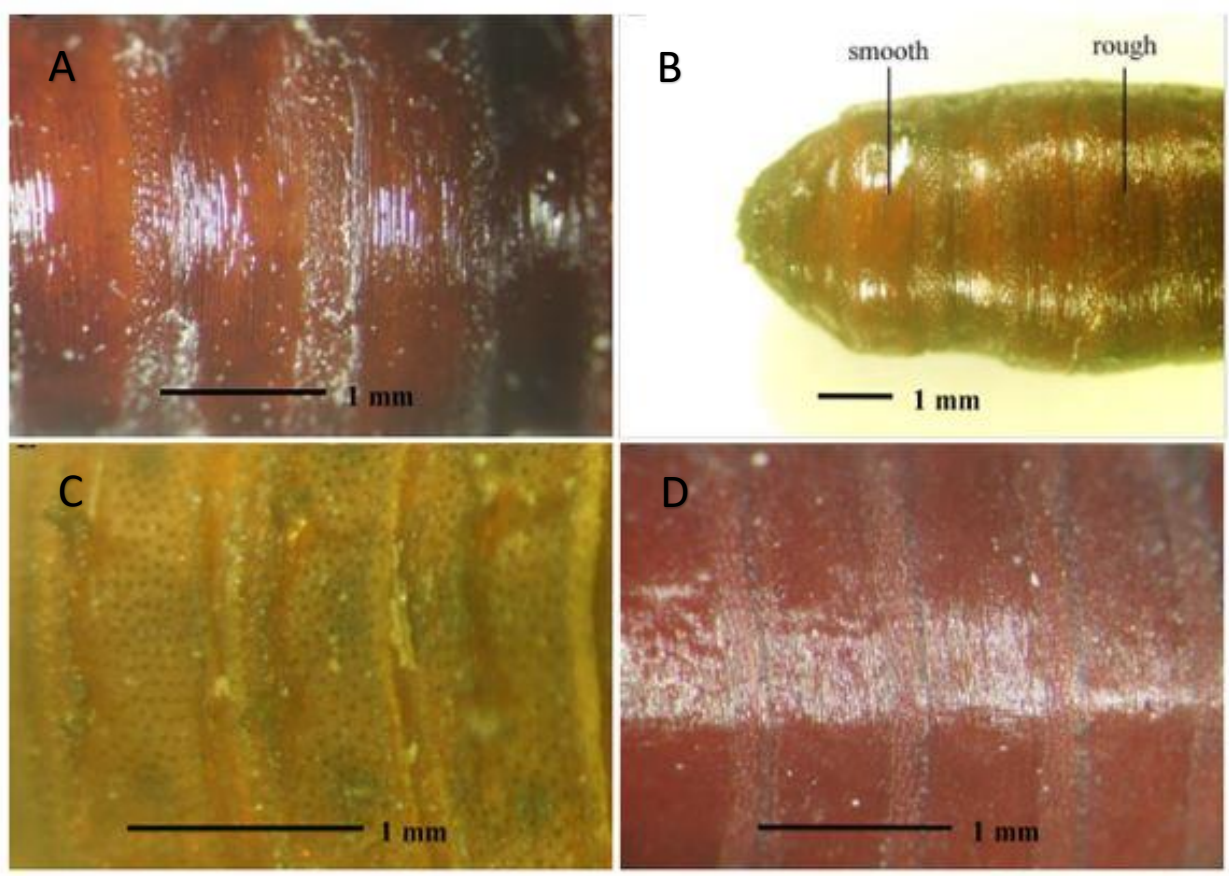

Figure 1.3: Light micrographs of the surface texture of the puparia of (A) *Chrysomya chloropyga*, (B) *Chrysomya marginalis*, (C) *Chrysomya albiceps*, (D) *Calliphora vicina* (Brink, 2009).

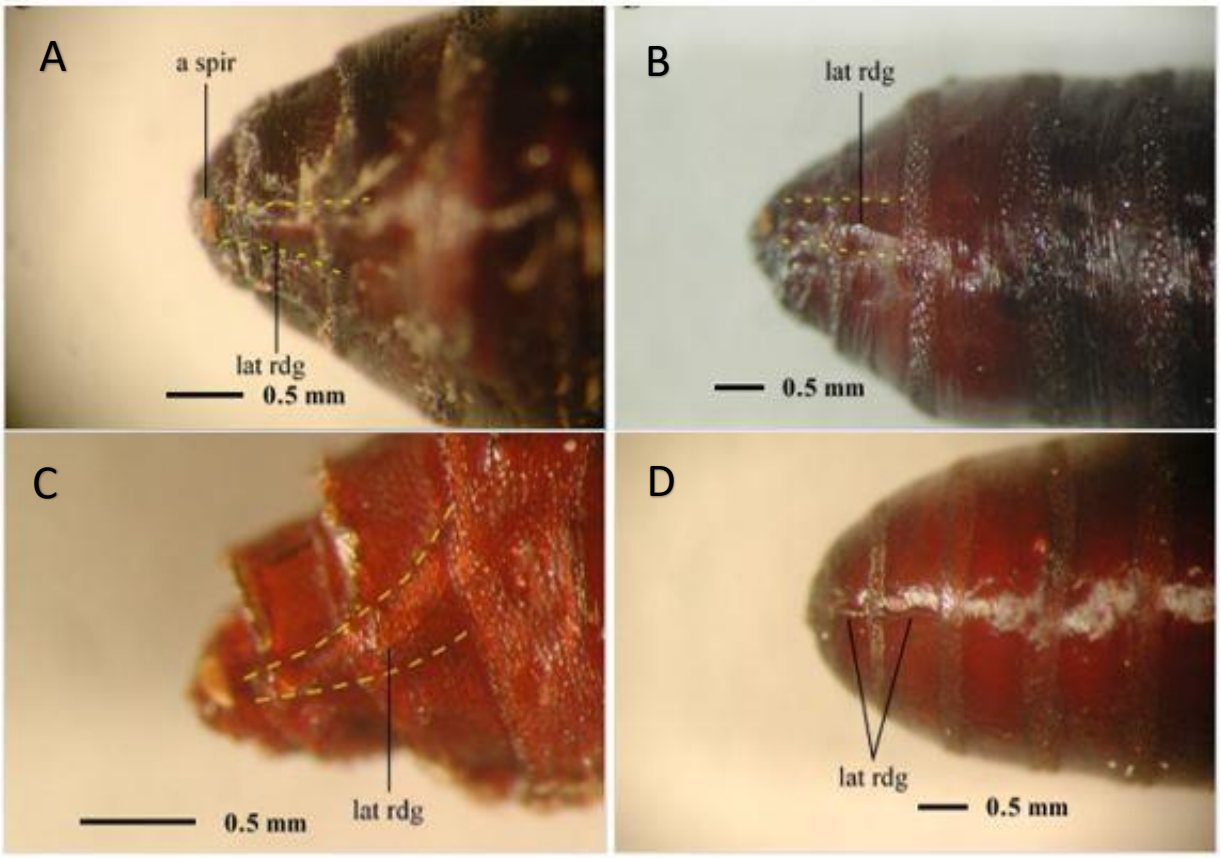

Figure 1.4: Light micrographs of the lateral ridge of the puparia of (A) C*hrysomya chloropyga*, (B) *Chrysomya marginalis*, (C) *Chrysomya albiceps*, (D) *Calliphora vicina* (Brink, 2009).

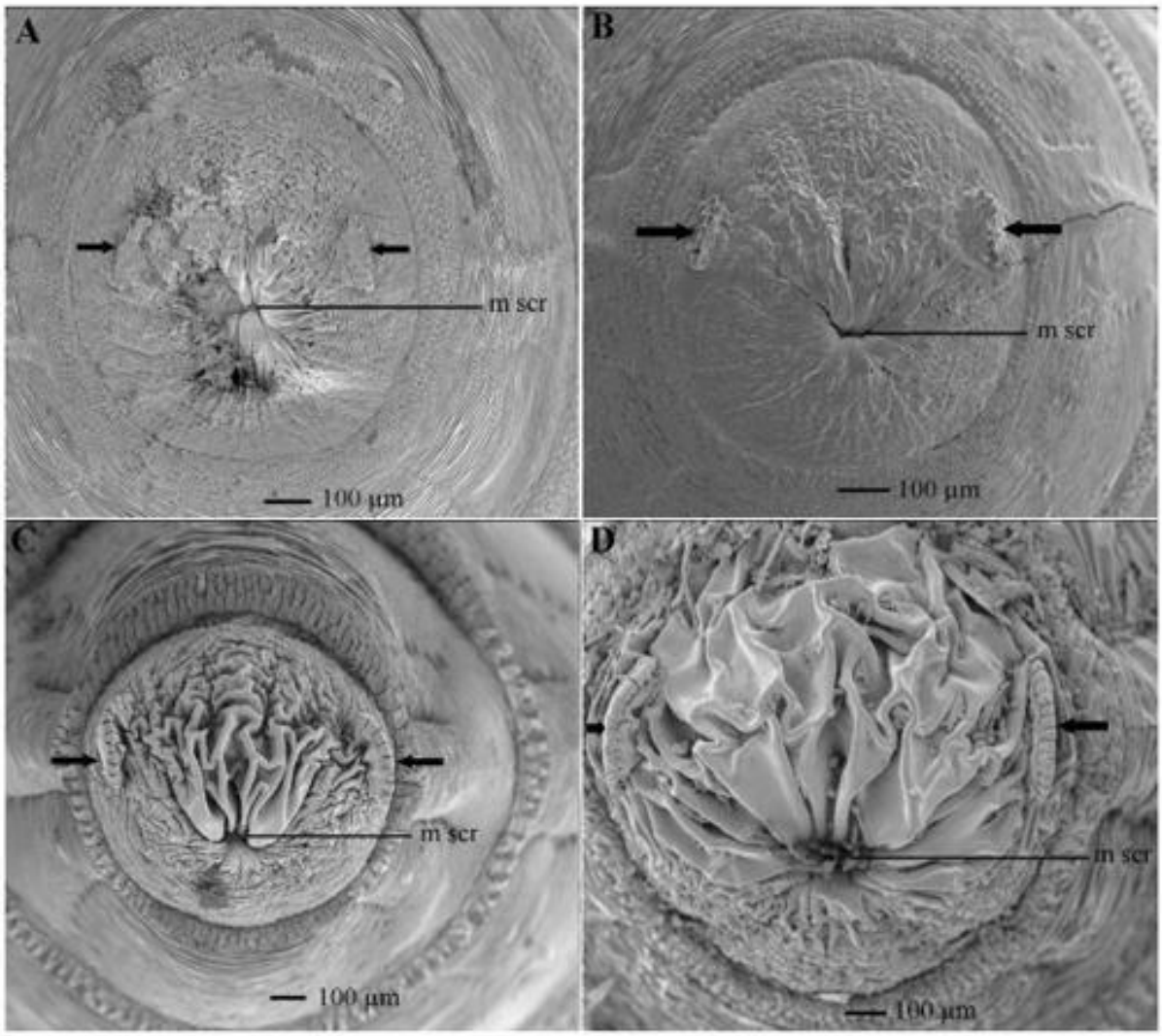

Figure 1.5: Scanning electron micrographs of the various folds in the frontal field of the puparia of (A) *Lucilia cuprina*, (B) *Lucilia sericata*, (C) *Chrysomya chloropyga*, (D) *Chrysomya marginalis* (Brink, 2009).

The pupae have little distinguishable morphological features at first (Pujol-Luz and Barros-Cordeiro, 2012; Barros-Cordeiro *et al.*, 2016). As time passes it will develop into a fully formed adult fly. At various stages of intra-puparial development different features will appear and continue to change until eclosion (Brown, Thorne and Harvey, 2012; Pujol-Luz and Barros-Cordeiro, 2012; Feng and Liu, 2013, 2014; Barros-Cordeiro *et al.*, 2016). These include basic structures like wing, legs, eyes, antennae, setae and mouth parts (Brown, Thorne and Harvey, 2015).

1.4.2 Stages of Intra-puparial Development.

Feng and Liu (2013,2014) explored the morphological changes of flies commonly found on indoor corpses in China. Feng and Lui concluded that the two different species (*Megaselia spiracularis* and *Megaselia scalaris*) of flies respectively completed 11 and 10 stages of development. Their conclusion was based on their observations of various morphological characteristics which could be associated with a developmental timeline. Pujol-Luz and Barros-Cordeiro (2012) and Barros-Cordeiro *et al*. (2016) observe and describe 5 distinct stages of intra-puparial development of three different blow fly species. These species included *Chrysomya albiceps*, *Cochliomyia macellaria* and *Lucilia cuprina*. The 5 distinct stages are: (1) pupariation, (2) larva-pupa apolysis, (3) cryptocephalic stage, (4) phanerocephalic stage and (5) pharate adult stage. These stages can also be associated with a developmental timeline.

Pupariation refers to the onset of the larval-pupal transition, also known as metamorphosis. This is a complicated process that involves changes in the structure and morphology of the third instar larvae (Denlinger and Zdarek, 1994). This process begins at the end of the third instar larval stage (Brody, 1995; Brink, 2009). Once the third instar larvae stop feeding, they wander away from the food source and begin to dig into the ground with the help of their mouth-hooks. During this process they begin to retract the segments of their bodies, thus reducing their size by about 50% (Pujol-Luz and Barros-Cordeiro, 2012). The cuticle of the larvae changes in colour (dark yellow to dark brown) and become more sclerotised. The posterior spiracles collapses and sink into the anal tubercle. At the end of this stage the larva will be in the shape of a barrel (Barros-Cordeiro *et al.*, 2016).

The second stage, larval-pupal apolysis, is initiated by the sudden release of a steroid hormone called ecdysteroid. This causes the larval cuticle to separate from the epidermis below to form the puparium that encloses the pupa until it emerges as a fully formed fly (Adams, 2009). During larval-pupal apolysis the legs and wings will begin to evert to some extent (Brody, 1995). The ends of the pupa will remain in contact with the puparium via the maxilla and mandible and the posterior spiracles (Pujol-Luz and Barros-Cordeiro, 2012; Barros-Cordeiro *et al.*, 2016). At this stage the pupa is referred to as a cryptocephalic pupa (Martín-Vega, Hall and Simonsen, 2016).

During the cryptocephalic stage the larval-pupal apolysis stops. The cuticle hardens and becomes the pigmented puparium that protects the pupa until eclosion (Pujol-Luz and Barros-Cordeiro, 2012). This is followed by the detachment of the mandible and maxilla from the rest of the cephalopharyngeal skeleton (the larval digestive system). The mandible and maxilla still remains attached to the puparium during this stage. At this stage the pupa does not have a defined form and the various section of the body are not distinguishable (Pujol-Luz and Barros-Cordeiro, 2012; Barros-Cordeiro *et al.*, 2016).

The next process is the eversion of the head and this happens during the phanerocephalic stage. The process happens rapidly and is coordinated by contractions of the abdominal muscles. During this stage imaginal discs undergo eversion to form the basic shape of the various parts of the adult body. These include the head, thorax and abdomen. The discs that form the wings and legs fuse to form the thorax. The discs of the eyes and antennas fuse to form the head and finally the head and thorax fuses with the abdomen to form the adult body (Brody, 1995). Imaginal discs are clusters of undifferentiated embryonic cells that form during the larval stage and separate and form various structures during the pupal stage after the release of the ecdysteroid hormone (Adams, 2009).

During the pharate stage, the final separation and maturation of the undifferentiated embryonic cells take place to form the wings and legs (Pujol-Luz and Barros-Cordeiro, 2012; Barros-Cordeiro *et al.*, 2016). During the next several days, various cell and tissue changes take place for the development of adult structures. The various adult structures include the development of setae, the layered adult cuticle as well as pigmentation of the eyes (Brody, 1995). This final stage can be divided into sub-stages according to the pigmentation of the eyes (Pujol-Luz and Barros-Cordeiro, 2012; Barros-Cordeiro *et al.*, 2016).

1.4.3 Utilising the pupae in PMI estimations.

It is evident above that the development of a pupa follows a systematic path, thus the appearance of these morphological changes has the potential to be used to estimate age of the pupa and therefore be used in PMI estimations. At times, pupae are the only insect evidence found at a crime scene. Therefore, it is important to also study pupae for their use in PMI estimation. However, a limited number of studies have explored methods of estimating pupae age with the aim of understanding the morphological changes that occur inside the puparium in order to improve the estimation of a PMI. Most studies done on pupal

age have focused on morphological analysis and histology of the pupae (Brown, Thorne and Harvey, 2015; Ma, Huang and Wang, 2015).

1.4.3.1 Morphology.

Various recent studies (within the last 10 years) have determined that morphological characteristics of a pupa can be associated with a developmental timeline (table 1.1). A fully developed adult fly is not formed in a single day, but rather develops over a period of time. This developmental timeline can aid in estimating PMI (Pujol-Luz and Barros-Cordeiro, 2012; Feng and Liu, 2013, 2014; Brown, Thorne and Harvey, 2015; Barros-Cordeiro *et al.*, 2016; Voss *et al.*, 2017; Karabey and Sert, 2018). The puparium is a structure that is opaque, therefore to help with the examination of the external morphological features of the pupa, the pupal case is removed.

Table 1.1: A comparison of the different studies utilising morphology (analysed under a microscope) to age pupae.

Author	Year	Species	Developmental temperatures and humidity	Method	Aging methods	Number of phases/stages	Number of characteristics identified/examined
Brown et al.	2012	*Calliphora vicina*	22 °C, 40– 60 % relative humidity	Hot-water killied and stored in 80 % ethanol at −20 °C followed by a morphological examination	ADH since oviposition	N/A	23
Pujol-Luz and Barros-Cordeiro	2012	*Chrysomya albiseps*	26°C ± 1°, 60% ± 10% relative humidity	Fixed in Carnoys solution and preserved in 70% ethanol followed by a morphological examination	Hours since pupation	8	N/A
Feng & Liu.	2013	*Megaselia spiracularis*	constant temperatures (21, 24, 27, 30 and 33 °C), 75% relative humidity	Submerged in 1% sodium alginate solution followed by a morphological examination	Hours since pupation	11	N/A
Feng & Liu.	2014	*Megaselia scalaris*	constant temperatures (18, 21, 24, 27, 30, 33 and 36 °C), 75% relative humidity	Submerged in 1% sodium alginate solution followed by a morphological examination	Hours since pupation	10	N/A
Barros-Cordeiro et al.	2016	*Cochliomyia macellaria and Lucilia cuprina*	23°C ± 1°, 60% ± 10% relative humidity	Fixed in Carnoy solution for 48 h, then transferred to 5% formic acid for another 48 h, and then transferred to and stored in 70% ethanol. This was followed by a morphological examination	Hours since pupation	5	N/A
Voss et al.	2017	*Calliphora dubia* and *Chrysomya rufifacies*	24°C ± 1 °, 60–70 % relative humidity	Immersed in hot water for 10 s and then preserved in 70% ethanol before undergoing morphological analysis.	Days since pupation	N/A	8
Karabey and Sert	2018	*Lucilia sericata*	constant temperatures (20, 25, and 30 °C)	Pupae were killed, dissected, analysed and then stored in 80% ethanol.	ADH since pupation	20	N/A

Due to the puparium being opaque, a clearing technique is used to view the different structure on it. Clearing is a technique that is used to lighten the colour of the puparium, to make the observation of external morphological features possible. This is done by transferring the empty pupal case into a beaker with 20% potassium hydroxide solution (KOH). After clearing, the puparium is wiped clean of any excess KOH and cut into sections before mounting onto glass slides (Sukontason *et al.*, 2007). Furthermore, removal of the pupa from the puparium allows examination the pupa. The development of appendages, setae and setae, and sensory features of the pupa can be examined using a stereomicroscope. Pigmentation of the pupa itself can also be observed (Brown, Thorne and Harvey, 2012). Recent studies have successfully used the presence/absence of legs, wings, labella, abdominal segments, antennae, thoracic setae, orbital/facial setae, arista and eye colour to estimate pupal age (Pujol-Luz and Barros-Cordeiro, 2012; Feng and Liu, 2013, 2014; Brown, Thorne and Harvey, 2015; Barros-Cordeiro *et al.*, 2016; Voss *et al.*, 2017; Karabey and Sert, 2018).

Even though all these studies have been successful in estimating pupal age, they all use different methods to age the pupa itself. Brown and colleagues identified 23 different morphological characteristics of *Calliphora vicina* puparia that correlated to age. From the 23 characteristics identified, 10 characteristics were identified that were significant for estimating pupal age. These 10 characteristics include the compound eye colour, facial setae, antennae colour, arista colour, labellum colour and shape, palp colour, labrum colour, wing folding, and leg width at full length. They made use of accumulated degree hours (ADH) to accurately age their pupae (Brown, Thorne and Harvey, 2015). Their ADH calculation started from time of oviposition and ended at the time of analysis. Voss et al. (2017) also identified characteristics of *Calliphora dubia* and *Chrysomya rufifacies* pupae, but aged their pupae in days (Voss *et al.*, 2017). The rest of the studies identified various different stages of development that corelated to age. These studies aged their pupa in hours (Pujol-Luz and Barros-Cordeiro, 2012; Feng and Liu, 2013, 2014; Barros-Cordeiro *et al.*, 2016). All of the above studies were able to create a developmental timeline using their various methods of aging (ADH, hours since pupation and days since pupation) and thus being able to estimate pupal age. All of the studies showed a similar trend in development; the legs and wings are first to develop and the colour of the compound eyes are last to develop. The rate of development is dependent on the temperature and the humidity (Feng and Liu, 2013, 2014).

Feng and Lui (2013 and 2014) did two experiments with two different species species (*Megaselia spiracularis* and *Megaselia scalaris*) at different temperatures, ranging from 21°C to 36 °C. This showed that the higher the temperature was, the quicker the pupae developed. When the temperatures were higher than 33°C and 36°C no development was noted for each respective species. This is due to the flies reaching their upper developmental threshold (Feng and Liu, 2013, 2014).

One study focussed on the pupal stage of *Lucilia sericata* for PMI estimation (Karabey and Sert, 2018). They focused on the different development stages of the pupae to aid in PMI estimation, utilising hours since pupation and ADH since pupation to age their pupa. This was the only study that utilised ADH since pupation to age their pupae. The experiments at different temperatures showed that 25°C was the optimal temperature for the growth of *L. sericata* pupae. They were successful in creating a timeline that consisted out of 18 different pupal development stages. These included 9 pre-existing stages and 9 new ones (Table 1.2) (Karabey and Sert, 2018).

Table 1.2: Stages of development in *Lucilia sericata* pupae (Karabey and Sert, 2018).

	Pre-existing	**Newly discovered**
1	Prepupae	Observation of the leg buds
2	Cryptocephalic stage	Wing formation
3	Phanerocephalic stage	Cephalopharyngeal skeleton embedded in the apical area Phanerocephalic
4	Segmentation of the abdomen	Stigma formation on the abdomen
5	Coloration of the posterior portion of the eye	Formation of maxillar palpus
6	Maroon red-coloured compound eyes	Yellow-coloured compound eyes
7	Setae darkened on head and thorax	Limbs darkened and thickened
8	Setae darkened on abdomen	Thinning of pupal cuticle
9	Adult emergence	Removal of pupal cuticle

Histological techniques have been utilised to observe the internal morphological changes of the pupa. These techniques identified various internal organs that could be used to estimate the age of various blow fly pupa. Research has shown that there are noticeable changes that take place in the brain, compound eye and thoracic muscles that can aid in age estimation of pupae (Davies and Harvey, 2013). Although these traditional methods of analyzing

morphology can be used to give accurate PMI estimation, they tend to destroy samples. Researchers have looked at Micro-computed tomography (micro-CT) to try and preserve samples, while still being able to identify and observe age-related morphological characters in pupae. Using these newer morphological analysis techniques in conjunction with traditional methods, can provide a better overall picture of the morphological changes that occur during the pupal stage (Martín-Vega *et al.*, 2017).

1.4.3.2 Molecular and chemical based techniques for PMI estimation of pupae.

Recently researchers have shifted their focus to molecular and chemical based techniques. These include the analysis of gene expression and analysing the presence of cuticular hydrocarbon (Gilby & McKellar, 1970) .

Hydrocarbons are comprised of n-alkanes, n-alkenes, as well as terminally and internally branched alkanes (Pechal *et al.*, 2014; Moore *et al.*, 2016). The analysis of cuticular hydrocarbons (CHCs) can be used for the age estimation of pupae. These cuticular hydrocarbons are stable structures that cover the insect cuticle. They have various functions including the prevention of dehydration and infiltration of microorganisms. They also function as pheromones (Moore, Adam and Drijfhout, 2013; Pechal *et al.*, 2014). Pechal et al. (2014) found these compounds to be species-specific. The hydrocarbons found on the cuticle of blow fly larvae and post-feeding larvae consist out of short chain hydrocarbons. These short chains hydrocarbons will develop into long chain compounds in the puparial and adult stages (Moore, Adam and Drijfhout, 2013; Moore *et al.*, 2016).

Pupae represent the oldest fly specimens present at a crime scene and in some cases they are the only source of entomological evidence available (Smith, 1986; Feng and Liu, 2013). In some cases where the body is in an advanced state of decomposition, empty puparial cases are the most common and sometimes only evidence at the scene (Zhu *et al.*, 2013). These empty cases are collected because they still contain the cephalopharyngeal skeleton and spiracles of the third instar larva. These features can aid in species identification (Buckland and Smith, 1986). Furthermore, a recent study has shown that Cuticular hydrocarbon analysis of empty puparial has been successful in estimating a PMI (Gilby & McKellar, 1970, Zhu *et al.*, 2013).

Other researchers have focussed more on the molecular aspects of puparial development. A study done by Brown (2012) found that age can by estimated by studying the gene-expression in blowfly pupae. The expression levels of a selected few genes were studied. It was found that the levels and ratios of these genes changed throughout the puparial stage. An age estimating method was created with the help of a regression analysis done on the expression ratios of the genes (Brown, 2012).

Finally, the steroid hormone, ecdysteroid, is important for insect development. It helps with insect metamorphosis by triggering transitions between the various developmental stages and controlling various metamorphic processes (Adams, 2009). Various researchers have studied the steroid hormone pathways, as well as the genes and proteins involved in metamorphosis. They have also studied how hormones control metamorphosis. A study by Gaudry *et al*. (2006) focussed on ecdysteriod. An Enzyme immunoassays (EIA) was performed to determine the levels of ecdysteriod present at different time points of the pupal stage. This study revealed that fresh and dried pupae have different levels of ecdysteroid present (Gaudry *et al.*, 2006). As steroid hormones have been indicated to be involved in the control of insect development, these hormones have the potential to be used as indicators of insect age.

1.5. Conclusion.

Flies in all stages of development can be of forensic importance when they are found at a death scene. Determining the age and species of insects in these various developmental stages are critical to any investigation. Previous research has largely focused on the developmental rate and morphological changes of the larval stages for estimation of PMI. This has left a gap in the field of forensic entomology and PMI estimation. Recent studies have started to explore the use of pupae in age estimation. Since fly species spend roughly half of their immature life-stage in the pupal stage, and that pupae are sometimes the only/oldest insect specimens found at certain crime scenes, it is important to understand the morphological changes during this stage of development.

To estimate PMI using developmental models, it is essential to determine the age and species of the insect. This is done by rearing the larvae to adulthood, determining the species and then using complicated calculations to determine how old the insect was at the time of collection. This method is time consuming and requires expertise in rearing insects. To be

able to determine the age of the pupae using only the puparium and not having to spend time rearing them to adulthood, could save valuable time in medico legal investigations. This study aims to identify reliable morphological markers to aid in a more accurate age estimation of *Lucilia sericata* during the pupal stage. *Lucilia sericata* was chosen for this study as they are one of the most common forensically important flies in South Africa and not many studies have been done on the species in South Africa (Tembe and Mukaratirwa, 2020).

2. Materials and methods.

Lucilia sericata flies were utilised from an already established colony at the University of Cape Town. In order to stimulate oviposition, flies were protein starved for seven days before they were given pig liver. Following oviposition, the eggs were collected and placed on a new piece of pig liver. This piece of liver was then placed on a piece of foil that was placed in a container filled about half with vermiculite. The container was then sealed with a lid that had holes in for ventilation. This container was subsequently placed in an incubator set at 25°C, 65% relative humidity and a photoperiod of 14:10 Light:Dark. These conditions were chosen to best represent the South African climate and day/night cycle during summer. After the larvae had hatched they were checked twice a day (at intervals of about 12 hours) and pig liver was provided as needed. The dried-out pig liver was removed when fresh liver was added.

2.1. Collection and preservation of specimens.

Once the larvae started to wander away from the food source, the containers were checked regularly for the presence of prepupae. Prepupae (referring to the onset of pupation) were identified by lack of movement and by a decrease in length compared to the third instar larvae. Once the first prepupae were spotted, all prepupae present at a given time were collected. The collections happened in intervals of 6 hours over 4 days. A total of 145 prepupae were collected and allowed to grow in the incubator until reaching a desired age. The age is represented by hours after onset of pupation, starting at 0 hours and increasing by 6 hours for each age group. No pupae were older than 168 hours. A total of 5 pupae were collected for each age group.

Once they reached the desired age, the pupae were removed from the incubator. The weight of pupae were recorded prior to being hot-water-killed by submergence in just boiled water for 30 seconds. Specimens were removed from the water and the puparium was pierced three times with an insect pin. They were then placed into 5ml tubes containing 70% ethanol for preservation. The tubed were kept in the fridge at 4° C until morphological analysis.

2.2 Morphological analysis.

2.2.1 Analysis of the external morphological characteristics of the puparium.

External morphological characteristics of the puparium were observed and photographed under a stereomicroscope (Zeiss Discovery V20, Zeiss, Oberkochen) coupled with an Axiocam

503 Colour (Zeiss, Oberkochen). Characteristics observed included the texture of the puparium, the presence of respiratory horns, the colour, and the presence of the lateral ridge.

Observation of the posterior spiracles and the cephalopharyngeal skeleton (CPS), could be better observed under the stereomicroscope after the puparium underwent a clearing technique. Prior to clearing, each pupa was dissected out of the puparium. This was done using a scalpel to cut through the outer puparium. The puparium was cut in the middle to produce a front and a back half, whereafter the pupae on the inside could be removed without any damage (Figure 2.1).

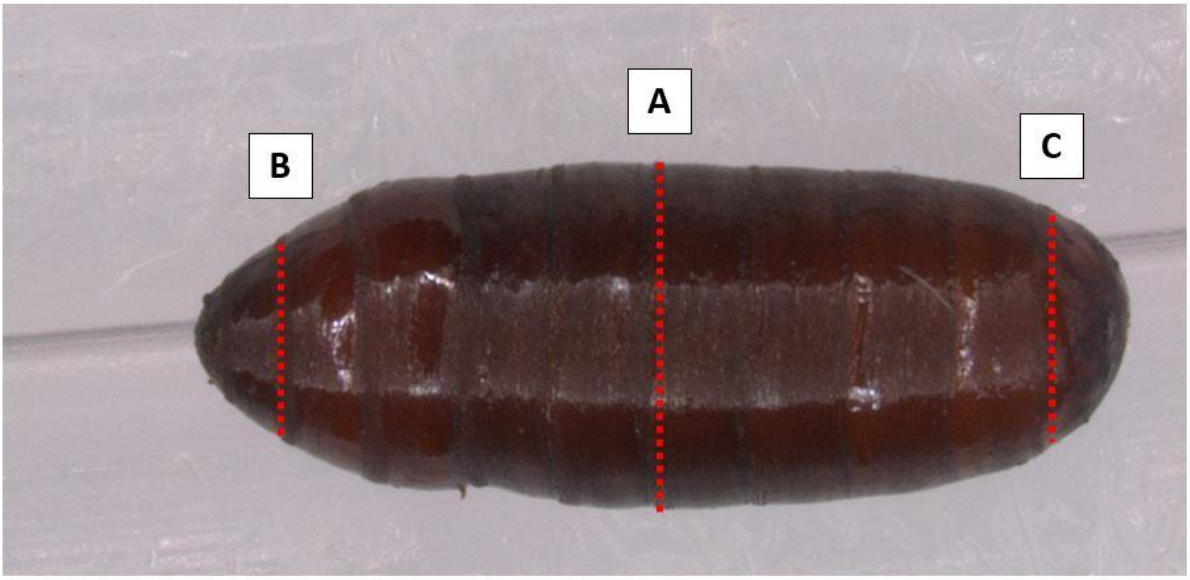

Figure 2.1: A puparium indicating where the cuts were made to dissect out the pupa (A) and removing the first and last segments of the puparium (B and C).

Once the pupa was removed it was placed into a 70% ethanol solution and stored in the fridge at 4° C. The first and last segment of the puparium (Figure 2.1) was separated from their respecting half before being placed in 20% potassium hydroxide (KOH) solution for 4 days. After 4 days the specimens were removed from the solution and patted dry with a paper towel. The specimens were then cut further to remove excess puparium, leaving behind only the parts that had either the posterior spiracles or the CPS. The different parts were then mounted on glass slides before being viewed under the stereomicroscope.

2.2.2 Analysis of the external morphological characteristics of the pupae.

After the external examination of the puparium, the pupae were examined under a stereomicroscope (as above). Specimens were retrieved from the fridge and removed from the 70% ethanol before examination. Before they were placed under the microscope they were patted dry with some paper towel. Twenty one morphological characteristics were identified prior to the analysis (Table2.1). Only 20 of these characteristics were successfully identified on the specimens and photographed. The labrum shape was the only one that was not successfully documented.

Table 2.1: Twenty one morphological characteristics identified for the external morphological analysis of the pupa.

Region of the body	Head	Thorax	Abdomen
Characteristics	Compound eye colour	Wing folding	Abdominal segments
	Orbital/facial setae	Wing colour	Abdomen macrosetae
	Antennae shape	Leg length	Abdomen microtrichia
	Antennae colour	Leg width at full length	
	Arista colour	Leg Bristle Colour	
	Labellum shape	Thoracic setae	
	Labella colour		
	Palp shape		
	Palp colour		
	Labrum colour		
	Labrum shape		
	Genal setae		

2.3 Aging of pupae.

After the documentation of all the external morphological characteristics of the puparia and pupae, timelines were created of characteristics vs time. With time being the dependent variable and the characteristics being the independent variables. The age was represented in hours since pre-pupation and accumulated degree hours (ADH). ADH is the amount of thermal energy needed to develop from one stage of development to the next. This is dependent on the ambient temperature and the relative humidity of the environment the insect is in. ADH was calculated with the following formula:

Temperature (°C) x Time (the age of the pupae in hours) = ADH

2.4 Statistical analysis.

Multiple linear regression analysis was performed to assess characteristics which were useful for estimating the age of the pupae (ADH) and to develop a regression equation based on the data collected. All characteristics were coded for analysis (Appendix A1). Initially a full model was created using all 20 characteristics. An analysis of multi-collinearity using the variance inflation factor (VIF) indicated unacceptable levels of covariance. Variables were removed from the model in a stepwise fashion after calculating the VIF and removing the variable with the highest VIF. Collinear variables were removed from the model until an acceptable level was achieved (VIF < 10). This created a second model consisting of 15 variables. Following this a final model was created using the 10 most significant coefficients in model 2. All statistical analysis was conducted using Stata Version 15 (Statacorp, TX, USA).

2.5 Ethical clearance.

This study forms part of a project which has received ethical clearance from the University of Cape Town (UCT) Ethics Committee (reference number: AEC: 018/027) (Appendix D).

3. Results.

A total of 145 pupae were reared and examined for external morphological changes over time. Five pupae were collected at each time point. A total of six external morphological characteristics of the puparia and twenty external morphological characteristics of the pupae were examined. These characteristics were correlated to age in ADH, with the aim of producing a timeline that can aid in the estimation of pupal age. The pupal ADH ranges from the youngest being 6550 ADH and the oldest being 11300 ADH. ADH was calculated from the time of oviposition till the time the pupae were killed.

ADH = total hours of development (time of oviposition to time of hot water kill) x temperature (25°C)

3.1. External morphological characteristics of the puparia.

Morphological features on the puparia appeared early on with little difference between the appearance of features. These features include the texture of the puparium (alternating between smooth and textured rings) (figure 3.1), the lateral ridge (figure 3.2), posterior spiracles (figure 3.3) and the cephalopharyngeal skeleton (CPS) (figure 3.4). Similarly, the colour and weight (Appendix C1) of the pupae were not found to be a discernible factor relating to age. The only exception to this were the respiratory horns (figure 3.5). The respiratory horns were observed for the first time at 30 hours (9200 ADH). Colour and weight varied across all the different ages. Only the prepupae were cream coloured, while the rest varied from light brown to black in colour. Most of the pupae collected were brown in colour.

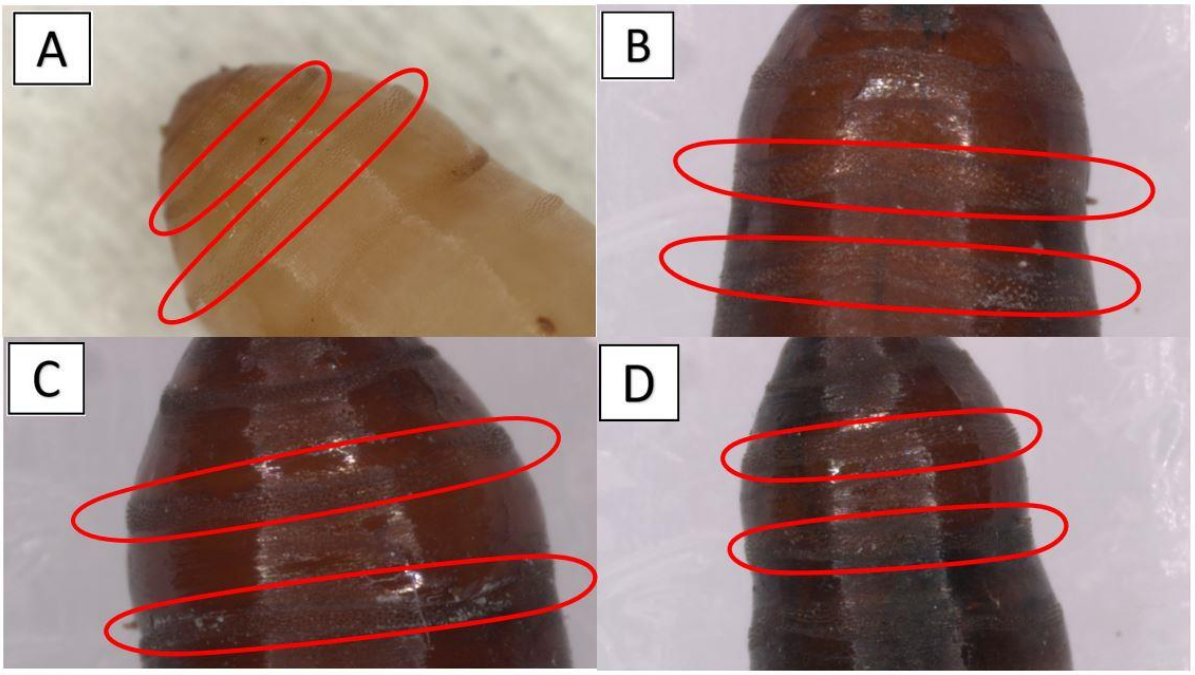

Figure 3.1: The texture of the puparium alternates between smooth and textured rings. No difference was seen between A) prepupae, B) 54 hours, C) 108 hours and D) 168 hours.

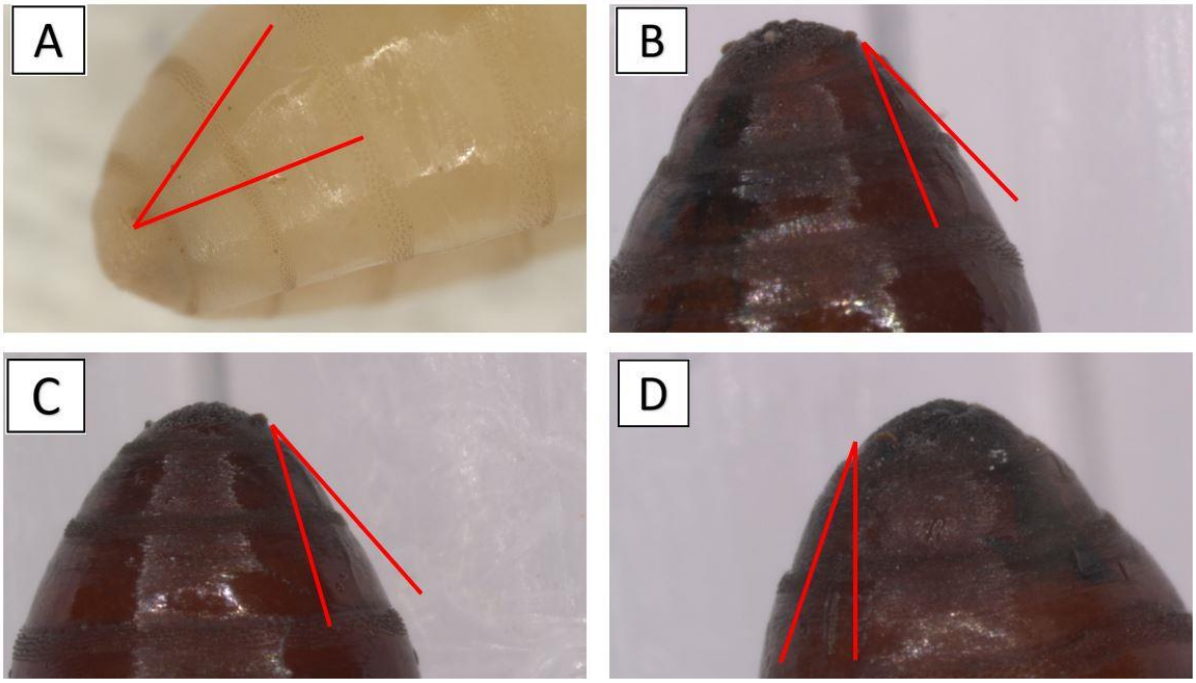

Figure 3.2: The lateral ridge was present on all pupae. No difference was seen between A) prepupae, B) 54 hours, C) 108 hours and D) 168 hours.

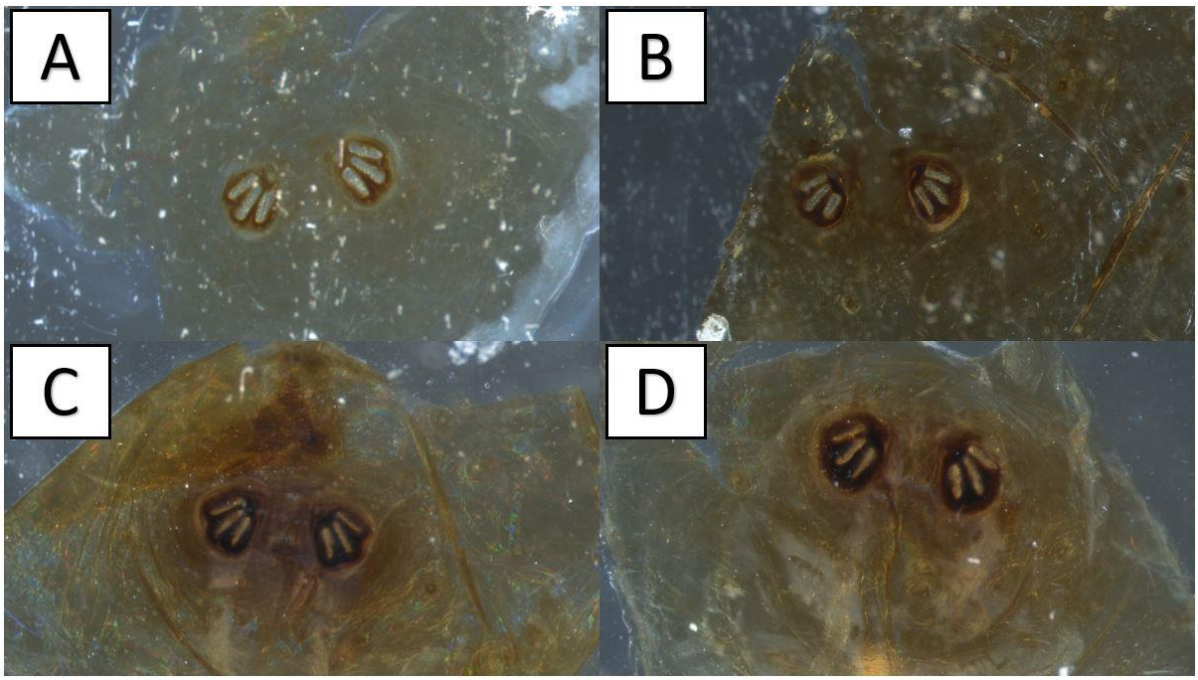

Figure 3.3: The posterior spiracles were present on all pupae. No difference was seen between A) prepupae, B) 54 hours, C) 108 hours and D) 168 hours.

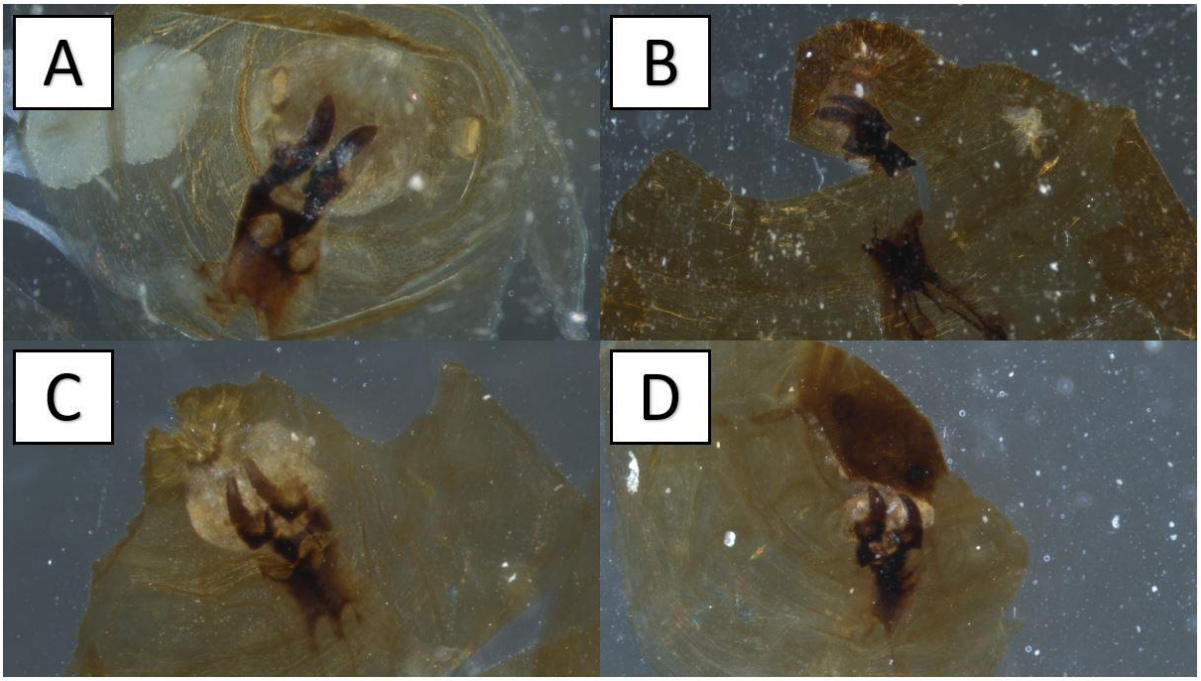

Figure 3.4: The cephalopharyngeal skeleton of the third instar larva within the puparium were present on all pupae. No difference was seen between A) 6 hours, B) 54 hours, C) 108 hours and D) 168 hours.

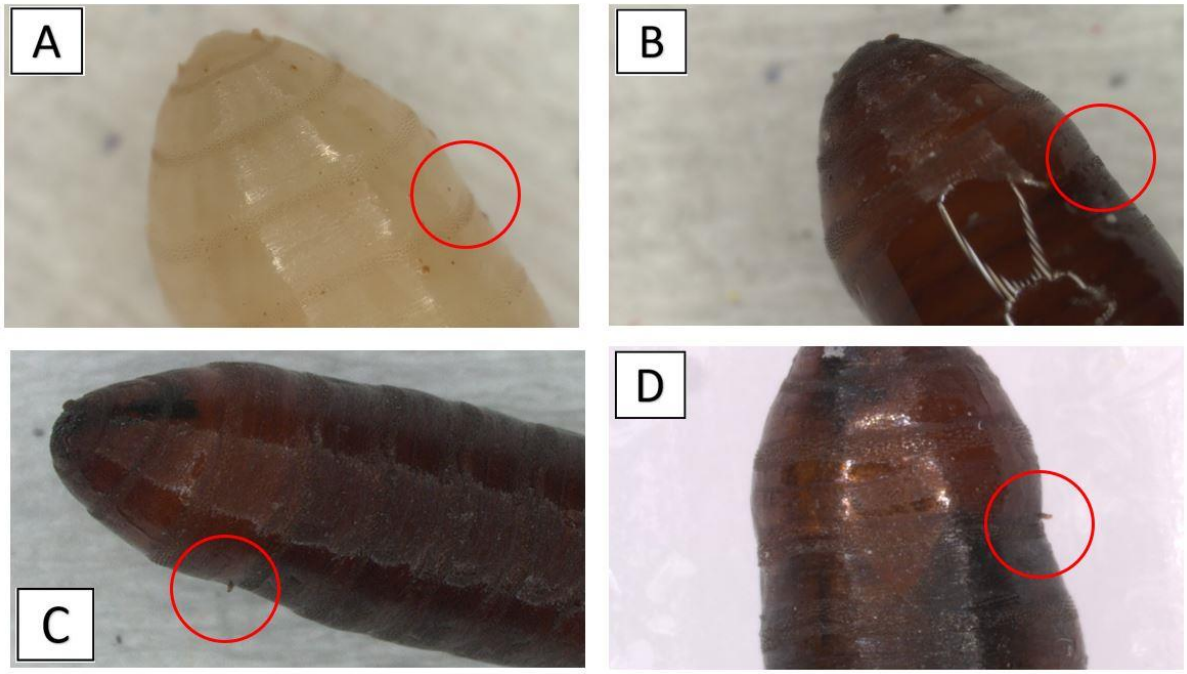

Figure 3.5: The respiratory horns are absent during the first few hours of development. respiratory horns were absent from A) prepupae stage to B) 24 hours. Respiratory horns were first noted at C) 30 hours and were present till D) 168 hours post pupation.

3.2. External morphological characteristics of the pupa.

To study the external morphological features associated with the developing pupae, they were carefully dissected from the puparia and analysed. The development of 20 features associated with the pupa were documented. The timing of appearance for each of these features can be seen in table 3.1.

Table 3.1. Changes that each feature undergoes and at what age in hours or ADH they occur.

Region	Feature	Change	Pupal age (hours)		ADH	
			min	max	min	max
Cephalic	**Compound eye colour**	Absent	6	24	6800	9175
		Cream	30	144	9200	10975
		Pale pink	138	162	10850	11150
		Pink	156	162	11150	11150
		Red	168	168	11150	11150
	Orbital/facial setae	Absent	6	144	6800	10975
		Brown	144	150	10975	11150
		Black	156	168	11150	11150

	Antennae shape	Absent	6	42	6800	9500
		Present	48	78	9650	10100
		Full length	84	168	10250	11150
	Antennae colour	Absent	6	42	6800	9500
		White	48	156	9650	11150
		Brown	156	162	11150	11150
		Black	162	168	11150	11150
	Arista colour	Absent	6	150	6800	11150
		White	156	162	11150	11150
		Half brown	156	162	11150	11150
		Brown	162	168	11150	11150
		Black	168	168	11150	11150
	Labellum shape	Absent	6	30	6800	9200
		Square	36	42	9350	9500
		Slightly lobed	48	66	9650	9950
		End lobed	72	150	9950	11150
		Oral hair developed	150	162	11150	11150
		Complete	162	168	11150	11150
	Labellum colour	Absent	6	36	6800	9350
		White	42	168	9500	11150
	Palp shape	Absent	6	54	6800	9650
		Round	54	54	9650	9650
		Slightly elongated	60	60	9800	9800
		Long	60	108	9800	10550
		Full length	114	168	10700	11150
	Palp colour	Absent	6	54	6800	9650
		White	54	150	9650	11150
		Black/brown	156	168	11150	11150
	Genal setae	Absent	6	150	6800	11150
		Brown	156	156	11150	11150
		Black	156	168	11150	11150
	Labrum colour	Absent	6	120	6800	10850
		White	126	132	10850	10700
		Light brown	138	156	10850	11150
		Brown	156	168	11150	11150
Thoracic	**Wing folding**	Absent	6	18	6800	9050
		Wing mass	18	24	9050	9175
		All unfolded	30	84	9200	10250
		Partially folded	90	120	10250	10850
		All folded	126	168	10850	11150
	Wing colour	Absent	6	24	6800	9175
		White	30	156	9200	11150

		Pale silver	156	162	11150	11150
		Silver	162	168	11150	11150
	Leg length	Absent	6	6	6800	6800
		Very short	12	18	8900	9050
		Short	24	30	9175	9200
		Full length	36	168	9350	11150
	Leg width at full length	Absent	6	24	6800	9175
		Inflated	30	42	9200	9500
		Fine	48	168	9650	11150
	Leg setae colour	Absent	6	132	6800	10700
		White	132	132	10700	10700
		Fine black	132	156	10700	11150
		Thick black	162	168	11150	11150
	Thoracic setae	Absent	6	96	6800	10375
		White	102	150	10850	11150
		Half brown	150	156	11150	11150
		Black	156	168	11150	11150
Abdominal	**Abdominal segments**	Larval	6	18	6800	9050
		Pupal	24	24	9175	9175
		Adult	30	168	9200	11150
	Abdomen macrosetae	Absent	6	150	6800	11150
		Brown	156	162	11150	11150
		Black	156	168	11150	11150
	Abdomen microtrichia	Absent	6	150	6800	11150
		Brown	156	156	11150	11150
		Black	156	168	11150	11150

3.2.1 Development of cephalic features.

The first feature on the head to develop is the eyes. The eyes are cream coloured at first and later undergo several changes from pale pink to pink and eventually turning red (figure 3.6). This is the last feature to change during the pupal development stage.

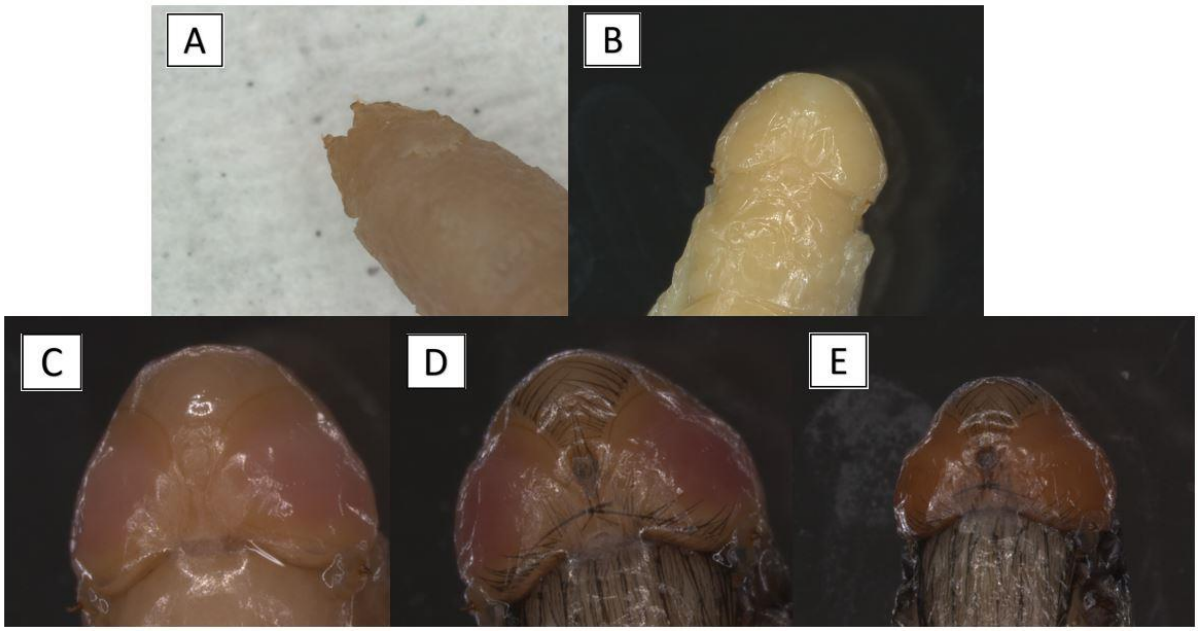

Figure 3.6: The development of the compound eyes and eye colour. Eyes are absent at first (A) and develop after head eversion. They are cream coloured when they first emerge (B) and change colour in the later stages from pale pink (C) to pink (D) to red (E).

This is followed by labellum shape and colour as well as antenna shape and colour. The labellum undergoes various changes from a square shape to being slightly lobular in shape to end lobed. The final change is the development of the oral hairs (figure 3.7). The colour stays white from the square shape till development is complete. The antennae are elongated at first but reach full length quickly (figure 3.8). From their first appearance they are white and then change to brown in the last stages of development (figure 3.9). In the last few hours of the pupal stage the arista appears and rapidly change colour from white to brown (figure 3.10).

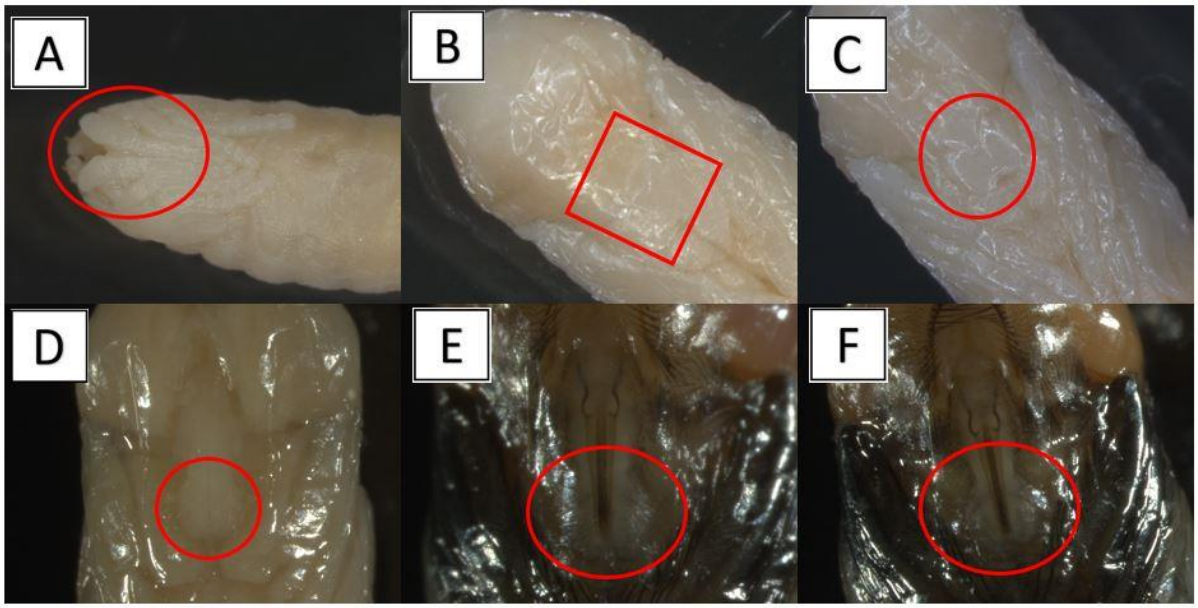

Figure 3.7: The development of the labellum. The labellum is absent at first (A). When it first develops it is square in shape (B) and becomes lobed at the end very quickly after that (C). As development progresses it becomes longer and lobed at the end (D) there after oral satae will begin to develop (E) until development is completed (F).

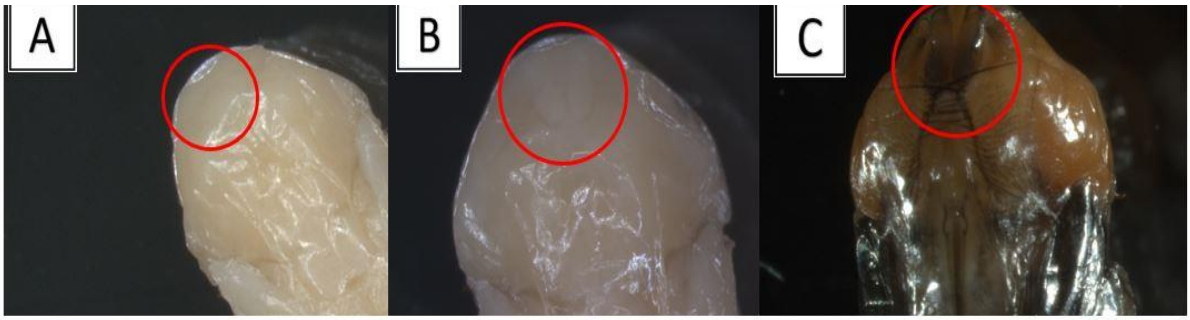

Figure 3.8: The development of the antennae. The antennae are absent at first (A) then appear quickly (B) before reaching their full length (C).

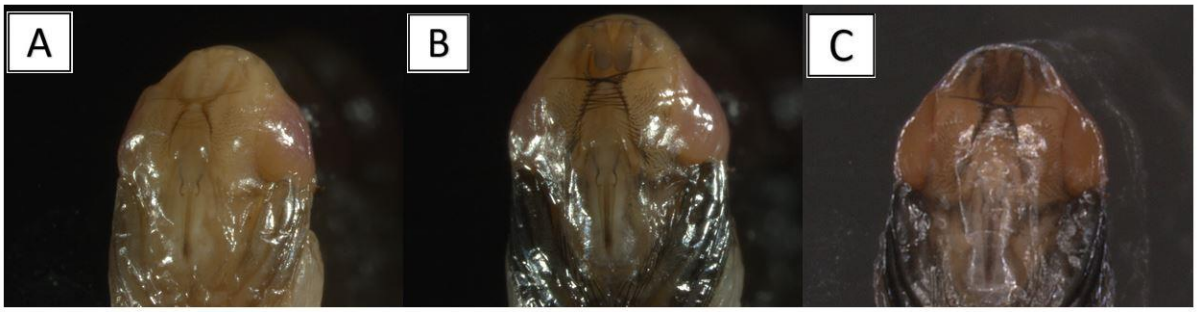

Figure 3.9: Colour change of the antennae. The antennae are white at first (A) then become more brown as development progresses. They are half brown (only the ends appear to be brown) (B) before turning fully brown in colour (C).

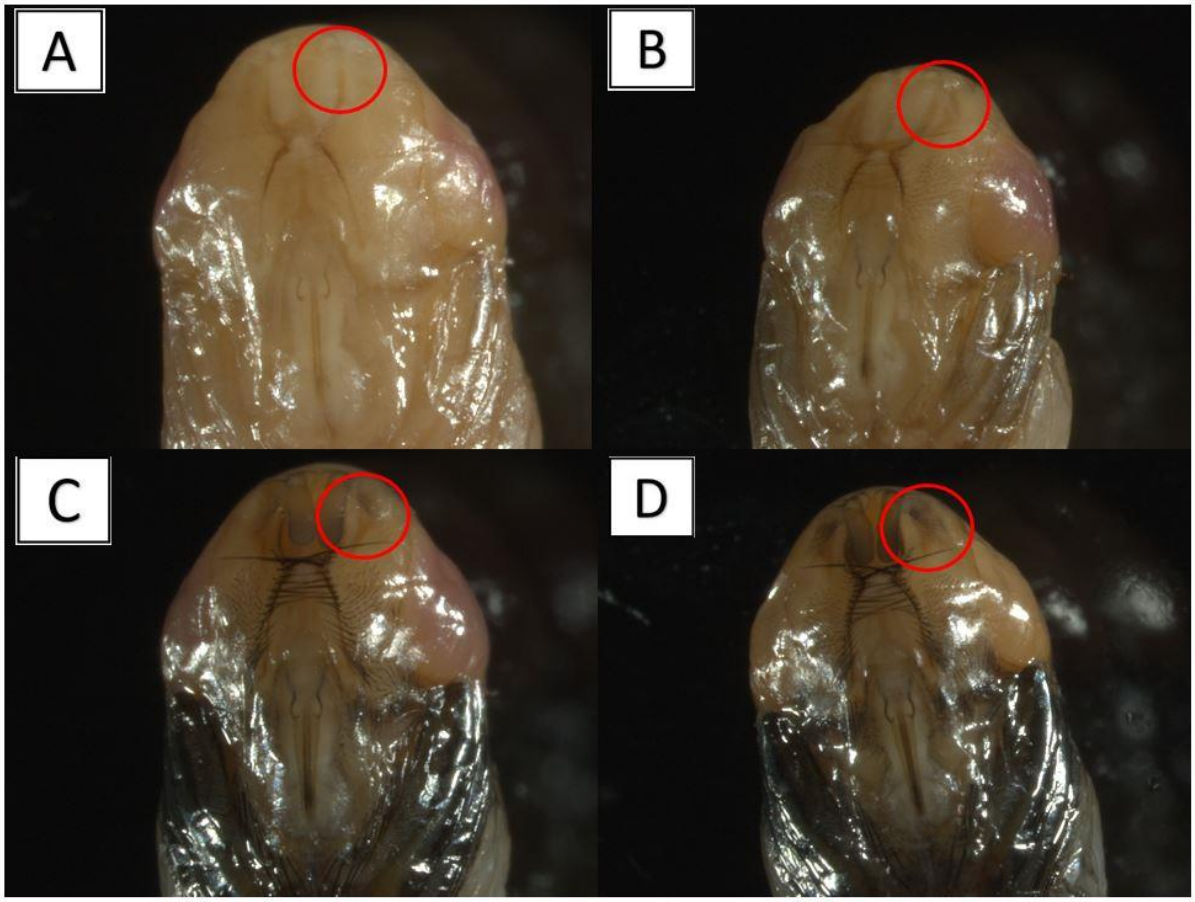

Figure 3.10: The Development of the arista. The arista are absent at first (A) then appear white in colour (B). They gradually become darker, appearing to be light brown at first (C) before turning a dark brown colour (D).

Similar to the antennae, the palp shape develops prior to its colour. The palp changes in length from being round then slightly elongated before reaching its full length (Figure 3.11). The colour stays white until the last few hours where they change to black/brown due to the setae being fully developed (Figure 3.12). The jowl and facial setae form at the end of the pupal development stage. Both the jowl and facial setae will appear brown at first and then become black (Figure 3.13 & 3.14). Unfortunately, it was not possible to distinguish the labrum shape at different times from the photos taken. The colour however was noted. In the later stages of development, the labrum starts off white then changes from light brown to dark (Figure 3.15).

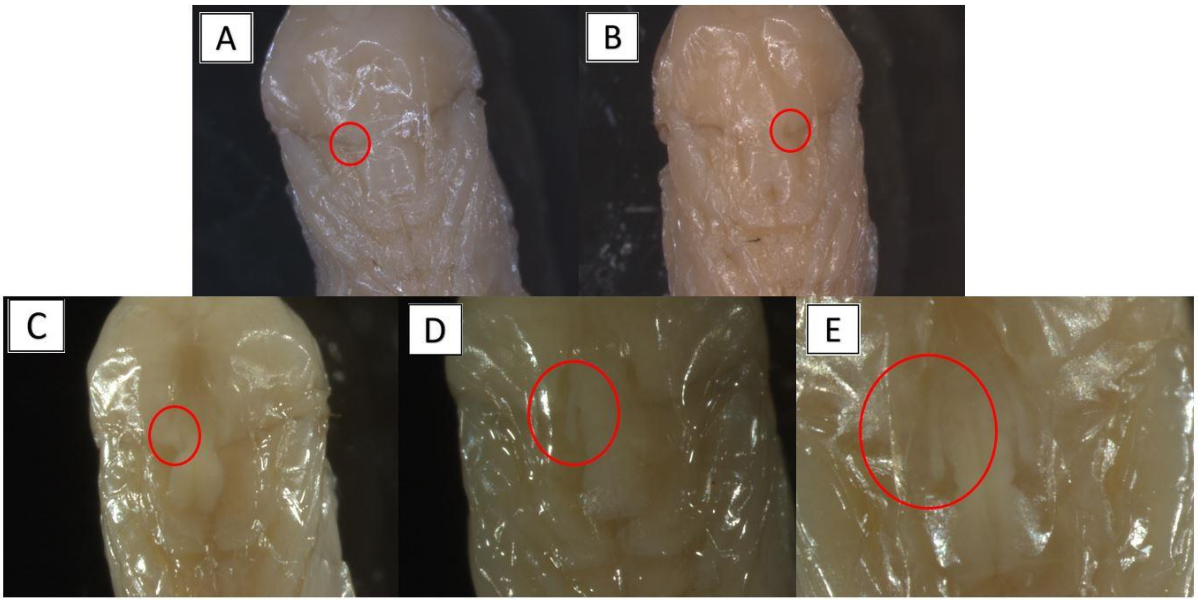

Figure 3.11: The development of the maxillary palps. The palp is absent at first (A). The palp changes in length from being round (B) then slightly elongated (C). The palp will appear long (D) before reaching its full length (E).

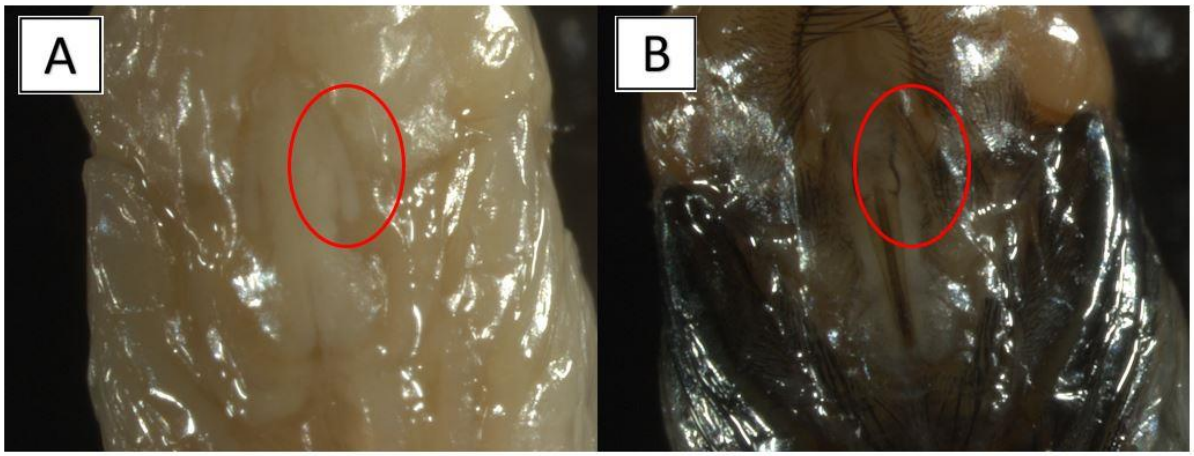

Figure 3.12: Colour change of the maxillary palp. The palp is white at first (A) and turns brown in the later stages of development (B).

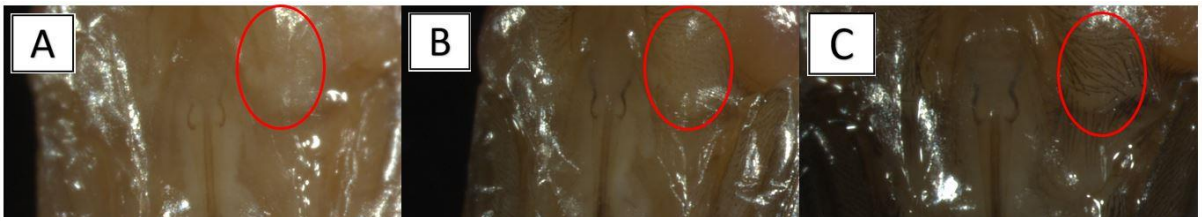

Figure 3.13: The development of genal satae. The genal satae are absent at first (A). When they appear, they are light brown in colour (B) and become black later in the development stage (C).

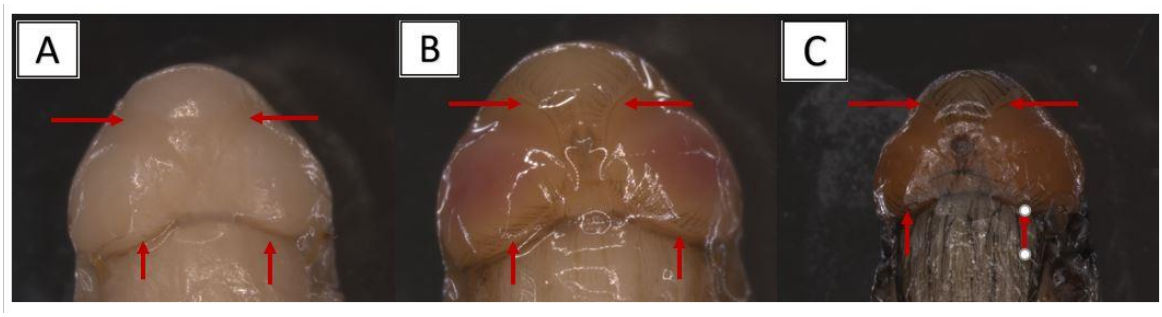

Figure 3.14: The Development of facial satae. The facial satae are absent at first (A). When they appear, they are light brown in colour (B) and become black later in the development stage (C).

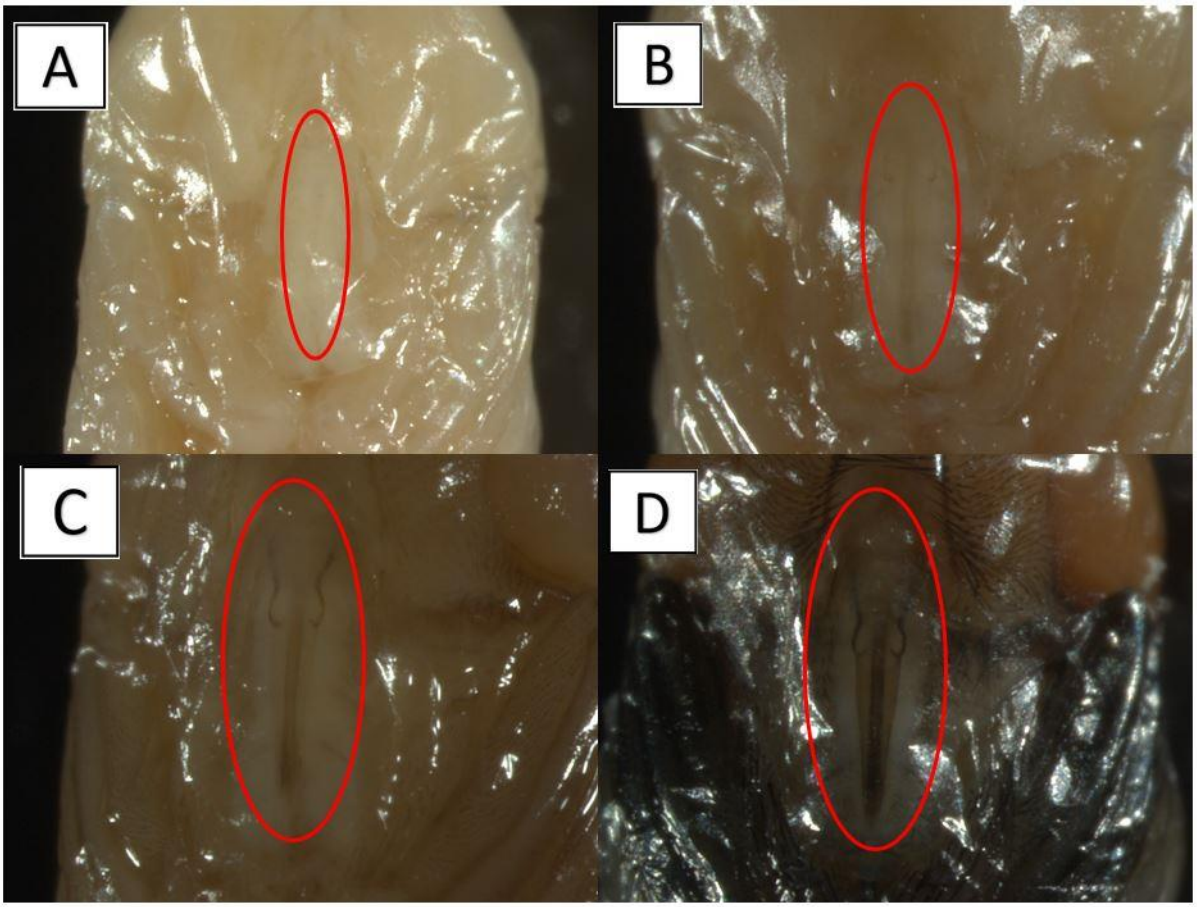

Figure 3.15: The development of the labrum. The labrum is absent at first (A) then appears white in colour (B). It gradually becomes darker, appearing to be light brown at first (C) before turning a dark brown (D).

3.2.2 Development of thoracic features.

The legs and wings are the first features to develop from the thoracic region. The legs grow to full length at a very early stage during pupal development (Figure 3.16). The legs are inflated (appear thicker when compared to those of an older age) for a very short time after reaching their full length before thinning out (Figure 3.17). The bristle on the legs develop very late. They start out as fine setae and develop into thick black setae in the final stage of development (Figure 3.18). The wings undergo various changes throughout the pupal development stage but only change colour in the last stage. They develop as a wing mass at first and are completely unfolded when they are fully developed. They will then start to fold until they are completely folded (Figure 3.19). The wing starts out white and change from a pale silver to dark silver colour (Figure 3.20). Finally, the thoracic bristle develops in the middle of the pupal stage. They change from white to black setae (Figure 3.21).

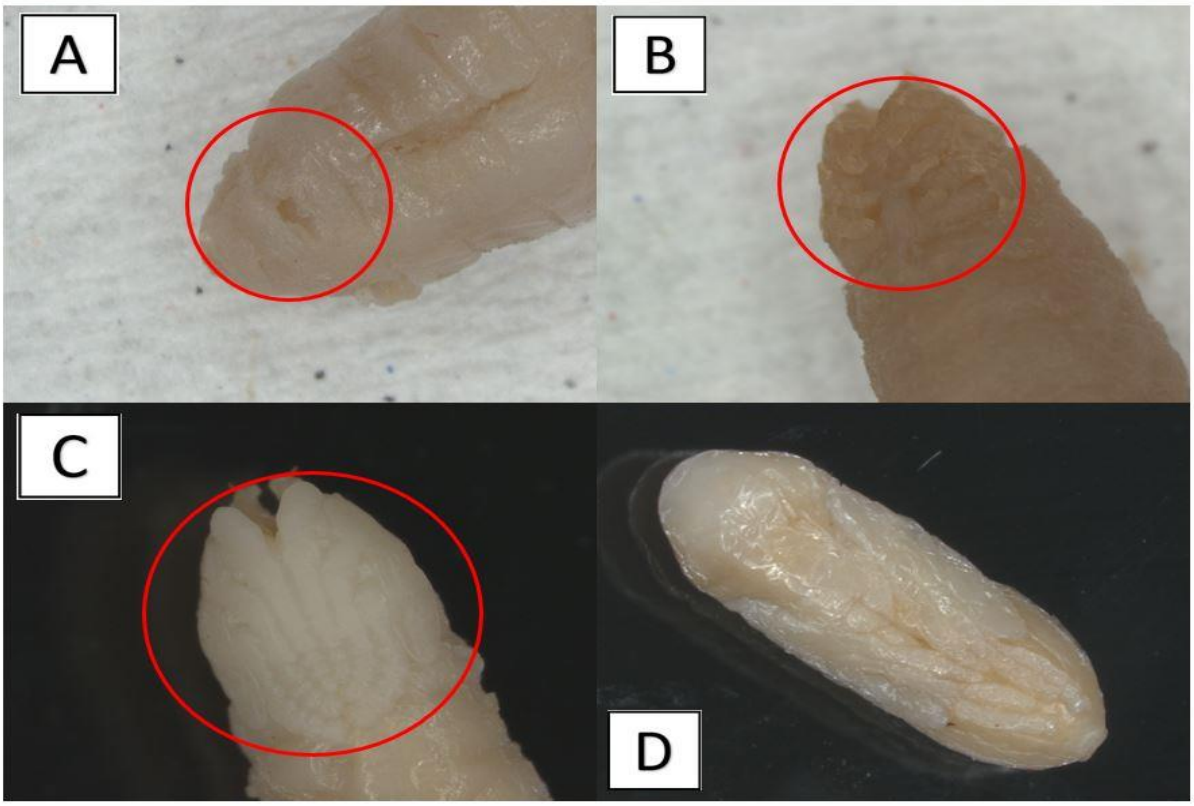

Figure 3.16: The development of the legs. Leg development happens rapidly. They are absent at first (A) and then develop from very short (B) to short (C) to full length (D) in a short amount of time.

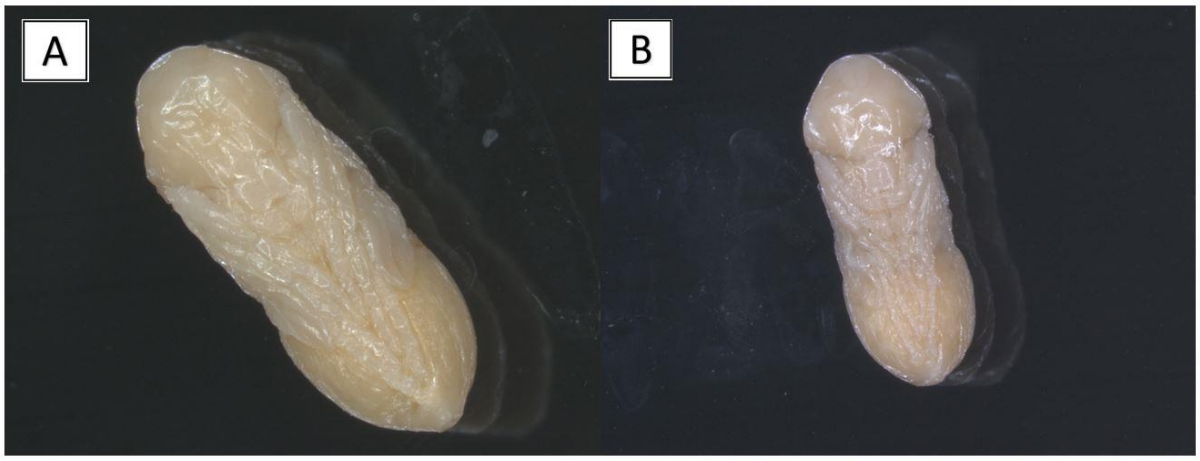

Figure 3.17: Leg width at full length. The legs appear to be inflated when they reach full length (A) and become fine as development progresses (B).

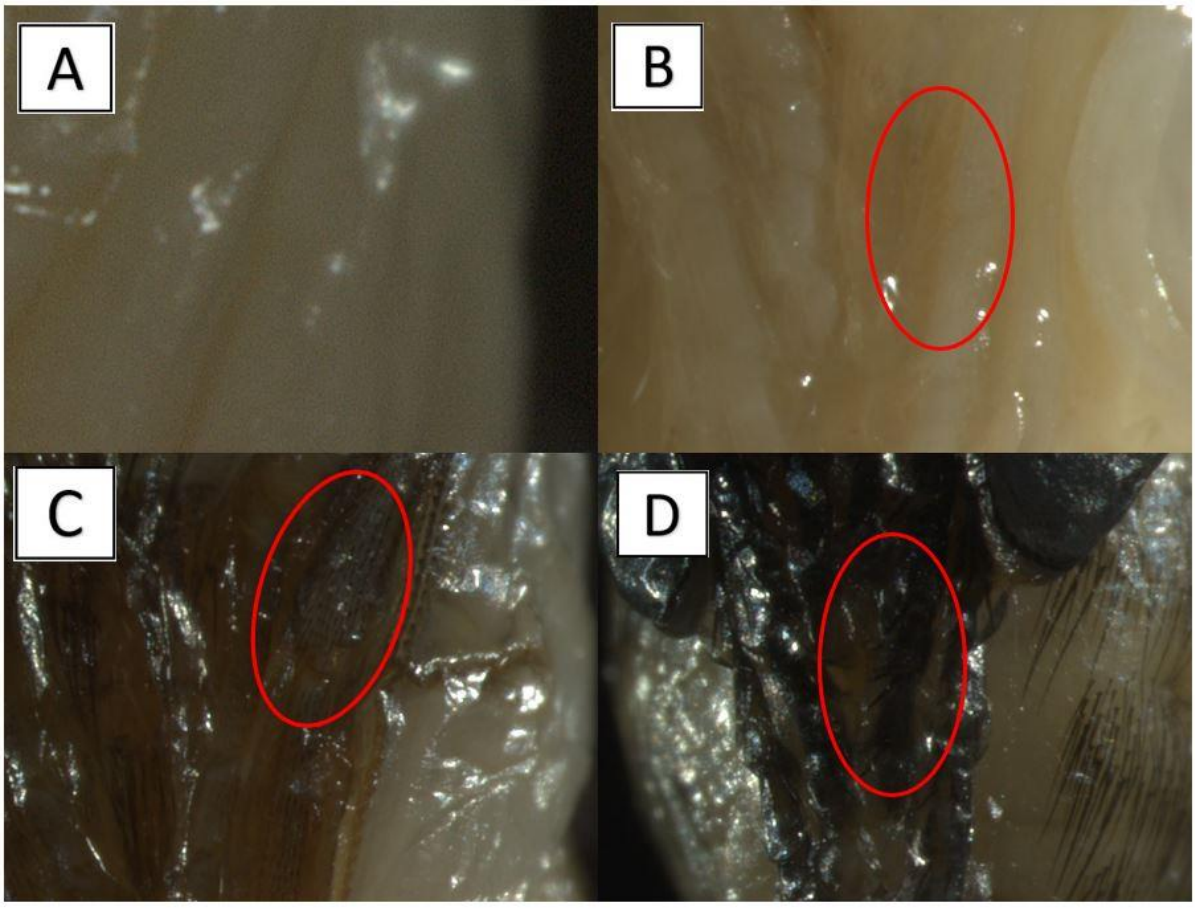

Figure 3.18: The development of the leg setae. Leg setae are absent at first (A). They develop as thin white setae (B) before becoming darker in colour (C). They will eventually develop into thick black setae (D)

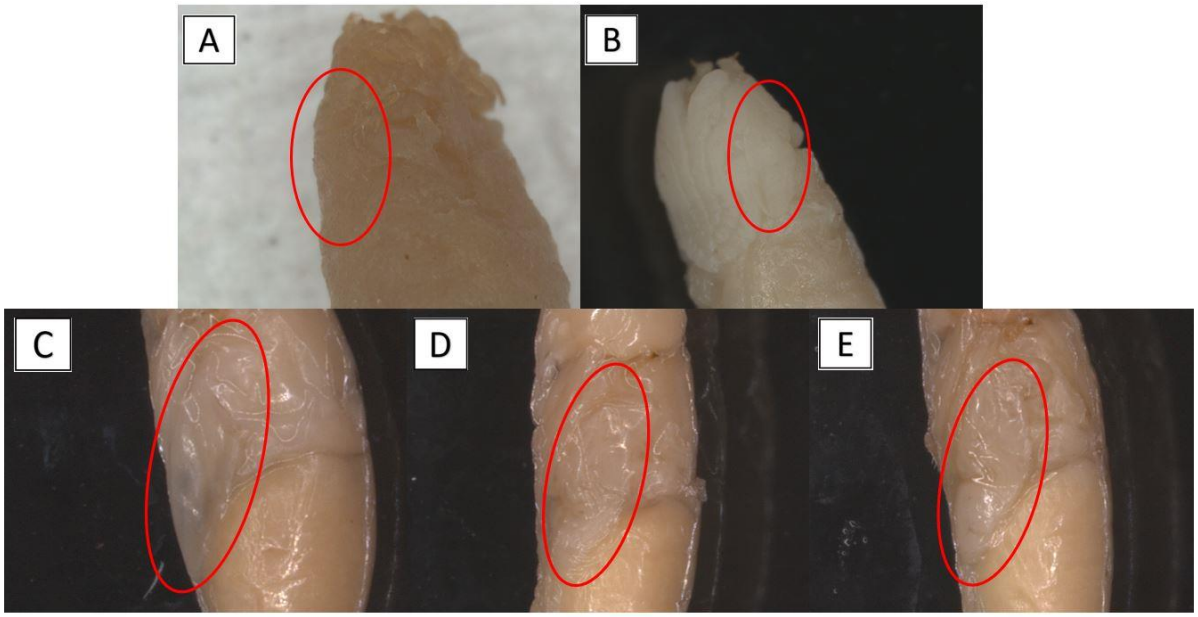

Figure 3.19: The development of the wings. The wings are absent at first (A). They appear as a wing mass at first appearance (B) and develop completely unfolded after that (C). They will gradually become partially folded (D) and end up being completely folded by the end of the pupal stage (E).

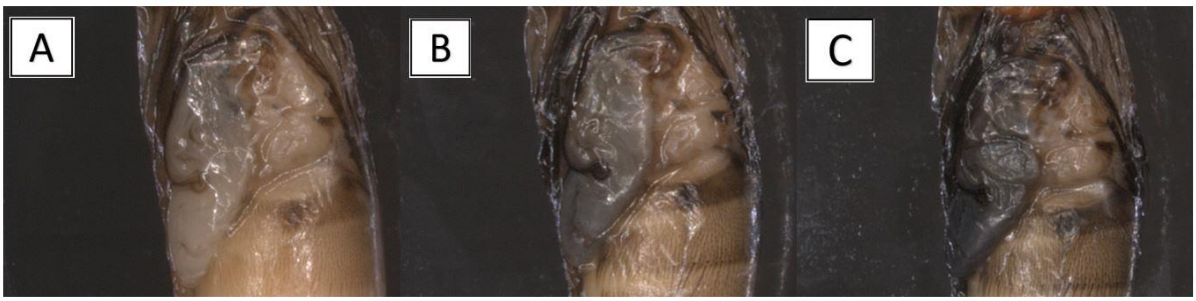

Figure 3.20: Changes in wing colour. Wings change from being white in colour (A) to pale silver (B) to dark silver (C).

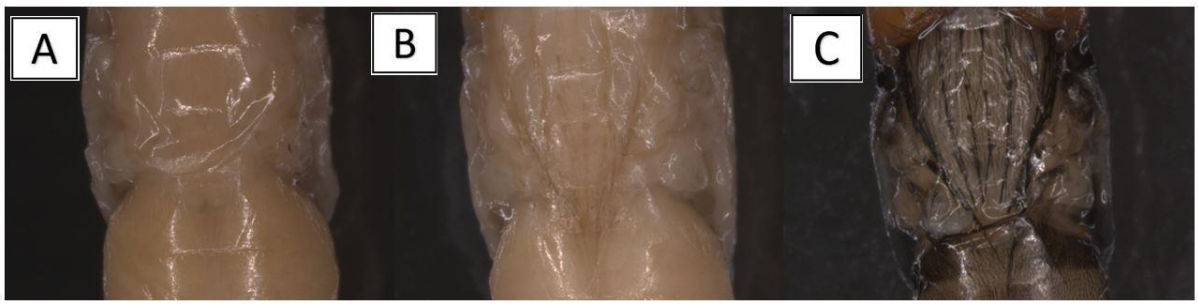

Figure 3.21: The development of the thoracic satae. The thoracic satae are absent at first. When they develop they are white in colour (A). They will then turn brown (B) before turning black in colour(C).

3.2.3 Development of abdominal features.

The abdominal segments are the first characteristic to develop in the abdominal region. At first they have a larval shape that eventually turns into the segments seen on the adult fly abdomen (Figure 3.22). The abdominal macrosetae and microtrichia are the last features to develop on the abdomen and only develop in the last stage. The microtrichia are fine and brown at first and develop into thick black setae. The macrosetae change colour from brown to black (Figure 3.23).

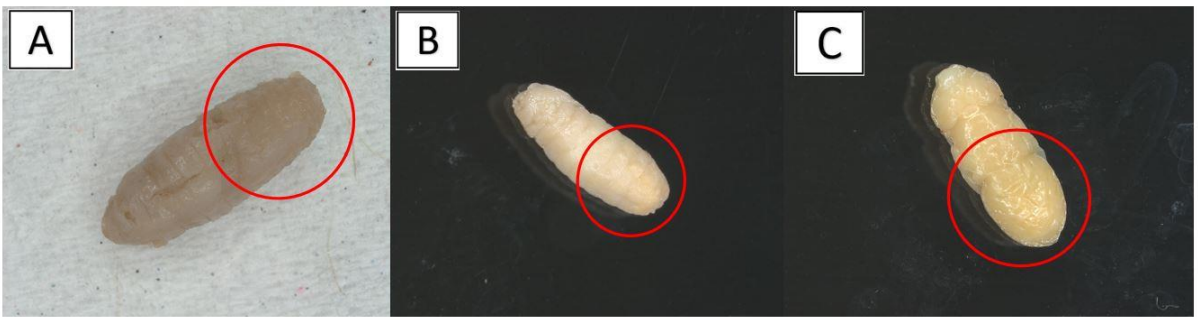

Figure 3.22: The development of the abdominal segments. The abdominal segments are first larval in appearance (A) before becoming more pupal in shape (B) and then finally adult (C).

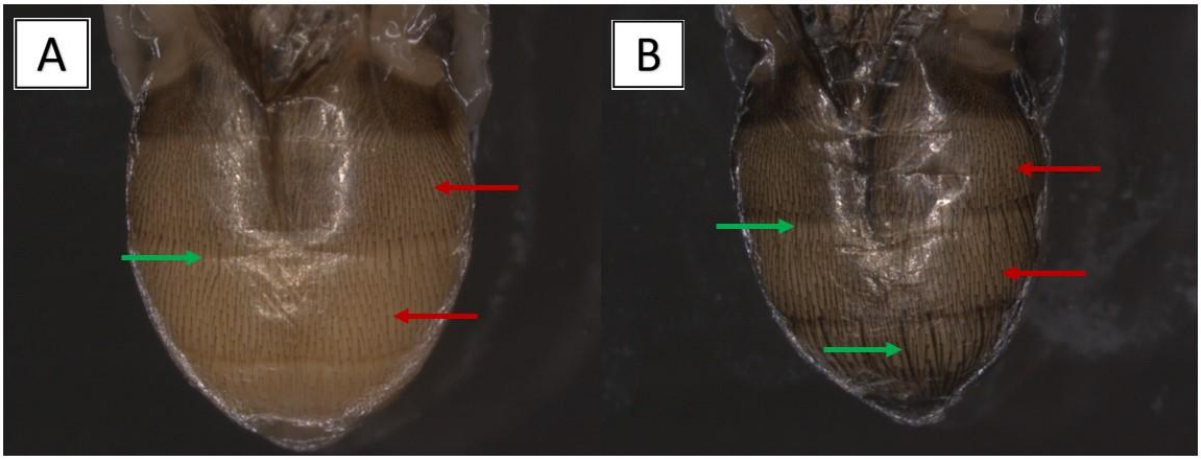

Figure 3.23: The development of abdominal macrosetae and microtrichia. Both types of setae are light brown at first (A) before turning black (B). Macrosetae are indicated by the green arrows and the microtrichia are indicated by the red arrows.

3.3. Developmental timelines.

This study made it possible to produce a timeline that can aid in the estimation of pupal age and identify characteristics which may assist in determining pupal age. Most of the features develop throughout the entire pupal stage. However, several features were identified as either being early (changes in shape or length and presence or absence) or late development features (colour changes). The early features include leg length and width and the development of the antenna shape and abdominal segments. Features that developed later were leg setae, abdominal macrosetae and microtrichia and compound eye, labrum and arista colour.

Timelines were created for all characteristics. The green timelines represent pupal age as ADH (Figure 3.24) and the red timelines represent pupal age in hours (Figure 3.25).

Figure 3.24: ADH timelines of each characteristic of the pupal development stage.

Figure 3.24 continued

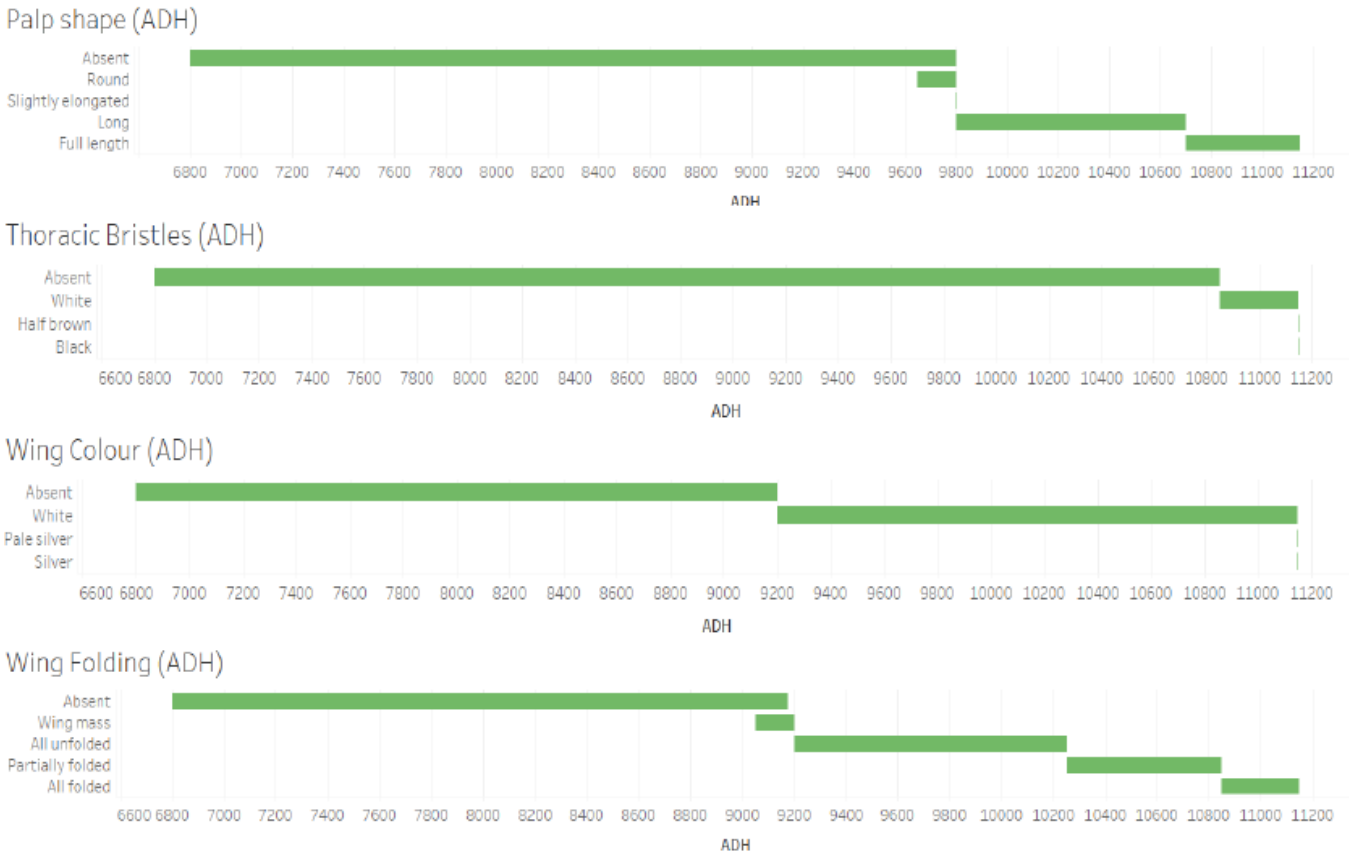

Figure 3.24 continued

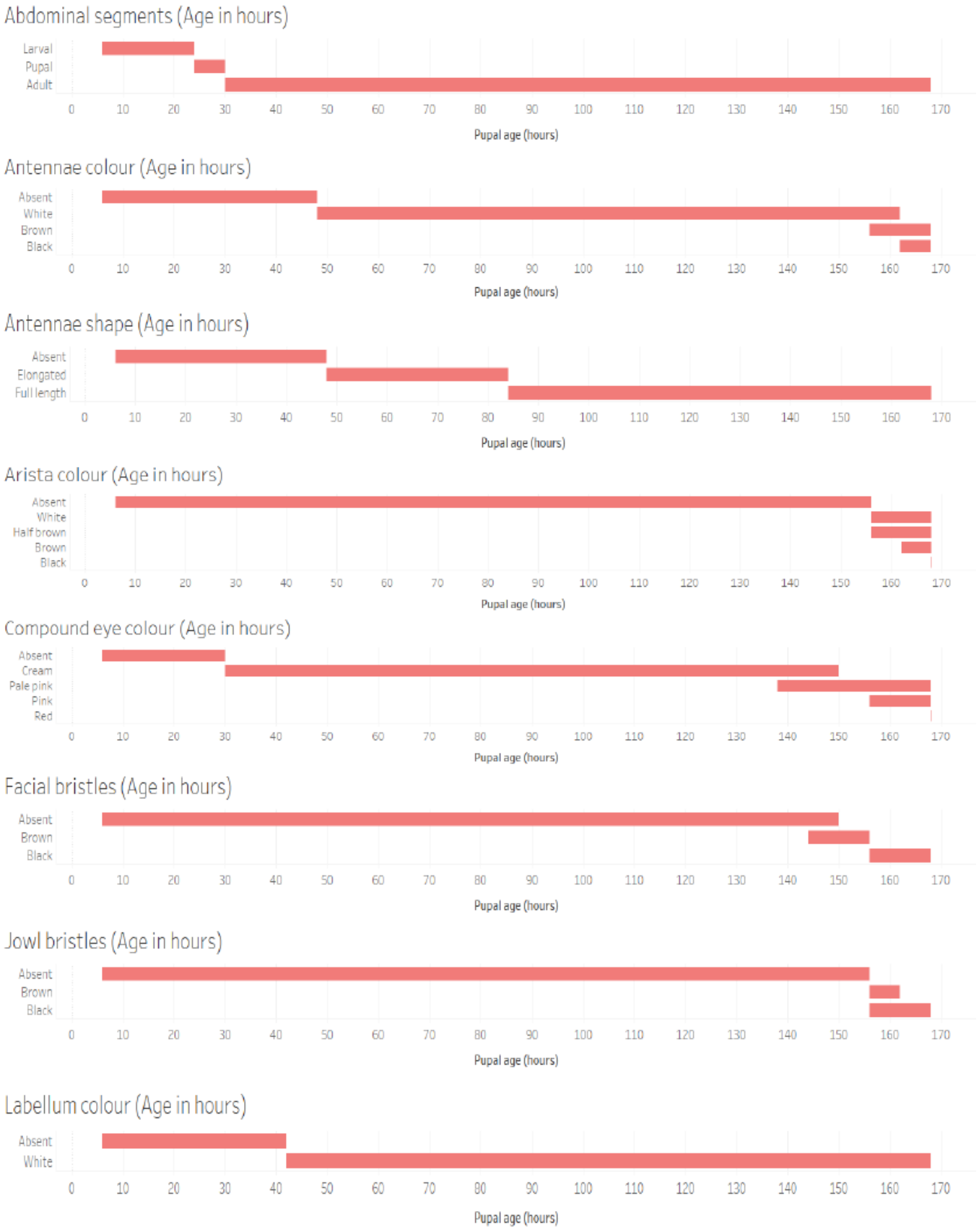

Figure 3.25: Age in hours timelines of each characteristic of the pupal development stage.

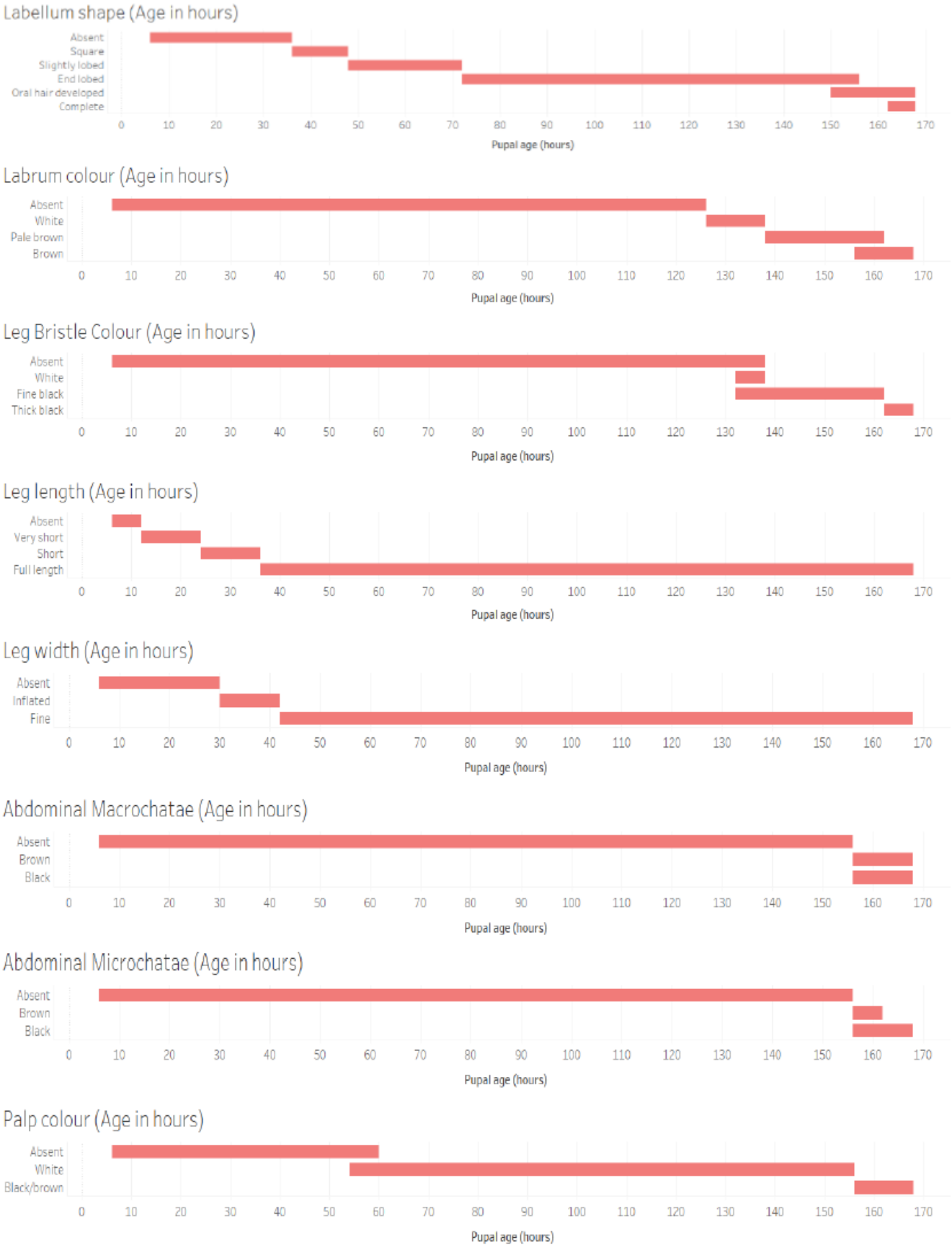

Figure 3.25 continued

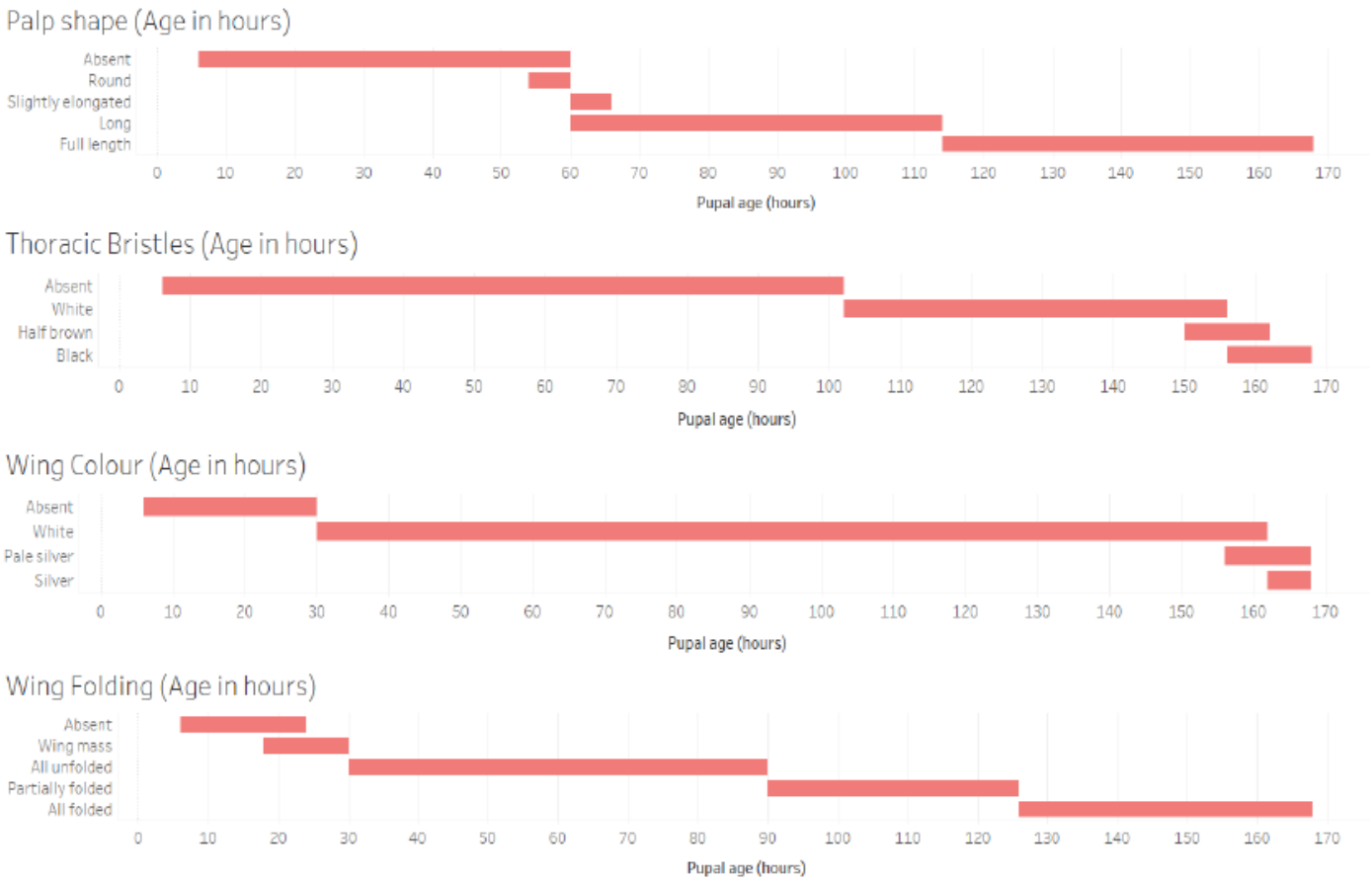

Figure 3.25 continued

3.4 Regression analysis.

A linear regression analysis was completed using a full model with the 20 characteristics as variables as well as a simplified model taking multi-collinearity into account (Appendix B1).

The full model had an adjusted-R^2 value of 0.9130 and the p-value for the F statistic was 0.0000. It was unsurprising that the analysis of the VIF indicated unacceptable levels of multicollinearity. VIF values higher than 10 are deemed to be unacceptable (O'Brien, 2007).

From the original 20 characteristics, antennae shape, arista colour, palp colour, wing colour and abdominal microtrichia were removed. This resulted in a second model that contained 15 variables (appendix B2). The second model had an adjusted-R^2 value of 0.9114 and the p-value for the F statistic was 0.0000. These values didn't differ much from the first model. However, the analysis of the VIF indicated acceptable levels of multicollinearity which is an improvement from model 1. Within model two, most of the coefficients were found to be significant except for compound eye colour, antennae colour, wing folding, leg setae colour and abdominal segments.

From model 2, the 10 most significant variables (variables with the lowest p-value) were selected to create a final regression model and equation that will assist in the estimation of pupal age (appendix B3). The 10 most significant variables were the following: labellum shape, labellum colour, leg length, leg width at full length, thoracic setae, facial setae, abdominal macrosetae, palp shape, genal setae and labrum colour. The third model had an adjusted-R^2 value of 0.9008 and the p-value for the F statistic was 0.0000. These values don't differ much from the first two models, however this model has more acceptable levels of multicollinearity. All models can be found in appendix B.

The regression equation for model 3 was as follows:

ADH = 4573.908 + (-126.6651 x labellum shape) + (1139.774 x labellum colour) + (518.023 x leg length) + (286.2846 x leg width at full length) + (231.7734 x thoracic setae) + (174.6361 x facial setae) + (242.4563 x abdominal macrosetae) + (74.74188 x palp shape) + (293.2403 x genal setae) + (68.81888 x labrum colour)

4. Discussion.

Previous research has demonstrated the ability to utilise external characteristics to estimate pupal age in *Chrysomya albiceps* (Pujol-Luz and Barros-Cordeiro, 2012)*, Megaselia spiracularis* (Feng and Liu, 2013)*, Megaselia scalaris* (Feng and Liu, 2014)*, Cochliomyia macellaria* and *Lucilia cuprina* (Barros-Cordeiro *et al.*, 2016)*, Phormia regina* (Greenberg 1985, 1991) and *Lucilia sericata* (Greenberg, 1985; Karabey and Sert, 2018). These studies have predominantly utilised hours since pupation is a dependent factor and mainly focussed on colour changes to identify the different stages of pupal development. However, they were all able to correlate the different stages to time and therefore they could estimate pupal age using their methods.

Some studies have however also utilised ADH as a dependent factor (Brown, Thorne and Harvey, 2015). The study done by Brown, Thorne and Harvey (2015) correlated changes in morphological characteristics on the pupae and ADH. The results obtained are similar to those seen in the current study. The same characteristics that were analysed in the study done by Brown, Thorne and Harvey (2015) were analysed in the current study. The current study did however exclude the following three characteristics (oral lobe hairs, labrum shape and cephalopharyngeal skeleton were not fully visible during the analysis of the pupae). These characteristics underwent the same changes, however the colour of some of the features did differ due to different species being used in each study (*Calliphora vicina* vs *Lucillia sericata*). Both studies identified 10 characteristics on the pupae as being significant in age estimation. Facial setae, labellum shape and colour, labrum colour and leg width and full length were identified as significant in both studies. However, the current study identified leg length, thoracic setae, abdominal macrosetae, palp shape and genal setae as significant too. This differs from the by Brown, Thorne and Harvey (2015) study where compound eye colour, antennae colour, palp colour and wing folding made up the 10 most significant characteristics. There was also a difference in the ADH values for each study. This difference is due to two different species being used in each study (*Calliphora vicina* vs *Lucillia sericata*). Each species has their own rate of development, even if it is at the same temperature and relative humidity. Therefore, the ADH values that Brown, Thorne and Harvey (2015) obtained in their study will differ from the ADH values obtained in the current study.

Despite, limited studies evaluating pupal morphological changes associated with ADH, it seems to be the preferred method of aging compared to hours after pupation. This is because

ADH takes fluctuation temperatures into account across the whole lifespan of the insect. In the current study it was noted that specimens incubated under the same conditions, pupated at varying times. Thus, utilising ADH, this difference in onset of pupation is taken into account, however utilising hours since pupation does not adequately reflect this intraspecies variation.

A sequential change in character states occurs as the puparium ages, however all of these characteristics in the puparium occur within the first few hours and are therefore not useful for age estimation beyond this point. This is most likely because some of these features are inherited from the third instar larvae. They include the posterior spiracles and the cephalopharyngeal skeleton. These features are fully formed by the end of the third instar stage and do not change any further during the pupal development stage. The other features are not connected to any features that change during the pupal development stage, so they remain unchanged from the start to end. Therefore, these features are not useful for estimating pupal age. The colour grading of the puparium is a subjective analysis and should therefore not be used as an indicator of age.

The only exception to this was the presence of respiratory horns. The respiratory horns are a pair of pointed structures which are found on both sides of the cephalic half of the pupae and puparia. They assist with the breathing of the pupae. They relate to the development of the different body segments of the adult fly (Roddy, 1955). Once the head started to evert and became more prominent, these respiratory horns started to appear. This happened at the 30 hour mark, around 9200 ADH. Greenberg (1991) determined that they are a useful marker which separates cryptocephalic from phanerocephalic pupae (Greenberg, 1991). However, this characteristic would only be useful in PMI estimation if the respiratory horns were absent. If the horns are present the time interval would be too big to be considered in a court of law.

Although all specimens were of the same species and reared under the same conditions, there were marked differences in the colour and weight of pupae which were not associated with age. This may be due to a difference in individual genetics. In two studies, it was noted that there were size differences between male and female pupae (Feng and Liu, 2013, 2014). This could account for the differences in weight documented in this study.

A study on *Lepidoptera* (butterflies) discovered that there are various factors that can influence the colour of the puparium. These include, temperature, humidity, photoperiod,

colour of the background and different wavelengths of light (Smith, 1980). Due to the conditions being constant throughout the rearing of the pupae none of these could have an influence on the difference in colours. Another study investigated the difference in pupal colour between subspecies of *Drosophila*. The study indicated that a difference in gene expression between the subspecies caused the puparia to be different colours (Ahmed-Braimah and Sweigart, 2015). It is possible that there is a difference in gene expression within a colony due to each pupa being a unique individual, but this will require more research to confirm.

Although, the external characteristics of the puparia (except for the respiratory horns) were not useful for determining age, the morphological characteristics of the pupa itself were. From the 21 characteristics identified for the analysis of the external morphological analysis of the pupae, only 20 were successfully analysed and documented. The labrum shape was the only one that was not successfully documented. The labrum is part of the adult fly's mouthparts. It is found on top of the labellum and this made it difficult to identify and photograph. The cuticle that encloses the pupae caused the light from the microscope to be deflected into the lens of the camera and this made it hard to distinguish the true shape of the labrum. Therefore, the changes in shape and size that the labrum undergoes during pupal development was not recorded. Similar difficulties have previously been noted by Brown, Thorne and Harvey (2015), however they were able to visualise these characteristics through manipulation of light on the microscope. They successfully documented the changes of the labrum of the species *Calliphora vicina*. They also documented two other characteristics; the hairs on the oral lobe and the eversion of the cephalopharyngeal skeleton (Brown, Thorne and Harvey, 2015). Neither of the two additional characteristics proved to be useful in age estimation of pupae in their study. For the cephalopharyngeal skeleton it is due to it being a hardened sclerotised structure that doesn't alter with time. The oral lobe hairs are only really useful diagnostically at the generic level and interspecifically is so lacking in variables that it provides little use in aging. Taking this into account it was decided to not visualise and analyse these features in the current study.

The data collected from the external characteristics of the pupae were easily converted into timelines for each characteristic (figure 3.24 and figure 3.25). The timelines that were created, clearly demonstrated a correlation between characteristics and time. These timelines clearly

show that each of the changes that happen during the pupal development stage, are related to a time period represented by either the ADH or age in hours. The timeline also indicated that some features are only present during the early stages of development (i.e. abdominal segments and leg length) and others in the later stages (compound eye colour and abdominal setae). These results were similar to those of Karabey and Sert (2018). Their results indicated that body parts develop early on and colour changes only occur later in the development stage. They noted that wings and legs were some of the first features to form. This was also the case in the current study. Both studies observed colour changes in the later stages as well as the formation and colour change of various setae. All of these changes were correlated to a point in time in both studies. This makes the timelines a useful tool in estimating pupal age. It will be possible to get an estimated age range from the timelines by physically comparing data to them in a manner similar to that demonstrated by Brown (2012).

Regression analysis was used to get a statistical sense of which characteristics may be useful for predicting age of the pupa. Regression analysis only focussed on the external characteristics of the pupa as the characteristics on the puparium were found to not vary with time. Ideally these characteristics should be modelled as factor variables. However, this resulted in high levels of multi-collinearity as well as underpowered models. To solve this, the variables were numerically coded (appendix A.1) and assessed using multiple linear regression methods. The initial model contained all 20 characteristics. While this model indicated a high adjusted R^2 value, analysis of the variance inflation factor indicated an unacceptable level of multi-collinearity.

Multi-collinearity is a strong correlation among a number of independent variables within a model. Multicollinearity will reduce the precision of the estimate coefficients, which in return weakens the statistical power of the regression model. This can cause the p-value to become untrustworthy; resulting in difficulty to be able to identify the variables that are significant (Ciaburro, 2018). Unfortunately, multi-collinearity was inevitable in this study. All characteristics analysed develop over time so it was no surprise that they all have some degree of correlation with one another. The reduction in R^2 value from model 1 to 2 was negligible, however the p-values and standard errors are more reliable due to more acceptable level of multi-collinearity present in the second model. However, the regression models presented above may be prone to overfitting. Overfitting is an error in the model that

occurs when the model is too complex. This will cause the function to fit a limited number of data points too closely and can result in inflated R^2 value (Ciaburro, 2018).

To limit overfitting a final model was developed utilising the 10 most significant characteristics seen in model 2. Using the regression equation that was derived from this final model it is possible to calculate an ADH value. Once a known ADH is calculated it is possible to work out an estimated PMI based on retrospective examination of temperatures in the area of the death scene over the days preceding the discovery of the body. As discussed previously, 5 of these variables were the same as a previous study and 5 were completely different. With entomological evidence being fragile, it is easy to damage parts (characteristics) during collection or sample prep. In some cases parts can degrade or even go missing. This makes it important to note which characteristics are the most significant to estimate pupal age. Precautions should be taken to preserve and document these characteristics. Without them the age estimation might become inaccurate or be unsuccessful.

When comparing the results of the regression analysis to the timelines, the most defined (little overlap between ages and specific to a development stage) timelines correlate with the most significant characteristics. The timeline for these characteristics indicates that these are changes specific to either the early or later stage with two characteristics (labellum and palp shape) developing throughout the pupal stage. However, one characteristic (wing folding) had a well-defined timeline but was not identified as significant by the regression analysis.

4.1. Limitations and future work.

As a pilot study the sample size was expectedly small. Thus, the regression analysis may be hindered by the lower sample size resulting in multi-collinearity, and low power. Furthermore, the low sample size prohibited the data being split into a training dataset and a validation set. It is important to note that the same data cannot be used for both sets. The training dataset is the data that the model will learn from and this will also be used to fit the model. The validation dataset needs to be different from the training set to be able to provide an unbiased account of the error and accuracy of the classifier as well as assess the fit the model (Brownlee, 2020).

It should be noted that the aim of this study was not to develop an accurate prediction model, but rather to identify characteristics that are significant in the estimation of pupal age. Thus, the results of this study are not affected by the fact that no training and validation set was used. Future work should however aim to increase the sample size to at least have 10 specimens per variable to be able to include both a training and validation set to be able to create an accurate prediction model.

Previous research indicated the rate of development is effected by temperature (Feng and Liu, 2013, 2014; Karabey and Sert, 2018). Rate of development is higher at higher temperatures but if the temperature is too high, no development will take place (Feng and Liu, 2013, 2014). The current study was only conducted at 25°C and therefore future research should aim to investigate the difference in pupal growth rate at different temperatures.

All pupae do not pupate at the same time. To have more pupae that pupate at the same time future research will have to increase the colony size. This will ensure that more pupae pupate at the same time. A bigger colony might influence temperatures due to an increase in maggot mass size. When there are more maggots present, they emit more heat. The temperature inside of the mass will vary from the ambient temperature and this can have an influence on the development rate (Heaton, Moffatt and Simmons, 2014). Taking this into account, it would be best to split the specimens into different container limit the size of the maggot mass.

The data collected from the external morphological analysis is subjective. Two people might not agree that a certain change is present at a time point. This can cause slight differences in the estimation of pupal age when using this method of aging. To minimise the errors caused by the subjective analysis, this method of aging can be used in combination with another one such as micro-CT analysis . More features can also be analysed to minimise subjectivity (Brown, 2012).

There have been studies that focused more on the use of internal morphological characteristics of pupae. One study identified 17 internal characteristics with the use of micro-computed tomography (micro-CT) (Martín-Vega *et al.*, 2017). These internal characteristics include the full eversion of the legs, wings and head, development of internal organs such as the oesophagus, midgut and crop and the completion of different stages of apolysis. They could successfully determine the age of the pupae by correlating these characteristics to an

age percentage. Future research should investigate the possibility of using both internal and external characteristics to estimate pupal age. This could possibly lead to creating a more accurate method of pupal age estimation.

5. Conclusion.

Fly species spend roughly half of their immature life-stage in the pupal stage. To be able to determine the age of the pupae by using an equation or timeline, could save valuable time in medico legal investigations. The aim of the current study was to determine what morphological characteristics could be useful for pupal age determination in *Lucilia sericata.* This study successfully identified 10 characteristics that were significant for pupal age estimation. They were the colour and shape of the labellum, leg length, leg width at full length, thoracic setae, facial setae, abdominal macrosetae, palp shape, jowl setae and labrum colour. Some of these characteristics like the leg length and width and abdominal macrosetae did provide important time-breaks on their respective timelines. However, the development and the pigmentation of the compound eye also provided valuable time-breaks it's timeline.

The results of this pilot study indicated that *Lucilia sericata* pupae can be useful for PMI estimation. The data clearly demonstrated a correlation between the development of external pupal characteristics and time. However, the characteristics of the external puparium, except for the development of the respiratory horns, did not demonstrate this correlation. They are not useful on their own for PMI estimation beyond a very early stage in development. Though using the development of the respiratory horns in conjunction with some of the characteristics of the pupae could be valuable for PMI estimation. Future work should aim to increase the sample size of this study to obtain more data and develop predictive models for estimating puparial age.

6. References.

Adams, M. E. (2009) 'Development, Hormonal Control of', in Resh, V. H. and Carde, R. T. (eds) *Encyclopedia of Insects*. Elsevier Inc., pp. 261–266. doi: 10.1016/B978-0-12-374144-8.X0001-X.

Ahmed-Braimah, Y. H. and Sweigart, A. L. (2015) 'A single gene causes an interspecific difference in pigmentation in Drosophila', *Genetics*, 200(1), pp. 331–342. doi: 10.1534/genetics.115.174920.

Amendt, J. *et al.* (2007) 'Best practice in forensic entomology - Standards and guidelines', *International Journal of Legal Medicine*, 121(2), pp. 90–104. doi: 10.1007/s00414-006-0086-x.

Amendt, J. *et al.* (2011) 'Forensic entomology: Applications and limitations', *Forensic Science, Medicine, and Pathology*, 7(4), pp. 379–392. doi: 10.1007/s12024-010-9209-2.

Amendt, J. (2018) 'Forensic entomology', *Forensic Sciences Research*, 3(1), p. 1. doi: 10.1080/20961790.2017.1403081.

Barros-Cordeiro, K. B. *et al.* (2016) 'Intra-puparial development of the Cochliomyia macellaria and Lucilia cuprina (Diptera, Calliphoridae)', *Revista Brasileira de Entomologia*. Elsevier Editora Ltda., 60(4), pp. 334–340. doi: 10.1016/j.rbe.2016.06.009.

Brink, S. L. (2009) *Key Diagnostic Characteristics of the Developmental Stages of Forensically Important Calliphoridae and Sarcophagidae in Central South Africa*. The University of the Free State.

Brody, T. B. (1995) *The Interactive Fly: Drosophila*, *Society for Developmental Biology*. Available at: https://www.sdbonline.org/sites/fly/aimain/1adult.htm (Accessed: 9 March 2020).

Brown, K. (2012) *Utility of the Calliphora vicina (Diptera: Calliphoridae) pupal stage for providing temporal information for death invesigations*, *University of Portsmouth*. University of Portsmouth.

Brown, K., Thorne, A. and Harvey, M. (2012) 'Preservation of Calliphora vicina (Diptera: Calliphoridae) pupae for use in post-mortem interval estimation', *Forensic Science International*, 223(1–3), pp. 176–183. doi: 10.1016/j.forsciint.2012.08.029.

Brown, K., Thorne, A. and Harvey, M. (2015) 'Calliphora vicina (Diptera: Calliphoridae) pupae: a timeline of external morphological development and a new age and PMI estimation tool', *International Journal of Legal Medicine*, 129(4), pp. 835–850. doi: 10.1007/s00414-014-1068-z.

Brownlee, J. (2020) *What is the Difference Between Test and Validation Datasets?* Available at: https://machinelearningmastery.com/difference-test-validation-datasets/ (Accessed: 12 February 2021).

Ciaburro, G. (2018) *Regression Analysis with R: Design and develop statistical nodes to identify ... - Giuseppe Ciaburro - Google Books*. 1st ed. Birmingham: Packt Publishing Ltd.

Davies, K. and Harvey, M. L. (2013) 'Internal Morphological Analysis for Age Estimation of Blow Fly Pupae (Diptera: Calliphoridae) in Postmortem Interval Estimation', *Journal of*

Forensic Sciences, 58(1), pp. 79–84. doi: 10.1111/j.1556-4029.2012.02196.x.

Denlinger, D. L. and Zdarek, J. (1994) 'Metamorphosis Behavior of Flies', *Annual Review of Entomology*. Annual Reviews, 39(1), pp. 243–266. doi: 10.1146/annurev.en.39.010194.001331.

Díaz-Aranda, L. M. *et al.* (2018) 'Annual variation in decomposition and insect succession at a periurban area of central Iberian Peninsula', *Journal of Forensic and Legal Medicine*. Elsevier, 56(February 2017), pp. 21–31. doi: 10.1016/j.jflm.2018.03.005.

Erzinçlioğlu, Y. Z. (1983) 'The application of entomology to forensic medicine', *Medicine, science, and the law*, 23(1), pp. 57–63.

Feng, D. X. and Liu, G. C. (2013) 'Pupal age estimation of forensically important Megaselia spiracularis Schmitz (Diptera: Phoridae)', *Forensic Science International*. Elsevier Ireland Ltd, 231(1–3), pp. 199–203. doi: 10.1016/j.forsciint.2013.05.008.

Feng, D. X. and Liu, G. C. (2014) 'Pupal age estimation of forensically important Megaselia scalaris (Loew) (Diptera: Phoridae)', *Forensic Science International*. Elsevier Ireland Ltd, 236, pp. 133–137. doi: 10.1016/j.forsciint.2014.01.002.

Gaudry, E. *et al.* (2006) 'Study of steroidogenesis in pupae of the forensically important blow fly Protophormia terraenovae (Robineau-Desvoidy) (Diptera: Calliphoridae)', *Forensic Science International*. Forensic Sci Int, 160(1), pp. 27–34. doi: 10.1016/j.forsciint.2005.06.014.

Gennard, D. E. (2012) *Forensic Entomology: An Introduction*. 2nd ed. Hoboken, New Jersey: Wiley-Blackwell.

Gilby, A. R. and McKellar, J. W. (1970) 'The composition of the empty puparia of a blowfly' *Journal of Insect Physiology*, 16(8), pp.1517-1529. doi:10.1016/0022-1910(70)90250-7

Greenberg, B. (1985) 'Forensic entomology: case studies', *Bulletin of the Entomological Society of America*, 31(4), pp. 25-28. doi:10.1093/besa/31.4.2

Greenberg, B. (1991) 'Flies as forensic indicators', *Journal of Medical Entomology*, 28(5), pp. 565-577. doi:10.1093/jmedent/28.5.565

Greenberg, B. and Kunich, J. C. (2002) 'Entomology and the law: flies as forensic indicators.', *Entomology and the law: flies as forensic indicators.* Cambridge University Press.

Harvey, M., Gasz, N. and Voss, S. (2016) 'Entomology-based methods for estimation of postmortem interval', *Research and Reports in Forensic Medical Science*. Dove Press, 6, p. 1. doi: 10.2147/RRFMS.S68867.

Heaton, V., Moffatt, C. and Simmons, T. (2014) 'Quantifying the temperature of maggot masses and its relationship to decomposition', *Journal of Forensic Sciences*. Blackwell Publishing Inc., 59(3), pp. 676–682. doi: 10.1111/1556-4029.12396.

Joseph, I. *et al.* (2011) 'The use of insects in forensic investigations: An overview on the scope of forensic entomology', *Journal of Forensic Dental Sciences*. Medknow, 3(2), p. 89. doi: 10.4103/0975-1475.92154.

Karabey, T. and Sert, O. (2018) 'The analysis of pupal development period in Lucilia sericata (Diptera: Calliphoridae) forensically important insect', *International Journal of Legal Medicine*, 132(4), pp. 1185–1196. doi: 10.1007/s00414-014-0968-2.

M, G. and C, R. (2002) 'Effect of temperature on development of the forensically important holarctic blow fly Protophormia terraenovae (Robineau-Desvoidy) (Diptera: Calliphoridae)', *Forensic science international*. Forensic Sci Int, 128(3), pp. 177–182. doi: 10.1016/S0379-0738(02)00199-8.

Ma, T., Huang, J. and Wang, J. F. (2015) 'Study on the pupal morphogenesis of Chrysomya rufifacies (Macquart) (Diptera: Calliphoridae) for postmortem interval estimation', *Forensic Science International*. Elsevier Ireland Ltd, 253, pp. 88–93. doi: 10.1016/j.forsciint.2015.06.005.

Martín-Vega, D. *et al.* (2017) 'Age estimation during the blow fly intra-puparial period: a qualitative and quantitative approach using micro-computed tomography', *International Journal of Legal Medicine*. International Journal of Legal Medicine, 131(5), pp. 1429–1448. doi: 10.1007/s00414-017-1598-2.

Martín-Vega, D., Hall, M. J. R. and Simonsen, T. J. (2016) 'Resolving Confusion in the use of concepts and terminology in intrapuparial development studies of cyclorrhaphous diptera', *Journal of Medical Entomology*, 53(6), pp. 1249–1251. doi: 10.1093/jme/tjw081.

Mona, S. *et al.* (2019) 'Forensic Entomology: A Comprehensive Review', *Advancements in Life Sciences*, 6(2), pp. 48–59.

Moore, H. E. *et al.* (2016) 'Age estimation of Calliphora (Diptera: Calliphoridae) larvae using cuticular hydrocarbon analysis and Artificial Neural Networks', *Forensic Science International*. Elsevier Ireland Ltd, 268(2016), pp. 81–91. doi: 10.1016/j.forsciint.2016.09.012.

Moore, H. E., Adam, C. D. and Drijfhout, F. P. (2013) 'Potential use of hydrocarbons for aging lucilia sericata blowfly larvae to establish the postmortem interval', *Journal of Forensic Sciences*, 58(2), pp. 404–412. doi: 10.1111/1556-4029.12016.

Pechal, J. L. *et al.* (2014) 'Hydrocarbon profiles throughout adult Calliphoridae aging: A promising tool for forensic entomology', *Forensic Science International*. Elsevier Ireland Ltd, 245(2014), pp. 65–71. doi: 10.1016/j.forsciint.2014.10.019.

Proença, B. *et al.* (2014) ' Intrapuparial Development of Chrysomya putoria (Diptera: Calliphoridae) ', *Journal of Medical Entomology*, 51(5), pp. 908–914. doi: 10.1603/me13205.

Pujol-Luz, J. R. and Barros-Cordeiro, K. B. (2012) 'Intra-puparial development of the females of Chrysomya albiceps (Wiedemann) (Diptera, Calliphoridae)', *Revista Brasileira de Entomologia*, 56(3), pp. 269–272. doi: 10.1590/S0085-56262012005000038.

Roddy, L. R. (1955) 'A Morphological Study of the Respiratory Horns Associated with the Puparia of Some Diptera, Especially Ophyra Anescens (Wied.)1', *Annals of the Entomological Society of America*. Oxford University Press (OUP), 48(5), pp. 407–415. doi: 10.1093/aesa/48.5.407.

Sharma, R., Kumar Garg, R. and Gaur, J. R. (2015) 'Various methods for the estimation of the post mortem interval from Calliphoridae: A review', *Egyptian Journal of Forensic Sciences*.

Forensic Medicine Authority, 5(1), pp. 1–12. doi: 10.1016/j.ejfs.2013.04.002.

Smith, A. . (1980) 'Environmental factors influencing pupal colour determination in Lepidoptera. II. Experiments with Pieris rapae, Pieris napi and Pieris brassicae', *Proceedings of the Royal Society of London. Series B. Biological Sciences*, 207(1167), pp. 163–186. doi: 10.1098/rspb.1980.0019.

Smith, K. G. V. (1986) *A Manual of Forensic Entomology*. London: The Trustees, British Museum.

Sukontason, K. L. *et al.* (2006) 'Surface ultrastructure of the puparia of the blow fly, Lucilia cuprina (Diptera: Calliphoridae), and flesh fly, Liosarcophaga dux (Diptera: Sarcophagidae)', *Parasitology Research*, 98(5), pp. 482–487. doi: 10.1007/s00436-005-0102-y.

Sukontason, K. L. *et al.* (2007) 'Identifying fly puparia by clearing technique: Application to forensic entomology', *Parasitology Research*, 101(5), pp. 1407–1416. doi: 10.1007/s00436-007-0660-2.

Tembe, D. and Mukaratirwa, S. (2020) 'Forensic entomology research and application in southern Africa: A scoping review', *South African Journal of Science*, 116(5–6), pp. 1–8. doi: 10.17159/sajs.2020/6065.

Vanin, S. (2018) 'Forensic Entomology:an overview', *Crime, Security and Society*, 1(1). doi: 10.5920/css.2018.05.

Voss, S. C. *et al.* (2017) 'Reflectance-based determination of age and species of blowfly puparia', *International Journal of Legal Medicine*. International Journal of Legal Medicine, 131(1), pp. 263–274. doi: 10.1007/s00414-016-1458-5.

Williams, K. A. and Villet, M. H. (2006) 'A history of southern African research relevant to forensic entomology', *South African Journal of Science*, 102(1–2), pp. 59–65.

Williams, K. A. and Villet, M. H. (2019) 'Spatial and Seasonal Distribution of Forensically Important Blow Flies (Diptera: Calliphoridae) in Makhanda, Eastern Cape, South Africa', *Journal of medical entomology*, 56(5), pp. 1231–1238. doi: 10.1093/jme/tjz056.

Zdarek, J. and Fraenkel, G. (1972) 'The mechanism of puparium formation in flie' *Journal of Experimental Zoology*, 179: pp. 315- 324. doi:10.1002/jez.1401790304

Zhu, G. H. *et al.* (2013) 'Time of Death Revealed by Hydrocarbons of Empty Puparia of Chrysomya megacephala (Fabricius) (Diptera: Calliphoridae): A Field Experiment', *PLoS ONE*, 8(9). doi: 10.1371/journal.pone.0073043.

7. Appendices.

Appendix A: Coding used for each characteristic identified in the study.

Table A1: The numerical code for each change of a feature.

Feature	Change	Numerical Code
Compound eye colour	Absent	1
	Cream	2
	Pale pink	3
	Pink	4
	Red	5
Orbital/facial setae	Absent	1
	Brown	2
	Black	3
Antennae shape	Absent	1
	Elongated	2
	Full length	3
Antennae colour	Absent	1
	White	2
	Brown	3
	Black	4
Arista colour	Absent	1
	White	2
	Half brown	3
	Brown	4
	Black	5
Labellum shape	Absent	1
	Square	2
	Slightly lobed	3
	End lobed	4
	Oral hair developed	5
	Complete	6
Labella colour	Absent	1
	White	2
Palp shape	Absent	1
	Round	2
	Slightly elongated	3
	Long or clubbed	4
	Full length	5
Palp colour	Absent	1
	White	2

	Black/brown	3
Genal setae	Absent	1
	Brown	2
	Black	3
Labrum colour	Absent	1
	White	2
	Pale brown	3
	Brown	4
Wing folding	Absent	1
	Wing mass	2
	All unfolded	3
	Partially folded	4
	All folded	5
Wing colour	Absent	1
	White	2
	Pale silver	3
	Silver	4
Leg length	Absent	1
	Very short	2
	Short	3
	Full length	4
Leg width at full length	Absent	1
	Inflated	2
	Fine	3
Leg setae colour	Absent	1
	White	2
	Fine black	3
	Thick black	4
Thoracic setae	Absent	1
	White	2
	Half brown	3
	Black	4
Abdominal segments	Larval	1
	Pupal	2
	Adult	3
Abdomen macrosetae	Absent	1
	Brown	2
	Black	3
Abdomen microtrichia	Absent	1
	Brown	2
	Black	3

Appendix B: Statistical output from regression analysis.

Multiple linear regression analysis was performed to assess characteristics which were useful for estimating the age of the pupae (ADH). Initially a full model was created using all 20 characteristics (figure B1). Analysis of multi-collinearity using the variance inflation factor (VIF) indicated unacceptable levels of covariance. Therefore, a second model (figure B2) was developed by removing collinear variables from the regression equation until such time that an acceptable level was achieved (VIF < 10). Following this a final model was evaluated using the 10 most significant coefficients in model 2 (figure B3).

Source	SS	df	MS	Number of obs	=	131
				F(20, 110)	=	69.18
Model	89718148.1	20	4485907.4	Prob > F	=	0.0000
Residual	7132815.67	110	64843.7788	R-squared	=	0.9264
				Adj R-squared	=	0.9130
Total	96850963.7	130	745007.413	Root MSE	=	254.64

ADH	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
Compoundeye_colour	27.99618	88.54771	0.32	0.752	-147.4846	203.477
Facial_bristles	157.5039	66.45969	2.37	0.020	25.79639	289.2114
Antennae_shape	305.6282	94.11855	3.25	0.002	119.1073	492.149
Antennae_colour	-39.77389	54.10404	-0.74	0.464	-146.9954	67.44761
Arista_colour	36.67297	154.4287	0.24	0.813	-269.3684	342.7143
Labellum_shape	-22.19865	42.71825	-0.52	0.604	-106.8562	62.45889
Labellum_colour	541.7929	230.6561	2.35	0.021	84.68657	998.8991
Palp_shape	-80.12606	66.774	-1.20	0.233	-212.4564	52.20433
Palp_colour	307.3053	117.4212	2.62	0.010	74.60405	540.0065
Labrum_colour	50.07876	40.06051	1.25	0.214	-29.31177	129.4693
Wing_folding	62.93992	35.16885	1.79	0.076	-6.756479	132.6363
Wing_colour	90.8733	105.1373	0.86	0.389	-117.4841	299.2307
Leg_length	510.9415	54.68008	9.34	0.000	402.5784	619.3045
Leg_width	287.9353	106.344	2.71	0.008	77.18653	498.6841
Leg_bristles	1.444096	63.09206	0.02	0.982	-123.5896	126.4778
Thoricic_bristles	174.4439	32.56518	5.36	0.000	109.9073	238.9804
Abdominal_segments	168.4878	71.78021	2.35	0.021	26.23627	310.7393
Abdominal_macrochatae	458.7135	276.166	1.66	0.100	-88.58272	1006.01
Abdominal_microchatae	-407.7966	367.4756	-1.11	0.270	-1136.047	320.4538
Jowl_bristles	651.2827	492.0408	1.32	0.188	-323.8268	1626.392
_cons	3923.527	521.8785	7.52	0.000	2889.286	4957.767

Figure B1: The statistical output of the regression analysis of the first model (model 1) containing 20 variables.

Source	SS	df	MS	Number of obs	=	131
				F(15, 115)	=	78.88
Model	88271390.9	15	5884759.39	Prob > F	=	0.0000
Residual	8579572.84	115	74604.9812	R-squared	=	0.9114
				Adj R-squared	=	0.8999
Total	96850963.7	130	745007.413	Root MSE	=	273.14

ADH	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
Compoundeye_colour	34.7664	78.60765	0.44	0.659	-120.9402	190.473
Facial_bristles	174.6361	66.86139	2.61	0.010	42.19658	307.0757
Antennae_colour	61.13521	44.58437	1.37	0.173	-27.17785	149.4483
Labellum_shape	-126.6651	33.26461	-3.81	0.000	-192.5559	-60.77428
Labellum_colour	1139.774	185.496	6.14	0.000	772.3422	1507.206
Palp_shape	74.74188	35.51942	2.10	0.038	4.384746	145.099
Labrum_colour	68.81888	40.55163	1.70	0.092	-11.5061	149.1439
Wing_folding	44.70839	35.52235	1.26	0.211	-25.65454	115.0713
Leg_length	518.023	57.63846	8.99	0.000	403.8523	632.1937
Leg_width	286.2846	70.34954	4.07	0.000	146.9357	425.6335
Leg_bristles	16.64361	66.00326	0.25	0.801	-114.0961	147.3834
Thoricic_bristles	231.7734	31.5551	7.35	0.000	169.2689	294.278
Abdominal_segments	-3.956359	62.66814	-0.06	0.950	-128.0899	120.1772
Abdominal_macrochatae	242.4563	95.50301	2.54	0.012	53.28316	431.6293
Jowl_bristles	293.2403	152.2394	1.93	0.057	-8.316649	594.7972
_cons	4573.908	391.7947	11.67	0.000	3797.838	5349.978

Figure B2: The statistical output of the regression analysis of the second model (model 2) containing 15 variables.

Source	SS	df	MS	Number of obs	=	131
				F(10, 120)	=	119.03
Model	87981192.7	10	8798119.27	Prob > F	=	0.0000
Residual	8869770.99	120	73914.7583	R-squared	=	0.9084
				Adj R-squared	=	0.9008
Total	96850963.7	130	745007.413	Root MSE	=	271.87

ADH	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
Labellum_shape	-121.2773	29.59051	-4.10	0.000	-179.8644	-62.69013
Labellum_colour	1291.173	141.8061	9.11	0.000	1010.406	1571.939
Leg_length	533.6306	55.79955	9.56	0.000	423.1514	644.1099
Leg_width	337.703	43.66157	7.73	0.000	251.2561	424.1498
Thoricic_bristles	218.8869	26.33209	8.31	0.000	166.7512	271.0226
Facial_bristles	183.1272	54.24442	3.38	0.001	75.72704	290.5274
Abdominal_macrochatae	232.3599	88.22204	2.63	0.010	57.6864	407.0334
Palp_shape	98.87512	31.4397	3.14	0.002	36.6267	161.1235
Jowl_bristles	280.012	121.1114	2.31	0.022	40.21992	519.804
Labrum_colour	66.10619	33.88942	1.95	0.053	-.9925049	133.2049
_cons	4537.75	241.4429	18.79	0.000	4059.71	5015.79

Figure B3: The statistical output of the regression analysis of the third model (model 3) containing 10 variables.

Appendix C: A table representing the age (in hours and ADH), colour and weight of each pupae collected.

Table C1: The hours since pupation, ADH, colour and weight of each pupae collected.

ID	Hours since pupation	ADH	Colour	Weight
1	T0	6550	Cream	0.0349g
2	T0	6550	Cream	0.0399g
3	T0	6550	Cream	0.0380g
4	T0	6550	Cream	0.0305g
5	T0	6550	Cream	0.0327g
6	T6	6800	Light brown	0.0347g
7	T6	6800	Light brown	0.0284g
8	T6	6800	Light brown	0.0370g
9	T6	6800	Light brown	0.0420g
10	T6	6800	Light brown	0.0429g
11	T12	8900	Light brown	0.0285g
12	T12	8900	Brown	0.0223g
13	T12	8900	Brown	0.0203g
14	T12	8900	Brown	0.0214g
15	T12	8900	Brown	0.0332g
16	T18	9050	Brown	0.0297g
17	T18	9050	Brown	0.0305g
18	T18	9050	Brown	0.0219g
19	T18	9050	Brown	0.0324g
20	T18	9050	Brown	0.0324g
21	T24	9175	Light brown	0.0336g
22	T24	9175	Light brown	0.0203g
23	T24	9175	Light brown	0.0343g
24	T24	9175	Brown	0.0337g
25	T24	9175	Brown	0.0263g
26	T30	9200	Light brown	0.0400g
27	T30	9200	Light brown	0.0291g
28	T30	9200	Brown	0.0281g
29	T30	9200	Brown	0.0255g
30	T30	9200	Brown	0.0305g
31	T36	9350	Brown	0.0347g
32	T36	9350	Light brown	0.0343g
33	T36	9350	Brown	0.0218g
34	T36	9350	Light brown	0.0249g

35	T36	9350	Brown	0.0293g
36	T42	9500	Brown	0.0415g
37	T42	9500	Brown	0.0291g
38	T42	9500	Brown	0.0270g
39	T42	9500	Brown	0.0252g
40	T42	9500	Brown	0.0336g
41	T48	9650	Brown	0.0253g
42	T48	9650	Dark brown	0.0254g
43	T48	9650	Brown	0.0299g
44	T48	9650	Brown	0.0369g
45	T48	9650	Brown	0.0274g
46	T54	9650	Brown	0.0243g
47	T54	9650	Brown	0.0405g
48	T54	9650	Brown	0.0270g
49	T54	9650	Brown	0.0390g
50	T54	9650	Brown	0.0218g
51	T60	9800	Brown	0.0343g
52	T60	9800	Brown	0.0227g
53	T60	9800	Brown	0.0279g
54	T60	10375	Brown	0.0352g
55	T60	10375	Brown	0.0213g
56	T66	9950	Brown	0.0341g
57	T66	9950	Brown	0.0363g
58	T66	9950	Brown	0.0343g
59	T66	9950	Brown	0.0263g
60	T66	9950	Dark brown	0.0335g
61	T72	9950	Light brown	0.0410g
62	T72	9950	Brown	0.0291g
63	T72	9950	Dark brown	0.0240g
64	T72	9950	Brown	0.0242g
65	T72	9950	Brown	0.0287g
66	T78	10100	Brown	0.0340g
67	T78	10100	Brown	0.0396g
68	T78	10100	Brown	0.0286g
69	T78	10100	Brown	0.0266g
70	T78	10100	Brown	0.0262g
71	T84	10250	Light brown	0.0351g
72	T84	10250	Brown	0.0312g
73	T84	10250	Brown	0.0238g
74	T84	10250	Brown	0.0385g
75	T84	10250	Brown	0.0264g
76	T90	10250	Brown	0.0324g
77	T90	10250	Brown	0.0276g

78	T90	10250	Brown	0.0332g
79	T90	10250	Dark brown	0.0324g
80	T90	10250	Brown	0.0254g
81	T96	10375	Brown	0.0328g
82	T96	10375	Brown	0.0220g
83	T96	10375	Brown	0.0358g
84	T96	10375	Brown	0.0188g
85	T96	10375	Brown	0.0293g
86	T102	10850	Dark brown	0.0458g
87	T102	10850	Brown	0.0261g
88	T102	10850	Light brown	0.0181g
89	T102	10850	Light brown	0.0247g
90	T102	10850	Brown	0.0296g
91	T108	10550	Brown	0.0279g
92	T108	10550	Brown	0.0409g
93	T108	10550	Brown	0.0345g
94	T108	10550	Brown	0.0293g
95	T108	10550	Brown	0.0238g
96	T114	10700	Brown	0.0286g
97	T114	10700	Brown	0.0378g
98	T114	10700	Brown	0.0391g
99	T114	10700	Brown	0.0310g
100	T114	10700	Brown	0.0245g
101	T120	10700	Brown	0.0382g
102	T120	10850	Brown	0.0349g
103	T120	10850	Brown	0.0256g
104	T120	10850	Light brown	0.0245g
105	T120	10850	Brown	0.0265g
106	T126	10850	Dark brown	0.0300g
107	T126	10850	Brown	0.0328g
108	T126	10850	Light brown	0.0225g
109	T126	10850	Dark brown	0.0213g
110	T126	10850	Brown	0.0252g
111	T132	11000	Brown	0.0297g
112	T132	11000	Brown	0.0378g
113	T132	10700	Brown	0.0318g
114	T132	10700	Brown	0.0293g
115	T132	10700	Light brown	0.0250g
116	T138	10850	Brown	0.0381g
117	T138	10850	Light brown	0.0314g
118	T138	10850	Brown	0.0234g
119	T138	10850	Brown	0.0255g
120	T138	10850	Light brown	0.0204g

121	T144	10975	Brown	0.0415g
122	T144	10975	Light brown	0.0271g
123	T144	10975	Light brown	0.0290g
124	T144	10975	Brown	0.0224g
125	T144	10975	Brown	0.0221g
126	T150	11150	Brown	0.0357g
127	T150	11150	Brown	0.0319g
128	T150	11150	Light brown	0.0290g
129	T150	11150	Brown	0.0227g
130	T150	11150	Light brown	0.0249g
131	T156	11150	Brown	0.0351g
132	T156	11150	Brown	0.0303g
133	T156	11150	Brown	0.0278g
134	T156	11150	Light brown	0.0348g
135	T156	11150	Brown	0.0438g
136	T162	11300	Dark brown	0.0329g
137	T162	11150	Dark brown	0.0326g
138	T162	11150	Dark brown	0.0267g
139	T162	11150	Dark brown	0.0163g
140	T162	11150	Dark brown	0.0306g
141	T168	11150	Dark brown	0.0338g
142	T168	11150	Dark brown	0.0220g
143	T168	10975	Dark brown	0.0203g
144	T168	10975	Dark brown	0.0287g
145	T168	10975	Dark brown	0.0318g

Appendix D: Letter of ethical approval.

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Animal Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Website: www.health.uct.ac.za/fhs/research/animalethics/forms

05 February 2020

FHS AEC REF NO: 018-027

Dr M. Heyns
Forensic Medicine
Room: 5.12 Falmouth Building

Dear Dr Heyns

PROTOCOL TITLE: INVESTIGATING AGE-RELATED INTRA-PUPARIAL CHARACTERISTICS OF NECROPHAGOUS FLY PUPAE TO IMPROVE THE ACCURACY OF POST-MORTEM INTERVAL ESTIMATION

Thank you for submitting your amendment to the Faculty of Health Sciences (FHS) Animal Ethics Committee (AEC) for review

I am pleased to inform you that the FHS AEC has approved the following amendments to the above-mentioned study:

- To add an additional research participant, Lisa Alberts

A Form for amendment (version October 2014) is also available at http://www.health.uct.ac.za/fhs/research/animalethics/forms
Yearly progress report submitted to the ethics office is a requirement for on-going approval of studies.
Notification of study closure is a requirement.
Ethics approval letter and copy of the application form to be submitted to the Animal Unit when commencing the study for release of animals.
The principal investigator has to:
Ensuring that all study participants perform within the confines of the procedures and experimental design of the protocol as approved, or as amended.
Ensuring that all study participants comply with all applicable national legislation, UCT policies, FHS AEC policies and standard operating procedures (SOPs) and national standards (SANS 10386: 2008).
Ensuring that you as the PI (principal investigator) immediately alert the FHS AEC to any event involving the welfare of the animals which has occurred during the course of the study, as well as the actions that were taken to respond to these events.

Ensuring that you as the PI (principal investigator) alert the FHS AEC to any new or unexpected ethical issues that arose during the course of the study, and how these issues were addressed.

Figure D1: Letter of ethical approval.

Ensuring that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of SAVC-registered veterinary professionals or SAVC-registered para-veterinary professionals.
If the principal investigator or any study participant is in any way uncertain how to respond to any of these obligations or deal with any of the issues referred to above, they must consult with FHS AEC.
All animals found dead must be reported to the RAF on the appropriate form:
http://www.health.uct.ac.za/fhs/research/animalethics/forms
All animals found in distress must be reported to the RAF on the appropriate form.

Please quote the REC. REF in all your correspondence

Yours sincerely

PROF G. LOUW
CHAIR, FHS AEC

Figure D1: Letter of ethical approval continued.