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Forensic aspects of the blow fly Calliphoria vicina

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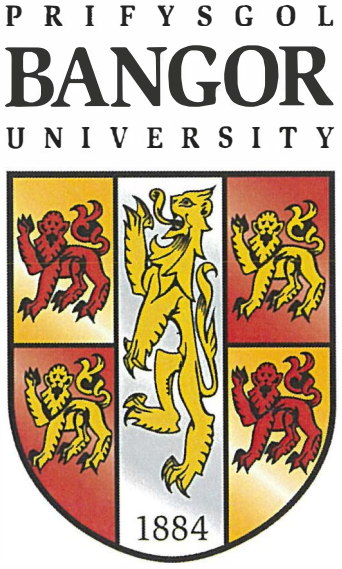

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by

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Summary

Estimations of the post mortem interval form the corner stone of forensic entomology. This is mainly accomplished with the help of larvae of blow flies. However, the wandering stage of 3rd instar larvae and the pupal stage of the important blow fly species *Calliphora vicina* constitute more than 60 % of the total length of the immature stages. These two stages have been very difficult to age until now.

The age of pupae of *C. vicina* can reliable be estimated without the need for extensive instrumentation, molecular procedures or the use of colour using a method that is exceptionally fast. The simple dissection of the yellow body allows an estimate of the pupal age with a resolution of 40 °D.

Ageing blow fly pupae based on morphological changes of external characters of the pharate developing adults is an even simpler method than using the yellow body of the metamorphosing midgut. It is also the simplest method currently available.

The minimum temperature of *C. vicina* has been for the first time established under laboratory conditions. It is 11.8 °C. A new, lower minimum temperature has been established for *C. vicina* for survival under diapaus. This is the first study that also includes arrest under diapause.

The first larval instar of *C. vicina* depends on bacteria as nutrition. Specific *Sphingomonas* species are associated with the egg surface and consumed by the first instar larvae.

The nuclei of the salivary glands of *C. vicina* larvae show sufficient change during the three days of the wandering stage to allow the ageing of the wandering stage. This is the first investigating in ageing the wandering stage based on morphology.

Forensic Aspects of the Blow Fly *Calliphora vicina*

Chapter One

Introduction

Forensic entomology is a science based on the interpretation of those insects, which successively infest a corpse, as decomposition continuous, and it monitors how these insects' progeny grows through various developmental stages and uses the ages of their immature stages as variable to estimate Post Mortem Interval (PMI). This branch of science is divided into three areas: medico-criminal, urban and stored product forensic entomology. The different arthropods studied in forensic entomology are flies (blow flies, flesh flies, house flies, and cheese flies), beetles (rove beetles, hister beetles, and carrion beetles), mites, moths, wasps, ants and bees.These insects are studied in succession depending on how long the corpse has been abandoned(Lord and Rodriguez, 1989; Bonacci *et al.*, 2009; Braig and Perotti, 2009; Perotti *et al.*, 2009; Goff, 2010; Amendt *et al.*, 2011; Joseph *et al.*, 2011). This study focuses on the medico-criminal part of the forensic entomology and with focus on blow fly.

1.1 Post Mortem Interval Estimations

The time since death, the post mortem interval (PMI) or post mortem period (PMP), is an important variable or parameter in criminal investigations and often part of the daily casework of forensic investigators. The dead body or corpse will undergo physical and biochemical changes and will be exposed to biological interactions summarized as taphonomic processes. Such processes can involve an endless list of variations, including intoxication, medication, pre-existing diseases, injuries, chemical impregnation, drowning, hanging, burial, burning, freezing, cannibalism, consumption by large vertebrate scavengers, clothing, concealment, environmental factors, arthropod colonization,and so on. All these processes can have a profound influence on the rate of decomposition, which explains why estimations of the post mortem interval can be very complex.

After death a body undergoes several physical and biochemical changes in a rapid succession that have been employed in estimating the time since death.

– Physical changes and supravital reactions

Cooling of the body – algor mortis

With death metabolism ceases and body temperature is no longer maintained. For the first 6.5 hours after death, the most reliable and precise technique is measuring the central brain temperature, then up to 80 hours after death, depending on body weight, measuring the rectal temperature becomes the most reliable and precise technique for humans(Henßge and Madea, 2004; Hubig *et al.*, 2011; Muggenthaler *et al.*, 2011) and for animals (Kaliszan *et al.*, 2005). Increasingly, the liver temperature is used instead of the brain or rectum temperature (Al-Alousi *et al.*, 2001, 2002). The development of infrared thermometers shifted measurements to the tympanic membrane of the ear (Rutty, 2005c, a, b; Cattaneo *et al.*, 2009). Measuring the temperature of the eyeball is still experimental (Kaliszan, 2013). In some countries based on an Anglo-American legal system, an interdiction of injuring examinations before autopsy exists, limiting the kind of measurements that can be performed at a crime scene.

Hypostasis or sacking of the blood – liver mortis

Blood settles in the capillaries of the skin and causes a purple or reddish purple discolouration of the skin. This process starts around 45 minutes after death and reaches its maximum around 9 hours after death (Henßge and Madea, 2004). The skin can now be measured with a spectrometer which extends the useful time period up to 100 hours post mortem (Sterzik *et al.*, 2014). However, if the corpse is exposed to cold temperature, the hemoglobin becomes re-oxygenated and the livid colour of the skin changes back to cherry red (Bohnert *et al.*, 2008).

Stiffening of the body – rigor mortis

With death the control of the muscles ceases; skeletal and smooth muscles become flaccid, the joints relax and sphincters loosen. The relaxation of the skeletal muscles can lead to an increase of a person's height by as much as 3 cm. The body might adopt

an unusual posture, urine and faeces might be released, and stomach and gut content mightleave the body.

Calcium is stored in sarcoplasmic reticulum of muscle cells. With the death of the muscle cells, the membrane around the sarcoplasmic reticulum breaks down, the released calcium displaces troponin and tropomyosin, which leads to a binding of actin and myosin causing the stiffening of the muscle. Rigor mortis sets in after around 3 hours post mortem, reaches complete rigidity after around 8 hours, and resolves after 76 hours. The rigidity of an elbow or knee joint can be broken by forcefully pulling a limb. In a third of cases, rigor mortis re-establishes itself after some 10 hours after an rearrangement(Henßge and Madea, 2004; Anders *et al.*, 2013).

Occasionally,an instant form of stiffness of particular muscle groups like a hand had been reported in the literature and in textbooks, often associated with gripping an object like a gun or a knife or grasping vegetation before drowning(Gunn, 2009). These cases have become known as cadaveric rigidity, cadaveric spams or instantaneous rigor. Recently, there is an increasing realisation that this phenomenon is an artefact and cadaveric rigidity has no veracity(Bedford and Tsokos, 2013; Fierro, 2013; Gill, 2013; Madea, 2013; Pirch *et al.*, 2013).

– Artificial excitation of muscles

Mechanical stimulation

Skeletal muscles can be mechanically stimulated to contract. When the biceps muscle of the upper arm is hit with three fingers, it contracts. This is known as Zsakó's phenomenon. It disappears 3 to 4 hours after death (Madea, 2002; Henßge and Madea, 2004; Warther *et al.*, 2012).

The local contraction at the site of a more forceful mechanical stimulation is called idiomuscluar contraction. This can be achieved, for example, by a single forceful percussion of the muscle's belly with a hard object such as the handle of a tool. The contraction is most pronounced directly after death and gradually weakens until it completely disappears after 13 hours (Warther *et al.*, 2012).

Electrical stimulation

When an electrode is inserted in the nasal part of the upper eyelid, facial muscles can be electrically stimulated to contract. Three hours after death, the hole half of the face contracts, whereas after 13 hours only the muscle at the side of the electrode still contracts (Henßge and Madea, 2004).

Pharmacological excitation

The muscle of the iris can be excitated with tropicamide up to 30 hours and with acetylcholine up to 46 hours after death, leading to a dilation of the pupal (mydriasis)(Madea and Henßge, 2002). Myotic drugs inducing a contraction of the pupil have also been proposed. All pharmacological methods involving the pupil have been questioned for their lack of reliability (Orrico *et al.*, 2008).

Other supravital reactions have been investigated during the early post mortem interval. The mucociliary motility of the nasal epithelium was measured under a phase-contrast microscope after being extracted with a rhinoprobe from a corpse (Romanelli *et al.*, 2012). Differences in the ciliary beat frequency were noticed between 5, 11, and 25 hours post mortem but not corrected for temperature. The g turbidity or opacity of the cornea changes significantly with time (Balci *et al.*, 2010). The intraocular pressure of the eye measured with a tonometer might provide a resolution of 2 hours (Balci *et al.*, 2010). The decrease in capacity reactance in electric impedance spectroscopy has been tested on rat spleens under temperature control (Mao *et al.*, 2011).

Traditional single physical methods have a confirmed range between 7 and 18 hours post mortem that can be extended slightly by combining several methods for a single estimate (Knight, 2002; Henßge and Madea, 2004; Mathur and Agrawal, 2011). Skin spectrophotometry can extend this range up to 100 hours post mortem (Sterzik *et al.*, 2014).

– Biochemical changes

With the arrest of the heart, cells will become deprived of oxygen and start dying. Brain cells will die within minutes and skin cells can survive up to 24 hours. Eventually every tissue in the human body will shown signs of autolysis. Every metabolite in the human body will show signs of chemical decay. With several tens of thousands metabolites available, each one could be used for a kinetic related to the post mortem interval. Henßge and Madea (2007) have complained about the great amount of papers just describing the time-dependence of an analyte or parameter. Such papers wouldn't make any sense or are not of any help because they would measure all the same.

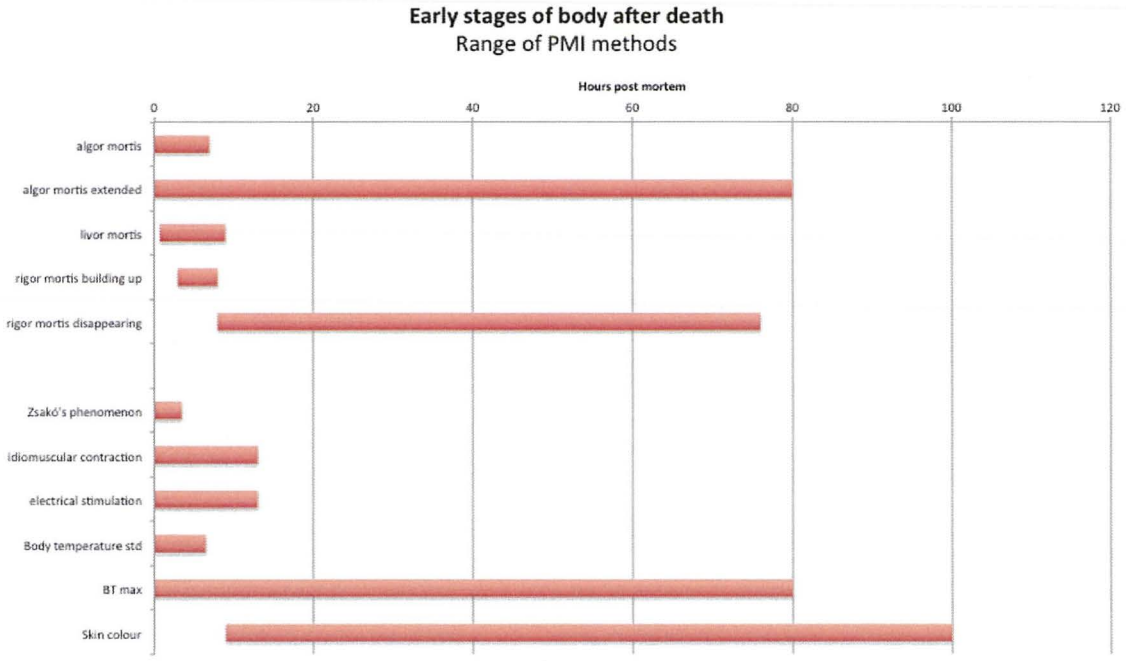


Figure 1.1: Traditional physical methods in routine practice allow PMI estimates during the first day of death. Measuring the brain temperature or the photospectrum of skin colour can extend these methods to 3 to 4 days post mortem.

Figure 1.1 shows the time limitations of classic physical methods for estimating PMI. For any longer periods, forensic entomology data are required.

1.2 *Calliphora vicina*Robineau-Desvoidy, 1830

Blow flies are very diverse, many species are found in the Neotropics and a large number in Africa and Southern Europe. For the family Calliphoridae, most of them are commonly found in India, Japan, Central America and Southern United States.Blow flies shows characteristics that differentiate them from flesh flies such as, they are metallic in colour and may appear to be shining green, blue or black while the other appears dull in colour respectively.Blow flies lay eggs into wounds of animals or openings on corpses such as eyes, nose, penis or vagina while flesh flies deposit live larva on the body. Among the blow flies, the ones from the family Calliphoridae are the first to arrive on corpses within minutes to several hours depending on the environmental conditions. And this is because they have the ability to smell death corpses from up to 10 miles (16 km) away. The blow flies of this family are known with certain features that differentiate them from others; they have blue bottle and green bottle colour and are also characterized by their three segmented antennae. There are many different *Calliphora* species but *C. vicina* and *C. vomitoria* are closely related.The blow fly *C. vicina* is a large fly of 9 – 11 mm in length and it was earlier recorded in older literatures as *C. erythrocephala*. The blow fly*C. vicina* is commonly calledbluebottle or blow fly, having its clear metallic blue-grey colouration on its thorax and abdomen. It has clear distinguishing features from its closest neighbour *C. vomitoria*. Where *C. vomitoria*has bright orange hairs on its occiput, the posterior part of its head capsule and *C. vicina* does not. And also on its third instar larvae it can be separated by the width of their posterior spiracles, for the posterior spiracles of *C. vicina* are within the range of 0.23 – 0.28 mm wide while that of *C. vomitoria* are within the range of 0.33 – 0.38mm (Smith, 1986; Erzinçlioğlu, 1996; Erzinçlioğlu, 2000; Goff, 2000; Petrašiūnas, 2007; Pohjoismäki *et al.*, 2010; Gennard, 2012). This study focuses on *C. vicina* for its unique characteristics of being among the first of blow flies to arrive on a corpse, its predictable rate of development, and it ability to be found both on open bodies and on bodies lying inside buildings in urban areas. It is also among the flies where their wandering third instar larvae move farthest away from a

corpse after they stops feeding and they are found mostly in temperate region (Petrašiūnas 2007).

A forensic entomologist uses successfully the age of blow flies to estimate the time of death of a corpse. For the larval stages of blow flies, the maximum lengths of the larvae are used as a variable to estimate the time interval the corpse was exposed to the natural environment. The three larval instars together last between 6-11 days depending on the ambient temperature. However, the longer the time interval, the more problematic these estimates become. Young 3rd instar larvae initially keep feeding and increasing their lengths with time until they have accumulated a sufficient amount of nutrients to develop into adults. At this stage, the 3rd instar larvae stop feeding and start to wander to find a place to pupate away from the corpse. During this process the larvae already start shrinking a little bit and the relationship between age of larvae and lengths is lost. The larvae now transform into pupae. The pupal stage lasts between 7-14 days depending on the ambient temperature. The pupal stage can be longer than all three larval stages together. The older a corpse becomes, the more important an entomological time estimate becomes for criminal investigations. Despite this fact, all morphological and molecular attempts to estimate the age of a given blow fly pupa have been unsatisfactory (Gomes *et al.*, 2006; Arnott and Turner, 2008).

The salivary glands and midgut of both the developmental stages and adult flies exhibit primary polytene chromosomnes (Doberskia, 1984; Stegnii *et al.*, 1999).The salivary glands of insects that undergo complete metamorphosis are very good tissues for examining physiological or programmed cell death (Levy and Bautz, 1985; Lockshin and Zakeri, 2004).

Apoptosis is the process of programmed cell death, which is defined as genetically controlled mechanism of cell death and that is involved in the maintenance of tissue homeostasis.The major markers of apoptosis are cell shrinkage; the salivary glands of blow fly *C. vicina* have long tubules of single-layered uniform epithelial cells, which span through the thoracic and abdominal portion of the fly (Oschman and Berridge, 1970; Rotte *et al.*, 2008).Polytene chromosomes are found nuclear blebs,

and condensation of chromatin and DNA fragmentation. Apoptosis is the most clearly expressed form of programmed cell death, though there are other non-apoptotic types of cell death, which might have biological importance (Kerr *et al.*, 1972; Salomon and Diaz-Cano, 1995; Leist and Jäättelä, 2001).

In this study, I seek to examine the degree of apoptosis in salivary glands of active feeding third instar larvae through the wandering third instar larvae to when pupariation start, to enable the ageing of the wandering stage of the third instar larvae.

Bacterial symbionts are a group of microorganisms that live in or on other organisms to make a living and/or contribute to the survival of its host (Buchner, 1965; Chanbusarakum and Ullman, 2008; Moya *et al.*, 2008).They are divided into two major groups, the extracellular also called ectosymbionts and the intracellular also called endosymbionts. They are generally found on or in arthropods as well as in or on other invertebrate animal phyla (Perotti and Braig, 2011).They might play very important functions in insect diversity and speciation(Bordenstein, 2003; Hurst *et al.*, 2003; Moran *et al.*, 2008; Harris *et al.*, 2010).

These symbionts have been shown to have specific influence on cellular functions of their host such as signal transduction, cell cycle progression, vesicular trafficking and programmed cell death (Tram *et al.*, 2003; Azzouna *et al.*, 2004; Veneti *et al.*, 2005; Bentley *et al.*, 2007; Ikeya *et al.*, 2009).They manipulate their way through the host immunity system and do that in one or two of these ways: firstly, preventing the activation of immune signalling by burying themselves inside a host vacuolar membrane and secondly by preventing the immune signalling by some unknown means (Rikihisa, 2006).The component (midgut epithelium) that harbours these bacterial symbionts is found to change its shape through the pupal development stage.

This study seeks to explore the changing shape of the pupal midgut epithelium to age the pupal stage of development and also molecular characterized the types of bacterial symbiont it habours.

1.2.1 Blow flies or bluebottles and death

One of oldest written records in Western literature about the association of flies and dead human bodies is likely Homer's Greek epic poem Iliad or the Song of Ilium describing the ten-year siege of Ilium (city of Troy) during the Trojan War:

> *forthwith to his mother he spake winged words:*
> *"My mother, the arms that the god hath given are such as the works of immortals should fitly be, such as no mortal man could fashion. Now therefore will I array me for battle; yet am I sore afraid lest meantime flies enter the wounds that the bronze hath dealt on the corpse of the valiant son of Menoetius, and breed worms therein, and work shame upon his corpse—for the life is slain out of him—and so all his flesh shall rot."*
> *Then the goddess, silver-footed Thetis, answered him:*
> *"My child, let not these things distress thy heart. From him will I essay to ward off the savage tribes, the flies that feed upon men slain in battle. "*
>
> Homer, The Iliad, Book XIX, lines 20-30 in the translation of A.T. Murray, London: William Heinemann, 1924.

Recently the Iliad was dated between 760 and 710 before Common Era by building a phylogeny of words in Homeric and Modern Greek (Altschule *et al.*, 2013). Papavero *et al.* (2010) proposed that this very passage inspired some two thousand three hundred years later the Italian physician and poet Francesco Redi to undertake the first experiments demonstrating that blow fly larvae on meat and decomposing carcasses originate from fly eggs and not from Aristotelian spontaneous generation (Redi, 1668). From there it is a small step to appreciate the general importance of blow flies in the decomposition of carcasses as a quote by Carl von Linné might illustrate:

> *Tres Muscae consumunt cadaver Equi aeque cito ac Leo.*
>
> [Three flies consume the carcass of a horse as quickly as a lion could do it]
>
> a Linné (1767)

The meaning of blow in blow flies is best illustrated with the help of William Shakespeare, which takes us back some two hundred years. One of the thirty seven different meanings in English of *to blow* is to lay or to deposit eggs from which larvae

(maggots) would emerge and feed on decomposing animal carcasses and human corpses (Hibbard, 1998; Papavero *et al.*, 2010; OED, 2015).

> *These sommer flies, Haue blowne me full of maggot ostentation*
>
> Shakespeare (1598)
>
> *Worms ... which are not bigger then such as Flyes blow in rotten flesh.*
>
> Topsell (1607)

From flies blow in rotten flesh to blow flies seems to have been a big step or over one hundred years:

> *The green blow-flies, attracted by the meat brought to our camp, exceeded everything that can be conceived.*
>
> Nuttal (1821)

However, bluebottle or blue bottle as a term appears to have been around much longer than blow fly:

> *A Man could not walk London-streets without having his Nose persecuted by Gnats, Wasps, or Blue-bottles.*
>
> Brown (1703)
>
> *A Fly upon the Chariot-Pole Cries out 'What Blue-bottle alive Did ever with such fury drive?'*
>
> Poem circa 1720 Prior (1741); (OED, 2015)

The first quotation for green bottle as a fly goes back only to 1828 (Deale, 1828). The adults of several blow flies species are characterised by a lustrous or metalic dark blue abdomen, particularly prominent for *Calliphora vomitoria* and *Protophormia terraenovae* (Calliphoridae), which explains the blue in bluebottle. Which leaves us withe the etymology of bottle in the word bluebottle. Surprisingly, the Oxford English Dictionary remains silent on the subject. Adams suggests that bottle is a diminutive of bot. He argues that certain larvae infesting sheep are called wor-bots or wor-bottles in Old English (Adams, 1859; Palmer, 1882). Not surprisingly, bot flies, Oestridae, constitute a sister family to the Calliphoridae

1.2.2 Taxonomy of *Calliphora vicina*

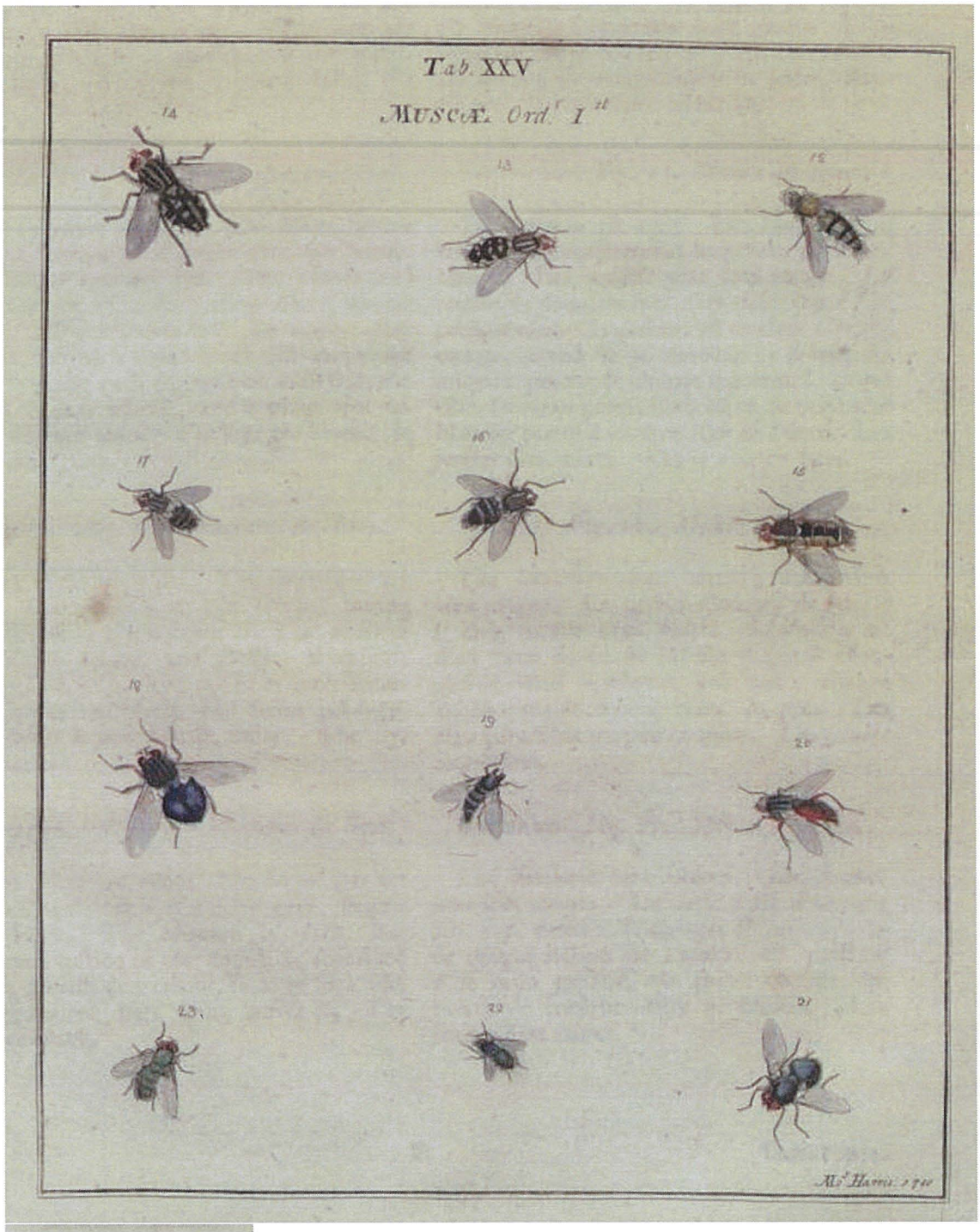
Calliphora vicina is a true fly characterized by a single pair of wings in adult stage on the second or middle segment of the thorax (mesosoma), the mesothorax, and a pair of halteres, knobbed structures that function as gyroscopes, on the third thoracic segment, the metathorax, and belongs to the order Diptera. Other flies such as alderflies, dobsonflies and fishflies (Megaloptera), butterflies (Lepidoptera), caddisflies (Trichoptera), damselflies and dragonflies (Odonata), fairyflies and sawflies (Hymenoptera), fireflies (Coleoptera), mayflies (Ephemeroptera), scorpionflies (Mecoptera), snakeflies (Raphidioptera), stoneflies (Plecoptera), and whiteflies (Hemiptera), have two pairs of wings as opposed to only one pair of the true flies. This rather long list of examples is also given for another reason. Thomson Reuters' Web of Science lists in January 2015 some 5,073 publications with the word 'blowfly' in the title and only 868 publications with the correct two words 'blow fly', whereas google gives a much better ratio with about 485,000 results for the search term blowfly and about 267,000 results for "blow fly", without quotation marks it grows to 8,640,000 results. This suggests that the professional literature follows conventions only in 1 in 6 cases but popular media in an astonishing 1 in 2 cases. Entomological convention is that species and groups of true flies or Diptera should be written as two words as in blow flies. This convention is mainly upheld in the entomological literature and becomes diluted in the applied fields of medical, agricultural and forensic sciences.

The higher taxonomy or phylogenetic relationships of what is now called a megadiverse order, the Diptera, is still subject to much diversity in opinions. Consistent quantitative morphological and molecular data support the following major clades or lineages (Wiegmann *et al.*, 2011; Lambkin *et al.*, 2013). Starting at the level of suborder, infraorder, section or subsection, blow flies belong to the Brachycera for having a frontoclypeal apotome among other characters, the Eremoneura for the fusion of the larval and antennal and maxillary lobes, the Cyclorrhapha (circular-seamed flies) for the circular aperture with the help of which adult flies escape the puparium, the Schizophora (split-bearers) for the ptilinium that inflates with the help of hemolymph to burst open the circular aperture, and the Calyptratae defined by the

two calipters that cover the gyroscopic halters in adults. The superfamily Oestroidea unites some six families and combines protelean parasitoid families with flesh fly and blow fly families. The blow fly family in the strict sense, Calliphoridae, is considered polyphyletic (Rognes, 1997; Kutty *et al.*, 2010). The family is home to carrion-associated blow flies, toad blow flies that cause primary obligate myiasis in amphibians, nest blow flies that are obligatory and intermittent bloodsucking parasites of nestling birds, screwworms, the tumbu fly, the Congo floor maggot, the deer and water buffalo skin maggots and the elephant skin maggot, in total some 1,450 species (Verves, 2005). The phylogenetic relationships at the family and subfamily level are mostly unresolved (Marinho *et al.*, 2012). *C. vicina* belongs to the subfamily Calliphorinae, which again is considered polyphyletic because it has the melanomyinae genera Angioneura and Opsodexia nested inside (Singh and Wells, 2013). The most recent taxonomic species description of *C. vicina* is as recent as 2012 (Whitworth, 2012).

Calliphora vicina has a long, rich and slightly twisted taxonomic history, which might have started in 1794. It is all the more surprising that after such a long time and many hundreds of publications, it still might be possible to raise questions and make new suggestions, in particular regarding the first illustration of this species.

The first description currently in use for *Calliphora vicina* as a new species derives from the Danish entomologist Johann Christian Fabricius, Figure 1.4, who in 1794 gave it the name *Musca carnivora* (carnivora L.: flesh eating), Table 1.2.



VOMITORIA MINIMUS. *Fig.* 22. *Measures three lines.*

The *larger eyes* are brown. The *thorax* is of a dark iron grey. The *abdomen* is of a fine deep blue; *legs*, black. It is like the Vomitoria, except that the *abdomen* has not the white glares.

Figure 1.2: Colour illustration of *Musca vomitoria minimus* (# 22 on page) and species description by Harris (1776), one of the earliest recognized descriptions for *Calliphora*

vicina. The figure also illustrates the paucity of discriminating characters typical for early species descriptions.

Because *C. vicina* was and is such a common fly, there might even be earlier discoveries of this species described under different names before 1794; however, the ever dwindling number of taxonomists makes it less likely that these earlier descriptions, if they exist, will ever be revealed. A candidate for presently the earliest description is by the hand of the English entomologist and engraver, Moses Harris, Figure 1.4, who in 1776 erected the species*Musca vomitoria minimus* (vomitoria L.: provoking vomiting), Figure 1.2. Possibly because the species name *vomitoria* had already been used for other fly species, his description did not receive much attention.

Possibly the earliest and one of the most detailed illustration of *C. vicina* might be found by the English natural philosopher, architect and polymath, Robert Hooke, in his book Micrographia, Figure 1.3. Because Hooke didn't give his fly a formal name, this description and illustration has been completely overlooked by the entomological taxonomists.

The Danish missionary and naturalist Otto Fabricius, Figure 1.4, working under extreme simple conditions in Greenland, might have described the fly as well in 1780 under the name *Volucella vomitoria*, but the species description is so brief that a confident identification is difficult (Fabricius, 1780). Bezzi and Stein (1907) are confident about the synonymy, Hall (1948) adds a question mark to it. Since the species name *vomitoria* had also been used earlier for another species, the name becomes a junior homonym and is considered preoccupied. Then in 1794, another Danish entomologist and professor at the Universities of Copenhagen and Kiel, also by the name of Fabricius, Johann Christian Fabricius, Figure 1.4, published the name *Musca carnivora.* With one exception explained further on, the species description of *M. carnivora* was overlooked for the next 196 years.

Unaware of Fabricius' book, the German entomologist Johann Wilhelm Meigen, Figure 1.4, described *C. vicina* in 1826 as *Musca erythrocephala* (erythrocephala Gr.: red head).

Figure 1.3: Illustration of a "blue fly" by Robert Hooke in Micrographia (Hooke, 1665). Is this the first illustration of *C. vicina*?

Four years later, the French physician and entomologist André Jean Baptiste Robineau-Desvoidy, Figure 1.4, erected a new genus, Calliphora, into which he moved *M. erythrocephala*, becoming *C. erythrocephala*. In 1948, David G. Hall of the US Bureau of Entomology uncovered that the species name *erythrocephala* had not only been used by Meigen butby two other taxonomists for different species and these different

species had been described before Meigen's description, Table 1.2. When different taxonomist give one and the same name to different fly species, these names become homonyms; the oldest name becomes the senior homonym and all the other names become junior homonyms, which eventually should receive new, distinct names. If the identical genus names and species epithets are in their original combination, it is known as a primary homonym; a secondary homonym is produced when fly species have the same species epithet but belong to different genera. The unrelated *erythrocephala* species described by De Geer, Fabricius and de Villers made the name *erythrocephala* preoccupied andunavailable for Meigen's species. Hall identified a type specimen of *C. vicina* of the Bigot Collection, then in Newmarket, England, as *C. erythrocephala*, making them synonyms and adopting the name *C. vicina* for *C. erythrocephala*(Hall, 1948). Robineau-Desvoidy was quite fond of the species name *vicina* (vicina L.: near, neighbour). In addition to Calliphora, he used it for new fly species in seven additional genera. The International Code of Zoological Nomenclature requires that all animal species names are unique, therefore none of the other *vicina* species of Robineau-Desvoidy did survive.

Root maggots (Anthomyiidae)
Delia vicina Robineau-Desvoidy, 1830
now*Hylemyia riparia* Robineau-Desvoidy, 1830

Blow flies (Calliphoriade)
Calliphora vicina Robineau-Desvoidy, 1830
Lucilia vicina Robineau-Desvoidy, 1830
now*Lucilia caesar* (Linnaeus, 1758)

Parasitoid and parasitic tachinids (Tachinidae)
Estheria vicina Robineau-Desvoidy, 1830
now*Estheria cristata* (Meigen, 1826)
Gonia vicina Robineau-Desvoidy, 1830
now*Gonia atra* Meigen, 1826
Myiobia vicina Robineau-Desvoidy, 1848
now*Solieria inanis* (Fallén, 1810)
Phryxe vicina Robineau-Desvoidy, 1863
now*Phryxe vulgaris* (Fallén, 1810)

Tachina vicina Robineau-Desvoidy, 1830
now*Exorista larvarum* (Linnaeus, 1758)

Table 1.1: Fly species named *vicinia* by Robineau-Desvoidy.

This meant that Robineau-Desvoidy missed that Meigen's species, *Musca erythrocephala*, which he moved into the new genus Calliphora that he just erected, and the new species, *Calliphora vicinia,* which he had described himself at the same, were one and the same. Not only are these two species the same, but another five species described by Robineau-Desvoidy in 1860 and 1863 are identical with *C. erythrocephala* and *C. vicina*, Table 1.2.

In 1990, Rognes discovered that the holotype of *M. carnivore* in the Fabrician Collection in the Zoological Museum of the University of Copenhagen was actually *C. vicina*. *M. carnivore*had been listed by others as a synonym of *C. vomitoria*(Bezzi and Stein, 1907; Schumann, 1986). Rognes dismissed the synonymy of *C. vomitoria* but made *C. erythocephala* a synonym of *M. carnivore*. When one and the same fly species is given different names by taxonomists, these different names become synonyms and the oldest of these names is the senior synonym; all other names become junior synonyms. Only senior synonyms should be used in naming species. Because Fabricius described *M. carnivore* before Robineau-Desvoidy described *M. vicina*, *C. vicina* should be changed to *C. carnivore*. To maintain stability of the taxonomy, prevent confusion, and based on the fact that apparently nobody had used Fabricius' name, *M. carnivore*, in any publication, Rognes andBlackith proposed to suppress the new name of *C. carnivore* and preserve the old name of *C. vicina;* the International Commission on Zoological Nomenclature (ICZN) agreed and upheld with 24 votes for, two against and one abstention the name *C. vicina* (Rognes and Blackith, 1990; ICZN, 1992).

It is a strict requirement of the International Commission on Zoological Nomenclature that a species name had not been used in any publication in order for it to agree to the suppression of a name.

Musca vomitoria minimus 1776

Moses Harris
(1730 – c. 1788)

Volucella vomitoria 1780

Otto Fabricius
(1744 – 1822)

Musca carnivora 1794

Johann Christian Fabricius
(1745 – 1808)

Musca erythrocephala 1826

Johann Wilhelm Meigen
(1764 – 1845)

Calliphora erythrocephala (Meigen) 1830

Calliphora vicinia 1830

André Jean-Baptiste Robineau-Desvoidy
(1799 – 1857)

Figure 1.4: The history of the European bluebottle in pictures of taxonomic authorities. The first five entomologists out of ten, in chronological order of discovery, who described independently from each other the species that is now known as *C. vicina*.

While reading all the original publications, I was surprised to discover that Robineau-Desvoidy had recognized the species description of Fabricius in one of his well-known publications and synonymized *Musca carnivore* with *Calliphora vomitoria* Linneus, Figure 1.5.

Musca vomitoria : De Géer-*Ins.* VI, 57, 4.
— — Rœsel.-*Ins.* II, pl. 9-10.
— — Geoff.-*Ins.* II, 524, 59.
— — Schrank.-*Ins. Aust.*, 926.
— — Har.-*Ex.*, 86, pl. 25, fig. 18.
— — Panz.-*Faun. Germ.* X, 19.
— — Zetterst.-*Ins. Lapp.*, 656, 14, et *Dipt. Skand.* IV, 1328, 1.
Musca carnivora : Fabr.-*Ent. Syst.* IV, 313, 4, et *Syst. Antl.*, 285, 5.
Musca mortuorum : Fabr.-*Syst. Antl.*, 290, 32.
Volucella vomitoria : Schrank.-*Faun. Boic.* III, 2488.
Musca erythrocephala : Meig.-*Dipt.* V, 62, 22; VII, 300, 2.
— — Walk.-*British Mus. Ins.* IV, 893.
Calliphora vomitoria : Rob. Desv.-*Myod.*, 435, 3.
— — Macq.-*Buff.* II, 262, 2.

♀. Frontis Facieique lateribus subauratis; Frontalibus fuscis aut fusco-fulvescentibus; Antennæ nigræ, basi fulvescente; Facialibus et Lateralibus subaureis : Epistomate, Medianeis, Palpis fulvis. Thorax

Figure 1.5: Top part of a book page by Robineau-Desvoidy (1863) on which the species name *Musca carnivora*(highlighted in yellow) is used in a taxonomic sense, showing that the species description of J.C. Fabricius had been prior recognized and used by a taxonomic authority.

Perhaps the suppression of *carnivore* as a species name was premature from a regulatory point of view. However, from a practical point of view, and especially considering the forensic importance of this species, taxonomic stability is very much desired.

Robineau-Desvoidy's specimen of *C. vicina* has survived and is currently as holotype in Bigot's Diptera Exotica collection in the Museum of Natural History of the University of Oxford (Dear, 1986). The term exotic refers to non-European specimens. Although *C. vicina* is known as the *European* bluebottle, the holotype, a female adult, originates from an unstated number of specimens collected in Philadelphia, Pennsylvania, USA; and the very first collection of the fly by Harris happened in somewhere in Massachusetts in the United States, not in Europe. The Philadelphia specimens ended up in the collection of Pierre François Marie Auguste Compte Dejean, a French entomologist and lieutenant general in Napoleon's army. Dejean became infamous for publishing some 22,000 species names of beetles, opposing the principle of priority. Robineau-Desvoidy studied the flies in Dejean's collection and named many species. Later, the collection was obtained by the French entomologist Jacques Marie Frangile Bigot. Eventually, the fly collection was purchased by the British entomologist and politician George Henry Verrall, Newmarket, Suffolk, before ending up in the Hope Entomological Collection of the University Museum in Oxford. It is not obvious and possible not known who collected all the specimens that Robineau-Desvoidy used for his eight species descriptions of what is now *C. vicina*. These specimens certainly come from desperate places, like France, the USA, Spitsbergen, and so on. This not only shows the already wide distribution of this species at that time, it also suggests that Robineau-Desvoidy might have seen small morphological differences in the specimens from the various locations that led him to describe them as separate species although there might be more cynical interpretations.

Thomson Reuters' Web of Science lists for the timespan 1950-2015 three publications for the name *Musca carnivora*, five publications for the name *Musca erythrocephala*, 1,546 records of publications with the name *Calliphora erythrocephala*, and 1,435 records with the name *C. vicina.* Although the name change from *erythrocephala* to *vicina* was proposed in 1948, the last five years (2010-2015) still saw 68 records of publications with the name *C. erythrocephala* compared to 270 records with the name *C. vicina.* Six publications on the cytology, biochemistry, genetics and cell biology of the fly still had the full name *Calliphora erythrocephala* in the title between 2010-2015, all of which came from Russia. Change is slow.

Date	Authority	Name	Taxonomic status	Reference, revision
1665	Hooke	blue fly		(Hooke, 1665)
1776	Harris	*Musca vomitoria minimus*	senior synonym	(Harris, 1776; Bezzi and Stein, 1907)
			junior primary homonym	
1776	De Geer	*M. erythrocephala*	unrelated fly, senior primary homonym, now*Cochliomyia macellaria* (Fabricius, 1775)(lectotype), Calliphoridae	(De Geer, 1776; Hall, 1948) (Thompson, 1973)
1780	O. Fabricius	*Volucella vomitoria*	senior synonym, uncertain identity	(Fabricius, 1780)
			also junior primary homonym	(Hall, 1948)
1787	J.C. Fabricius	*M. erythrocephala*	unrelated fly, junior primary homonym, still unrevised	(Fabricius, 1787; Hall, 1948)
1789	de Villers	*M. erythrocephala*	unrelated fly, junior primary homonym,	(de Villers, 1789; Rognes and Blackith, 1990)
			still unrevised	
1794	J.C. Fabricius	*M. carnivora*	senior synonym, suppressed	(Fabricius, 1794; Rognes, 1990; Rognes and Blackith, 1990; ICZN, 1992)
1826	Meigen	*M. erythrocephala*	junior primary homonym	(Meigen, 1826; Hall, 1948)
1830	Robineau-Desvoidy	*Calliphora*	new genus	(Robineau-Desvoidy, 1830)
1830	Robineau-Desvoidy	*C. erythrocephala* (Meigen)	new combination, secondary homonym	(Robineau-Desvoidy, 1830)
1830	Robineau-Desvoidy	*C. vicina*	senior synonym,	(Robineau-Desvoidy, 1830)
			new and current, valid species name	(Hall, 1948; ICZN, 1992)
1830	Robineau-Desvoidy	*C. littoralis*	junior synonym	(Robineau-Desvoidy, 1830; Bezzi and Stein, 1907)
1830	Robineau-Desvoidy	*C. monspeliaca*	junior synonym	(Robineau-Desvoidy, 1830; Bezzi and Stein, 1907)

1830	Robineau-Desvoidy	*C. musca*	junior synonym	(Robineau-Desvoidy, 1830; Bezzi and Stein, 1907)
1830	Robineau-Desvoidy	*C. nana*	junior synonym	(Robineau-Desvoidy, 1830; Bezzi and Stein, 1907)
1830	Robineau-Desvoidy	*C. spitzbergensis*	junior synonym	(Robineau-Desvoidy, 1830; Bezzi and Stein, 1907)
1833	Macquart	*M. scutellata*	junior synonym	(Macquart, 1833; Bezzi and Stein, 1907)
1949	Walker	*M. lilaea*	junior synonym	(Walker, 1849; Bezzi and Stein, 1907)
1849	Walker	*M. thuscia*	junior synonym	(Walker, 1849; Schumann, 1986)
1851	Macquart	*C. rufifacies*	junior synonym	(Macquart, 1851; Hall, 1948)
1852	Walker	*M. aucta*	junior synonym	(Walker, 1852; Schumann, 1986)
1863	Robineau-Desvoidy	*C. insidiosa*	junior synonym	(Robineau-Desvoidy, 1863; Bezzi and Stein, 1907)
1926	Rohdendorf	*C. turanica*	junior synonym	(Rodendorf, 1926; Rognes, 1990)

Table 1.2: Taxonomic history of *Calliphora vicina*. Citations are only given for the original description and for revisions that changed the taxonomic status. Revisionary literature that confirmed a taxonomic status, which is extensive for *C. erythrocephala*, has been omitted.

1.2.3 Model animals for blow fly decomposition studies: Size does matter

To study decomposition, a wide variety of model animals of desperate size differences have been employed. At one extreme end are retail-sized slabs of meat, muscle or liver, and rodents, one the other end, decomposition studies on elephants, and in between human body farms at anthropological research facilities.

Most researches do not have access to human bodies and make use of domestic pigs, *Sus scrofa*, as the best approximation for a human body. The bloating stage (putrefaction) of decomposition is governed by the intestinal microbiota. Omnivorous pigs have an intestinal track that houses a similar intestinal flora to humans. Bloating in pigs and humans proceeds at a similar rate (Campobasso *et al.*, 2001). The pig skin resembles the skin of human so closely that it can be used on burn victims.

Human corpses analysed in forensic investigations range from neonatal remains to obese adults. Relatively few data are available for human infants (Archer, 2004). By far, most forensic cases involve adults. Although pigs are the most used model animal for studying the decomposition of adult humans, full-sized adult pigs in a weight range of 60 to 90 kg have rarely been used. Most often piglets below a weight of 20 kg are employed. This difference in size makes a difference for the stages of decomposition and the speed of decomposition in terms of accumulateddegree days (ADD). A full-sized pig enters a plateau phase during the advanced stage of decomposition. This plateau stage complete disappears in pigs of less than 20 kg. On average a piglet decomposes almost three times faster than an adult pig(Spicka *et al.*, 2011; Sutherland *et al.*, 2013). It is necessary to differentiate between a large body characterised mainly by muscle tissue and an obese body characterised mainly by fat tissue. Obese corpses lose weight and body mass faster than more emaciated bodies. In part this might be caused by the thermal isolation of the fat, allowing fly larvae to consume body mass at a higher rate (Tracqui, 2000). This suggests that the fly larvae are the rate limiting factor. Comparing decomposition rates in the presence and absence of fly larvae for increasing larger carcasses, Simmons *et al.* (2010) confirm this hypothesis. Obese bodies might also liquefy fats during decomposition,which are then

lost with the leachate, accelerating decomposition overall. This has as a consequence that a 110 kg corpse decomposes much faster than a 65 kg corpse (Mann *et al.*, 1990). Particularly putrefaction, the bloating stage, in human foetuses and newborn babies has been shown to be slower than for larger body sizes (Campobasso *et al.*, 2001). However, larger bodies reveal a longer period of butyric fermentation (advanced or post-decay stage), slowing down the overall decomposition. This lead Archer (2004) to argue that newborns decompose at three to five times the rate of adults. This emphasises that there is not an easy, linear relationship between body size and the rate of decomposition and that body size and body weight are not necessarily equivalent. Two threshold values can be identified. Human corpses and pig carcasses above a weight of 20 kg are subject to a plateau stage. Between 5 kg and 20 kg, smaller carcasses (i.e., 8 kg) might or might not show a faster initial rate of decomposition compared to larger carcase (i.e., 16 kg) (Hewadikaram and Goff, 1991). Below 5 kg, variability increases. In very small piglets, microbial decomposition might dominat and cases might occur, where the small pig carcase will not be colonised by flies (Sutherland *et al.*, 2013). The density of flies is directly related to the size of the carcase (Denno and Cothram, 1975).

Increasing the number of carcases or corpses at a study site does not have an effect on either the composition of the necrophagous fauna nor on the rate of decomposition. This has been clearly shown on human body farms (Shahid *et al.*, 2003; Schoenly *et al.*, 2005).

1.3 Aims of Objectives of the Thesis

The older a corpse becomes, the more important an entomological time estimate becomes for criminal investigations. But for older corpses, no reliable methods are available to be applied in age estimations in forensic investigations. Blow flies could be used, but reliable methods to age the the wandering third instar larval and pupal stage of development of blow flies have not yet been developed. For the larval stage, when it start to wander, it does shrink and it makes the relationship between length and age

to be lost while for the pupal, there is no morphological feature to hold on to for ageing it, see Figure 1.6.

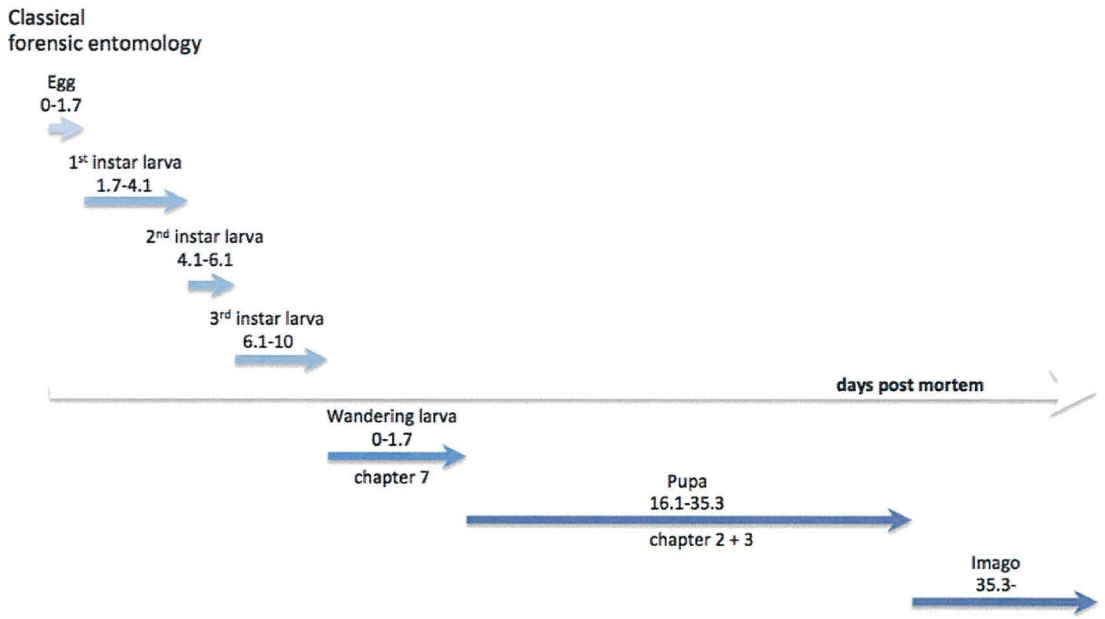


Figure 1.6: Post mortem intervals are currently estimated through the ageing of 1st to 3rd instar larvae of blow and flesh flies. However, the longest periods in the development of the fly, the wandering stage and the pupal stage, are not yet utilized for time estimations. By comparing the time lines above and below the central arrow, an idea of the potential practical impact the methods developed in these chapters might be obtained. The number above the arrows gives the days post mortem for the individual life stages for *Calliphora vicina* at 16 ˚C.

The aim of this thesis is to develop methods of ageing both for the wandering stage of third instar larvae and for the pupae. This is achieved in chapters 2 and 3 for pupal ageing and in chapter 7 for ageing of the wandering stage. Crucial for the practical application of these methods is the relationship of developmental time and ambient temperature. Calculating time based on temperature requires a constant called the basal developmental temperature of the species. This constant has never been determined in a realist and physiological meaningful way. This is achieved in chapter 4. The method developed in chapter 2 relies on a structure or organ called the

yellow body. The biology of the yellow body in relation to midgut bacteria is investigated in chapter 5. In chapter 6, the bacteria identified in the midgut are compared to bacteria associated with the eggs of blow flies to explore whether or not any bacteria are permanently associated with blow flies or essential for blow flies.

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Mouth. Lilly the Fortune-Teller, to Coley the Almanack-Maker, in Baldwin's Garden. Tony Lee, to Cave Underhil. Harry Purcel to Dr. Blow. Mrs. Behn to the Famous Virgin Actress. Madam Creswell, to her Sister in Iniquity Moll. Quarles. Several between an Attorney and a Dead Person. And several others, with their Answers (new edition). Benj. Bragg, London.

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Chapter Two

Ageing pupae of the blow fly *Calliphora vicina* with the yellow body of the midgut for post mortem interval estimations

2.1 Abstract

Forensic entomology uses the age of immature stages of blow flies as a marker to estimate a minimum post-mortem interval (PMI_{min}) of corpses. While measuring the length of larvae is well established, puparia are lacking visible morphological changes other than colour, making estimates for older corpses particularly difficult, especially in cases where rearing of pupae to the adult stage is difficult or impossible, or facilities to perform instrumental analyses are not available. Here, as a proof of principle,morphological changes are described in 24 hour intervals in the internal anatomy of the pupal midgut that allow the ageing of pupae. Particular emphasis was given to the ease of recognising these changes without the need for highly specialized instrument facilities. Landmarks of the degeneration the larval midgut epithelium, the yellow body, correlate with the development and age of the pupa. This inexpensive method promises to extend the time period during which blow flies can provide forensically important data.

2.2 Introduction

Forensic entomology is the study of insects and their arthropod relatives as evidence in legal investigations (Smith, 1986; Catts and Goff, 1992; Goff, 2000; Benecke, 2001; Amendt *et al.*, 2004; Wyss and Cherix, 2006; Haskell and Williams, 2008; Byrd and Castner, 2009; Frost *et al.*, 2010; Goff, 2010; Amendt *et al.*, 2011; Gennard, 2012; Hall *et al.*, 2012; Rivers and Dahlem, 2014). Insects such as blow flies (Calliphoridae, Diptera), flesh flies (Sarcophagidae, Diptera) and beetles (Coleoptera) are mostly encountered in forensic work, however, other arthropods like mites (Acari) are increasingly recognised as valuable resources in cases where insects are absent or cannot provide sufficient information (Braig and Perotti, 2009; Perotti *et al.*, 2009; Perotti and Braig, 2010; Perotti *et al.*, 2010; Alasaad *et al.*, 2012; Gonzalez Medina *et al.*, 2013; Mašán *et al.*, 2013; Barton *et al.*, 2014). An estimation of the time since death or a minimum post-mortem interval (PMI_{min}) based on a minimum period of insect activity forms currently the main body of forensic or mediocriminal

entomology(Wells and LaMotte, 2009; Villet and Amendt, 2011; Michaud *et al.*, 2014; Wells, 2014). Blow flies are often the first insects to colonise a corpse or remains and the most dominant species group to arrive(Mégnin, 1894; Marchenko, 1988; Greenberg, 1991; Campobasso *et al.*, 2001; Fremdt and Amendt, 2014). Entomological estimates are commonly based on measuring the length, width or weight of the oldest larvae of blow flies as a marker for their age of development(Nishida, 1984; Reiter, 1984; Wells and LaMotte, 1995; Grassberger and Reiter, 2001; Day and Wallman, 2006). Blow flies will lay eggs, which hatch into first instar larvae, moult subsequently into second and third instar larvae, then leave the corpse, metamorphose into pupae and finally eclose as adults (imagines). Knowing the temperature and species, the age of larvae can be calculated to within a few hours, making PMI estimates for early stages of decomposition highly accurate(Villet *et al.*, 2009; Ieno *et al.*, 2010). For later stages of decomposition, problems arise. The pupal stage accounts for roughly 60 % of the whole developmental time of the blow fly *Calliphora vicina*, which is longer than all three larval instars together (Kamal, 1958; Greenberg and Kunich, 2002), yet pupae are difficult or impossible to stage or age with standard methods. However, morphological changes of dissected pupa have recently been identified providing six to nine developmental landmarks (Zajac and Amendt, 2012; Defilippo *et al.*, 2013). The outer cuticle or epicuticle of the final larval instar forms the puparium, which rapidly darkens and becomes completely sclerotized; it obstructs the observation of the external changes to the developing imago inside the pupa, and observation of the internal metamorphosis that involves the breakdown of larval tissues and organs including the consumption of fat and glycogen stores, the formation of a pupal cuticle and finally the formation of adult tissues and organs. The formation of the puparium, pupariation, starts many hours before the pupa starts to develop, which represents pupation (Fraenkel and Bhaskaran, 1973). The pigmentation or tanning of the puparium from white to dark brown progresses very fast, often in less than a day. The degree of tanning of the puparium has been measured as percentage of light reflectance for a flesh fly but there seems very little if any reference data available for any other flies (Sivasubramanian and Biagi, 1983). Greenberg and Kunich (2002) described a double

murder case where colour photographs of darkening pupae of the blow fly *Phaenicia sericata* were firstly and successfully entered as entomological testimony. For the subsequent 8 to 27 days of the pupal stage, depending on temperature (25 to 12 °C) (Greenberg and Kunich, 2002; Zajac and Amendt, 2012), no reliably changing characters are available on the surface of the puparium. The puparium is often referred to as a black box (Greenberg and Kunich, 2002). Best practice for pupae has been the collection of live specimens for rearing to adulthood in the laboratory, an option that is not always available or not always successful (Amendt *et al.*, 2007). The importance of PMI estimates at later stages of decomposition and the potential that the determination of the age of blow fly pupae promises, has recently spurred research into new methods. Very recently, studies engaged in gene expression analyses, histological sectioning methods for internal morphological analysis, gas chromatography methods to discriminate volatiles and 3D micro-computed tomography used for describing internal and external morphological changes (Tarone and Foran, 2011; Frederickx *et al.*, 2012; Pujol-Luz and Barros-Cordeiro, 2012; Richards *et al.*, 2012; Ubero-Pascal *et al.*, 2012; Boehme *et al.*, 2013; Davies and Harvey, 2013; Zhu *et al.*, 2013; Boehme *et al.*, 2014). These methods are mostly complex research techniques that either are quite time-consuming and labour-intensive and/or demand advanced instrumentation and intensive training, both of which are out of reach for routine forensic entomological work.

The proof of principle is presented for a method to age blow fly pupae that only requires a low-power stereo or dissecting microscope with a 10 x magnification. It takes advantage of the formation and changing features of the yellow body in the midgut section of the pupa. During metamorphosis, embryonic replacement cells migrate laterally between the basal lamina and the midgut epithelium of the larva forming the new digestive tube of the adult midgut. While the single layer of the adult epithelium forms, some replacement cells start to envelope the degenerating larval epithelium, which results in the formation of a yellow body in the lumen of the gut. The yellow body changes in colour and in size during the course of the pupation. The yellow body is easy to recognise and of great length. In blow flies it has first been

described in detail for*Calliphora vicina* Robineau-Desvoidy 1830 under its synonym *C. erythrocephala* (Pérez, 1910; de Priester, 1972).*C. vicina* is a model organism with one of the longest pupal periods of forensically important flies and we propose the yellow body as a marker for the ageing of blow fly pupae.

2.3 Material and Methods

2.3.1 Laboratory culture

An established *Calliphora vicina* colony was used for the experiments. The identity of the flies was confirmed with the key by Erzinçlioğlu (1996). The blow flies were reared in screened breeding boxes (44 cm x 44 cm x 44 cm) in a temperature control room. Larvae were fed on pig liver, adults on a sucrose solution. Eggs were collected with the help of 5 g pieces of pig liver placed on petri dishes placed in a box for 24 hours. The importance of using fresh liver has been recognized (Niederegger *et al.*, 2013; Richards *et al.*, 2013). The food supply was constantly monitored to prevent premature wandering of third instar larvae. When the third instar larvae started to moult, they were transferred to a new recipient inside the breeding box. Fine wood chips and sawdust were offered as a substratum for wandering larvae. Temperature was maintained at 22.3 ± 1 ^{o}C, relative humidity of 93 ± 7 %, with a light cycle of 12 hours. The experiment was repeated at 15.8 ± 1 ˚C.

2.3.2 Sampling of pupae

Wandering 3rd instar larvae were monitored every 24 hours and when movement became sluggish, the wandering larvae were separated from the large population pool. This was to enable an almost equal age of the pupae. After a further 24 hours, the separated wandering larvae were checked and any larvae yet to initiate pupariation were removed from that container, leaving only the pupae of almost equal age. Then five specimens were picked out for dissection for each time point. This process of pupae collection for dissection continued until adult blow fliers started to emerge. The experiments were repeated four times. Pictures of the yellow body were taking,

coded, and scoring of the yellow body stage done blindly by seventeen volunteers following proposal of blind evaluation by Tarone and Foran (2011) and Boehme *et al.* (2014).

2.3.3 Dissection of pupae

Each living pupa was placed in a small petri dish containing phosphate-buffered saline (PBS) for dissection. The puparium was initially held with a pair of forceps and an incision was made at the constriction at the anterior first quarter with a pair of micro-scissors (Vannas spring scissors, FST Fine Science Tools, InterFocus, Cambridge, UK) and then dissection continued with a pair of sharp dissection needles (FST Fine Science Tools) under a dissecting microscope (Leica Zoom 2000) at a magnification of 10.5 x. The yellow body was found inside the lumen of the gut. Pictures were taken with a Panasonic HX-DC2, 15 x zoom camera.

2.3.3 Accumulated degree days and statistical analysis

Accumulated degree days (ADD) [°D] were calculated with a lover development threshold of 1.6 °C for larval development and 5.1 °C for pupal development(Michaud and Moreau, 2011). For ease of comparison, the standard literature value of 2 °C for *Calliphora vicina* according to Marchenko (1988); (Marchenko, 2001) has also been calculated. The lower developmental threshold values were experimentally determined for the larval and pupal stages separately by growing flies at 11.8, 15.8 and 22.3 °C and calculating the temperature with zero development by linear regression: $y = 0.0048 x - 0.0076$, $R^2 = 0.9972$, for the larval developmental stages only and $y = 0.0053 x - 0.027$, $R^2 = 0.9995$, for the pupal developmental stage only. This threshold temperature refers to the development from egg to eclosion of adults.

2.3.4 Statistics

Mircosoft Excel 2010, IBM SPSS Statistic version 22 and R for statistical analyses were used for statistical analysis.

A total of 17 volunteers were given a training data set consisting of 11 pictures showing yellow body development in increments of 20 °D. Then these volunteers were given a test data set consisting of 27 pictures of yellow bodies of random age and in random order. The order of picture was different for every volunteer. All pictures were randomly coded. The coded pictures were chosen from both temperature schemes and had the following time points: 220, 220, 232, 240, 244, 255, 260, 267, 279, 280, 291, 300, 302, 314, 320, 326, 338, 340, 349, 360, 361, 379, 380, 384, 396, 400, 408, 420, 420 °D. The time points were initially calculated with a literature-derived overall developmental threshold temperature of 2 °C. The volunteers were asked to estimate the age of the yellow body in each of the 27 pictures of the test set based on the training set on a continuous scale from 220 to 420 °D.

The results were analysed with a TwoStep Cluster analysis as implemented in SPSS version 22. The estimates for each test picture were treated as 27 continuous variables and standardised. Outliers were recognized as being over a 25 % noise level. The distances between clusters were estimated based on log-likelihood. Schwarz's Bayesian Criterion was applied for cluster analysis. A fixed number of 1 to 11 clusters were analysed. For each cluster analysis, the cluster quality was evaluated as a silhouette measure of cohesion and separation based on the method of Kaufman and Rousseeuw.

2.4 Results

The puparium of *Calliphora vicina* changes from white to a bright orange-read in less than six hours, it then gradually darkens over the next 24 hours. During the next 10 days, the dark red changes over a dark brown to an almost black colour. After the initial reddening, the colour changes are subtle, surreptitious and very difficult to categorise, Figure 2.1. The puparia of Figure 2.1 are exactly from the same time points as the yellow bodies shown in Figure 2.2

During day one of metamorphosis, the yellow body represents a closed, translucent tube filled with a pale yellow gel-like substance. A small white body is just forming inside the tube.

At day two, the tube shortens to a barrel form and the white body inside is at its greatest length and volume. Often, the white body is off centre. The tube is very fragile.

At day three, the tube has become more robust and is at its shortest size with the white body at its centre. The yellow substance is at its brightest.

At day four, the tube starts expanding unilaterally, which increasingly puts the white body at a centric position.

At day five, the unilateral extension has doubled in size and is now longer than the section harbouring the white body. The yellow substance starts to darken. A small, fragile, transparent and colourless tube becomes apparent on the other side of the white body; it is breaks off during dissection.

At day six, the transparent and colourless tube becomes tougher and increases in length until it is the size of the white body section. The white body becomes gradually smaller.

At day seven, the yellow substance has further darkened. The transparent and colourless tube has doubled in length.

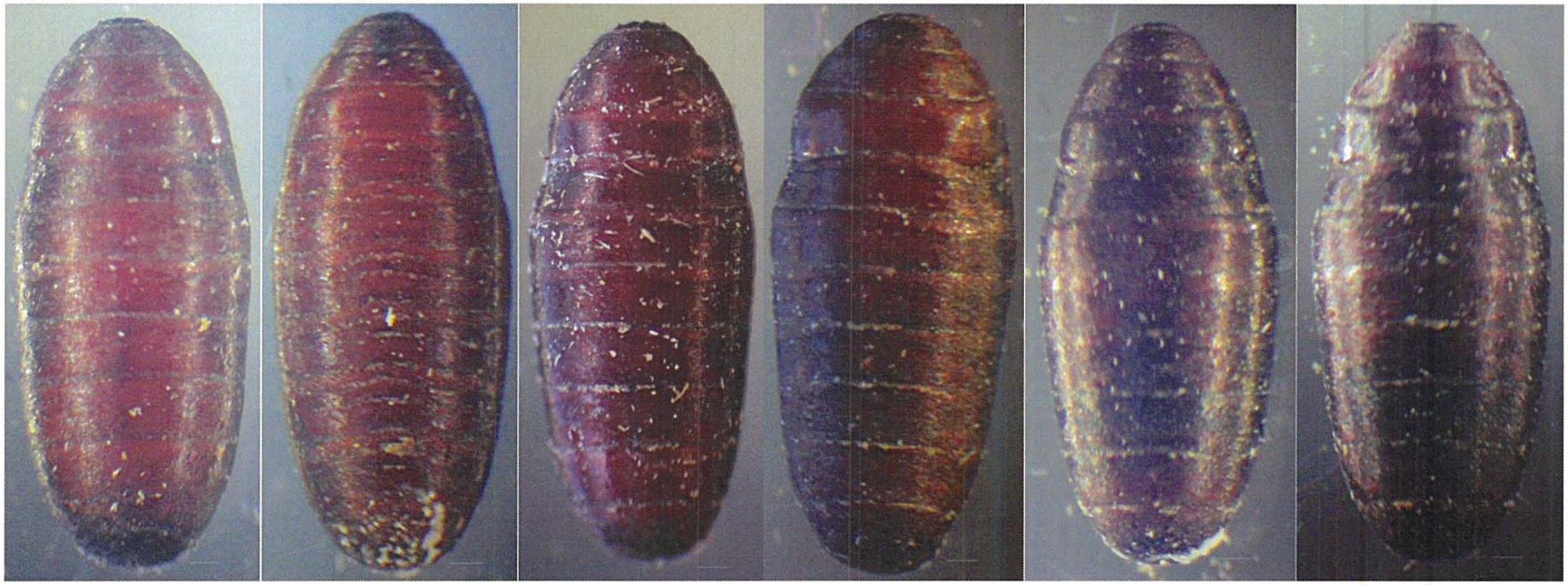
At day eight, the yellow substance is now a dark brown. The white body starts to fragment.

At day nine, the yellow substance starts to become black. The white body has now disintegrated.

At day ten, the white body has disappeared and only yellow substance is left at its former position.

At day eleven, all remaining yellow substance at the position of the former white body has turned black as well. The black substance contracts, leaving behind transparent and colourless tube sections. The tube itself starts to break apart.

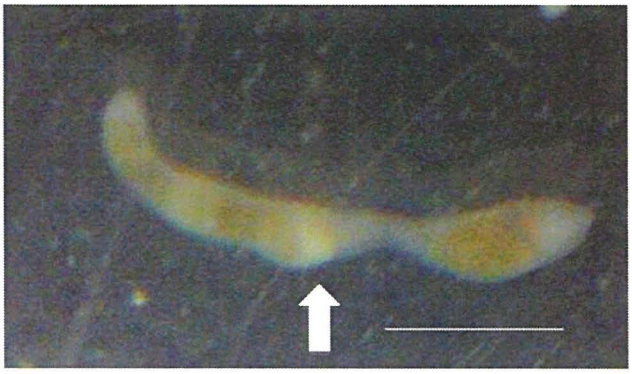
day 1, 224 (220) ˚D day 2, 241 (240) ˚D day 3, 259(260) ˚D day 4, 276 (280) ˚D day 5, 293 (300) ˚D



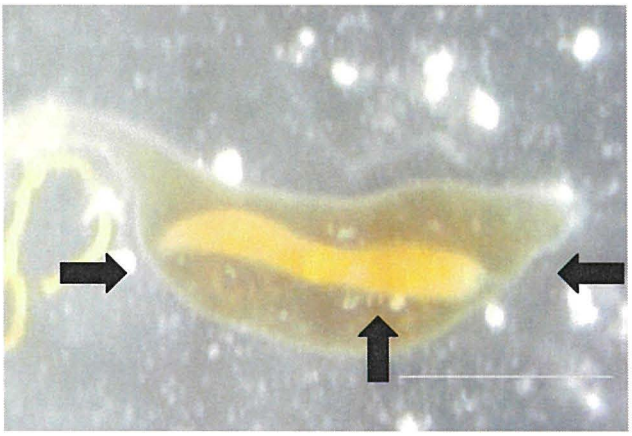
day 6, 310 (320) ˚D day 7, 327(340) ˚D day 8, 345(360) ˚D day 9, 362(380) ˚D day 10, 379(400) ˚D day 11, 396(420) ˚D

Figure 2.1: Colour changes of puparia of *C. vicina* during the course of metamorphosis. Age expressed as day of pupal stage and as accumulated degree days (ADD) fora temperature of 22.3 ˚C and mixed developmental threshold temperatures (in parentheses: ADD based on a lower developmental threshold (LDT) of 2 ˚C).

day 1, 224(220) ˚D



day 2, 241(240) ˚D



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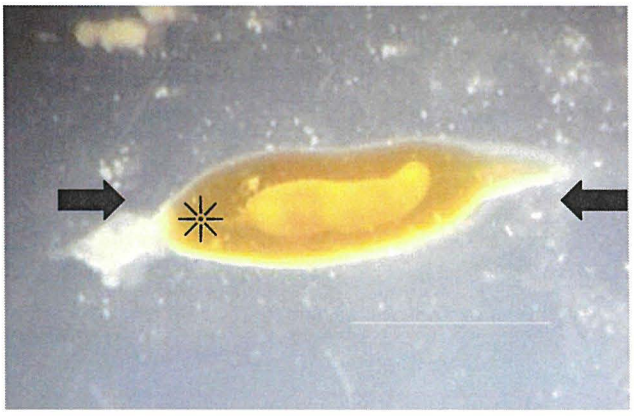

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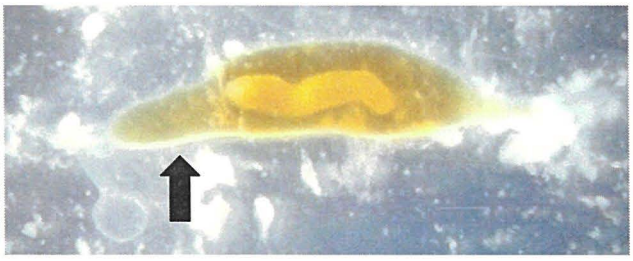

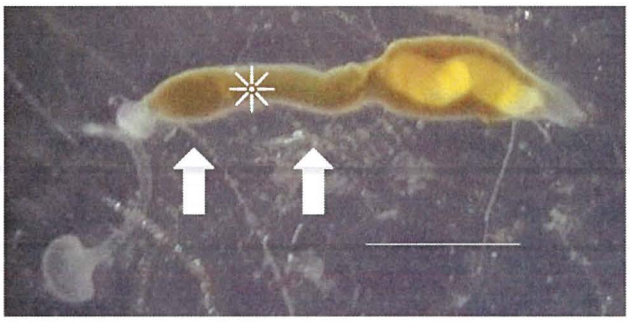

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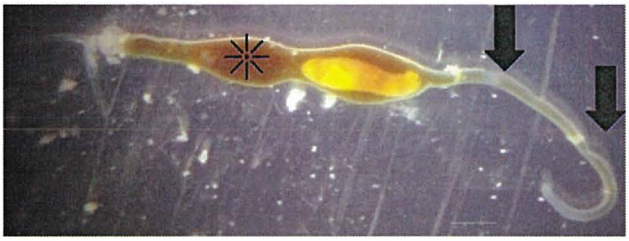
day 6, 310(320) ˚D



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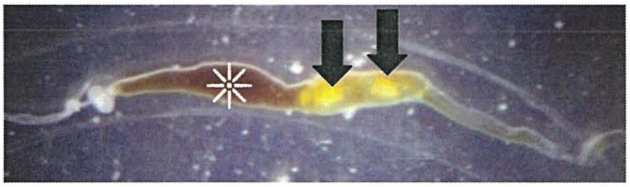

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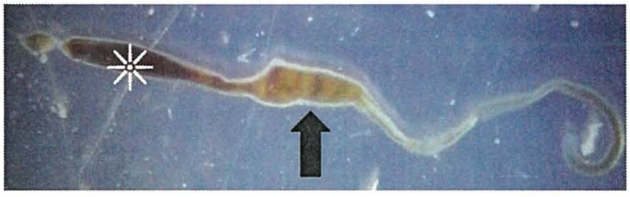

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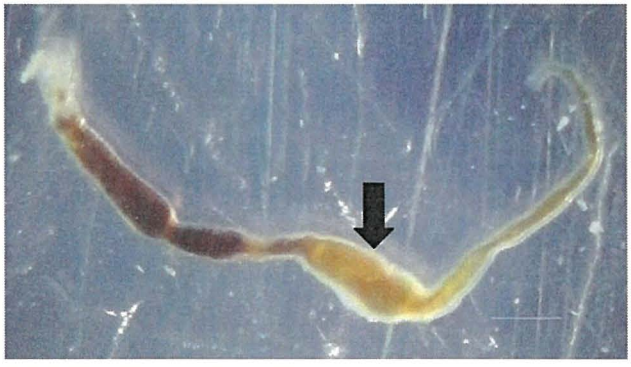

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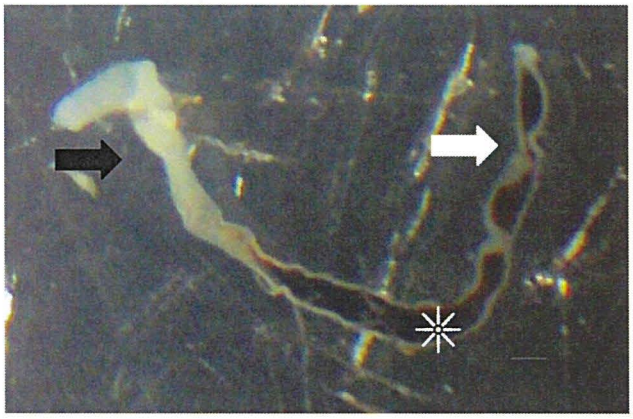

day 11, 396(420) ˚D

Figure 2.2: Yellow body dissected from pupae of *C. vicina* during the course of metamorphosis; the corresponding puparia are depicted in Figure 2.1. Age expressed as day of pupal stage and as accumulated degree days (ADD)for a temperature of 22.3 ˚C and mixed developmental threshold temperatures (in parentheses: ADD based on a LDT of 2 ˚C). Arrows refer to important changes explained in the text, stars indicate marked changes in colour. Scale bar is 1 mm.

Cluster analysis confirmed that seven different clusters or time points can reproducible be retrieved by volunteers in the ageing of pupae.

Cluster Distribution

		N	% of Combined	% of Total
Cluster	1	5	29.4%	14.7%
	2	2	11.8%	5.9%
	3	1	5.9%	2.9%
	4	3	17.6%	8.8%
	5	4	23.5%	11.8%
	6	1	5.9%	2.9%
	7	1	5.9%	2.9%
	Combined	17	100.0%	50.0%
Excluded Cases		17		50.0%
Total		34		100.0%

Cluster membership and profiles

		P220		P232		P240		P244		P255		P260	
		Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
Cluster	1	220.00	.000	220.00	.000	242.00	10.954	224.00	8.944	252.00	10.954	260.00	.000
	2	220.00	.000	220.00	.000	240.00	.000	250.00	.000	240.00	.000	260.00	.000
	3	220.00	.	220.00	.	260.00	.	240.00	.	260.00	.	280.00	.
	4	230.00	10.000	220.00	.000	260.00	.000	246.67	11.547	253.33	11.547	246.67	5.774
	5	220.00	.000	236.25	4.787	240.00	.000	241.25	2.500	256.25	4.787	260.00	.000
	6	220.00	.	220.00	.	360.00	.	340.00	.	240.00	.	260.00	.
	7	220.00	.	220.00	.	220.00	.	240.00	.	260.00	.	240.00	.
	Combined	221.76	5.286	223.82	7.401	251.18	30.390	243.82	27.359	252.06	9.529	257.65	9.034

P267		P279		P280		P291		P300		P302		P314	
Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
256.00	8.944	260.00	.000	280.00	.000	280.00	.000	320.00	.000	300.00	28.284	328.00	17.889
260.00	.000	279.00	.000	280.00	.000	280.00	.000	360.00	.000	265.00	.000	315.00	.000
260.00	.	270.00	.	290.00	.	240.00	.	360.00	.	280.00	.	350.00	.
260.00	.000	273.33	5.774	286.67	5.774	273.33	11.547	313.33	11.547	340.00	.000	340.00	17.321
265.00	5.774	280.00	.000	280.00	.000	292.50	5.000	300.00	.000	300.00	.000	313.75	4.787
280.00	.	260.00	.	240.00	.	320.00	.	260.00	.	340.00	.	240.00	.
240.00	.	240.00	.	260.00	.	260.00	.	260.00	.	300.00	.	280.00	.
260.00	9.354	268.71	11.548	278.24	11.851	280.59	17.128	314.12	28.952	304.12	27.570	318.53	27.994

P320		P326		P338		P340		P349		P360		P361	
Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
324.00	8.944	340.00	.000	320.00	14.142	320.00	.000	336.00	8.944	364.00	8.944	392.00	10.954
310.00	.000	340.00	.000	340.00	.000	340.00	.000	350.00	.000	400.00	.000	412.50	3.536
380.00	.	340.00	.	300.00	.	390.00	.	340.00	.	360.00	.	380.00	.
320.00	.000	340.00	.000	360.00	.000	366.67	40.415	353.33	5.774	386.67	11.547	353.33	11.547
320.00	.000	326.25	4.787	340.00	.000	340.00	.000	347.50	5.000	360.00	.000	360.00	.000
302.00	.	320.00	.	300.00	.	220.00	.	340.00	.	340.00	.	400.00	.
360.00	.	300.00	.	320.00	.	320.00	.	320.00	.	360.00	.	400.00	.
324.82	18.922	333.24	11.311	331.76	20.073	333.53	38.720	342.94	10.467	369.41	17.489	380.29	22.533

P380		P384		P396		P400		P408		P420	
Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
402.00	24.900	400.00	.000	400.00	.000	396.00	21.909	420.00	.000	420.00	.000
420.00	.000	410.00	.000	370.00	.000	400.00	.000	430.00	.000	420.00	.000
400.00	.	320.00	.	400.00	.	380.00	.	420.00	.	420.00	.
400.00	.000	386.67	11.547	393.33	5.774	393.33	23.094	420.00	.000	420.00	.000
380.00	.000	381.25	2.500	400.00	.000	400.00	.000	407.50	5.000	421.25	2.500
400.00	.	400.00	.	400.00	.	360.00	.	420.00	.	420.00	.
390.00	.	380.00	.	380.00	.	380.00	.	380.00	.	420.00	.
397.65	17.511	388.53	20.899	394.12	10.641	392.94	17.235	415.88	11.757	420.29	1.213

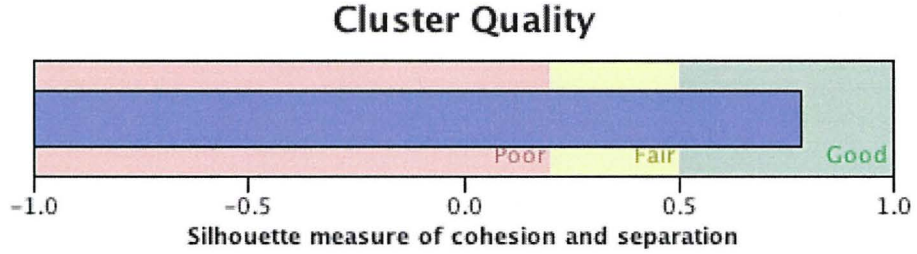

Figure 2.3: Results of statistical analysis using iterated TwoStep Cluster Analyses, testing the ability of volunteers to assign pictures of yellow bodies to clusters of time points of pupal age, showing here the results for dividing pupal development in seven clusters.

2.5 Discussion

The pupal stage covers the metamorphosis from the larval stage to the adult stage. It comprises two transitions with two manifestations of the pupa.

The first transition is the larval-pupal apolysis that creates a cryptocephalic pupa inside the larval puparium; the head structures of the pupa are not yet recognisable. The larval stage in the larval-pupal apolysis is called a prepupa. The moment a wandering larva settles down to become sedentary, the prepupal stage begins. The processes and chances during the prepupal period constitute pupariation. Already during the prepupal stage, the lining of the foregut (stomadeum) and hindgut (proctodeum) plus rectumare extruded; they get stuck to the inside of the puparium at either end. At the same time, in the midgut (mesenteron) nidi of progenitor cells at the basal side of larval epithelium near the basal lamina take up replication. With the larval-pupal aploysis,pupation starts and separates the pupa from the puparial wall of the larva so that it is free inside the puparium. This separation starts at the anterior end or head region. Continuous changes in the cryptocephalic pupa eventually lead to a phanerocephalic pupa, where the head structures have become evident in a process of evagination. The transformation between cryptocephalic and phanerocephalic is an uninterrupted process. The nidi of progenitor cells in the midgut are made up of two different cell types. The peripheral cells first proliferate into a monolayer of cells under the larval epithelial layer forming a tube that takes up the midgut of the larva into its lumen. The larval midgut is highly contracted and folds suggest the formation of pseudo compartments. The tube formed by the peripheral progenitor cells is actually a pupal midgut epithelium(Takashima *et al.*, 2011). In most dipteran species that have been studied, including *C. vicina* and *D. melanogaster*, larval and pupal epithelia remain attached to each other and establish together the sac structure of the yellow body. With little delay, the central cells in the nests of progenitor cells form a second, monolayer tube that will become the adult midgut epithelium with in it the now free-swimming yellow body. The larval intestinal cells in the yellow body start to degenerate through histolysis caused by the pupal epithelium(Andries, 1976). The epithelial cells of the larval midgut degenerate at a fraction of the speed at which the

epithelial cells of the epidermis do, but the mechanisms are similar. In *C. vicina*, parts of the microvilli of the larval epithelium are still visible eight days after pupation at 25˚C(Bautz, 1979). Eventually the pupal epithelium will self-digest as well.

In flesh fly species such as *Parasacrophaga ruficornis*, the formation of the pupal and adult midgut epithelium are more separated in time. In *P. ruficornis*, the degenerating larval midgut epithelium forms an amorphous yellow body during the prepupal stage while a pupal epithelium forms. Ten hours into the pupal stage, the pupal epithelium started to degenerate starting from the posterior site and progressed until the time of head eversion. The pupal epithelium became part of the yellow body and the imaginal epithelium formed (Singh and Srivastava, 1980). The process of the yellow body degeneration is proposed as a measure for the age of the pupa.

The phanerocephalic pupa eventually undergoes a second transition, the pupal-adult apolysis that generates a pharate adult inside the puparium. Separation between pupa and adult starts at the posterior end or anal region. For most of the pupal period, the pupa is at the stage of the pharate adult. Finally, the imago ecloses from the puparium. The remnants of the yellow body leave the adult fly as the meconium (Romoser *et al.*, 2000; Klowden, 2013). The two transformations are moulting processes that are governed by moulding hormones, ecdysteroids.

Gaudry *et al.* (2006) explored the possibility of determining the age of pupae by measuring ecdysteroid levels in *Protophormia terraenovae* pupae with an enzyme immunoassay and high-pressure liquid chromatography. The assay can only indicate a post-pupariation period of between 2 and 3 days. Analysing the cuticular hydrocarbons of puparia and pupae was proposed by Drijfhout (2010). Hydrocarbon profiles depend on environmental factors as diet, temperature and microorganism associated with the insect. The possibility of staging puparial hydrocarbons has not yet been taken up, but it has recently been used to estimate the age of *Lucilia sericata* larvae (Moore *et al.*, 2013). However, the changes of hydrocarbons and cuticular lipids during weathering of empty puparia over longer time periods of 90 days to several years has been explored. Experiments on the larval predatory *Hydrotaea aenescens* (American black dump fly, Muscidae) in France and of the blowfly *Chrysomya*

megacephala in China show lipids and hydrocarbons as new indices for the estimation of post-mortem intervals (Zhu *et al.*, 2013; Frere *et al.*, 2014). Both experiments require extensive instrumentation in the form of gas chromatography linked with mass spectroscopy.

Changes in the profile of volatile organic compounds (VOCs) emitted by pupae of *Calliphora vicina* were monitored with gas chromatography coupled to mass spectrometry (Frederickx *et al.*, 2012). Twenty of the VOCs released by pupae were also identified as cadaveric VOCs, among which ten might constitute markers for the ageing of pupae. The influence of environmental conditions on the composition of the pupal VOCs was seen as a potential problem in the sense that the environmental signal might be greater than the genetic signal of the developmental stage of the pupa. Early attempts to identify differentially expressed genes (DEGs) during pupal development of *Calliphora vicina*(Ames *et al.*, 2006) and *Lucilia sericata*(Mösch, 2005; Tarone *et al.*, 2007) were unsuccessful. Recently, a study on pupae of *L. sericata* showed that when data on nine DEGs (*acetylcholine esterase, chitin synthase, ecdysone receptor, heat shock protein 60* and *90, resistance to organophosphate 1, ultraspiracle, white, slalom*) were added to a generalized additive model of blow fly development and traditional morphological data, only a 3 - 8 % decrease in age overestimation was achieved when evaluated blindly (Tarone and Foran, 2011). The possible effect of a fluctuating environment on the DEGs has to be studied in more detail. Boehme *et al.* (2013) have identified four DEGs in *C. vicina* pupae (*15_2, 2014192, actin, arylphorin receptor*). The expression profile versus pupal age of three of the four genes varied strongly with temperature. Temperature is not the only environmental factor having a profound impact on DEGs. Boehme *et al.* (2014) have shown in three blind experiments that levels of mRNA of these four DEGs can successfully been used in inverse predictions of estimated pupal age.

The pupal stage is characterized by the most extensive changes to the internal morphology of the organ systems, larval organs and structures and partially replaced by pupal structures, which themselves are replaced by adult tissues. This suggests that a histological analysis of the internal anatomy would provide the highest possible

resolution for ageing pupae (Davies and Harvey, 2013). Zajac and Amendt (2012) present such a histological analysis of pupae of *C. vicina* and *Lucilia sericata* in steps of one day or roughly 20 °D. Drawbacks of a histological approach are several. To obtain high quality sections, the pupae should be fixed and/or cleared for at least one week, then dehydrated, embedded, sectioned, stained, and mounted. The interpretation of the slides is complex and requires knowledge of insect histology. Standard sectioning equipment has problems with cutting highly sclerotized tissues of the pupa and standard embedding media such as paraffin are incompatible with the fat body (Davies and Harvey, 2013). This leaves histological techniques as labour- and time-intensive, requiring better than standard equipment and advanced knowledge for the interpretation of the results. All of these problems of sectioning pupa can be overcome by 3D micro-computed tomography. This technique has been applied to pupae of *C. vicina* by (Richards *et al.*, 2012). The results are fascinating from an academic point of view but the costs of a micro-CT scanner is out of reach of many University departments and the instrument requires specialized software. The requirement for staining the samples remains, and this can easily take a week. Compared with classical light microscopy histology, not all insect tissues are visible in CT scans. Increasing financial pressure on many institutions might spur a demand for more economical methods.

Pupae don't change in length but they do change in weight. Pupae of *C. vicina* loose on average 21 % and pupae of *L. sericata* loose roughly 16 % of their weight in a near linear mode (Zajac and Amendt, 2012). However, the absolute weight of blow fly pupae varies by a similar magnitude depending on competition for food resources during the larval stages under natural conditions. The time-dependent degeneration of the yellow body of the midgut might be an easy and fast to score indicator for the age of blow fly pupae. The yellow body should be formed, in principal, in all holometabolous insects, all insects that have a pupal stage (Snodgrass, 1954). The yellow body was originally discovered by August Weissmann in the common flesh fly, *Sarcophaga carnaria* (Diptera:Sacrophagidae) and in the bluebottle *Calliphora vomitoria* (Diptera: Calliphoridae), he named the structures in German gelbe Körper

(yellow body) (Weissmann, 1864). In hindsight, features observed by Swammerdam and Dufour can be interpreted as yellow bodies as well. The physiological origin of the yellow body has first been realized by Ganin(Ganin, 1876; Kowalevsky, 1887). Table1.1 lists some of the species for which a yellow body have been described. The yellow body of the midgut should not be confused with the yellow body of the ovaries, which is connected with the autolysis of the follicular epithelium of the ovaries of adult muscid flies at each gonotrophic cycle(Bhide and Sahai, 1986). In some insects such as the onion maggots, *Delia antiqua*(Diptera: Anthomyiidae), the yellow body in the midgut of the pupa is visible without dissection, when the puparium is removed (Li *et al.*, 2012).

Yellow body formation during pupation

Lepidoptera			
Bombyx mori	silk worm	Bombycidae	(Casagrande, 1887)
Galleria mellonella	greater wax moth	Pyralidae	(Uwo *et al.*, 2002; Khoa *et al.*)
Malacosoma castrense	ground lackey	Lasiocampidae	(Deegener, 1908)
Pieris brassicae	large white	Pieridae	(Henson, 1929)
Trichoptera			
Plectrocnemia conspersa	caddisflies	Polycentropodidae	(Lübben, 1907)
Coleoptera			
Cybister roseli	diving beetle	Dytiscidae	(Deegener, 1904)
Dytiscus marginalis	great diving beetle	Dytiscidae	(Korschelt, 1924)
Ptinus ocellus	spider beetle	Anobiidae	(Mansour, 1927)
Stegobium paniceum	drugstore beetle	Anobiidae	(Karawaiew, 1899)
Tenebrio molitor	mealworm beetle	Tenebrionidae	(Rengel, 1897)
Diptera			
Calliphora vicina	urban bluebottle	Calliphoridae	(Lowne, 1890-1892, 1893-1895; Pérez, 1910)
C. vomitoria	bluebottle		(Weissmann, 1864; Kowalevsky, 1887; van Rees, 1889)
Delia antiqua	onion maggot	Anthomyiidae	(Li *et al.*, 2012)
Parasacrophaga ruficornis	flesh fly	Sacrophagidae	(Singh and Srivastava, 1980)

Sarcophaga carnaria	common flesh fly	Sacrophagidae	(Weissmann, 1864)
S. haemorrhoidalis	red-tailed flesh fly		(Dufour, 1846)
S. peregrina	flesh fly		(Nakajima *et al.*, 1997; Price *et al.*, 1999)
Stratiomys chamaeleon	clubbed general	Stratiomyidae	(Swammerdam, 1737-1738)
Drosophila melanogaster	vinegar fly	Drosophilidae	(Skaer, 1993; Takashima *et al.*, 2011)

Table 2.1

Wigglesworth (1972) reported the formation of yellow bodies for the spurge hawk-moth, *Hyles euphorbiae*, citing Deegener (1909), and the Japanese beetle, *Popillia japonica*, citing Ludwig and Abercrombie (1936),but this could not be corroborated; only larval moulds had been studied.

There is some heterogeneity in the formation of the yellow body within orders, likely constituting derived features. In Coleoptera, the yellow body of weevils (Curculionidae) like *Sitophilus oryzae* and *Anthonomous pomorum* is more of a homogenous gel than a discrete body; it contains no cellular remains of the larval midgut epithelium. An advancing midgut tube has pushed the larval cells into the hindgut before the yellow mass forms (Mansour, 1927). Another derived feature can be seen in mosquitoes (Diptera: Culicidae). In species such as *Anopheles punctipennis*, *Culex pipiens* and *Aedes aegypti*, a meconial peritrophic membrane or matrix (MPM1) surrounds the larval midgut epithelium instead of a pupal epithelium during yellow body formation. In some individuals of all three species, a second meconial peritrophic membrane or matrix (MPM2) forms around the degenerating yellow body around the time of adult emergence (Romoser *et al.*, 2000; Moll *et al.*, 2001). The meconial yellow body is used to differential teneral and nulliparous from non-teneral and parous female mosquitoes (Hugo *et al.*, 2008). The yellow body meconium remains in adult mosquitoes for up to 49 h after emergence (Rosay, 1961).

C. vicina larvae reach their greatest length at the moment they stop eating. Feeding X-ray contrast medium to third instar larvae, Reiter and Hajek (1984) could show that

the midgut is emptied immediately after the larvae reach their maximum length. At that stage, the midgut of *C. vicina* has a length of around 30 mm. After emptying, the midgut start contracting during the wandering stage. At the prepupal stage when the yellow body forms, the larval midgut has almost reached its adult length of only 4 mm. This process varies markedly between individuals. This is one of the reasons why otherwise attractive length measurements were not considered here as marker for pupal ageing. The yellow body first contracts slightly before it lengthens during disintegration of the larval epithelium. For these reasons, only qualitative landmarks are proposed for the ageing of blow fly pupae.

The proposed method achieves a resolution in the ageing of *C. vicina* pupa comparable with the methods proposed by Zajac and Amendt (2012) and Defilippo *et al.* (2013). While the aforementioned methods score morphological changes on the outside after removing the puparium, the proposed method relies on internal changes, making it an independent method that can underpin or confirm age estimates based on external features.

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Chapter Three

Aging pupae of the blow fly *Calliphora vicina* based on external morphology of developing imagines for post mortem interval estimations

3.1 Abstract

The importance of forensic entomology to convicting crime offenders, especially those linked to murder, cannot be over emphasised. Forensic entomology deals with the estimation of the time of death of corpses, using the age of immature stages of blow flies as a marker. The ageing of the larval stage is well established, whereas ageing the pupal stage poses some difficulties. The quest for getting a more reliable and more cost effective marker for ageing the pupal stage is the aim of this study. This study involved the removal of the outer puparia of the pupae of the blow fly *Calliphora vicina* reared at three different temperature regimes, 22.3 ± 1.0 ^{o}C, 15.8 ± 0.7 ^{o}C and 11.8 ± 0.3 ^{o}C. The developmental features of the maturing pharate imagines inside the pupae for each day were noted and allocated points. The developmental landmarks fit a regression line that at a temperature of 22.3 ± 1.0 ^{o}C has an R^2 = 0.9647 and p-value: 7.612 x e^{-08} while rearing temperatures of 15.8 ± 0.7 ^{o}C and 11.8 ± 0.3 ^{o}C shared the same R^2 = 0.9799 and p-value of < 2.2 e^{-16}. The developing features of the imagines shown to have the potential to estimate the age of blow fly *Calliphora vicina* with high degree of accuracy and reliability. And this is a technique that can be carried out in any forensic entomology laboratory.

3.2 Introduction

Insects found on dead bodies are very important to forensic entomologists, where the age of the immature stages are used for estimation of the minimum post mortem interval (PMI_{min}). Insects are known to colonize corpses both indoors and outdoors but how fast they do this is dependent on the ambient temperature of the surrounding where the dead body is located. Flies are the first colonizers of corpse(s) and these flies are often blow flies of the family Calliphoridae with species of the genera*Calliphora*, *Protophormia* and *Lucilia* being most prominent. The Calliphora species are found mainly in temperate regions and two of the most important species,*Calliphora vicina*Robineau-Desvoidyand *Calliphora vomitoria* (Linnaeus), are found throughout Europeand America (Lane, 1975; Donovan *et al.*, 2006; Gomes *et al.*,

2006; Pohjoismäki *et al.*, 2010). The age of immature stages of the blow fly has been used as a marker for estimation of minimum PMI of a corpse, in as much as the estimation of the age of larvae is well established by measuring length, width or weight. Ageing the pupal stage is still problematic for quantitation as the resolutiontypical for the larval investigationscannot be obtained with only the colour changes of the pupaein routine forensic entomology applications(Goffeau *et al.*, 1996; Amendt *et al.*, 2000; Greenberg and Kunich, 2002; Byrd and Castner, 2009).

As the blow flies are cold blooded, the development of their immature stages is dependent on ambient temperature. The age of a specimen can be deduced by summing the thermal input they accumulated during growth (Sharpe and DeMichele, 1977; Greenberg and Kunich, 2002; Byrd and Castner, 2009; Goff, 2010). The standard method used for estimation of the rate of development of immature stages of insects over a period of time with temperature compensation is called accumulated degree hours (ADH) or accumulated degree days (ADD). It puts into consideration the lower development threshold (LDT), which is the temperature value, below which an insect's development stops. This is specific to species and can vary between life stages (Defilippo *et al.*, 2013). Blow flies also show geographical differentiation. Life history traits of *C. vicina* can even differ slightly at the small scale of urban versus rural populations (Hwang and Turner, 2009). On a bigger scale of latitudes, geographical variation is seen in *C. vicina* for incidence and duration of larval diapause (Chernysh *et al.*, 1995; McWatters and Saunders, 1998; Saunders, 2000). The photoperiod has a major impact on the mother regarding larval diapause, although eggs and larvae themselves are photosensitive (Vaz Nunes and Saunders, 1989).

The pupal stage of the blow fly takes about 60% of the total life cycle of the immature stages of development, which also comprise the eggs and three instars of the larvae. How long it takes the pupa to emerge as an adult is dependent on the temperature of rearing the flies. This stage of development of the blow fly has a unique feature that is the initially translucent nature of the puparia covering the pharate or maturing imagine. This is the longest immature stage and a stage in-between two active feeding stages (Kamal, 1958; Davies and Harvey, 2013). Although

this is a motionless stage of development of the blow fly, a lot of activities are going on within it, which include vast external and internal developmental changes. Internal changes are carried by processes like histolysis, histogenesis and several other biochemical and cellular pathways required to provide all the energy and substrate for the development of the pupa to an adult blowfly(Fraenkel and Bhaskaran, 1973).

Regarding the pupa of blow flies, several different approaches have been made to age this part of the life cycle to improve estimates of minimal post mortem interval (PMI_{min}). Most recently, studies involved gene expression analyses, histological sectioning methods for internal morphological analysis, removal of the puparia to expose external morphological features, gas chromatographic techniques to discriminate volatiles emitted and 3D micro-computed tomography describing external and internal morphological changes (Tarone and Foran, 2011; Frederickx *et al.*, 2012; Pujol-Luz and Barros-Cordeiro, 2012; Richards *et al.*, 2012; Ubero-Pascal *et al.*, 2012; Zajac and Amendt, 2012; Boehme *et al.*, 2013; Davies and Harvey, 2013; Defilippo *et al.*, 2013).

An approach that requires the removal of the puparium and allocates points to visible developing features of the pharate developing imago of the pupa at three temperature regimes is presented here. The aim of this study is to develop a template that could be used for the estimation of PMI_{min}.

3.3 Material and Methods

3.3.1 Laboratory culture

Eggs of *Calliphora vicina* (Diptera: Calliphoridae) Robineau-Desvoidy 1830 were collected with the help of pieces of pork liver in Bangor, Gwynedd, Wales, United Kingdom, latitude 53° 13' 040.8" North and longitude 4° 07' 040.8" West, and used to establish a permanent colony at the School of Biological Sciences of Bangor University. Flies of this colony were used for this study. The identity of the blow flies was confirmed with the help of the key of Erzinçlioğlu (1996). The blow flies were reared in screened breeding boxes (44 cm x 44 cm x 44 cm) in a temperature control room. Eggs

were collected with the aid of pieces of pig liver placed on a petri dish placed in the box where the blow flies were for 24 hours. The petri dish with pig liver having the blow fly eggs was then transferred into a rectangular basin with fine sawdust, which offered as a substratum for wandering larvae. The pig liver supply was monitored constantly to prevent insufficient food, which might lead to premature wandering of the third instar larvae. This experiment was carried out at three different temperatures of 22.3 ± 1 ^{o}C, 15.8 ± 0.7 ^{o}C, 11.8 ± 0.3 ^{o}C and relative humidity levels between 45 and 65%, between 87 and 99%, between 77 and 99%, respectively. The light cycle was set 12 hours light and 12 hours darkness.

3.3.2 Sampling of pupae

The active 3rd instar larvae were observed every 24 hours and the observation continued until the wandering 3rd instar larvae movement became very slow. Then those larvae were separated from the rest of the population of larvae, to enable obtaining an almost equal age of the larvae. After 24 hours, those wandering 3rd instar larvae that had initiated pupariation, were removed from that container into another container to have an almost equal age pupae. Then five specimens were picked out for dissection after 24 hours. This process of pupae collection for dissection continued until adult blow flies started to emerge. The experiment was repeated two times to give a total of three experiments.

3.3.3 Dissection of pupae

For the preparatory stages for puparium removal, the methods of Zajac and Amendt (2012)was used with modification. The collected pupae were placed in a 1.5 ml test tube with tap water three quarter filled. It was then incubated at 70 ^{o}C for three hours in a heating block (Techne, DRI-Block DB.2A, England). The pupae were removed from the test tubes and placed in small petri dish, then kept in freezer for 30 minutes and thawed with hot water of about 70 ^{o}C for 3 minutes; after which the pupae were removed and placed on a tissue paper to soak up the water on the pupae.

The pupae were placed in a petri dish and the puparia were removed under a stereo light microscope (Leitz Wetzlar, Germany) with the aid of dissection kits. The pictures of the imagines were taken using a digital camera (Panasonic HX-DC2, 15X I. Zoom).

3.3.3 Accumulated degree days and statistical analysis

Accumulated degree days (ADD) [°D] were calculated following Grassberger and Reiter (2001)with a lover development threshold of 2 ^{0}C for *Calliphora vicina* according to Marchenko (2001). Mircosoft Excel 2010, IBM SPSS Statistic version 20 and R were used for statistical analyses.

3.4 Results

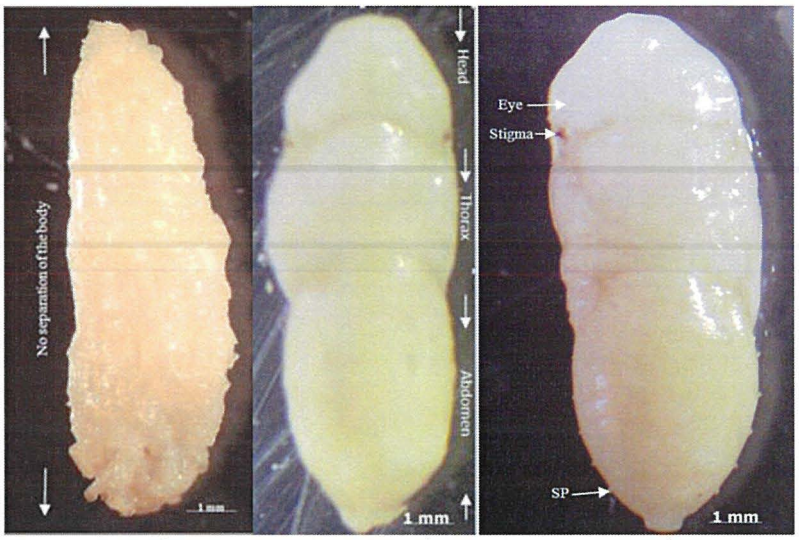
As the aim was to develop a very practical and simple method for ageing pupae, the decision was taken to only observe and describe the morphological changes that are visible on the dorsal side. The ventral side offered far less morphological changes than the dorsal side, and the lateral sides even less. The few characters that are uniquely visible on the ventral and lateral sides such as the development of the legs would complicate the procedure more than it would contribute to the increase in resolution if added to the morphological characters visible on the dorsal side.

3.4.1 Development at 22.3 ˚C

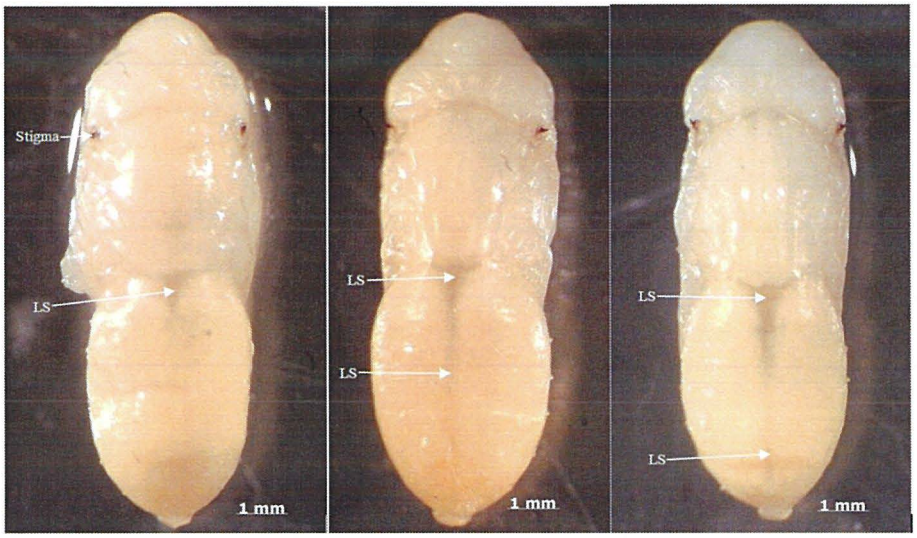
The developing features of pharate imagines of pupae of the blow fly *Calliphora vicina* reared at temperature of 22.3 ± 1^{0}C, relative humidity between 45 and 65% are shown in Figure 3.1.

On day one of pupariation after the removal of puparium, the only visible features were the mouthparts of the larval stage. For day two, segments of the body were formed, i.e. head, thorax and abdomen, with stigma and eye. On day three, stigmata prolongation was present while on day four, the formation of the longitudinal strip started. Both, day five and six showthe full longitudinal strip formed but the

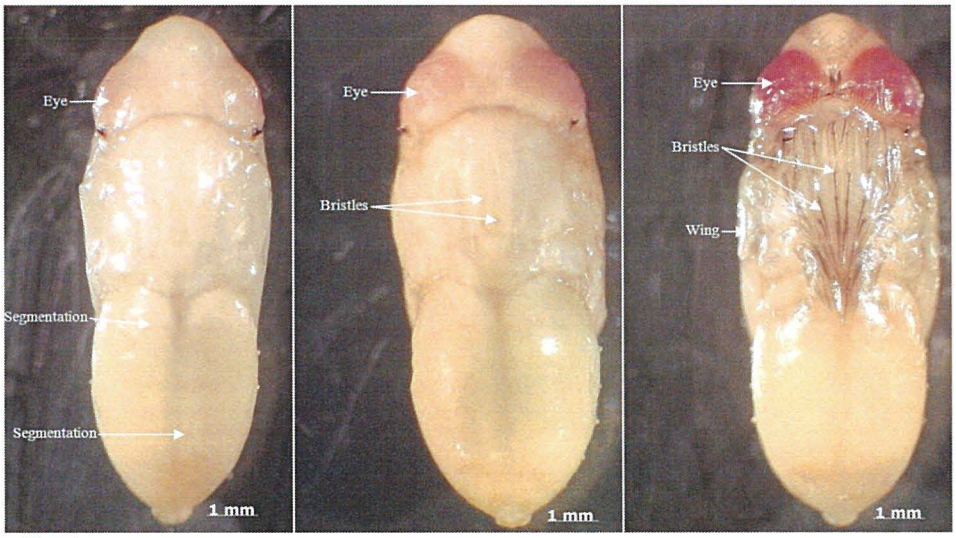
shape of the lower part of the thorax differed a little bit. For day seven, eye colouration started and segmentation on the abdomen became more visible. On day eight, the colouration of the eye deepened and the visible appearance of the bristles on the thorax became clear. On day nine, eye colouration deepened continuously, bristles became more visible and wings started to darken.



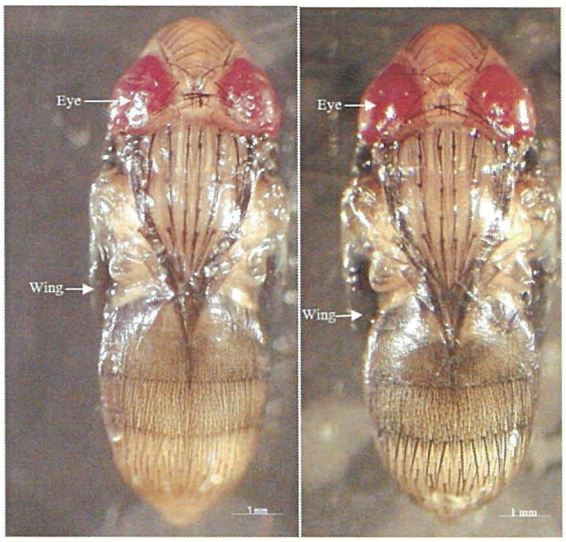
Day 1 168.0 ^{o}D day 2 184.8 ^{o}D day 3 207.6^{o}D



day 4 223.6 ^{o}D day 5 240.8 ^{o}D day 6 259.5 ^{o}D



day 7 275.2 ^{o}D day 8 294.1 ^{o}D day 9 311.4^{o}D



day 10 336.3 ^{o}D day 11 356.0 ^{o}D

Figure 3.1: Morphological characteristics in one day intervals of dissected developing pharate adults of *C. vicina* maintained at 22.3 ± 1 ^{o}C. LS stands for longitudinal strips and SP stands for stigmata prolongation.

For day ten and eleven, the pharate adults have similar eye colouration, wings darkened and bristles on the thorax darkened as welland different bristles formed on the abdomen.

These observed developmental features led to the generation of a scoring table that encompasses fifteen different morphological features, to which between 1 and 7 points are assigned, depending on their degree of development, resulting in a maximum overall score of 43 points, Table 3.1.

Points for development features of pharate adults of *Calliphora vicina*

Landmark	Points
Whole body	
larval mouth parts clearly visible	
larval mouthparts partially visible	1-4
only remnants of larval mouthparts visble	
larval mouthparts clearly absent	

Head	1
Thorax	1
Abdomen	1
Stigma	1
Eye	1
Stigma prolongation (SP)	1
Longitudinal strips (LS) slight expression or uncoloured moderate expression or not fully coloured strong expression or fully coloured	1-3
Segmentation	1
Abdomen thorax separation (ATS) beginning of separation complete separation	1-2
Neck region	1
Lower thorax barely visible partially visible clearly visible clearly visible and darkenend very pronounced	1-5
Eye colour white cream (cornsilk, FFF8DC) lightsalmon (FFA07A) coral (FF7F500 orangered (FF4500) red bright red	1-7

Wings wings barely visible wings partially visible wings clearly visible wingsstarted darkening wingspartially dark wingscompletely dark wings very pronounced	1-7
Bristles bristle channels barely visible bristle channels partially visible bristle channels clearly visible bristles barely visible bristles partially visible bristles clearly visible bristles very pronounced	1-7

Table 3.1 Colours were given X11 (W3C) standard colour names and for some the hex Code in brackets.

The quantification system of Table 3.1 has been applied to the morphological characters visible in Figure 3.1, the results of which are shown in Table 3.2.

The progression of characters during development is summarised in the bar chart of Figure 3.2.

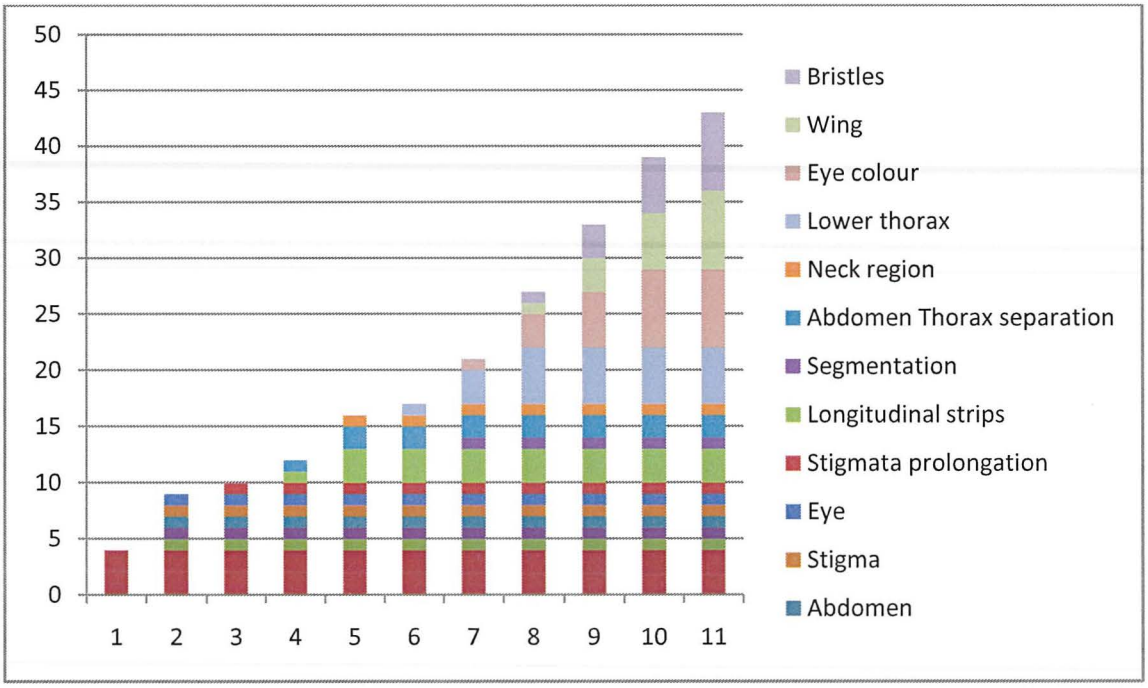


Figure 3.2: Bar chart showing the features of external morphology of developingimagines of pupae of*C. vicina* reared at temperature of 22.3 ± 1 ^{0}C from start of pupation to eclosion of adults. The abscissa indicates the days of pupation and the ordinate the sum of points of development according to Table 3.2.

The repetitions of the experiment came to the same results. The point scores at each day of the pupation were graphed against age expressed as degrees ADD and then a regression analysis performed. The regression lines did not show any significant difference. The standard deviation in point scores was less than 10 % of the score value.

After the success of this method in differentiation the pupal development, the procedure was repeated for two more temperature regimes, focussing on the lower temperatures for this temperate species.

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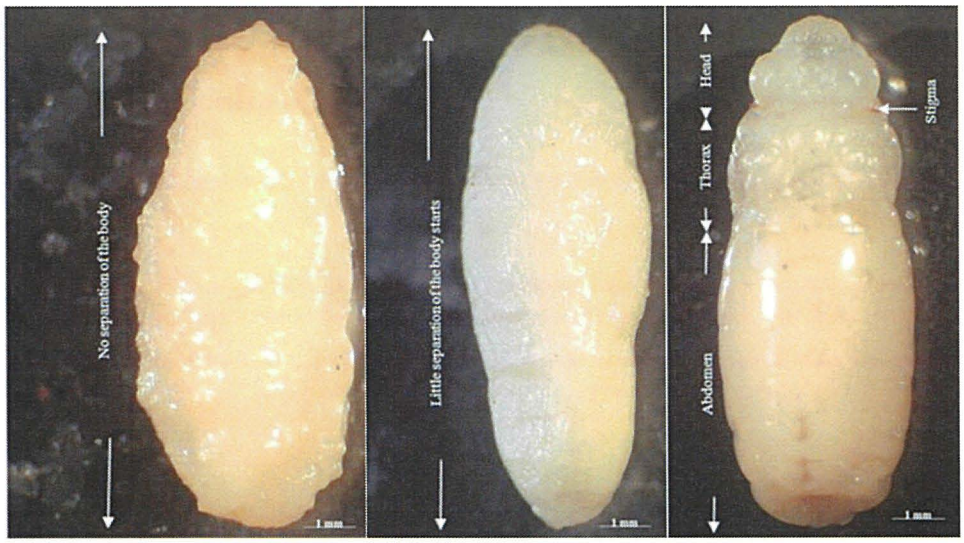
*Calliphora vicina*at 22.3 ˚C																
Age		Whole body	Head	Thorax	Abdomen	Stigma	Eye	SP	LS	Segmentation	ATS	Neck region	Lower thorax	Eye colour	Wing	Bristles	Points
Days	ADD																
1	168.0	4															4
2	184.8	4	1	1	1	1	1										9
3	207.6	4	1	1	1	1	1	1									10
4	223.6	4	1	1	1	1	1	1	1		1						12
5	240.8	4	1	1	1	1	1	1	3		2	1					16
6	259.5	4	1	1	1	1	1	1	3		2	1	1				17
7	275.2	4	1	1	1	1	1	1	3	1	2	1	3	1			21
8	294.1	4	1	1	1	1	1	1	3	1	2	1	5	3	1	1	27
9	311.4	4	1	1	1	1	1	1	3	1	2	1	5	5	3	3	33
10	336.3	4	1	1	1	1	1	1	3	1	2	1	5	7	5	5	39
11	356.0	4	1	1	1	1	1	1	3	1	2	1	5	7	7	7	43

Table 3.2: Scoring table for morphological changes in pharate adults of Figure 3.1. SP = Stigmata prolongation, LS = Longitudinal strips, ATS = Abdomen Thorax separation.

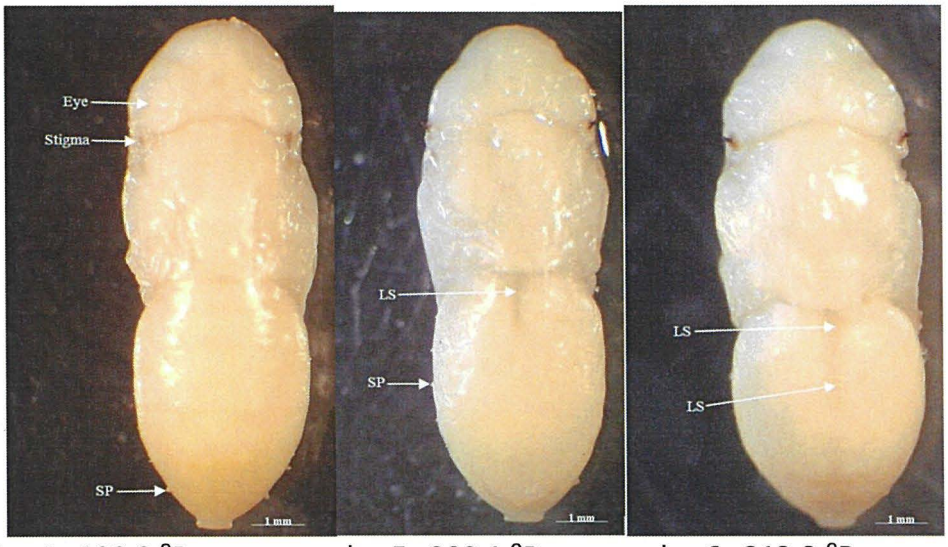
3.4.2 Development at 15.8 ˚C

The developing features of pharate imagines of pupae of the blow fly *Calliphora vicina* reared at temperature of 15.8 ± 1^{0}C, relative humidity between 87 and 99 % are shown in Figure 3.3.

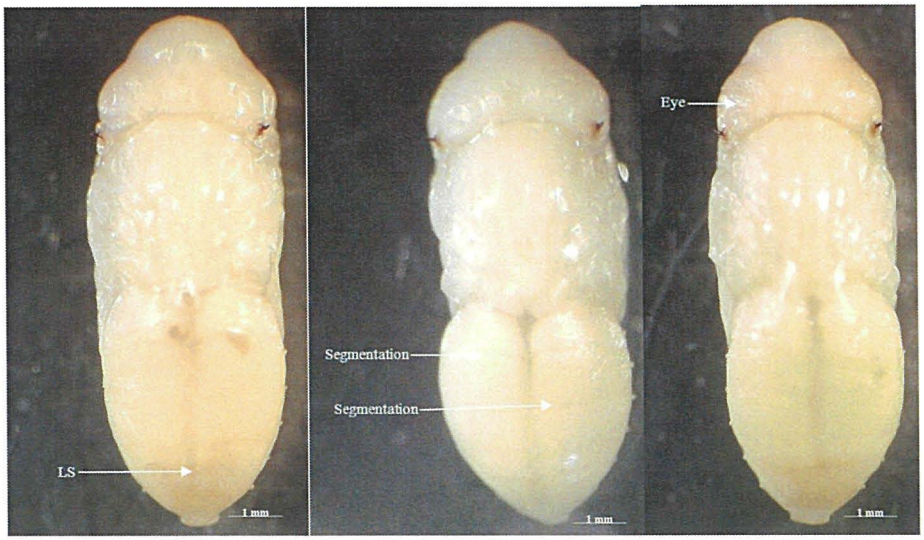
On day one, to remove the puparium was a little difficult because the puparium has not completely separated itself from the pharate imago, that means the shape of the imago most be tempered with in the process. For day two, to remove the puparium was much easier compared to day one and with little or no damage to the shape of imago and have the mouthpart of the larval stage. For day three, segmentation of the entire imago starts with a small head with eye region and stigma. The thorax too was small but the abdomen was big. On the fourth day, segmentation was complete with every part taken it normal size. On this day stigmata prolongation appeared. For day five, formation of the longitudinal strip started. For day six, seven and eight, all pupae have full longitudinal strip but differ in the shape formed at the lower part of the thorax and at the abdomen thorax separation; while on day eight, the imagines have visible segmentation on the abdomen. On day nine, eye colouration started. For day ten, eye colouration deepened. On day eleven, eye colouration deepened continuously and the bristles on the thorax became visible. For day twelve, the eye colouration deepened further and the bristles became more visible. For day thirteen, the bristles continued to be more visible and the wings got darken. On day fourteen, the wings darkened further and bristles formed and the segmentation of the abdomen shows different degree of formation. For day fifteen, sixteen, seventeen and eighteen, pupae have almost similar features but differences could be seen in the formation of bristles and in the segmentation of the abdomen.



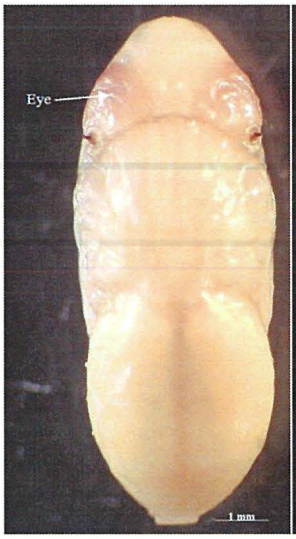
Day 1 155.3 ^{o}D day 2 168.8 ^{o}D day 3 182.2 ^{o}D



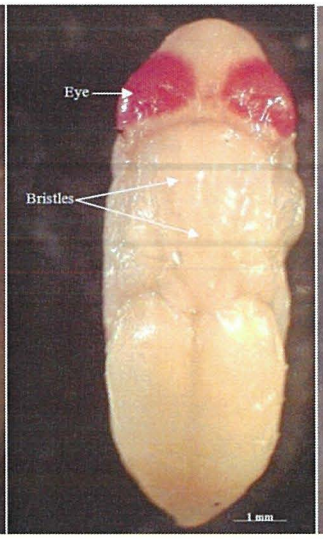
day 4 198.9 ^{o}D day 5 208.1 ^{o}D day 6 213.0 ^{o}D



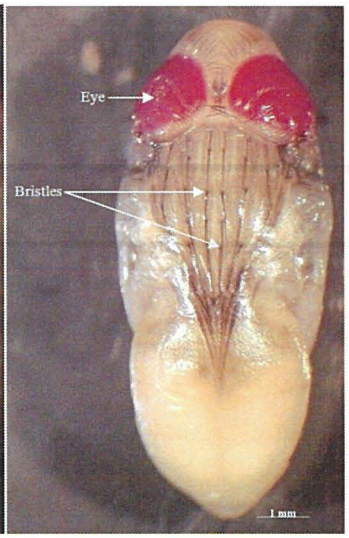
day 7 223.7 ^{o}D day 8 231.0 ^{o}D day 9 248.4 ^{o}D



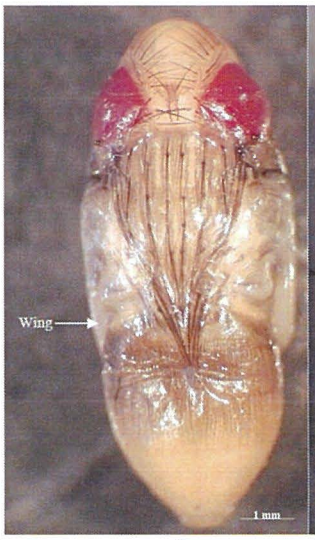
day 10 254.4 ^{o}D



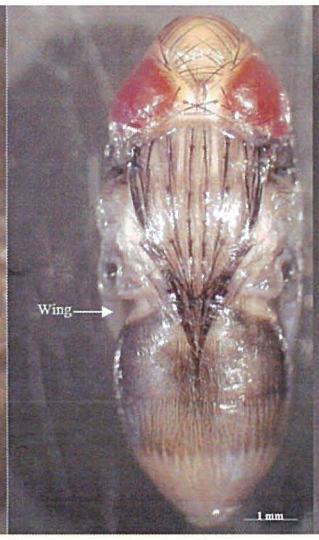
day 11 267.5 ^{o}D



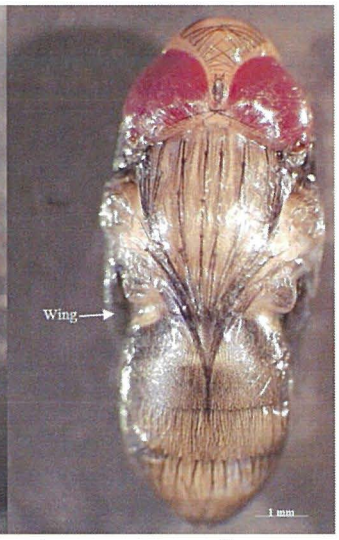
day 12 293.3 ^{o}D



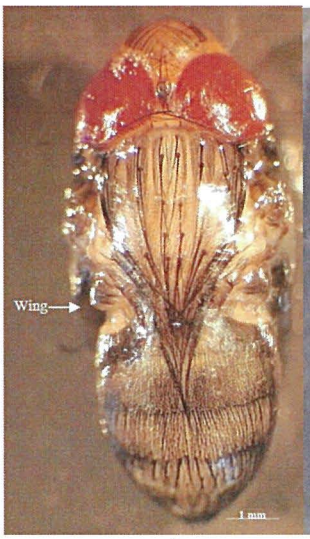
day 13 295.7 ^{o}D



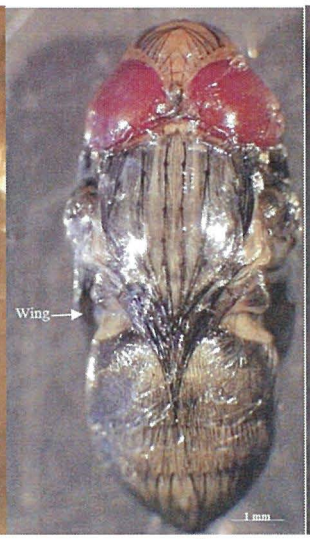
day 14 299.6 ^{o}D



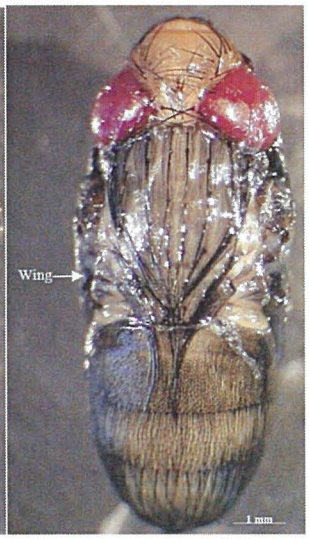
day 15 320.5 ^{o}D



day 16 340.5 ^{o}D



day 17 341.0 ^{o}D



day 18 358.4 ^{o}D

Figure 3.3: Morphological characteristics in one day intervals of dissected developing pharate adults of *C. vicina* maintained at 15.8 ± 0.7°C. LS stands for longitudinal strips and SP stands for stigmata prolongation.

These observed developmental features in Figure 3.3 were scored according to Table 3.1 and resulted in the matrix of Table 3.3.

The progression of characters during development is summarised in the bar chart of Figure 3.4.

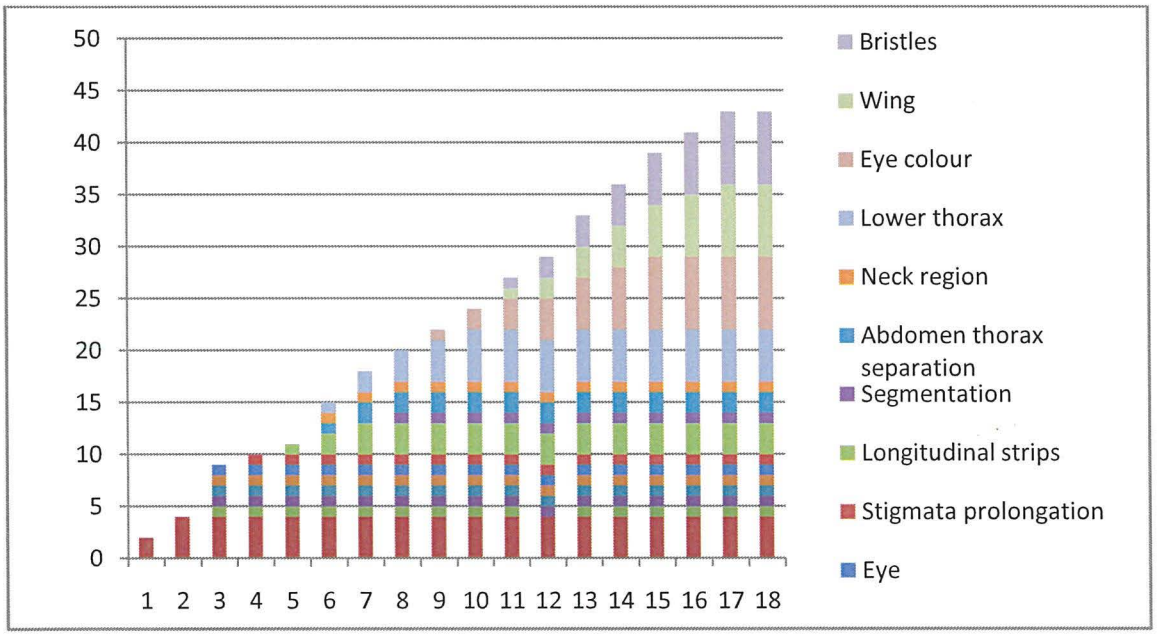


Figure 3.4: Bar chart showing the features of external morphology of developing imagines of pupae of*C. vicina* reared at temperature of 15.8 ± 0.7°C from start of pupation to eclosion of adults. The abscissa indicates the days of pupation and the ordinate the sum of points of development according to Table 3.2.

Calliphora vicina at 15.8 °C																
Age		Whole body	Head	Thorax	Abdomen	Stigma	Eye	SP	LS	Segmentation	ATS	Neck region	Lower thorax	Eye colour	Wing	Bristles	Points
Days	ADD																
1	155.3	2															2
2	168.8	4															4
3	182.2	4	1	1	1	1	1										9
4	198.9	4	1	1	1	1	1	1									10
5	208.1	4	1	1	1	1	1	1	1								11
6	213.0	4	1	1	1	1	1	1	2		1	1	1				15
7	223.7	4	1	1	1	1	1	1	3		2	1	2				18
8	231.0	4	1	1	1	1	1	1	3	1	2	1	3				20
9	248.4	4	1	1	1	1	1	1	3	1	2	1	4	1			22
10	254.4	4	1	1	1	1	1	1	3	1	2	1	5	2			24
11	267.5	4	1	1	1	1	1	1	3	1	2	1	5	3	1	1	27
12	293.3	4	1	1	1	1	1	1	3	1	2	1	5	4	2	2	30
13	295.7	4	1	1	1	1	1	1	3	1	2	1	5	5	3	3	33

14	299.6	4	1	1	1	1	1	1	3	1	2	1	5	6	4	4	36
15	320.5	4	1	1	1	1	1	1	3	1	2	1	5	7	5	5	39
16	340.5	4	1	1	1	1	1	1	3	1	2	1	5	7	6	6	41
17	341.0	4	1	1	1	1	1	1	3	1	2	1	5	7	7	7	43
18	358.4	4	1	1	1	1	1	1	3	1	2	1	5	7	7	7	43

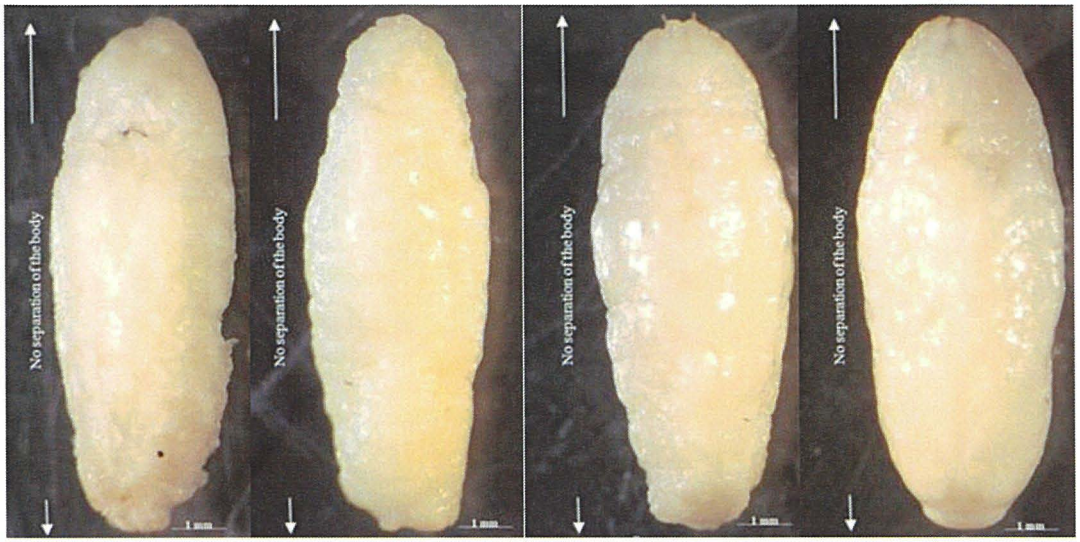
Table 3.3: Scoring table for morphological changes in pharate adults of Figure 3.3. SP = Stigmata prolongation, LS = Longitudinal strips, ATS = Abdomen Thorax separation.

3.4.3 Development at 11.8 ˚C

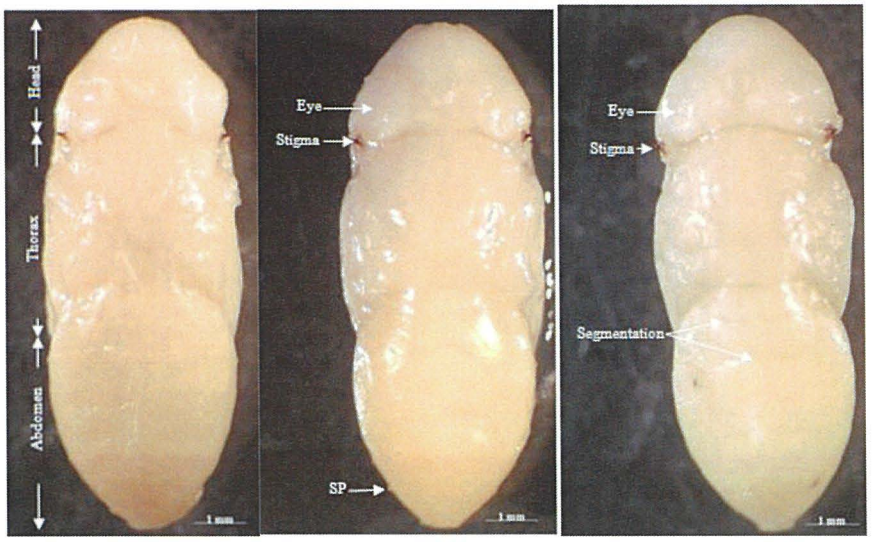
The developing features of pharate imagines of pupae of the blow fly *Calliphora vicina* reared at temperature of 11.8 ± 0.3^{0}C, relative humidity between 77 and 99 % are shown in Figure 3.5.

On day one, to remove the puparium was difficult again which resulted in a little damage of the imagines.The mouthparts of the larval stage were retained. On day two, the removal of the puparium was easy and it had a more organised shape and it also had the mouthpart of the larval stage. On day three, removal of puparium got much easier, had two spikes on head region that looked like antenna and had mouthparts. On day four, proper body formations started with a sign seen at the bottom of the abdomen. On day five, body segmentation took place with the formation of the head carrying the eye and stigma, thorax and abdomen. On day six, the stigmata prolongation was evident. On day seven, visible segmentation was observed on the abdomen. On day eight, the formation of the longitudinal strip started. For day nine, ten, eleven, twelve and thirteen, all had full longitudinal strip formed but differed in the groove shape formed at abdomen thorax separation. And on day thirteen, segmentations on the abdomen became visible. For day fourteen, fifteen and sixteen, all had similar features with the exception of shape formed at the lower thorax, which became more visible as can be seen through these days. On day seventeen, eye colouration started. On day eighteen, the eye colouration continued to deepen further. On day nineteen, eye colouration deepened further with a visible appearance of the wings also on that day. For day twenty, eye colouration deepened further and the appearance of bristles became evident. On day twenty one, eye colouration deepened further and the bristles get more visible. On day twenty two, eye colour deepened further, wings got darker and bristles more visible. For day twenty three, eye colour deepened further, bristles got more visible, wings got darker and segmentation on the abdomen showed signs of bristles forming. On day twenty four, wings get darker and bristles formed on segmentation of the abdomen. For day twenty five, twenty six, twenty seven and twenty eight, all specimens had almost

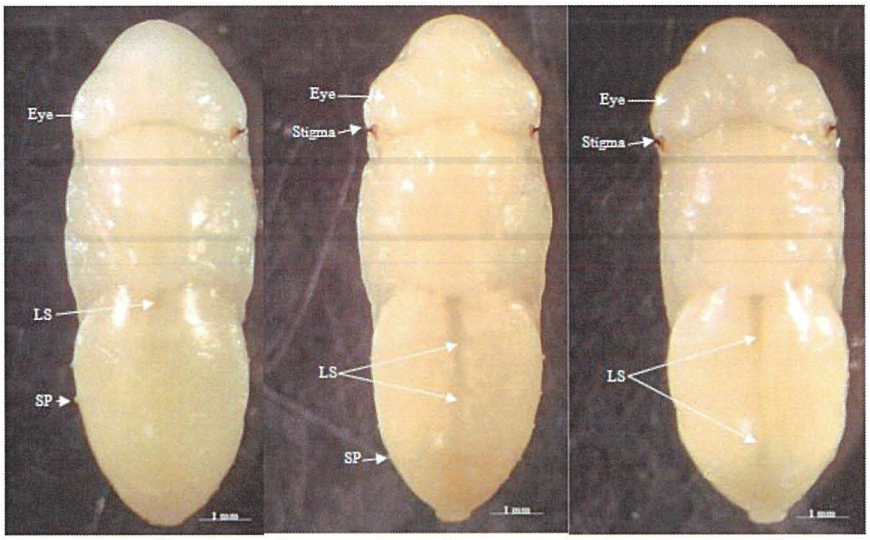
similar features but differed a little in the darkening of the wings and the formation of bristles on the thorax and abdomen.



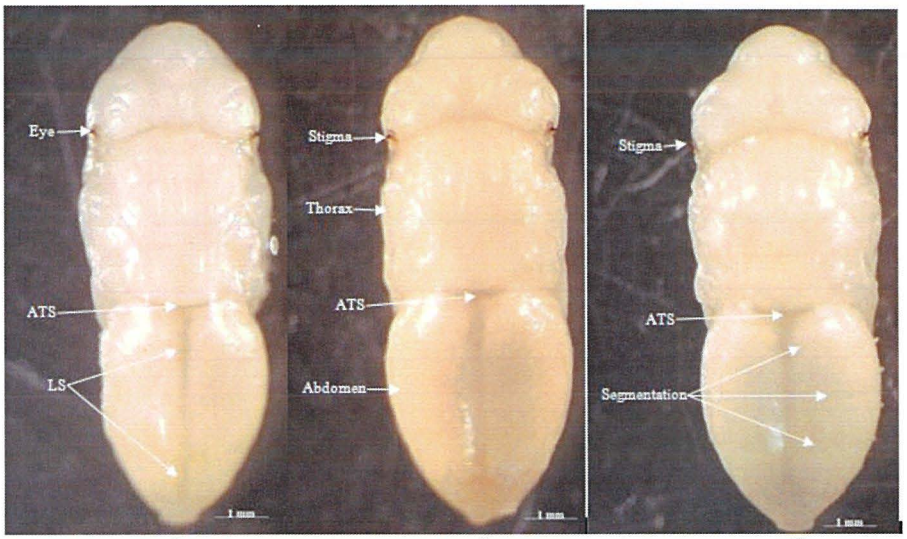
Day 1 125.1 ^{o}D day 2 121.6 ^{o}D day 3 140.0 ^{o}D day 4 147.0^{o}D



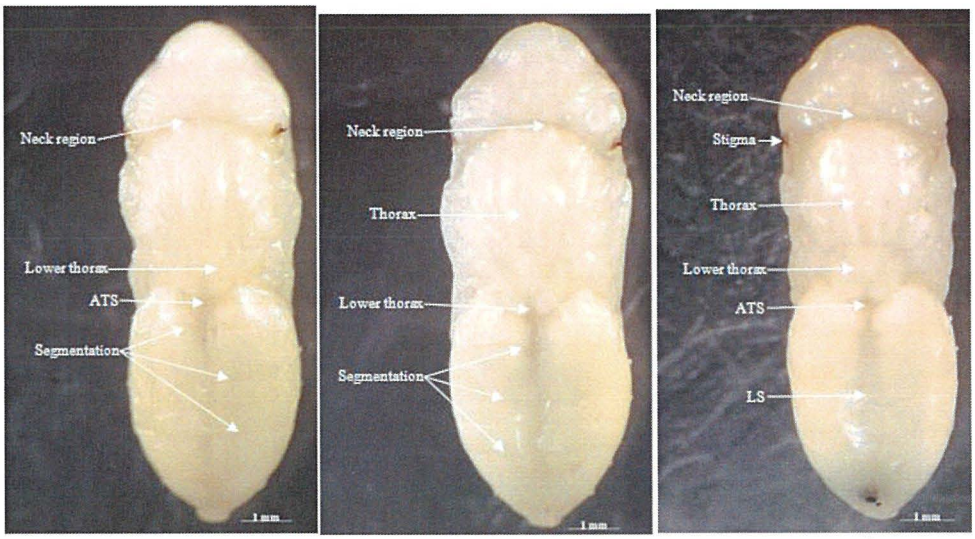
day 5 146.3 ^{o}D day 6 157.6 ^{o}D day 7 165.6^{o}D



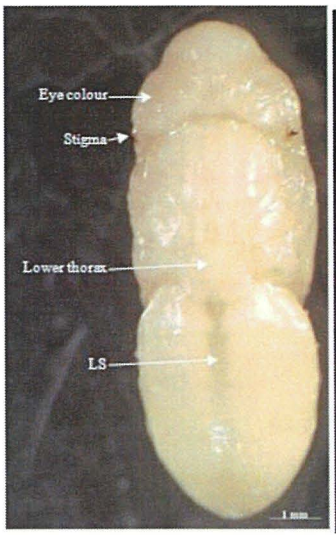
day 8 165.0 ^{o}D day 9 171.6 ^{o}D day 10 180.9^{o}D



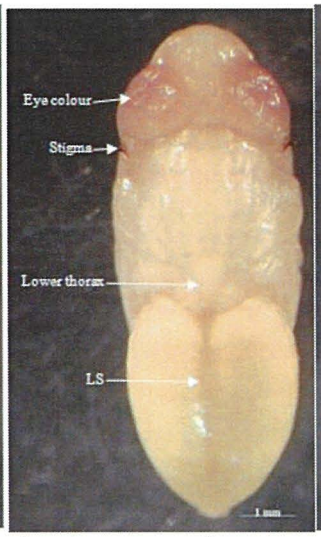
day 11 179.2 ^{o}D day 12 205.8 ^{o}D day 13 193.5^{o}D



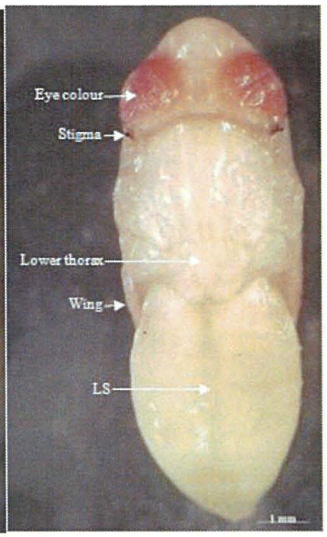
day 14 210.8 ^{o}D day 15 217.6 ^{o}D day 16 216.2^{o}D



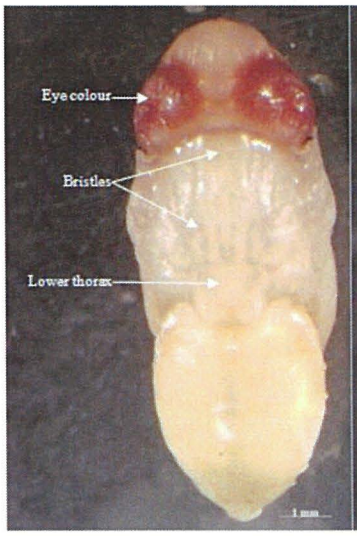
day 17 231.2 ^{o}D



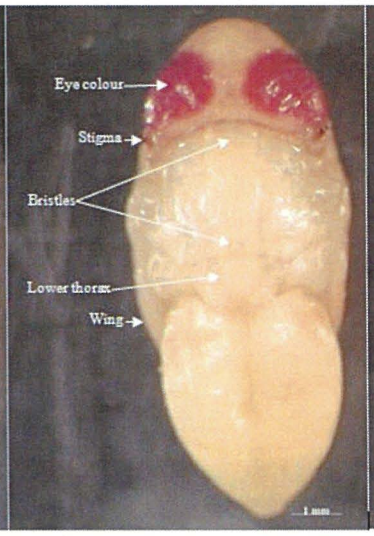
day 18 238.0 ^{o}D



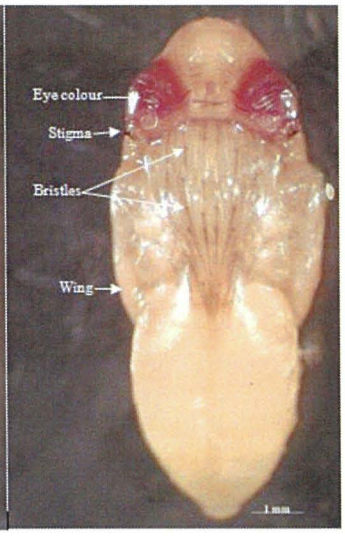
day 19 241.2^{o}D



day 20 251.6 ^{o}D



day 21 256.5 ^{o}D



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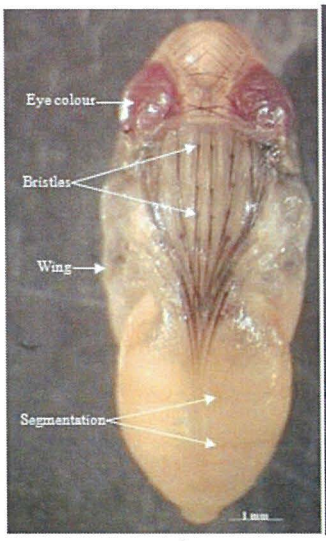

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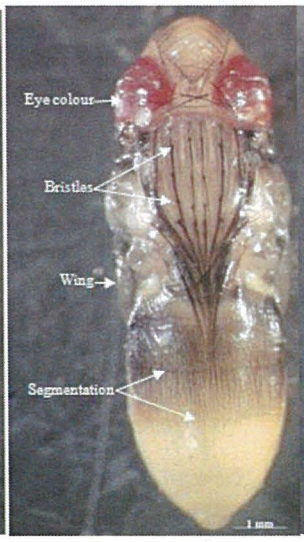

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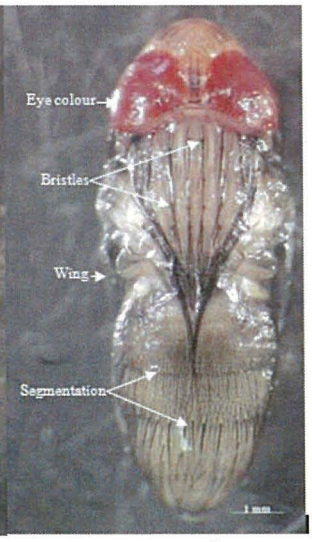

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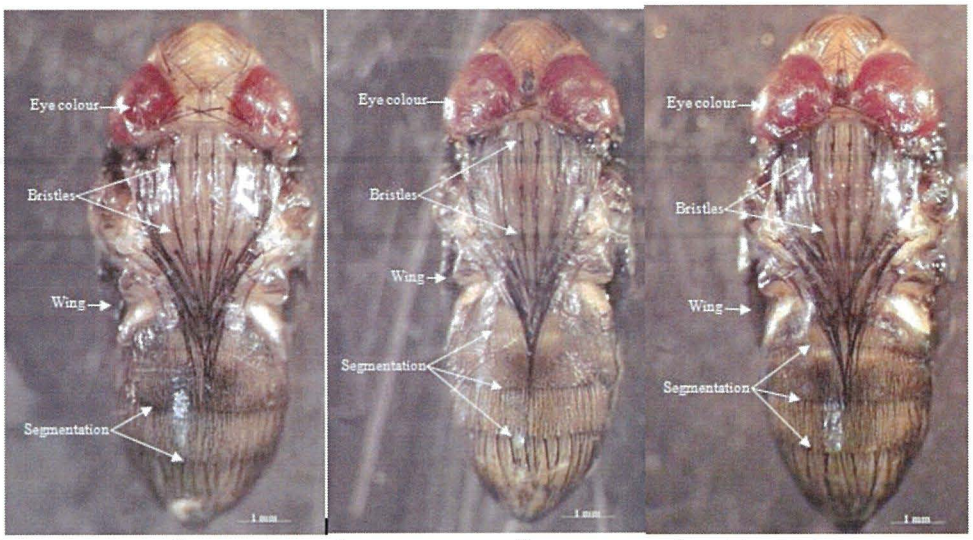

day 26 311.8 ^{o}D day 27 328.5 ^{o}D day 28 324.3

Figure 3.5: Morphological characteristics in one day intervals of dissected developing pharate adults of *C. vicina* maintained at 11.8 ± 0.3^{o}C. LS stands for longitudinal strips and SP stands for stigmata prolongation.

These observed developmental features in Figure 3.5 were scored according to Table 3.1 and resulted in the matrix of Table 3.4.

The progression of characters during development is summarised in the bar chart of Figure 3.6.

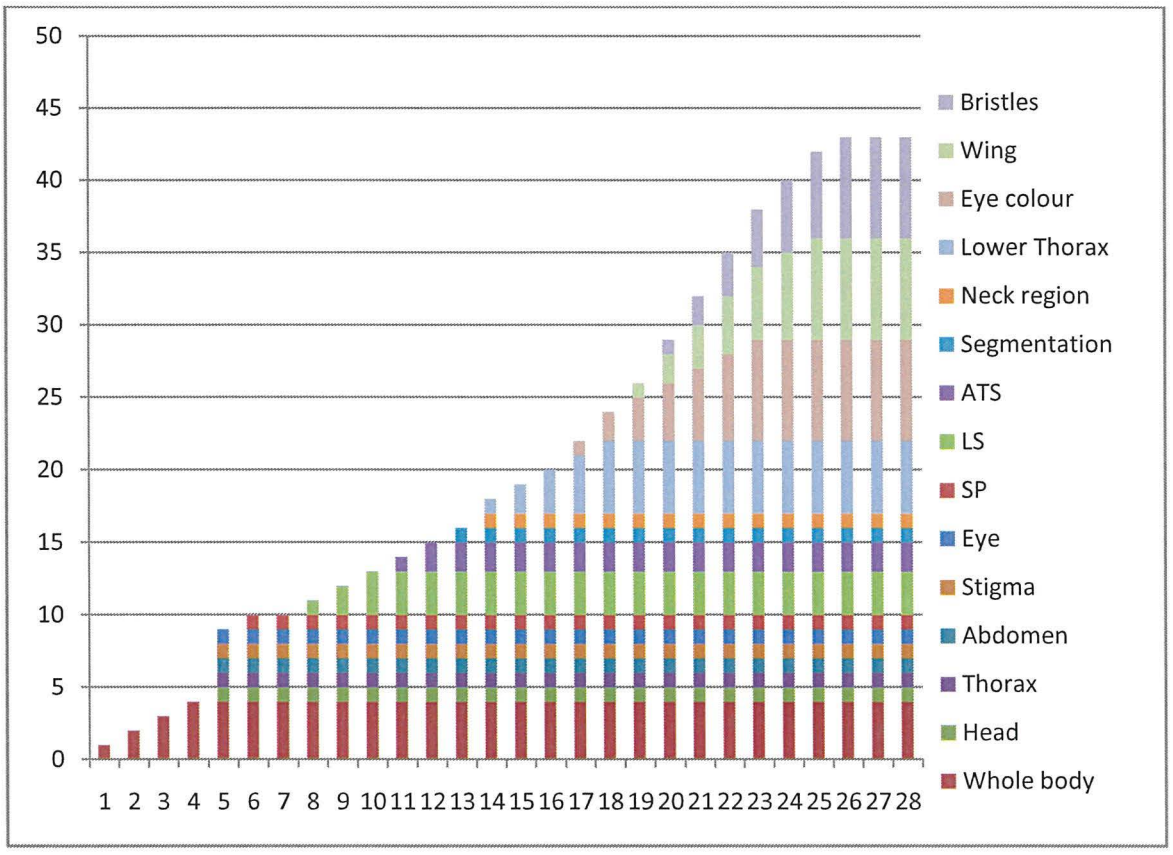

Figure 3.6: Bar chart showing the features of external morphology of developing imagines of pupae of*C. vicina* reared at temperature of 11.8 ± 0.3°C from start of pupation to eclosion of adults. The abscissa indicates the days of pupation and the ordinate the sum of points of development according to Table 3.4.

*Calliphora vicina*at 11.8 °C																
Age		Whole body	Head	Thorax	Abdomen	Stigma	Eye	SP	LS	Segmentation	ATS	Neck region	Lower thorax	Eye colour	Wing	Bristles	Points
Days	ADD																
1	125.1	1															1
2	121.6	2															2
3	140.0	3															3
4	147.0	4															4
5	146.3	4	1	1	1	1	1										9
6	157.6	4	1	1	1	1	1	1									10
7	165.6	4	1	1	1	1	1	1									10
8	165.0	4	1	1	1	1	1	1	1								11
9	171.6	4	1	1	1	1	1	1	2								12
10	180.9	4	1	1	1	1	1	1	3								13
11	179.6	4	1	1	1	1	1	1	3		1						14
12	205.9	4	1	1	1	1	1	1	3		2						15
13	193.5	4	1	1	1	1	1	1	3	1	2						16
14	210.8	4	1	1	1	1	1	1	3	1	2	1	1				18
15	217.6	4	1	1	1	1	1	1	3	1	2	1	2				19
16	216.2	4	1	1	1	1	1	1	3	1	2	1	3				20

17	231.2	4	1	1	1	1	1	1	3	1	2	1	4	1			22
18	238.0	4	1	1	1	1	1	1	3	1	2	1	5	2			24
19	241.2	4	1	1	1	1	1	1	3	1	2	1	5	3	1		26
20	251.6	4	1	1	1	1	1	1	3	1	2	1	5	4	2	1	29
21	256.5	4	1	1	1	1	1	1	3	1	2	1	5	5	3	2	32
22	257.4	4	1	1	1	1	1	1	3	1	2	1	5	6	5	3	35
23	280.0	4	1	1	1	1	1	1	3	1	2	1	5	7	6	4	38
24	295.2	4	1	1	1	1	1	1	3	1	2	1	5	7	7	5	40
25	273.0	4	1	1	1	1	1	1	3	1	2	1	5	7	7	6	42
26	311.8	4	1	1	1	1	1	1	3	1	2	1	5	7	7	7	43
27	328.5	4	1	1	1	1	1	1	3	1	2	1	5	7	7	7	43
28	324.3	4	1	1	1	1	1	1	3	1	2	1	5	7	7	7	43

Table 3.4: Scoring table for morphological changes in pharate adults of Figure 3.4. SP = Stigmata prolongation, LS = Longitudinal strips, ATS = Abdomen Thorax separation

Replicates of the growth experiments for all three temperatures exhibited the same number of days for pupal development despite small fluctuations in temperatures, Table 3.5.

Minimum development times of *C. vicina* from pupariation to eclosion

Temperature [°C]	Number of days from pupariation to eclosion	Error in ADD [°D]
22.3 ± 1.0	11 ± 0	< 22.7
15.8 ± 0.7	18 ± 0	< 23.5
11.8 ± 0.3	28 ± 0	< 19.4

The values are expressed as mean days ± standard deviation (n = 3).

Table 3.5

3.5 Discussion

The pupal stage takes longer than the time it takes for the three larval stages together. The development of the pupal stage in *C. vicina*is divided into five stages by Defilippo *et al.* (2013): first, the cryptocephalic pupa, where the head is still invaginated, second, the phanerocephalic pupa, which shows complete eversion of the head and thoracic appendages, third, the pharate adult, where segmentation in the thorax, legs and abdomen becomes visible, fourth, the "Yellow eyed" stage, where the colouration of the eye starts changing, and fifth, the "Tanned chaetae" stage, where all bristles, both macro- and microchaetae are tanned. This division follows the practice in flesh flies (Sivasubramanian and Biagi, 1983). In addition, Defilippo *et al.* (2013) describe a further junction, "Tanned legs", which they denote as a landmark but not as a stage, it is characterised by all the bristles, legs, and wings being fully tanned. The tanning pattern also corresponds with the development in Drosophila (Bainbridge and Bownes, 1981).

The pupal development observed in *C. vicina* follows an antero-posterior course. Tanning also proceeds in an antero-posterior order. Tanning on the appendages is from proximal to distal.

The reliability of this method over three different temperature regimes was analysed by comparing the regression values. Figure 3.7.

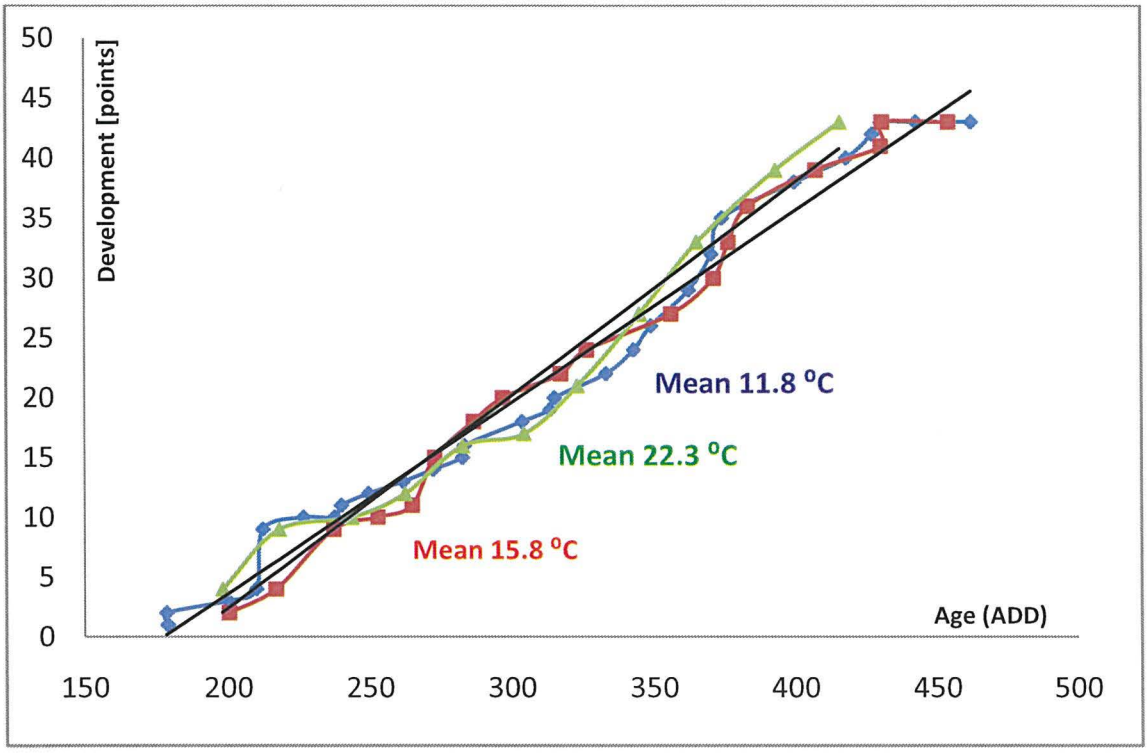

Figure 3.7: Regression analysis of pupal development at three temperatures, age against morphological characters of pharate adults.

Development at 22.3 ˚C versus morphological change resulted in a regression function of y = = 0.1777x – 33.166 and R^2 = 0.9647, while development at 15. 8 ˚C and 11.8 ˚C was indistinguishable and resulted in a single regression function of y = 0.16x – 28.389 and R^2 = 0.9799. Both regression lines pretty much overlap, showing that this method of ageing pupa is applicable over a range of temperatures.

To make a start in validating this method, the group of Dr Jens Amendt at the Institute for Legal Medicine of the University of Frankfurt in Germany was contacted.

The idea was to compare the results of this study with the work Zajac (2012). Zajac had already left the laboratory in Frankfurt and the *C. vicina* colony had been lost.

The rate of development during the pupal stage was compared with the two other studies by Zajac and Amendt (2012) and Defilippo *et al.* (2013), which wererecently published for *C. vicina*, Figure 3.8.

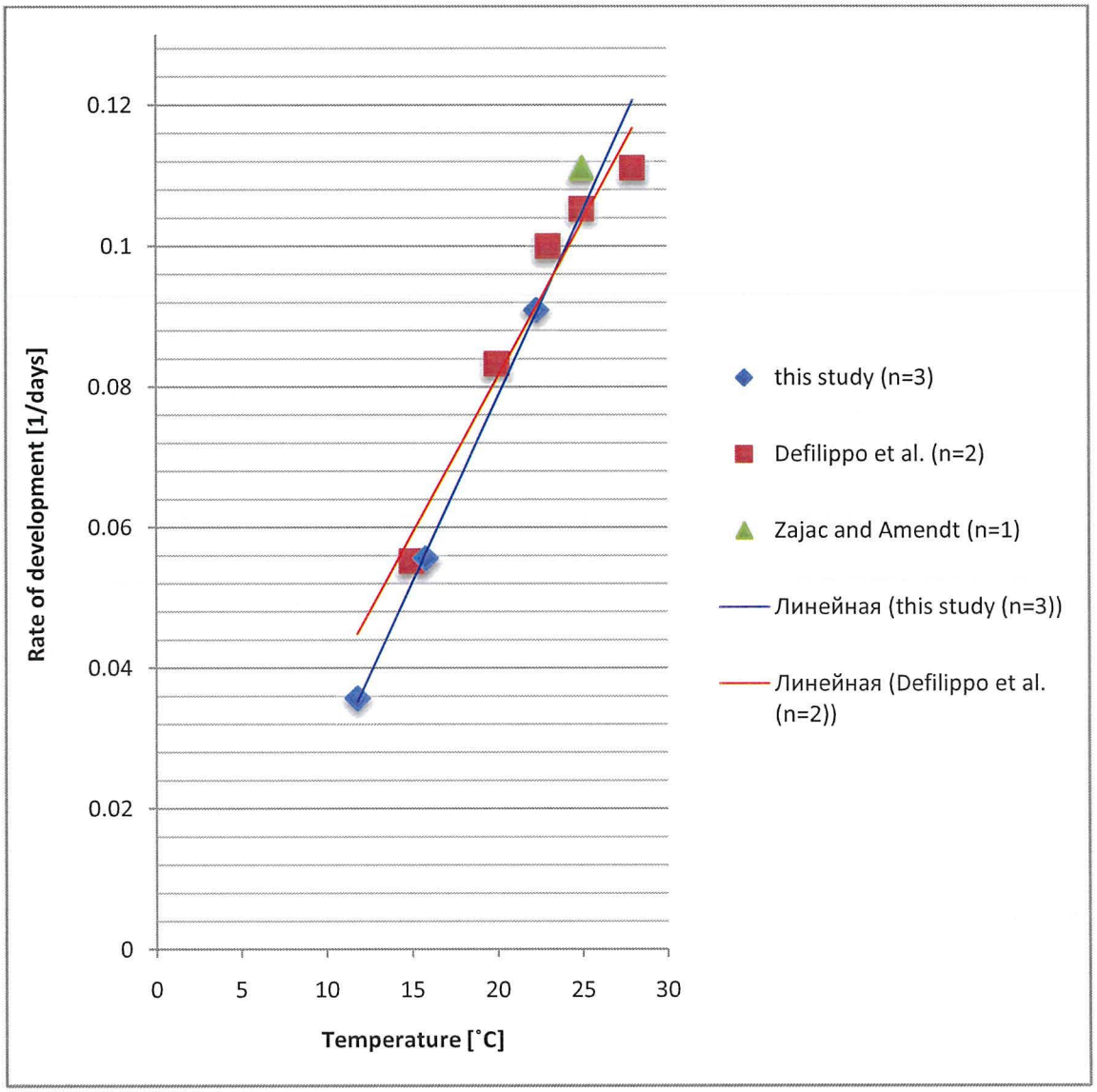

Figure 3.8: Rate of development of *C. vicina* pupae versus rearing temperature. N indicates the number of replicate experiments for each temperature regime. The blue regression line for this study is y = 0.0053 x - 0.027 with R^2 = 0.9995 and the red regression line for Defilippo et al. is y = 0.0044 x - 0.0076 with R^2 = 0.9586.

Zajac and Amendt (2012) used only a single temperature for their study without any replicates. The regression lines of the rate of development in Figure 3.8 for Defilippo et al. and this study approach both y = x, showing a strong linear relationship between developmental rate and environmental temperature with $p < 0.01$.

The results from our study demonstrated that this method of puparia removal could be used to monitor changes on external features of developing imagines, which take place during metamorphosis and then be used to estimate a PMI_{min}. This method has number of advantages; requiring only a stereo microscope as infrastructure, non-destructive aside from the puparium, very fast, and less expensive than any other method. Because of these advantages, it can be carried out in any forensic entomology laboratory.

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Chapter Four

Minimal developmental temperatures for the blow fly *Calliphora vicina*

4.1 Abstract

Temperature being the major environmental factor affecting the function and activity of cold-blooded animals is a key factor in the forensic application of mimimal post mortem interval estimates using larvae of the blow flies *Calliphora vicina*. *C. vicina* is an important blow fly species adapted to survive at low temperatures. It has been extensively studied. Nevertheless, the minimum temperature at which the fly will oviposit and the eggs will develop has surprisingly not been investigated. The theoretically limited for development for development of immature stages of *C. vicina* have been reported by various researchers as between 1 and 6 ˚C.

Here, the minimum temperature for oviposition and subsequent development has been investigated at temperatures of 5.0 ± 1.0 ˚C, 8.6 ± 0.6^{0}C, 10.0 ± 0.3^{0}C, and at 11.8 ± 0.3^{0}C, studying developmemt to larvae, pupa and adults.

No oviposition occured at 5 ˚C. Rare events of oviposition were achieved at 8.6 ˚C with subsequent development of a very eggs to first instar larvae, which eventually died. At 10.0 ˚C, oviposition was reliable and most eggs hatched. The larvae developed through all three instars but did pupate. Only at 11.8 ˚C, development progressed to the pupal and adult stage. It was possible to induce pupation of larvae arrested at 10.0 ˚C.

4.2 Introduction

Blow flies are cold-blooded and cannot regulate their own body temperature but rather dependent on the temperature of the surroundings. For the temperature of the surroundings influences their development, behaviour and physiology. This is a major environmental factor that affects development of the adult and immature stages of development of blow flies(Niederegger *et al.*, 2010; Hückesfeld *et al.*, 2011). The age of immature stages of blow flies are used as marker for estimation of minimum post mortem interval (PMI_{min}) of a corpse in case of a homicide and suicide(Catts, 1992; Goff, 1993; Goff, 1994; Goff and Win, 1997; Goff, 2010; Richards *et al.*, 2012). This

study seeks to look at the behaviour of the adult blow fly with respect to eggs lying and development of the other immature stages under extreme temperatures.

The blow fly of the family Calliphoridae are the flies found on corpses at extreme temperature conditions. One of species of blow fly is *Calliphora vicina* and these were also found to have higher metabolic rate compared to blow flies found in warmer temperatures(Faucherre *et al.*, 1999). That temperature been the most important environmental factor that affects the behaviour, development and physiology of blow flies was demonstrated in other studies that the blow fly *C. vicina* has limit of temperature it can bear.

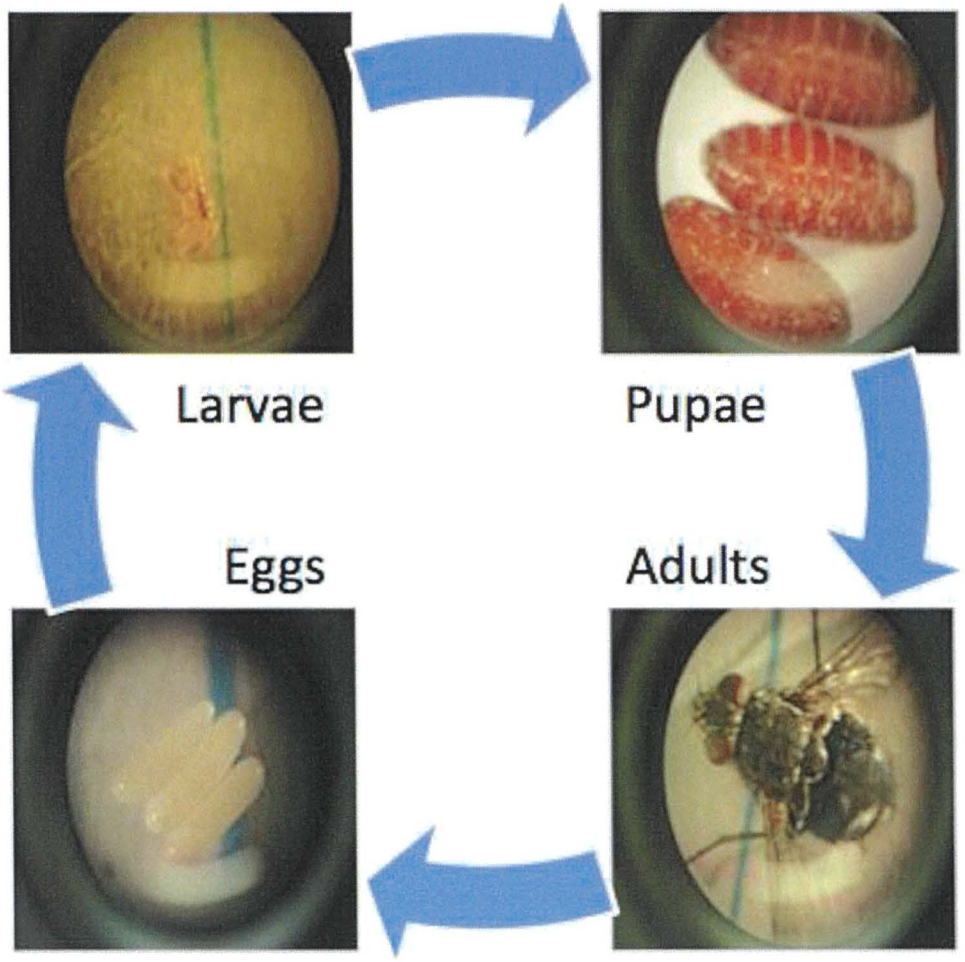


Figure 4.1: Life cycle of the blowfly *Calliphora vicina*

Study show the temperature range for which *C. vicina* can tolerate is between 15 ^{0}C and 30 ^{0}C for complete development from egg to adult fly. Studies also show that the highest and lowest temperatures that allow the pupal development of *C. vicina* lies between 15 and 28 ^{0}C Any temperature outside this range has to be considered deleterious to *C. vicina* pupal development. The lower the temperature, the longer it takes to complete the pupal stage (Defilippo *et al.*, 2013).

The literatures about development of markers for ageing immature stages of blow flies, especially the pupal stage, are many, but amazingly only few experiments are carried out below the temperature of 15 ^{0}C (Grassberger and Reiter, 2001; Defilippo *et al.*, 2013). All these experiments were carried out in such a way that the eggs were either collected at a higher temperature and then transferred to a temperature control room of high temperature and then monitored the development of the immature stages (Davies and Ratcliffe, 1994) or the eggs were collected at higher temperature and at reared at lower temperature (Faucherre *et al.*, 1999).

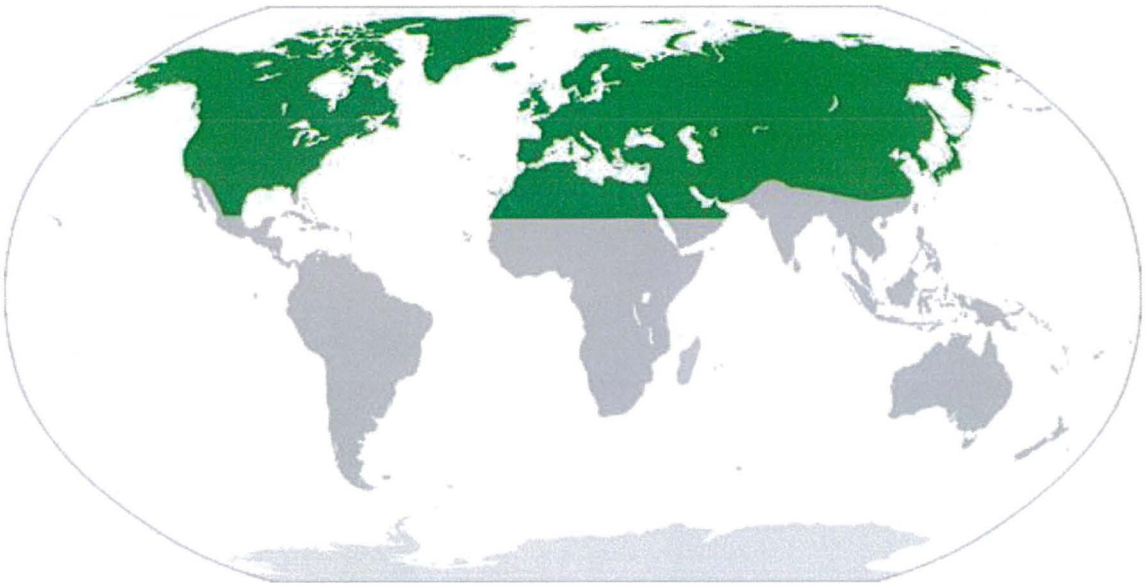


Figure 4.2: Original distribution of *Calliphora vicina*highlighted in green covering the whole of the holartic region. As a synanthropic species it has followed humans into South America, the Afrotropical region, Northern India, Japan, and Australia and New Zealand.

To quantify human association of blow flies, Nuorteva (1963) devised a formula that incorporates the number of specimens trapped at three different locations.

$$\text{Synanthropic index} = \frac{2a + b - 2c}{2}$$

a: percentage of fly species in a dense human settlement or city relative to all specimens of that species collected at all three sites,

b: percentage of the same fly species in sparsely inhabited place or at a rural house relative to all specimens of that species collected at all three sites,

c: the percentage of the same fly species in uninhabited area or in the wild relative to all specimens of that species collected at all three sites.

The index ranges from + 100 as most synanthropic to – 100 as avoiding man most. The index is dependent on the location. *Lucila cuprina* is a highly synanthropic blow fly with an index of + 79.1 and *Paralucilia xanthogeneiates* is a strongly non-synanthropic species with an index of – 84.6 (Vianna *et al.*, 1998).

Synanthropic indices of *C. vicina*

– 32.6	Buenos Aires, Argentina	average	(Schnack *et al.*, 1995)
– 14.0	Mariehamn, Finland	average	(Nuorteva, 1963)
+ 49.4	Rio Grande do Sul, Brazil	year average	(Vianna *et al.*, 1998)
+ 55.2	Valdivia, Chile	year average	(Figueroa-Roa and Linhares, 2002)
+ 83.1	Bogota, Colombia	year average	(Pinilla Beltran *et al.*, 2012)
+ 77.1	Suez, Egypt	spring only	(Gabre and Abou Zied, 2003)
+ 86	Hungary	average	(Nuorteva, 1963)

The index is also highly dependent on the season. At certain times of the year, flies might not be around in certain habitats or at all. Even in regions were there is no break due to overwintering or diapause, flies might be absent due to exceedingly high temperatures. The fluctuation in the synanthropic index over two years for *C. vicina* in

Pelotas in the state of Rio Grande do Sul (Brazil) is shown in Figure 1.3.2; it ranges between + 100 and – 66.7 (Vianna *et al.*, 1998).

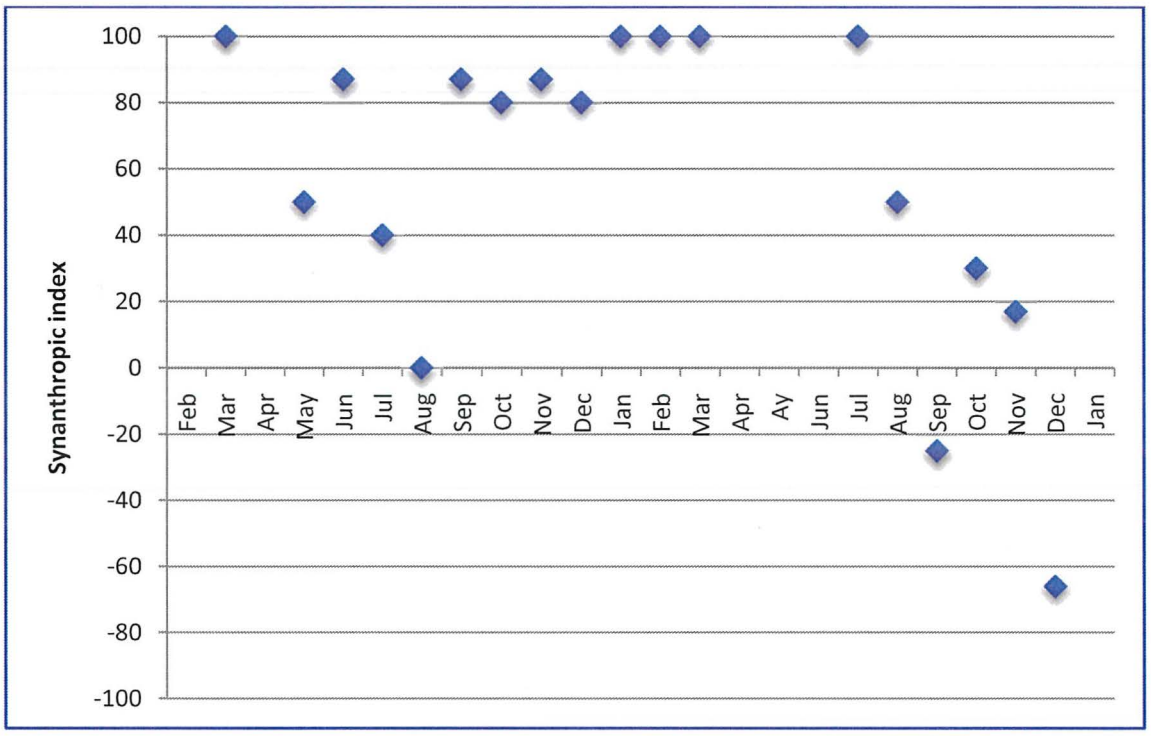


Figure 3.1.2: Variation in synanthropic index of *C. vicina* during the seasons. Data extracted from Vianna *et al.* (1998).

Not only is the geographical distribution of *C. vicina* almost spanning the entire northern globe with presumably the coldest occurrence reported from the Kerguelen Islands of the Antartic (Frenot *et al.*, 2005), this blow fly species is equally wide distributed in terms of altitude. The distribution of *C. vicina*between lowland and upland habitats in England and Wales have been studied by Davies (1990). Increasing slightly in altitude, Morris and Titchener (1997) studied *C. vicina* in moorlands and highlands of Scotland. Although *C. vicina* is a very rare blow fly for Japan compared to *C. vomitoria*, *C. vicina* has been collected on Hokkaido island at altitudes of 1,000 m (Nissho Pass), 1,150 m (Mt. Tsurugi), and 1,850 m (Mt. Kurodake), however, *C. nigribaris* dominated at higher elevations (Iwasa *et al.*, 2012). *C. vicina* was collected in Colorado, USA, from rabbit carcassesatGuanella Pass at 3,566 m and at Mt. Evans at

4,191 m during summer (June and July) (De Jong and Chadwick, 1999). The highest record for *C. vicina* from a human corpse also comes from Colorado. An adult hiker was killed by a fall in the Rocky Mountain National Park at an elevation of just over 3,475 m and exclusively colonised by *C. vicina*(Adair, 2008).

The aim of this study is to find a minimum temperature under extreme conditions where massive eggs are laid, massive hatching of these eggs to larvae and the moulting of these larvae into pupae and subsequently, emergence of these pupae to adult flies.

4.3 Material and Methods

4.3.1 Laboratory culture

An established *Calliphora vicina* (Diptera: Calliphoridae) Robineau-Desvoidy 1830 was used for this study. The identity of the blow flies was confirmed by the key ofErzinçlioğlu (1996). The blow flies *Calliphora vicina* were reared in screened breeding boxes (44 cm x 44 cm x 44 cm), first at an ambient temperature of 8.57 ± 0.56 ^{o}C with relative humidity between 70 and 96%, secondly, at an ambient temperature of 9.99 ± 0.27 ^{o}C with relative humidity between 88 and 97% and thirdly, at an ambient temperature of 11.84 ± 0.30 ^{o}C with relative humidity between 77 and 99 %. Eggs were collected at each temperature with the aid of pieces of pig liver placed on petri dish placed in the box where the blow flies were at different duration of time. The petri dish with pig liver having the blow fly eggs was then transferred into a rectangular basin with fine sawdust, which offered as a substratum for wandering larvae. The pig liver supply was monitored constantly to prevent insufficient food, which might lead to premature wandering of the third instar larvae.

4.3.2 Sampling of pupae

The active 3rd instar larvae were observed every 24 hours and the observation continued until the wandering 3rd instar larvae movement became very slow. Then those larvae were separated from the rest of the population of larvae, to enable

obtaining an almost equal age of the larvae. After 24 hours, those wandering 3rd instar larvae that had initiated pupariation, were removed from that container into another container to have an almost equal age pupae. Then five specimens were picked out for dissection after 24 hours. This process of pupae collection for dissection continued until adult blow flies started to emerge. The experiment was repeated two times to give a total of three experiments.

4.3.3 Dissection of pupae

For the preparatory stages for puparium removal, the methods of (Zajac and Amendt, 2012)was used with modification. The collected pupae were placed in a 1.5 ml test tube with tap water three quarter filled. It was then incubated at 70 ^{0}C for three hours in a heating block (Techne, DRI-Block DB.2A, England). The pupae were removed from the test tubes and placed in small petri dish, then kept in freezer for 30 minutes and thawed with hot water of about 70 ^{0}C for 3 minutes; after which the pupae were removed and placed on a tissue paper to soak up the water on the pupae.

The pupae were placed in a petri dish and the puparia were removed under a stereo light microscope (Leitz Wetzlar, Germany) with the aid of dissection kits. The pictures of the imagines were taken using a digital camera (Panasonic HX-DC2, 15X I. Zoom).

4.3.3 Accumulated degree days and statistical analysis

Accumulated degree days (ADD) [°D] were calculated following Grassberger and Reiter (2001)with a lover development threshold of 2 ^{0}C for *Calliphora vicina* according toVinogradova and Marchenko (1984); (Marchenko, 2001). Mircosoft Excel 2010, IBM SPSS Statistic version 20 and R for statistical analyses were used for statistical analysis.

4.4 Results

At an ambient temperature of 5 ± 1 ˚C, no single event of oviposition was observed.

At an ambient temperature of 8.57 ± 0.56 ^{o}C, the minimum temperature was 7.60 ^{o}C while the maximum was 9.65 ^{o}C among the three replicate experiments, with relative humidity between 70 and 96%. Approximately 100 eggs were laid. Out of that 4 days later one larva was found, then subsequently, within a month 5 others emerged. All first instar larvae died after several weeks.

At an ambient temperature of 9.99 ± 0.27 ^{o}C, the minimum temperature was 9.50 ^{o}C while the maximum was 10.35 ^{o}C among the three replicate experiments with relative humidity between 88 and 97 %. Approximately 200 eggs were laid. Out of that 30 hatched to larvae within one month. All the larvae became arrested as 3rd instars. After three months at the arrested stage, the ambient temperature was raised to 11.84 ± 0.30 ^{o}C, the batch of eggs and larvae in the temperature control room were maintained. After three months three replicate experiments had been performed.

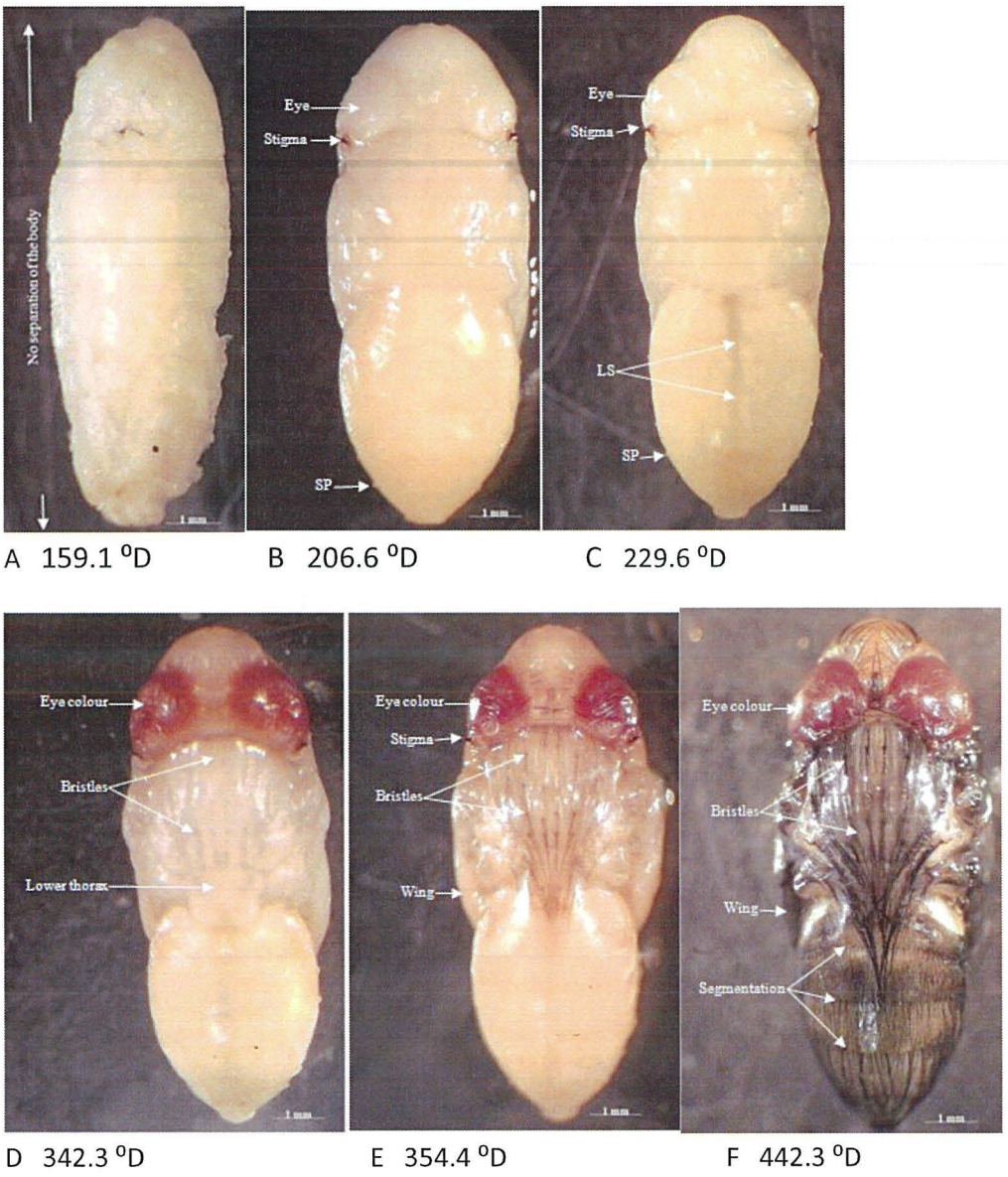
Larvae = 53 ± 4

Pupae = 66 ± 6

Puparia = 14 ± 2

Total = 123 ± 5

At an ambient temperature of 11.84 ± 0.30 ^{o}C, the minimum temperature was 11.40 ^{o}C while the maximum was 12.35 ^{o}C for the three replicate experiments with relative humidity of between 77 and 99%. Massiveegg laying, massive hatching of eggs to larvae but the wandering 3rd instar larvae moult into pupae gradually not as observed at higher temperature of 15 ^{o}C and above. It took 18 days from egg lying to pupa and took 28 days from pupa to adult fly. The temperature of 11.84 ± 0.30 ^{o}C is the minimum temperature for the complete development of *C. vicina* under "natural" or conditions, meaning oviposition occurring at the same temperature as subsequent development. Figure 4.2 showing the six stages of pupae obtained at this minimal temperature.



A 159.1 ^{o}D B 206.6 ^{o}D C 229.6 ^{o}D

D 342.3 ^{o}D E 354.4 ^{o}D F 442.3 ^{o}D

Figure 4.2: Pictures showing the six stages of the pupa where *Cryptocephalic pupa* [A] (the head still invaginated), *Phanerocephalic pupa* [B] (complete eversion of the head thoracic appendages), *Pharate adult* [C] (visible segmentation in the thorax, legs and abdomen), *Yellow eyed* [D], *Tanned bristles of thorax* [E], and *Tanned bristles of abdomen* [F] at an ambient temperature of 11.84 ± 0.30 ^{o}C.

4.5 Discussion

A wide range of base temperature at which development of immature stages of *C. vicina* will progress have been reported in the literature or been calculated from the

available data. These base temperatures have been determined or calculated with the help of regression analysis; they have not been measured directly.

Base temperature [˚C]	Authors
1.6	Defilippo *et al.* (2013)
1-1.5	Donovan *et al.* (2006)
2	Vinogradova and Marchenko (1984)
3.5	Davies and Ratcliffe (1994)
5	Greenberg (1991)
5	Diaz Martín *et al.* (2014)
5.3	calculated from data of Chapter 3
6	Greenberg and Kamal according to Higley and Haskell (2000)

In the new edition of Forensic Entomology, The Utility of Arthropods in Legal Investigations, a standard work in the field, Higley and Haskell (2009) provide detailed calculation for the developmental minima based on four data sets of *C. vicina*. Their data suggest that the minimal temperature lies between – 7.71 and – 32.9 ˚C for the development from egg to adult. They also suggest that the developmental temperature changes drastically based on which developmental stage is used for the regression analysis, for example pupae only – 35.7 ˚C, egg-pupa + 4.6 ˚C and egg-adult – 32.9 ˚C. Using the data of Defilippo *et al.* (2013), the following minimal variations were observed, pupae only + 1.7, egg-pupa + 1.6 ˚C and egg-adult + 1.8 ˚C. It was impossible to repeat or verify any of the calculations in the three page long table 10 of that book chapter. Considering that this book might used as a reference, extreme caution should used regarding the data in Table 10.1-3.

The critical step is the experimental set up. Possibly being afraid to cause maternally induced diapause of any of the immature stages, but this has never been stated in any of the publications, all but onegroup have maintained their flies at room temperature.

Overwintering diapause in *C. vicina*

Stage	Reference
Larvae	(Vinogradova, 1986; Saunders, 1987; McWatters and Saunders, 1998; Saunders and Hayward, 1998)
Larvae and pupae	(Graham-Smith, 1916)
Adults	(Vinogradova, 1986; Rognes, 1991; Aak *et al.*, 2011)

At a temperature of 10 ˚C, diapause was induced in 3rd larvae instar. The experiment showed that the normal requirements for inducing diapause such as gradual changes in temperature or gradual changes in photoperiod are not absolutely necessary. Diapause can be induced also under conditions of very abrupt change from room temperature to minimal physiological temperature and without any change in photoperiod.

Experiments to establish the minimal developmental temperature of *C. vicina* have been conducted so far under very artificial experimental conditions.

Authors	Experimental set up
Reiter (1984)	oviposition and eclosion at room temperature
Greenberg (1991)	no material and methods in publication
Davies and Ratcliffe (1994)	oviposition at room temperature
Anderson (2000)	oviposition at room temperature
Ames *et al.* (2006)	oviposition at room temperature
Donovan *et al.* (2006)	oviposition and eclosion at room temperature
Defilippo *et al.* (2013)	oviposition at room temperature
Diaz Martín *et al.* (2014)	oviposition at room temperature

The only exception is the work of Vinogradova and Marchenko (1984) in Russia who over a long time period conducted field experiments. The lowest temperature with

which they worked was 11 ˚C; however, they did not determine whether this was the lowest possible temperature for *C. vicina* under natural condition. Until, no laboratory or field experiments have been carried out to establish the lowest oviposition and developmental time for *C. vicina*. The field work of Vinogradova and Marchenko (1984) is in excellent agreement with the findings of this first study under precisely controlled laboratory condition, which found for a British population of *C. vicina* a minimum temperature of 11.8 ˚C for complete development to the adult stage and a newly established minimum temperature that includes diapausal arrest at the 3rd instar larval stage of 10 ˚C.

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Chapter Five

The midgut bacteria of pupae of the blow fly*Calliphora vicina*

5.1 Abstract

The pupal stage of development of the blow fly *Calliphora vicina* is the stationary and non-feeding stage of the fly. The pupal stage can be longer than all the three larval stages put together. Generally, living things are known to harbour in them bacteria, which they gain from either their nutrition, their environment or through a maternal line. Since the yellow body of the midgut of pupae is formedvfrom the accumulation of the degenerated larval midgut, then the hypothesis is that it should carry in it bacteria. The species of bacteria harboured by the yellow body of the midgut of pupae was investigated using both culture-dependent and culture-independent methods. Fluorescent in situ hybridization (FISH) was used to localize the bacteria on and in the yellow body of the pupal midgut. The results of the culture-independent molecular methodidentified three different genera of bacteria to be dominant:*Providencia* species related to*Providencia heimbachae* (94%), *P. rustigianii* (94%), *P. alcalifaciens* (94%), *P. vermicola* (94%), *P. rettgeri* (94%) and *P. stuartii* (94%);*Proteus* species related to*Proteus mirabilis* (95%), *P. mirabilis* (95%), *P. penneri* (95%), *P. vulgaris* (94%) and *P. myxofaciens* (95%); and *Escherichia* species related to*Escherichia fergusonii* (91%), *Shigella flexneri* (91%), *E. coli* (91%) and *E. albertii* (91 %); the percentage identity is given in parentheses. The culture-dependent method detected only a*Providencia* species complex, which is comprised of*Providencia rustigianii* (100%), *Providencia alcalifaciens* (100%), *Providencia vermicola* (100%), *Providencia heimbachae* (100%), *Providencia rettgeri* (100%) and *Providencia stuartii* (100%); the percentage identity given in parentheses. FISH analysis showed that the bacteria were located within the groove on the yellow body of the midgut of the pupae.

5.2 Introduction

All through the evolution of the class Insecta, bacteria have been a consistent and powerful force in the environment. Insects became successful by making available countless opportunities for the bacteria to occupy niches created by them. The gut of insects provides many of such niches where the insects are able to benefit from the

products of the bacterial metabolism and the adaptability of the prokaryotes(Dillon and Dillon, 2004). In addition to these, insects were found to be amongst the most successful animals, in terms of both diversity and colonization of all ecological niches(Rajagopal, 2009).

The adult blow fly *Calliphora vicina* (Diptera: Calliphoridae) and its immature stages have been found to have its own share of the interaction with bacteria(Greenberg, 1969). The immature stage of the blow fly of focus in this study is the pupae. This is because it takes more than 60% of the immature stages of the blow flies lifecycle and it is stationary, immobile with no feeding habit. All the changes that occur at this stage are mostly internal changes, which include histolysis and histogenesis(Kamal, 1958; Wigglesworth, 1972; Klowden, 2013). The blow fly puparia havea cuticle that is highly chitinous and sclerotized (Davies and Harvey, 2012).

The pupal midgut forms the yellow body and this has been reported in model fly *Drosophila melanogaster* (Diptera: Drosophilidae) (Micchelli and Perrimon, 2006; Micchelli *et al.*, 2011; Takashima *et al.*, 2011)and other insects such as *Hyalophora cecropia* (Lepidoptera: Saturniidae), *Bombyx mori* (Lepidoptera: Bombycidae), *Tomocerus minor* (Entomobryomorpha: Tomoceridae), *Manduca sexta* (Lepidoptera: Tomoceridae), *Galleria mellonela* (Lepidoptera: Pyralidae), *bees* (Hymenoptera: Apidae), *Heliothis virescens* (Lepidoptera: Noctuidae)(Judy and Gilbert, 1970; Waku and Sumimoto, 1971; Humbert, 1979; Uwo *et al.*, 2002; Tettamanti *et al.*, 2007). It was first identify in blow fly by Lowne (Lowne, 1890-1892) without any explanation on what it is made up of and it importance to the blow fly immature stage.

The gut of any insect, whether it be adult or immature stage, carries in it diverse microbial communities since it is in an active feeding stage. Although the microbial communities might differ due differences in feeding habit, but in most cases plays its own function. These bacteria colonizing a particular gut depend on the variety of distinct structures the gut possesses and depends on other conditions too such as pH, redox conditions, digestive enzymes present and type of food ingested. It has also been observed that those species of insects with simple and straight digestive tract might possess less diverse microorganisms, compared to the ones with more complex

structures like paunches, diverticula and caeca do have more diverse mutualistic microorganisms(Bignell, 1984; Tanada and Kaya, 1993; Dillon and Dillon, 2004). Another parameter that has been used to distinguish the amount of microorganisms a species of insect gut could have, was by looking at the insect feeding state, where those insects that feed regularly could have more diverse microorganisms than those insects that has not feed for many days (Dillon, Dillon 2004).

The bacterial diversity of the midgut has been studied of several fly species. *Phlebotomus duboscqi* (Diptera: Psychodidae) the midgut of fourth instar larvae just before pupation, pupae and newly emerged adult females carry the bacterium*Ochrobactrum* sp.(Volf *et al.*, 2002), the bacteria isolates of the screw-worm *Cochliomyia macellaria* (F.) (Diptera: Calliphoridae) contained*Providencia* sp., *Escherichiacoli* O157:H7, *Enterococcus faecalis* and *Ochrobactrum* sp.(Ahmad *et al.*, 2006), in females of *Lutzomyia* sand fly species, plant-related bacteria were found including *Erwinia* sp. and putative *Ralstonia* sp.(Sant'Anna *et al.*, 2012).

The microorganisms in the gut of insects have the ability to immediately accommodate any changes in the insect diet by the introduction of enzymes and population changes in the microbial community (Kaufman and Klug, 1991; Santo Domingo *et al.*, 1998). A typical example is when cockroaches change their diet to low-protein and high-fibre content, their foregut microbial community *Streptococci* and *Lactobacilli* decrease in number thereby leading to decrease in the production of lactate and acetate(Kane and Breznak, 1991). Other characteristics the microorganisms have are them possessing metabolic properties not present in insects they occupy and serving as "microbial brokers" making it possible for plant-feeding insects to overcome biochemical barriers to herbivory(Jones, 1984; Douglas, 2013).

In this study the hypothesis was tested that if the yellow body of midgut of pupae is an accumulation of degenerated larval gut then it carries in it bacteria that were present in the larval gut. The work was arranged to achieve the following objectives: (1) dissect the pupae for the yellow body; (2) to detect bacteria by squashing the yellow body on slide and cover slip; (3) identify the bacteria by culture independent method and (4) identify the bacteria by culture dependent method.

5.3 Material and Methods

5.3.1 Rearing of *Calliphora vicina*

Adult blow fly *Calliphora vicna* Robineau-Desvoidy 1830 were already available in a wooden breeding box (Bangor University) in a temperature control room. Eggs were obtained by putting approximately 5 g of pig liver (bought from Morrison store) on a Petri dish (Sterilin, UK) and placed it in the breeding box with the adult blow flies. The adult blow flies feeds on sugar and do require protein to lay eggs. While feeding on the pig liver, they also lay their eggs on it. The Petri dish was left there for approximately 3 hours. Then removed and placed in a rectangular rubber basin which has sawdust in it. After 24 hours the eggs hatched to give 1st instar larvae, although dependent on the ambient temperature. After 20 hours the 1st instar larvae moult into 2nd instar larvae and in another 24 hours it moults into 3rd instar larvae. At the 3rd instar larvae they have the active feeding stage where they take in enough food that will take them through the remaining stage of the life cycle and also wandering stage where they moved away from the food source (pig liver). Within the 3rd instar larvae food source was monitored, to avoid premature wandering, in the case where their food finishes. It was monitored carefully to make sure they have enough food (pig liver). From the 3rd instar larvae moult to pupae, where they remain for a longer time than the time for the three instars larvae all together. After that they moult to give adult fly. All through the life cycle temperature and humidity of the room were noted. The average temperature and relative humidity recorded are 15.8 ± 0.7 ^{o}C and 93 ± 7 %, respectively. From 1st instar larvae to the 3rd instar larvae takes about 6-11 days, though dependent on the ambient temperature while the pupae stage which is the dormant stage takes between 7-14 days to give adult blow fly.

5.3.2 Sampling collection

Samples were collected from the eggs to help in determining the mode of transmission of the bacteria. Subsequently, samples of pupae were collected after every 24 hours for dissection until the adult fly emerges. For the samples were collected and taken for dissection directly without preservation or placing in any chemical. If placed in alcohol for instance, during dissection no organ was visible and everything was a mesh (Saloña Bordas, personal communication).

5.3.3 Dissection of pupae

Dissection was carried out in PBS (phosphate buffered saline) of pH 7.4, under the microscope (Leica, Zoom 2000, USA). This is to prevent the tissue from drying during the process of dissection and which might lead to breakage. The dissection was to remove the yellow body that harbours the bacteria and straight to DNA extraction.

5.3.4 Staining of yellow body

The dissected yellow body was quashed on glass slide with coverslip. Fixed in 100 % ethanol and allowed to evaporate. Then mount with 90 % glycerol (SIGMA-ALDRICH, Batch No. 03336CB, USA) in PBS and PI. The slides were viewed using Confocal Laser Scanning Microscope.

5.3.5 DNA extraction

The DNA extraction was carried out using the Qiagen® Blood and Tissue extraction kit (Qiagen® Germany, Cat. No. 69506) based on the manufacturer's instructions. Samples were incubated in Proteinase K Solution (Promega, Germany, Cat. No. 71049-3) for a minimum two (2) hours (Braig, personal communication).

5.3.6 Primer design

Universal bacterial and archaeal primers were selected and modified and send for synthesis, using eurofins mwg operon and SIGMA.

Bacteria

I

F27 Modified – 1391R Modified primer pair

F27M 16S UB 5' **W**GA **S**TT TGA T**YM** TGG CTC AG 3'

Eubacteria, primer design (Lane, 1991) and modified (Thomas *et al.*, 2011).

1391RM 16S 5' GAC GGG C**Y**G **W**GT GT**R** CA 3'

RC TG**Y** ACA C**W**C **R**GC CCG TC

Universal primer design (Lane *et al.*, 1985) and modified (Marchesi *et al.*, 1998; Turner *et al.*, 1999) and Braig, unpublished. The non-modified primer combination was recently used in the human oral microbiome (Bik *et al.*, 2010).

Annealing temperature: 55 ^{0}C (Bik *et al.*, 2010).

II

HDA1 M (338-358) – 1350R (1407-1389) primer pair

338FM 16S UB 5' AC**W** CCT ACG GG**W** GGC **W**GC AGT 3'

Eubacterial primer, primer design (Walter *et al.*, 2000) and modified (Thomas *et al.*, 2011).

1350R 16S UB 5' GAC GGG CGG TGT GTA CAA G 3'

Eubacterial primer, primer design(Lane, 1991).

This primer combination has the highest coverage of eubacteria diversity according to(Thomas *et al.*, 2011).

Annealing temperature: 56 ^{0}C(Walter *et al.*, 2000).

Archaea

Archaeal endosymbionts are rare and mainly found in termites, ruminants and humans (Moissl-Eichinger and Huber, 2011).

I

A571F – UA1204R primer pair

A571FM 16S UA 5' GC**Y** TAA AG**SRIY** CGT AGC 3'

Archaeal primer, primer design (Baker and Smith, 2003)modified(Thomas *et al.*, 2011).

UA1204R 16S UA 5' TT**M** GGG GCA T**R**C **IK**A CCT 3'

Archaeal primer, primer design (Baker and Smith, 2003).

Annealing temperature: 55 ^{o}C (Baker and Smith, 2003).

II

A751F – UA1406R primer pair

A751F 16S UA 5' CCG ACG GTG AG**R** G**RY** GAA 3'

Archaeal primer, primer design (Baker and Smith, 2003)

UA1406R 16S UA 5' ACG GGC GGT G**W**G T**R**C AA 3'

Universal primer, primer design (Baker and Smith, 2003).

Annealing temperature: 55 ^{o}C (Baker and Smith, 2003).

5.3.7 PCR

Reaction Mix for eubacteria and archaea. The standard 1X PCR reaction mix. The conventional PCR was carried out using a Master cycler gradient (Eppendorf, Hamburg, Germany). A single reaction contained 5 X Green GoTaq® Flexi buffer (Promega, USA), 25 mM MgCl2 (Promega, USA), 200 µM dNTPs, 5 u/ul of GoTaq® DNA Polymerase (Promega, USA), 300 nM of Forward Primer (euro fins MWG Operon, UK), 300 nM of Reverse Primer (Euro fins MWG Operon, UK), DNA template of 20 nM to 50 nM were used and water Molecular Biology Grade (MELFORD, Bat. No. D23566, UK).

PCR Procedure for eubacteria and archaea. These were the times and temperatures used for the PCR: The protocol consist of an initial denaturing for 3 minutes at 94 ^{o}C, followed by 60 seconds at 94 ^{o}C (denaturing), 30 seconds at 56 ^{o}C (annealing) and 2 minutes at 72 ^{o}C (extension), repeated from the second step for 35 cycles. After which it undergoes a final extension of 10 minutes at 72 ^{o}C and hold at 4 ^{o}C.

Run the PCR for positive control archaea. These were the times and temperatures used for the PCR: The protocol consist of an initial denaturing for 5 minutes at 95 ^{o}C, followed by 30 seconds at 95 ^{o}C (denaturing), 30 seconds at 62 ^{o}C (annealing) and 1 minute 30 seconds at 72 ^{o}C (extension), repeat from the second step

for 30 cycles. After which it underwent a final extension for 10 minutes at 72 ^{o}C and hold at 4 ^{o}C.

5.3.8 Agarose gel electrophoresis

The gel was prepared with 1 g of Agarose Molecular Biology Grade (Melford, UK, Batch No. D23374) in conical flask of 500 ml with 100 ml of 1X TAE. After heating the mixture in autoclave, 1 µl of ethidium bromide Molecular Biology Grade (Promega, USA, Cat. # H5041) was added. After conducting the PCR reaction, 5.0 µL was taken out of the reaction mixture and run on an agarose gel with a 1 kb DNA ladder (Promega, USA, G571A 24724304) to check the success of the reaction.

5.3.9 Purification of PCR product

The desired bands were excise from the agarose gel with a clean, sharp scalpel and purified using the Qiagen QIAquick Gel Extraction Kit (Qiagen® Germany, Cat. No. 28704).

5.3.10 Cloning

Preparing the PCR Product.

The purified fragment was diluted 10:1.

Ligating the insert.

The ligation mixture was prepared by combining (in order) the following components:

3 µl StrataClone Cloning Buffer

2 µl of PCR product (5-50 ng, typically a dilution of a robust PCR reaction)

1 µl StrataClone Vector Mix amp/kan

The mixture above was mixed gently by turning the tip of the pipette and then incubates the ligation reaction at room temperature for 5 minutes. After incubation was completed the reaction was returned to ice.

Transforming the Competent cells.

One StrataClone Solo Pack competent cells were thaw on ice for each ligation reaction. 1 µl of the cloning reaction mixture was added to the tube of thawed competent cells, was mixed gently (without pipetting).

The transformation mixture was incubated on ice for 20 minutes. During the incubation period, LB broth medium was pre-warm at 42 ^{o}C.

The transformation mixture was then heat-shock at 42 ^{o}C for 45 seconds i.e. the mixture was taken from ice to heating block (Techne, DRI-BLOCK, DB.2A, USA) at 42 ^{o}C for 45 seconds then back to ice.

The transformation mixture was again incubated on ice for 2 minutes.

The pre-warmed LB broth medium, 250 µl was added to the transformation reaction mixture. The competent cells were allowed to recover for 2 hours at 37 ^{o}C with agitation. The tubes of the cells were laid horizontally on the incubator with shaker (INFORS HT, Multitron standard, Switzerland) for better aeration.

During the outgrowth period, LB agar – ampicillin plates were prepared for blue-white colour screening by spreading 40 µl of 2% X-gal on each plate.

150 µl of the transformation mixture was spread on the colour-screening plates and incubated overnight at 37 ^{o}C in an incubator (Binder, USA).

Confirmation of Clones.

PCR eppendorfs tubes were prepared (ten for each ligation reaction) with 3 µl of nanopure H_2O and falcons tubes (also ten for each ligation reaction) with 2 ml of LB broth (with ampicillin).

White colonies were picked with using pipette tips. Microscope was used to be sure of what colony one pick.

After colonies were picked, plates return to fridge.

The falcons were incubated overnight at 37 ^{o}C with vigorous shaking (~250/300 rpm).

To each of the PCR eppendorf tubes of 0.2 µl of each primer (T3/T7) and 5 µl of Master Mix added.

PCR runs with the following program. These were the times and temperatures used for the PCR: The protocol consist of an initial denaturing for 5 minutes at 96 ^{o}C, followed by 60 seconds at 96 ^{o}C (denaturing), 30 seconds at 55 ^{o}C (annealing) and 1 minute at 72 ^{o}C (extension), repeat from the second step for 35 cycles. After which it undergoes a final extension of 7 minutes at 72 ^{o}C and hold at 4 ^{o}C.

Agarose gel was run to confirm sample with the right insert.After confirmation and after overnight incubation, falcons were removed and centrifuge at 6000 rpm for two (2) minutes.

Purification of plasmids

Plasmid mini kit was used for the purification of plasmids.

5.3.11 DNA sequencing

Direct Sequencing of PCR products were carried out first. Later clones samples were taken for sequencing. Each sample sends for sequencing were with both the forward and reverse primers separately. All sequencing was carried out by Macrogen Europe.

5.3.12 Bioinformatics

The NCBI software was used for blasting the both the forward and reverse sequence for each PCR product. The software Geneious 5.6 was used to create a consensus sequence which was later blast again in NCBI. Primer premier 6.1.2 was used to design primers and specific probes for FISH. RNAfold was used to locate where the fragment design by Primer Premier for specific primers are on the RNA 3D (three dimensional) structure, to help for probe design. Bio edit was also employed.

5.3.13 Fluorescent inn situ hybridization

Fixation

Collected the pupae on petri dish, dissected to remove the yellow body, fixed in 75% - 100% ethanol 2 – 3 hours (Use pure ethanol not methylated), removed the ethanol, placed the specimen in 50 % Xylol and 50 % ethanol (This is a clearing agent, prepared

50 % xylol should be on ice before use). Kept in fridge overnight, washed 3 X with 100 % ethanol (For every washing allow to stay in ethanol for 30 minutes before the next wash), placed the specimen in 100% ethanol for 1 hour, removed the ethanol, added 80 % acetone to specimen and keep in freezer for 30 minutes (acetone be prepared and keep in freezer before use), removed acetone, washed 3 X with 100 % ethanol, removed ethanol, added PBST to the specimen and leave for 45 minutes, removed PBST.

Hybridization.

Working in darkness, added hybridization buffer (HB) and leave for 30 minutes, removed HB, added HB + probes and incubate on heating block covered with foil paper at 46 ^{o}C overnight, removed HB + probes (can be kept for further work in micro centrifuge tube rapped with foil paper and stored in fridge, it can last for about 2 days)

Washing:

Added large amount of HB about 1 ml to wash the specimen, place back on the heating block and leave for 45 minutes, removed HB, added HB again, place in a test rack for 45 minutes, removed HB, added PBST leave for 20 minutes.

Detection:

Added 50% PBS and 50% glycerol with PI to mount the specimen on slide with cover slip, viewing with confocal laser scanning microscopy.

5.3.14 Culturing of bacteria

The dissected yellow bodies from the pupae were squashed in PBS in a 1.5 ml test tube and pour plate on LB agar, incubated at 37 ^{o}C overnight. Using microscope to pick distinct colonies and sub-culture into two different plates. Each of those two plates was sub-culture again four (4) times to obtain a pure culture. Thereafter the colony obtained were inoculated into LB broth and incubated at 37 ^{o}C overnight in an incubator with shaker. The bacteria were harvested and DNA extracted using the Qiagen® Blood and Tissue extraction kit (Qiagen® Germany, Cat. No. 69506) based on the manufacturer's instructions. PCR was run and send for sequencing for the both primers. The sequences were blast in NCBI andGeneious was also used to create

consensus sequence, which were later blast. The above was repeated using LAB M Tryptone soy agar (U.S.P) (England).

5.4 Results

5.4.1 Yellow body

The yellow body of a *Calliphora vicina*pupa was dissected out, Figure 5.1.

Figure 5.1: The yellow body on day six (6) of pupa development at a temperature of 15.8 ± 0.7 ^{o}C and relative humidity of 93 ± 7 %.

The yellow body in Figure 5.1 was squashed and stained with propidium iodide, Figure 5.2.

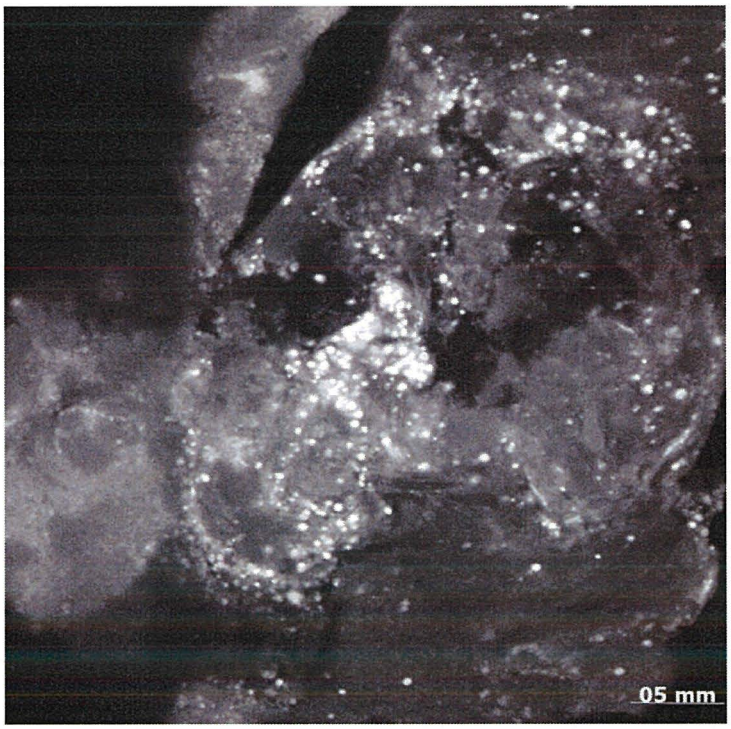

Figure 5.2: The picture of a squashed yellow body showing bacteria present viewed with Confocal Laser Scanning Microscopy (CLSM 510, Zen 2009) at Plan-Neofluar 40X/1.3OilDIC.

The electrophoretic gel in Figure 5.3 shows the presence of eubacteria and absence of archaea in the yellow body.

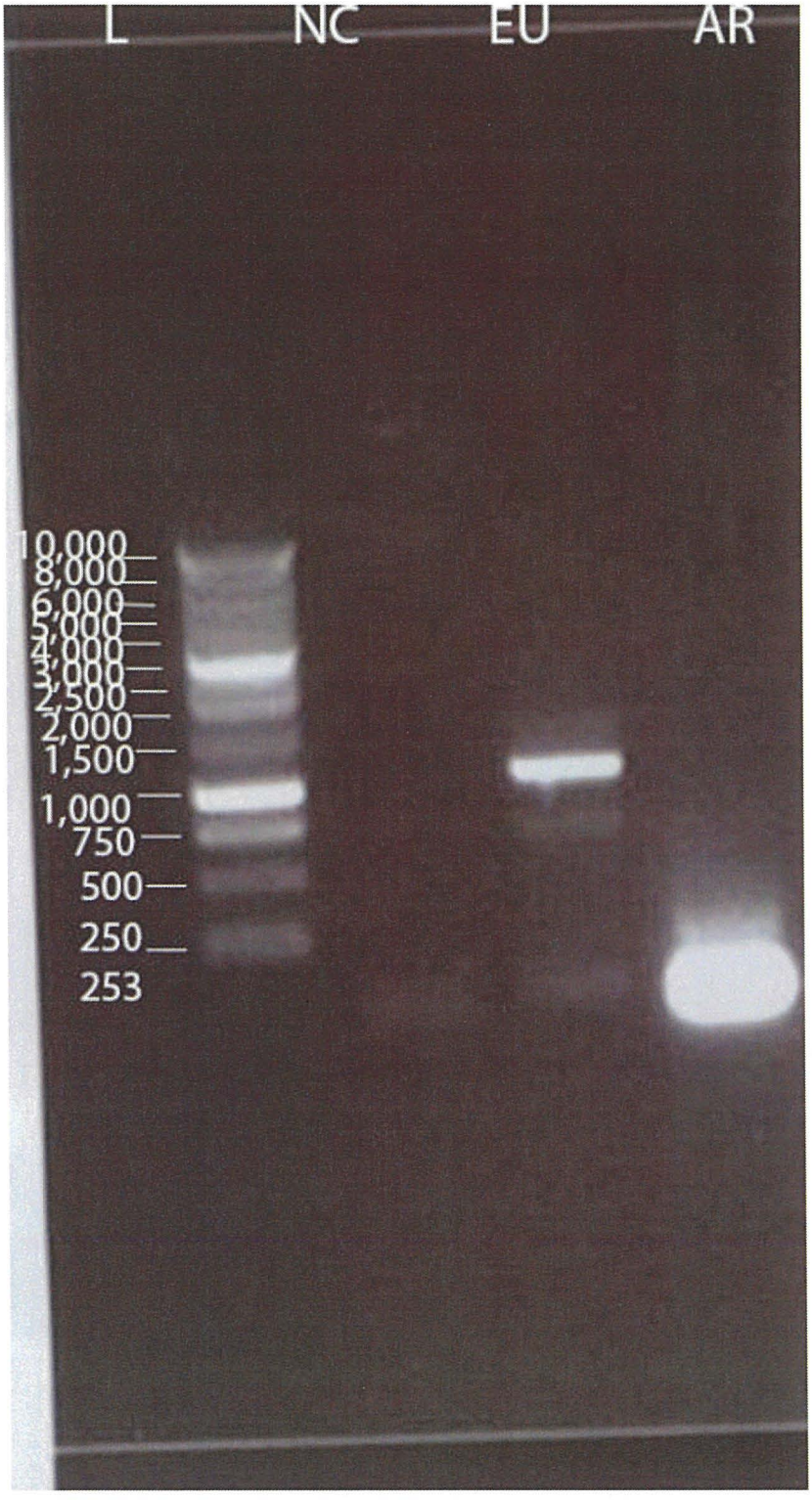

Figure 5.3: Showing band for eubacteria and with no band for archaea, where L = Ladder (1 kb ladder Promega), NC = negative control, EU = Eubacteria with universal primer (338FM 16S UB & 1350R 16S UB) and AR = Archaea with universal primer (A751F 16S UA & UA1406R 16S UA), 1% agarose

This was further confirmed by using positive archaea samples with the archaea universal primer to show the primers are working. Figure 5.4 shows that archaea universal primers are working further confirming the absence of archaea in the yellow body.

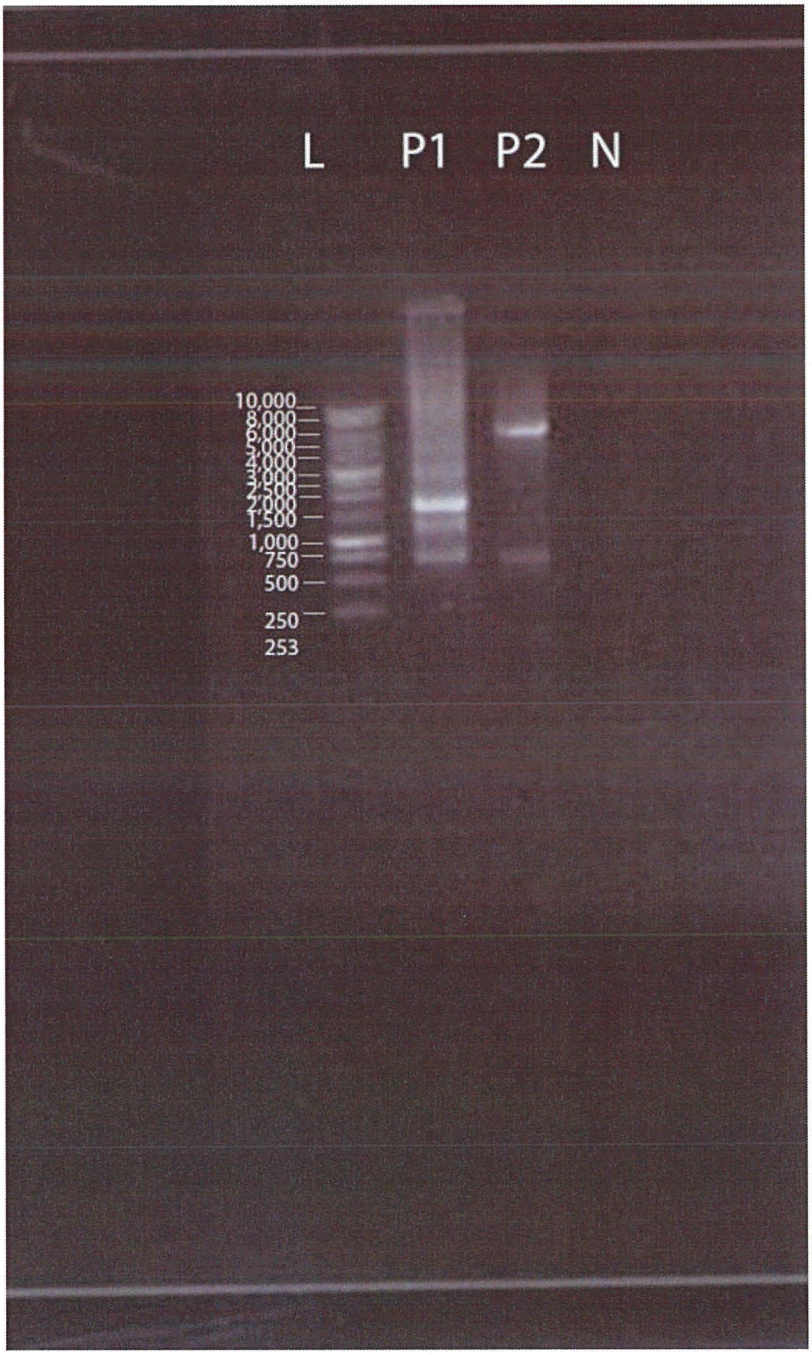

Figure 5.4: Showing bands for positive archaea PCR product using the archaea universal primers, where L = ladder (1 kb ladder Promega), P1 = Positive archaea with universal primer (A751F 16S UA & 1406R 16S UA), P2 = Positive archaea with universal primer (A571FM 16S UA & UA1204R 16S UA) and N = Negative control, 1% agarose

5.4.2 Bacteria on yellow body

From the results obtained it could be said that the bacteria were found within a cavity of the yellow body of the midgut and some could be found scattered on the surface of the yellow body of the midgut, though in an organized manner, Figure 5.5. It was also observed that they were not found all over the yellow body and nothing was seen on the transient midgut or the developed body from the regenerative cells.

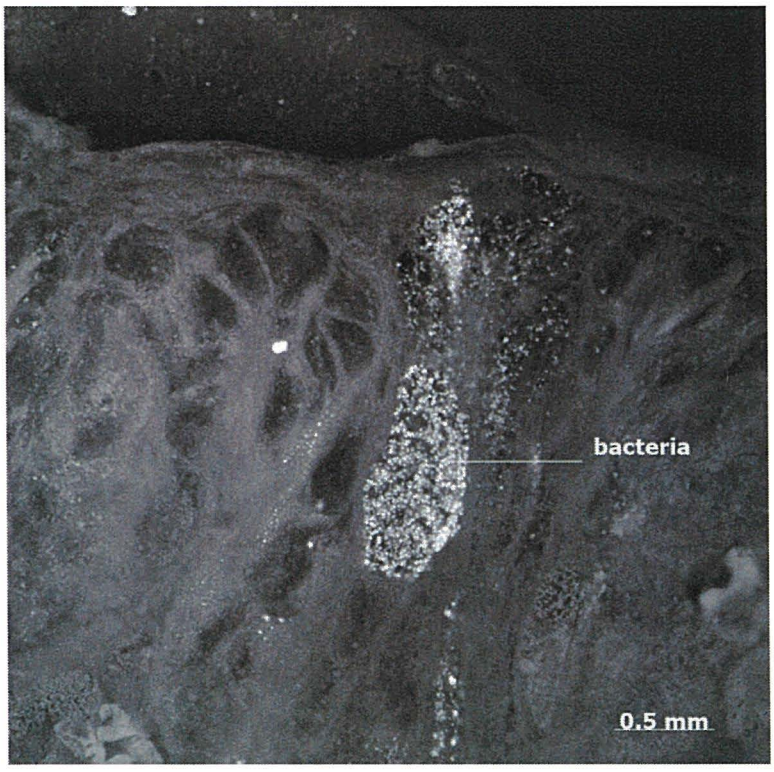

Figure 5.5: The above FISH picture shows the appearance of bacteria on the yellow body using universal probes, viewed with Confocal Laser Scanning Microscope (CLSM 510, Zen 2009) at Plan-Neofluar 40X/1.3OilDIC.

4.4.3 Molecular identification of bacteria

The results of the culture-independent molecular method detect three different genera of bacteria to be dominant: *Providencia* species realted to *Providencia heimbachae* (94 %), *P. rustigianii* (94 %), *P. alcalifaciens* (94 %), *P. vermicola* (94 %), *P. rettgeri* (94 %) and *P. stuartii* (94 %), *Proteus* species realted to *Proteus mirabilis* (95 %), *P. mirabilis* (95 %), *P. penneri* (95 %), *P. vulgaris* (94 %) and *P. myxofaciens* (95 %) and *Escherichia* species related to *Escherichia fergusonii* (91 %), *Shigella flexneri* (91 %), *E. coli* (91 %) and *E. albertii* (91 %); the percentage identity is given in parentheses.

The culture-dependent method detected only a *Providencia* species complex, which is comprised of *Providencia rustigianii* (100 %), *Providencia alcalifaciens* (100 %), *Providencia vermicola* (100 %), *Providencia heimbachae* (100 %), *Providencia rettgeri* (100 %) and *Providencia stuartii* (100 %); the percentage identity given in parentheses.

5.5 Discussion

In this study, both culture-dependent and culture-independent methods were used to identify the microbial community in the yellow body (midgut epithelium) of pupae of blow fly *Calliphora vicina* Robineau-Desvoidy 1830. This study demonstrated that the microbial community of the pupae yellow body is similar to the once found in the larvae gut. This is an indication that the yellow body, which is the degenerated larval gut, carries in it the bacteria that were in the larvae and transfer same to the pupae in that form. Although the microbial community found in this study is not very large, even with the both methods complementing each other. Because it has to be recognize that no complex microbial community has ever been completely sampled and thus might have low estimates. A fly having high species richness and diversity of microbial community tells a lot about it in terms of feeding and reproduction behaviours (Graczyk *et al.*, 2001; Gupta *et al.*, 2012)

The results revealed that the bacteria are of Proteobacteria and mainly of the class Gammaproteobacteria, which support other studies on microbial community in other species of arthropods such as honey bee, *Apis mellifera*; the deer tick, *Ixodes*

scapularis; the orbivirus vector, *Culicoides sonorensis*; the gypsy moth *Lymantria dispar* L.; wild *Culex quinquefasciatus* mosquito midgut; the ant lion, *Myrmeleon mobilis*; bee species (*Apoidea*); *Drosophila melanogaster* and the gut of house flies, *Musca domestica* L. (Jeyaprakash *et al.*, 2003; Benson *et al.*, 2004; Broderick *et al.*, 2004; Campbell *et al.*, 2004; Pidiyar *et al.*, 2004; Dunn and Stabb, 2005; Mohr and Tebbe, 2006; Corby-Harris *et al.*, 2007; Cox and Gilmore, 2007; Gupta *et al.*, 2012).

The results of the culture-independent method of identifying bacteria demonstrate that there three dominant species of bacteria present in the yellow body and they are of the class Gammaproteobacteria. A study has shown that Gammaproteobacteria are the most diverse group of bacteria isolated from flies (Gupta *et al.*, 2012). The three dominant species of bacteria are first *Providenciaspecies*: *Providencia heimbachae*, *Providencia rustigianii*, *Providencia alcalifaciens*, *Providencia vermicola*, *Providencia rettgeri* and *Providencia stuartii*, while the second is *Proteusspecies*: *Proteus mirabilis*, *Proteus mirabilis*, *Proteus penneri*, *Proteus vulgaris* and *Proteus myxofaciens,* and the third is *Escherichia species*: *Escherichia fergusonii*, *Shigella flexneri*, *Escherichia coli* and *Escherichia albertii*. Several other studies have shown that the present of these species of bacteria in the gut of either immature stage such as larvae or adult of the fly. In house flies *Musca domestica* (Diptera: Muscidae) *Providencia species*, *Proteus species* and *M. morganii* were isolated with *Providencia* and *Proteus* been the dominant culture species(Gupta *et al.*, 2012). For the screwworm *Cochliomyia macellaria* (Diptera: Calliphoridae) the isolated species of bacteria were of four species: *Providencia species*, *Escherichia coli* O157:H7 (Escherich), *Enterococcus faecalis* (Orla-Jensen) and *Ochrobactrum species* (Holmes)(Ahmad *et al.*, 2006).

However, the results of the culture dependent method only show the present of one species out of the three species identified using the culture independent method. This species is Providencia species: *Providencia rustigianii*, *Providencia alcalifaciens*, *Providencia vermicola*, *Providencia heimbachae*, *Providencia rettgeri* and *Providencia stuartii*. The other two species could have mutated to the extent that they have becomes endosymbionts so do not have the capability to grow on culture media.

Study has demonstrated that *Proteus mirabilis* is an endosymbiont of screwworms and of several blow fly species(Erdmann, 1987).

In this study the bacterial composition of yellow body of the transitional midgut in the pupae was investigated. It carries in them bacteria which are similar to the once that have been seen in newly emerged house flies and screwworms. In the screwworm *Cochliomyia macellaria* (Diptera: Calliphoridae) bacteria were found in the gut of newly emerged adults, that is an indication that these bacteria in the gut of the larvae can survive pupation and colonize the gut of the adult flies(Ahmad *et al.*, 2006). For the house fly, live bacteria such as *M. morganii*, *Providencia* spp., and *Proteus* spp. were identified in the gut of newly emerged adults, which shows that these bacteria found in the larval have the capability to withstand the pH level of the pupa midgut and recolonize the adult gut(Su *et al.*, 2010). This study clearly identifies the tissue that harbours these bacteria during the pupa stage of metamorphosis of blow fly *Calliphora vicina*.

The pH of midgut of blow fly and housefly has been determined to be acidic within the range of 2-3, this simply means only few bacteria could withstand such condition. One of such bacteria that have been studied is *Proteus mirabilis,* which has a sterilizing effect because of its ability to produce antibacterial agents such as phenyacetic acid (PAA) and phenyacetaldehyde (PAL). The bacteria used it to inhibit the multiplication of many other types of Gram-positive and Gram-negative bacteria(Greenberg and Klowden, 1972; Erdmann, 1987).

The biological function of these bacteria was not addressed in this study, but suggestions have been made that they could be providing an additional protection for their host against pathogenic microorganisms and other allochthonous microbes. This has been observed on *Proteus mirabilis* where it has been found to suppress the growth of *Salmonella typhimurium* and *Pseudomonas aeruginosa* under laboratory conditions (Su et al. 2010, Greenberg, Klowden 1972). Another study with the same *Proteus mirabilis* in blow fly *Lucilia sericata* shows that the two aromatic compounds produce by this bacterium is responsible for the suppression against the other microorganisms. It was also point out that for bacteria that were found in the larval

midgut and were later found in the newly emerged adult fly could be that they are not digestible and contribute almost nothing to the larval development and survival(Greenberg and Klowden, 1972; Erdmann, 1987; Su *et al.*, 2010).

This study has shown that the translucent yellow body is the degenerated larval gut for which it carries in it bacteria that were detected in the larval gut. It shows that some of the bacteria even when it does withstand pupation could still grow on culture media for example the *Providencia species*. While some of the bacteria could not grow on culture media for instance the *Proteus species*. This goes with other what other studies has reported that *Proteus mirabilis* is an endosymbiont of blow fly and screw worm.

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Chapter Six

Bacteria on thesurface of *Calliphora vicina* eggsand the midgut of first and second instar larvae

6.1 Abstract

In insects, the female deposits its eggs in an environment that is optimal for the development of the larvae. For blow fly species (Calliphoridae), the larvae feed on decomposing animal carcasses. However, the mouthparts of first instar larvae are so small that they cannot feed on animal tissue. The external surfaces of all living organisms are covered by bacteria and fungi. To investigate whether or not bacteria are a likely food source of first instar larvae of *Calliphora vicina*, the bacteria on the surface of eggs and in the midgut of first and second instar larvae were compared molecularly with culture-independent methods. Bacteria were localised with the help of fluorescent in situ hybridisation (FISH). The bacteria found on the surface of the eggs were*Sphingomonas* species closely related to: *Sphingomonas dokdonensis*, *S. mucosissima*, *S. pituitosa*, *S. koreenis* and *S. truperi*. In the midgut of first instar larvae, the same species of bacteria were detected as on the surface of the eggs, but a different set of bacterial species were found in the gut of second instar larvae; these were *Providencia* species closely related to: *Providencia heimbachae*, *P. rustigianii*, *P. alcalifaciens*, *P. vermicola* and *P. stuartii*.

In search for where these bacteria could have come from, the species of bacteria that the adult female blow fly carries on its legs, mouthpart and abdomen were investigated, but the bacteria detected were of the *Escherichia* and *Shigella* species, which were closely related to: *Escherichia fergusonii*, *Shigella flexneri*, *S. boydii*, *E. coli* and *E. alberti*.

The bacteria on the surface of eggs of laboratory-reared flies were compared with wild-caught flies. The same species of *Sphingomonas*were identified in free-living blow flies and in a laboratory colony that has been inbred for at least ten years.

6.2 Introduction

Generally, living things are found with different behaviours that suit and benefit them in a way; such behaviour could be their feeding habit, reaction to opposite sex or where they lay their eggs in case of birds, insects and other animals. Looking at insects

to be specific on where they lay their eggs. Studies have shown that insects select their sites for laying eggs. This does happen because for stable development of a fly it does depend on a live microbial community in the natural habitat and this is demonstrated by the way female flies are able to select an oviposition site, depending on the microbial derived stimuli that shows the place has the required substrate for proper larval development(Leroy *et al.*, 2011). This has been shown in other fly species (Nordlund and Lewis, 1976; Martinez *et al.*, 1994; Knols *et al.*, 2004; Ma *et al.*, 2012; Mukhopadhyay *et al.*, 2012), but blow fly *Calliphora vicina* could not have been an exception in this behaviour. With the importance of blow fly to forensic entomologist and to biological sciences at large, the investigation of the type of bacteria on the eggs surface is of utmost importance in this study.

The bacteria on the surface of the eggs could either come from any part of the fly (gut, mouthpart etc.) or might be found on the oviposition site of the fly. But of what importance are these bacteria to the immature stage of the fly? These bacteria could serve as food or have any kind of interaction with the fly that will improve its development. It is very crucial to know what the first instar larvae of the blow fly feed on, for this is a crucial stage in the development of the fly. If anything happens to that stage then development of the fly will be terminated. The bacteria found on the surface of the eggs in this study are *Sphingomonas* species (*Sphingomonadaceae, Alphaproteobacteria*). Where these bacteria come from will be investigated to know if it does come from any part of the female fly or from where the eggs are laid. Furthermore, it is known that both insects and bacteria emit chemical substances that help direct insects to their oviposition sites. These chemical substances are called semiochemical and they do induce behavioural or physiological responses in other individual(Heuskin *et al.*, 2011).

A semiochemical (semeon in Greek which means signal) is a common term used for a chemical substance or a combination of chemical substances that carries a message. They served as messengers within or between species. These compounds are grouped into two: the intraspecific (pheromones) or interspecific (allelochemicals) mediators. Nevertheless, a single chemical signal may perform the function of both

pheromone and allelochemical. The pheromones are further classified into sex pheromones, aggregation, alarm, trail, host marking pheromones, while the allelochemicals are also classified into allomones positive emitter, kairomones positive receptor, synomones positive emitter and receptor and apneumones(Schulz and Dickschat, 2007; Klein and Roberts, 2010; Leroy *et al.*, 2011).

These semiochemical compounds, which are volatile in nature, have bacteria involved in their production. And insects on another hand were reported to respond to specific volatiles compounds emitted by specific bacteria which could either be hosted by the insect itself (e.g. gut, mouthparts etc.) or are found within the natural environment where the insects evolves. The commonest families of bacteria that interact well with insects in this respect are the *Enterobacteriaceae*(*Gammaproteo-bacteria*), *Pseudomonadaceae*(*Gammaproteobacteria*) and *Bacillaceae* (*Firmicutes*).These bacteria were found to have the ability to modify products like fatty acids derivatives, aromatic compounds such as aromatic amino acids (L-phenylalanine, L-tyrosine and L-tryptophan), pyrazines, (sesqui) terpenes, sulphur compounds or carbohydrates through the shikimate pathway. Another report shows a collection of 300 volatile compounds from different bacteria, which prove that 75 are from fatty acids derivatives, 50 from aromatic compounds, 74 from nitrogen-containing compounds, 30 from sulphur compounds and 18 from halogenated compounds(Schulz and Dickschat, 2007; Klein and Roberts, 2010; Leroy *et al.*, 2011).

Different insects have their different preferred habit for laying of eggs: some mosquitoes preferred to lay their eggs on unmodified substrates from natural larval habitats containing live microorganisms(Knols *et al.*, 2004), while female houseflies, *Musca domestica* (Diptera: Muscidae) prefer to lay eggs in ephemeral resources such as animal manure(Lam *et al.*, 2007; Lam *et al.*, 2009). For *Calliphora vicina* that oviposit on rotten garbage and carrion could have particular microorganisms that motivate them. Although this study is not dealing with rotten garbage or carrion but using pig liver for the blow flies to have enough protein to lay their eggs and investigate the type bacteria on the eggs. In general, bacteria are found to appear in what habitat an insect will prefer to lay its eggs.

A number of studies have shown that bacteria at the oviposition site of insects served different functions for the newly emerging larvae. The following studies shows how the presence of bacteria at the oviposition site affect different insects; that bacteria are essential dietary constituents for larvae of housefly(Schmidtmann and Martin, 1992; Rochon *et al.*, 2004), for face fly larvae the presence of bacteria in bovine faeces was essential for normal growth and development(Hollis *et al.*, 1985); for sand flies the bacterial community of the larval habitat affects oviposition and larval development, however,it was shown not to be essential for successful development of *L. longipalpis*(Peterkova-Koci *et al.*, 2012); for *Cochliomyia macellaria* (Diptera: Calliphoridae) the presence of bacteria do not have much effect on the development of the larvae, for they believe that some bacterial isolates negatively impact larval development (Ahmad *et al.*, 2006). In the gut of females of the Old World sand fly species *Phlebotomus paptasi*, three species of bacteria were identified that were implicated in inducing oviposition behaviour: *Bacillus licheniformis*, *B. pumilus*(*Bacillaceae*), and *Staphylococcus saprophyticus* (*Staphylococcaceae*), all belonging to the phylum *Firmicutes*(Mukhopadhyay *et al.*, 2012).

In this study the hypotheses were tested that: (1) flies select site for oviposition that carries the microbial community for the survival of its larvae; (2) the mouthparts of the first instar are so small that they cannot feed on the food source (pig liver) rather the larvae feed on bacteria on the surface of the egg. The work was organized to achieve the following aims and objectives: (1) molecularly identify the type of bacteria on the surface of blow fly egg; (2) molecularly identify and locate the type of bacteria in the gut of first instar larvae; (3) molecularly identify the type of bacteria in the gut of second instar larvae; (4) compare the bacteria on the surface of eggs in the laboratory and the wild type and (5) molecularly identify and locate the type bacteria at the legs, mouthpart and abdomen of an adult female blow fly.

6.3 Material and Methods

6.3.1 Sample collection

The adult blow fly *Calliphora vicina* were reared in wooden breeding box in a temperature control room. The eggs were collected by placing pig liver in a petri dish and subsequently placed into the wooden breeding box where the flies were being reared. Then eggs were picked with clean spatula into a test tube with PBS then taken for DNA extraction.

Adult female blow fly *Calliphora vicina* were collected from the wooden breeding box, where the flies were being reared. Their legs, mouth and the abdominal parts were removed and collected into different test tubes with PBS for DNA extraction.

First and second instar were collected from the breeding room. Dissected and removed the guts for both separately. For both first and second instars were taken for DNA extraction while for the first instar, some were used for FISH.

6.3.2 DNA extraction

The DNA extraction was carried out using the Qiagen® Blood and Tissue extraction kit (Qiagen® Germany, Cat. No. 69506) following manufacturer's instructions. It was incubated in Proteinase K Solution (Promega, Germany, Cat. No. 71049-3) for a minimum two (2) hours (Braig, personal communication).

6.3.3 Culture-independent identification of bacteria

The 16S rDNA gene was amplified by PCR using the universal eubacteria primers 338FM 16S UB 5' ACW CCT ACG GGW GGC WGC AGT 3', eubacteria, primer design (Walter *et al.*, 2000) modified by (Thomas *et al.*, 2011) and 1350R 16S UB 5' GAC GGG CGG TGT GTA CAA G 3', eubacteria, primer design (Lane, 1991) with annealing temperature of 56 ^{o}C(Walter *et al.*, 2000).

The conventional PCR was carried out using a Master cycler gradient (Eppendorf, Hamburg, Germany). A single reaction contained 5 X Green GoTaq® Flexi buffer (Promega, USA), 25 mM MgCl2 (Promega, USA), 200 µM dNTPs, 5 U/µl of

GoTaq® DNA Polymerase (Promega, USA), 300 nM of Forward Primer (Eurofins MWG Operon, UK), 300 nM of Reverse Primer (Eurofins MWG Operon, UK), DNA template of 20 nM to 50 nM were used and water Molecular Biology Grade (MELFORD, Bat. No. D23566, UK).

These were the time and temperatures used for the PCR: The protocol consist of an initial denaturing for 3 minutes at 94 ^{o}C, followed by 60 seconds at 94 ^{o}C (denaturing), 30 seconds at 56 ^{o}C (annealing) and 2 minutes at 72 ^{o}C repeat from the second step for 35 cycles. After which it undergoes a final extension of 10 minutes at 72 ^{o}C and hold at 4 ^{o}C.

The gel was prepared with 1 g of Agarose Molecular Biology Grade (Melford, UK, Batch No. D23374) in 100 mls of 1 X TAE. Then 1 µl of ethidium bromide Molecular Biology Grade (Promega, USA, Cat. # H5041) was added. After conducting the PCR reaction, 5.0 µL was taken out of the reaction mixture and run on an agarose gel with a 1 kb DNA ladder (Promega, USA, G571A 24724304) to check the success of the reaction.

The desired bands were excise from the agarose gel with a clean, sharp scalpel and purified using the Qiagen QIAquick Gel Extraction Kit (Qiagen® Germany, Cat. No. 28704). The samples were then sent for sequencing to Europe Macrogen.

The NCBI software was used for blasting the both the forward and reverse sequence for each PCR product. The software Geneious 5.6 was used to create a consensus sequence which was later blast again in NCBI. Primer premier 6.1.2 was used to design primers and specific probes for FISH. RNAfold was used to locate where the fragment design by Primer Premier for specific primers are on the RNA 3D (three dimensional) structure, to help for probe design. Bio edit was also employed.

6.3.4 Fluorescent in situ hybridization

The yellow body fixed in absolute alcohol and cleared with 50% xylol and 50% alcohol. Washed with absolute alcohol and further clean with 80% acetone. Phosphate buffered solution Triton-100 (PBST) was used for further washing to prepare the tissue for penetration by probes. Hybridization buffer (HB) was added first then hybridization

buffer with Cy5 universal probes. It was further washed with HB to remove excess or unattached probes then finally with PBST. The tissue was then mounted in 50% PBS (NaCl, BDH, England; KCl, BDH, England; Na_2PO_4, BDH, England & KH_2PO_4, BDH, England) and 50 % glycerol with Propidium iodide (PI) on a slide cover with cover slip. Detection of signal was by confocal laser scanning microscopy (CLSM).

6.4 Results

6.4.1 Identification of bacteria

The culture-independent analysis of bacteria on the surface of the eggs using 16S rDNA (see Figure 6.1) showed the following species: *Sphingomonas dokdonensis* (99%), *Sphingomonas mucosissima* (99%), *Sphingomonas pituitosa* (99%), *Sphingomonas koreenis* (99%) and *Sphingomonas truperi* (99%); the percentage identity is in parentheses.These bacteria were different form the ones identified from the yellow body. PCR was performed on the gut of the first instar larvae (see Figure 6.2). A similar set of bacteria as listed above was obtained but here query coverage was 100% and the fourth species is *Sphingomonas asaccharolytica* while *Sphingomonas koreenis* moved to the fifth position. PCR on the gut of second instar larvae (see Figure 5.2) was performed, but the following species of bacteria were identified: *Providencia heimbachae* (100%), *Providencia rustigianii* (99%), *Providencia alcalifaciens* (99%), *Providencia vermicola* (99%) and *Providencia stuartii* (100%).

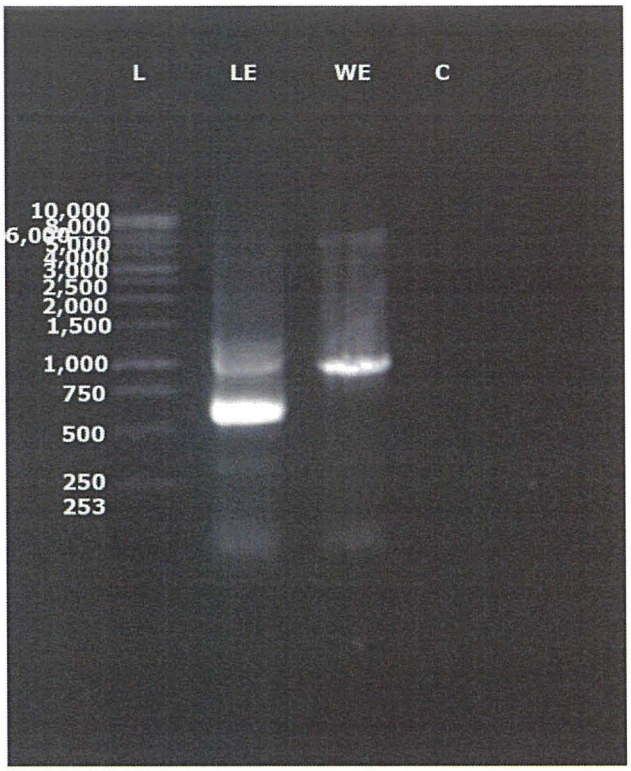

Figure 6.1: The agarose gel (1% agarose) showing bands of L = Ladder (1 kb ladder Promega), LE = Laboratory eggs, WE = Wild eggs and C = Control

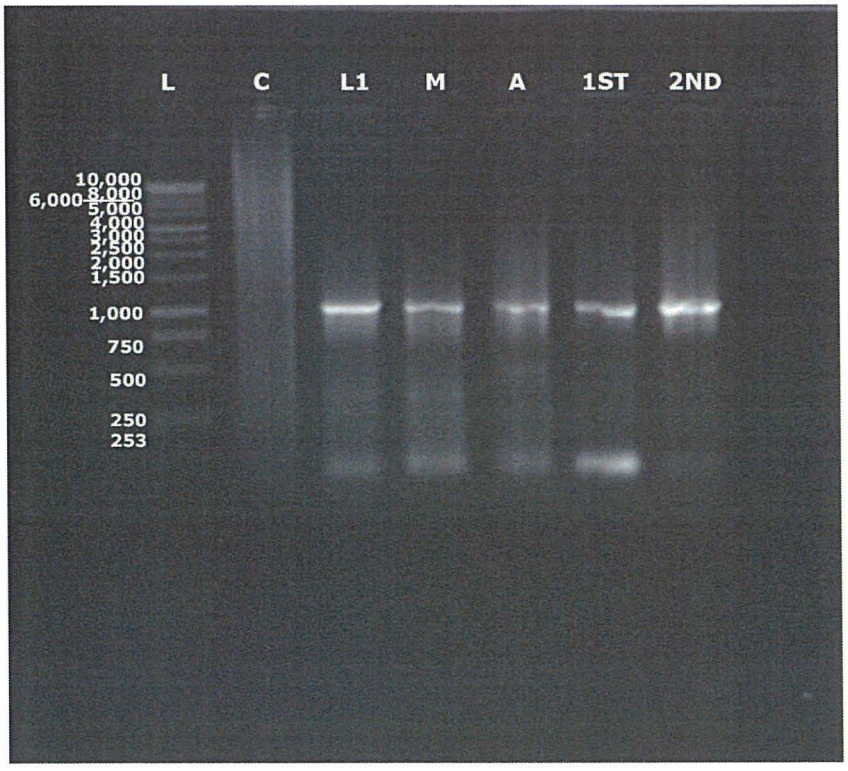

Figure 6.2: The agarose gel (1% agarose) showing bands of L = Ladder (1 kb ladder Promega), C = Control, L1 = Legs, M = Mouthpart, G = Abdomen, 1st = First Instar and 2nd = Second Instar

Wild eggs were collected of the blow fly *Calliphora vicina*, and extracted the DNA of the bacteria on it and ran PCR using 16 S rRNA gene (see Figure 6.1). Blast in NCBI, used Geneious software to create consensus sequence which was later blasted in NCBI and the bacteria found are related to the following: *Sphingomonas dokdonensis* (89%), *Sphingomonas mucosissima* (89%), *Sphingomonas pituitosa* (89 %), *Sphingomonas asaccharolytica* (89%) and *Sphingomonas koreenis* (89%).

6.4.2 Fluorescent in situ hybridization

Bacteria were located in the digestive tract and were localized as ingested food; see Figure 6.3.

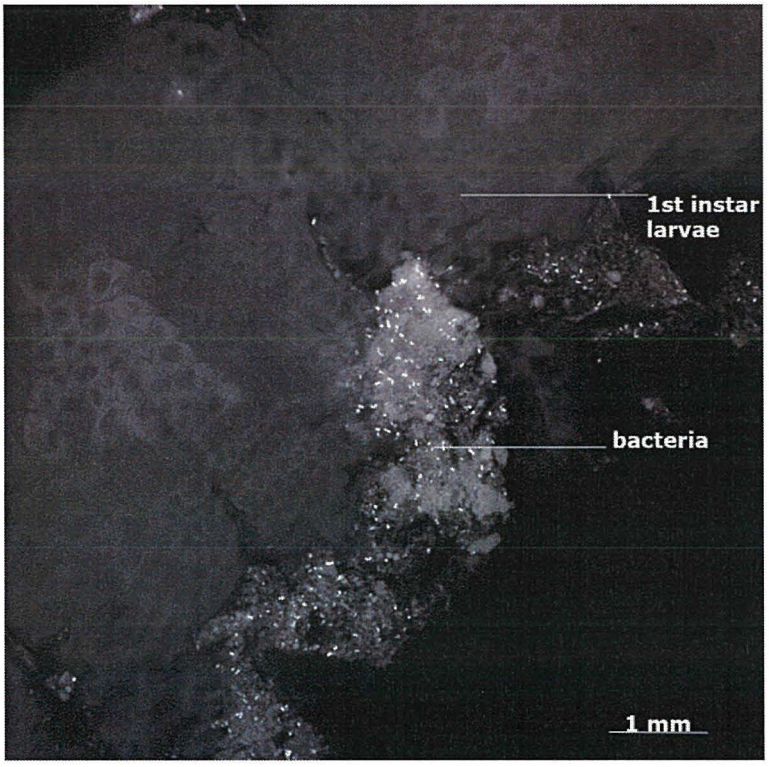

Figure 6.3:Visualization of bacteria to first instar larvae based on fluorescent In Situ Hybridization (Cy5 labelled universal bacterial robes) with Confocal Laser Scanning Microscope (CLSM 510, Zen 2009) at Plan-Neofluar 40X/1.3OilDIC.

6.5 Discussion

The results show the presence of microorganisms on the surface of *Calliphora vicina* eggs are of the *Sphingomonas* species. They also show that the same species of microorganisms occupy the gut of first instar larvae of the blow fly but a different set of microorganisms species occupy the gut of second instar larvae of the same blow fly. This raised our curiosity to know what role these bacteria play at this particular stage of development of the blow flies.

Studies on association of flies with bacteria to mention a few are; the work of Hobson (1932) on the nutrition of blow fly larvae, the work of Greenberg (1969) on *Salmonella* suppression by known populations of bacteria in flies, the work of Romero *et al.* (2006) on the role of bacteria in the oviposition behaviour and larval development of stable flies and that of Ma *et al.* (2012) who looked at *Proteus mirabilis* interkingdom swarming signals attract blow flies.

An initial work on the yellow body (midgut epithelium) of pupae was carried out to identify the type of bacteria it harbours. It was suspected that the bacteria it carries might be endosymbionts, which was not the case, so this necessitated our extraction of bacteria DNA on the eggs. But the bacteria identified were of the *Sphingomonas species*. These bacteria are likely to be on surface of the eggs, but what significant are them to the larvae or the blow fly itself. The hypothesis which said, the mouthpart of the first instar larvae are so small that they do not feed directly on the food available at the moment rather they feed on available microbial community. Investigation went ahead into the type of bacteria the first instar gut carried. In the first instar larvae *Sphingomonas* species were identified. This supported other works that said bacteria may serve as direct food for early stage of flies(Merritt *et al.*, 1992;

Radjame *et al.*, 1997; Zurek *et al.*, 2000; Romero *et al.*, 2006; Wasserberg and Rowton, 2011; Peterkova-Koci *et al.*, 2012).

The source of the bacteria became another fundamental issue to address, which could either come from the female fly herself (Lam *et al.*, 2007; Mukhopadhyay *et al.*, 2012) or by what other studies point out that flies in the families of Muscidae, Calliphoridae and Sarcophagidae lay their eggs and larvae in decay organic matter such as animal faeces/manure, carrion and food waste/trash containing a large and diverse bacterial community(Spiller, 1964; Damato *et al.*, 1980). Legs, mouthparts and abdomen of adult female blow flies *Calliphora vicina*were removed and identified the bacteria but not same as the ones seen on the eggs surface but could not conclude that they are completely absent rather it was expected to be dominated by these.

Study has shown that flies lay their eggs in habitat that harbours a diverse number of bacteria but the larvae and the flies interact with specific bacteria for specific purpose(Ahmad *et al.*, 2006; Lam *et al.*, 2007; Lam *et al.*, 2009; Peterkova-Koci *et al.*, 2012). So the absent of bacteria in the PCR product for the different parts of the adult fly does not mean it might be present, but could be it was dominated by other bacteria. There are two ways to confirm this; by cloning or by running FISH with specific probes design with sequence gotten from the bacteria on the surface of the eggs.

To verify the absent or present of the *Sphingomonas species*, fluorescent in situ hybridization(FISH) was performed. The bacteria from the surface of the eggs are of the *Sphingomonas* spp which before 1990 were classified as *Pseudomonas* and *Flavobacterium* spp(Cavicchioli *et al.*, 1999). For in 1990 Yabuuchi *et al.* (1990) defined them as *Sphingomonas* genus from the family *Sphingomonadaceae,* order *Sphingomonadales,* class *Alphaproteobacteria*. They are found to be widely distributed in nature and also possess the ability to survive and grow at different conditions which include: low temperature, low nutrient concentration and also toxic chemical environments(Cavicchioli *et al.*, 1999; Tabata *et al.*, 1999; Koskinen *et al.*, 2000; Zhang *et al.*, 2005; Yoon *et al.*, 2006; Reddy and Garcia-Pichel, 2007; Mahani *et al.*, 2009; Sun *et al.*, 2013). These *Sphingomonas* species are found to produce exopolysaccharides

that have so many applications in food, pharmaceuticals, petroleum and other industries (Matsuyama *et al.*, 2003; Seo *et al.*, 2004), and they also carry in them genes that degrade a wide range of polycyclic aromatic hydrocarbons (PAHs), which has vast biotechnological applications in bioremediations, wastes water treatment contaminated with xenobiotics(Pinyakong *et al.*, 2003).

The *Sphingomonas* species have the capability to degrade wide range of polycyclic aromatic compounds shows it might be playing a dual function for the first instar larvae as: serving as food and also helping in the degrading the liver or the corpse for easy consumption by the second instar larvae.

Sphingomonas species are increasingly found in the midgut of insect larvae. *Sphingomonas paucimobilis* has been detected in the midgut of larvae of the malaria mosquito *Anopheles stephensi*(Diptera)(Djadid et al., 2011), *Sphingomonas*were also found in the midgut of the yellow fever mosquito *Aedes aegypti* (Diptera) (Terenius *et al.*, 2012), in the midgut of larvae of the pine bark beetle *Dendroctonus armandi* (Coleoptera) (Hu *et al.*, 2014), the midgut of larvae of the cotton bollworm *Helicoverpa armigera* (Lepidoptera) (Priya *et al.*, 2012), in several species of the cochineal scale insect genus *Dactylopius* (Hemiptera) (Ramirez-Puebla *et al.*, 2010), the anterior foregut of the glassy-winged sharpshooter*Homalodisca vitripennis*(Hemiptera) (Rogers and Backus, 2014), just to give some recent examples. Although it has been questioned that bacteria on the surface of the fly eggs might serve as the sole food source for first instar larvae, the fact that the *Sphingomonas* bacteria of the egg surface end up in themidgut of the first instar larvae and disappear in second instar larvae strongly suggests that these bacteria play at least an essential role in the development of the fly. In addition, the fact that the same *Sphingomonas* bacteria have been detected in wild-caught flies and long-established laboratory colonies underpins the point of view.

6.6 References

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Chapter Seven

Degeneration of the salivary glands of the blow fly *Calliphora vicina*'s larvae for ageing of its wandering stage

7.1 Abstract

Forensic entomology uses the age of immature blow flies as a marker to estimate the minimum post-mortem interval (PMI_{min}) of corpses. While measuring the length of larvae from the first instar through the active feeding stage of the third instar is well established, the wandering stage of third instar larvae is posing a problem for it shrinks as it wanders away from its source of food. The degeneration of its salivary glands is explored as a potential marker for the age of the wandering stage, looking at the cells of the salivary gland with a particular emphasis on the changing morphology of the nucleus and its polytene chromosomes. Flies were reared; eggs collected, allowed the larvae to moult to the active feeding third instar and monitored, for when they started to wander. Samples were collected for dissection of the salivary glands every 24 hours. Salivary glands were kept in alcohol, rehydrated in Phosphate Buffer Solution Triton-100 (PBST), stained with Propidium Iodide (PI) and viewed using a Confocal Laser Scanning Microscope (CLSM 510, Zen, 2009). The changing morphology of the nucleus revealed a predictable pattern, from the active feeding stage through the wandering larval stage until moult into pupae. Therefore, this has been recognized as a very good marker for ageing the wandering larvae of the blow fly *Calliphora vicina,* which can be used to estimate post-mortem interval of older corpses.

7.2 Introduction

Forensic entomology is the study of insects and their arthropod relatives as evidence in legal investigations (Goff, 2000; Benecke, 2001; Donovan *et al.*, 2006; Bonacci *et al.*, 2009; Goff, 2010). The insects of interest here are blow flies (also known as carrion flies, blue bottles, green bottles or cluster flies) (Diptera: Calliphoridae), used to estimate the time of death of a corpse. The forensic entomologist uses these insects because their life stages follow a set pattern and therefore when seen at a crime scene can yield information about time and place of death (Donovan *et al.*, 2006). The blow fly species used in this study was the blue bottle *Calliphora vicina* Robineau-Desvoidy 1830 for its unique characteristics of being among the first flies to arrive on a corpse

and found both on corpses indoors and outdoors. Its larvae were found to be among other larvae that travelled farther away from their food source after they have taken sufficient nutrients, looking for a place to pupate. *Calliphora vicina* are found in temperate regions(Gomes *et al.*, 2006; Pohjoismäki *et al.*, 2010).

Forensic entomology uses the length and size of blow fly larvae to estimate the time of death of younger corpses, which is well established for first, second and early third instars, when the larvae are actively feeding and growing. However, it is a little different with the late third instar larvae, because of changes in morphology due to changes in feeding habit. This is observed when third instars stop feeding and wander about looking for a place to pupate, they begin to shrink and show increased differences in body size. However, far little attention has been paid to this part of the life cycle, even though it does take the flies two to three days to wander about and start pupating, depending on the ambient temperature (Greenberg, 1991; Anderson, 2000; Grassberger and Reiter, 2001; Arnott and Turner, 2008).

The blow fly *Calliphora vicina* uses a pair of salivary glands for its larval stages and degenerate completely some few days into the pupal stage. And a new set of salivary glands form from the imaginal cells to be used in the adult flies(Berridge *et al.*, 1976; Levy and Bautz, 1985). But for this study the interest is on the internal events that occur in the nucleus of the cells of the salivary glands, when the larvae stop feeding to when they start pupating, to help in ageing the wandering stage of the larvae. Levy and Bautz (1985) described the salivary glands to appear in vivo appear as two long translucent tubes besides the oesophagus. From the thorax the salivary glands extend to the abdominal part. A few adipose or fat cells are attached at the posterior ends of the glands. The glands cells themselves have a hexagonal shape. A subspherical nucleus lies round a central lumen. The degeneration of the cells of the salivary glands of the blow fly has been proved to be classical case of apoptosis but at the later stages the degeneration shows signs of autophagy. Some of the chromosomes in the salivary glands of the larvae of this blow fly are polytene chromosomes (Levy and Bautz, 1985).

Zhimulev and Koryokov (2009)defined polytene chromosomes as specific chromosomes at interface which consist of thousands of individual deoxyribonucleic acid (DNA) strands. And these are usually very large and show a characteristic band-interband morphology that occurs in tissues and organs during development stages were an organ or tissue requires a rapid development for an unaltered function. In normal chromosomes replication cycle (mitosis), chromosomes condense, replicate, divide and segregate to daughter cells but the polytene chromosome undergoes replication cycles but do not divide.Polytene chromosomes are commonly found in larval salivary glands of dipterans, especially flies from the families Drosophillidae, Chironomidae, Cecidomyiidae, Calliphoridae and Sciaridae. They also found in other tissues and organs of same flies such as their midgut and fat body. Polytene chromosomes undergo a special process called endopolyploidy, were the chromosome number increase within the nuclei of the tissue without a breakdown of the nuclear membrane. The growth of these tissues or organs is by enlargement not by cell division (White, 1973; Hoy, 2013).

Another phenomenon exhibited by polytene chromosomes is their ability to swell or puff. Puffing involves the opening up of DNA within a region of chromosomes to allow extensive transcription. This process is controlled by the hormone ecdysterone, heat shock and other environmental conditions. The region of the polytene chromosomes with the largest puffs has DNA that code for genes for proteins needed in large quantity in the salivary glands, such as the salivary glands secretions and silk. The puffing patterns differ in different instars of *Drosophila melanogaster*, showing that different instars have different active genes (Hoy, 2013).

Apoptosis is an active metabolic pathway to cell death that is genetically encoded and evolved over time (Wu *et al.*, 2001). This condition occurs either physiologically or pathologically, for salivary glands of blow flies it does occur under physiological conditions. This condition is quite necessary at this point because the pair of larval salivary glands has outlived their usefulness (Cairrão and Domingos, 2010).

The programmed cell death in apoptosis is an active enzymatic process where the enzymes are been activated first either extrinsically or intrinsically. The activated

enzymes are called caspases (Cysteine – aspartic proteases or Cysteine – dependent aspartate – directed proteases). These caspases are classified as initiators and executioners (having long prodomains and short prodomains, respectively) (Fan *et al.*, 2005; Fan and Bergmann, 2010). These caspases have been well studied in mammals (humans), nematodes (*Caenorhabditis elegans*) and dipterans (*Drosophila melanogaster*) (Shaham, 1998; Kumar and Doumanis, 2000; Fan and Bergmann, 2010; Denning *et al.*, 2012).

Propidium iodide (PI), a nucleic acids dye, is used for the first time to stain the salivary glands of the larvae of blow fly *Calliphora vicina* which intercalates with the DNA in the chromosomes in the nucleus. These interactions between the dye and the nuclear material help to observe any changes of polytene chromosomes or the changes in the morphology of the nuclear material due to ageing of the larvae as it turns to pupae. The markers in this study were changes in polytene chromosomes and changing morphology of the nuclear material.

7.3 Material and Methods

7.3.1 Laboratory culture

Adults of the blow fly *Calliphora vicina* Robineau-Desvoidy 1830 were already available in a wooden breeding box in a temperature control room. Eggs were obtained by putting approximately 5 g of pig liver on a Petri dish and placing it in the breeding box (44 cm X 44 cm X 44 cm) where the adult blow flies were. The adult blow flies feed on sugar and do require protein to lay eggs. While feeding on the pig liver, they also lay their eggs on it. The Petri dish was left there for approximately 3 hours. Then it was removed and placed in a rectangular rubber basin (44 cm X 13 cm X 25 cm), which had saw dust in it. The eggs hatched to give larvae, which were the 1st instar then molted to give the 2nd instar and molted to give the 3rd instar. At the 3rd instar, larvae have an active feeding stage where they take in sufficient nutrients that will take them through the remaining stage of the life cycle and eventually entered a wandering stage where they moved away from the food source (pig liver). For the 3rd instar larvae, the

food source was monitored to avoid premature wandering, in the case where their food had diminished. All through the life cycle, a temperature of 22.3 ±1 ^{o}C and a relative humidity of 47.1 ± 2% were maintained.

7.3.2 Sampling of larvae

The larvae at the active stage of the 3rd instar where monitored closely within twenty-four hours intervals. When larvae started wandering away to find a place to pupate, sampling started. The biggest of the larvae on the pig liver were selected as day zero, while the wandering ones were separated from the main population to help having larvae of almost equal age. Then from the separated wandering larvae samples were picked for dissection for day one, day two, day three and so on. Four of the larvae were picked life without any preservation for dissection.

7.3.3 Dissection of larvae

Each larva was placed in small petri dish containing PBS (phosphate buffer saline) for dissection. Fine forceps were used to hold the larvae at both ends and pulled gently and the two salivary glands were seen attached to the head and removed. All procedures were carried out with aid of a light microscope (Leica Zoom 2000, USA) at a magnification of 10.5 X. The salivary glands were then placed in a 1.5 ml test tube with 90 % alcohol and 10 % glycerol.

7.3.4 Histological methods

The removed pairs of salivary glands were stored in an alcohol-glycerol solution. For processing the salivary glands were placed in a 1.5 ml test tube with Phosphate buffer saline triton-100 (PBST) overnight for rehydration. A piece of the rehydrated salivary gland was cut and placed in another 1.5 ml test tube with PBST and propidium iodide (PI) added to it and left for three hours. The PBST and PI were removed and more PBST were added to wash the remaining PI that might not have been absorbed by the piece of salivary gland. The washing was done two times at an interval of one hour each. The piece of salivary gland was removed and placed on a glass slide and further

in order to cut it into two or three pieces depending on its length. Then the pieces on the glass slides were squashed with the aid of a coverslip and taken for imaging.

7.3.5 Imaging the nuclei of the salivary glands

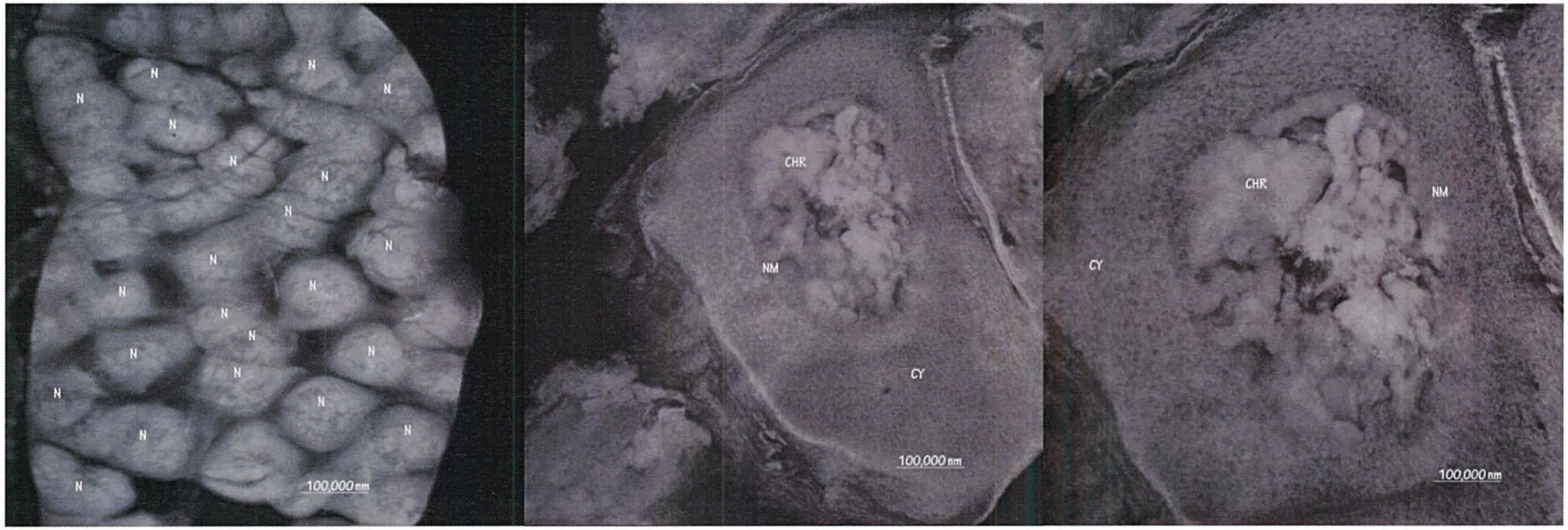
The slides were viewed using a Fluorescence Confocal Laser Scanning Microscope (CLSM 510 using Zen 2009 software) to monitor changes in the nucleus of the salivary gland at magnifications of 10X using a Plan-Neofluor 0.3 objective, 40X using aPlan-Neopfluor 1.3 oil DIC objective, and 63X using a Plan-Apochromat 1.4 oil DIC objective.

7.3.6 Ageing the wandering stage of larvae

Ageing of the wandering stage of the blow fly larvae was based on the changing features of the nucleus of the salivary gland as observed from pictures taken with CLSM 510 (Zen 2009).

7.4 Results

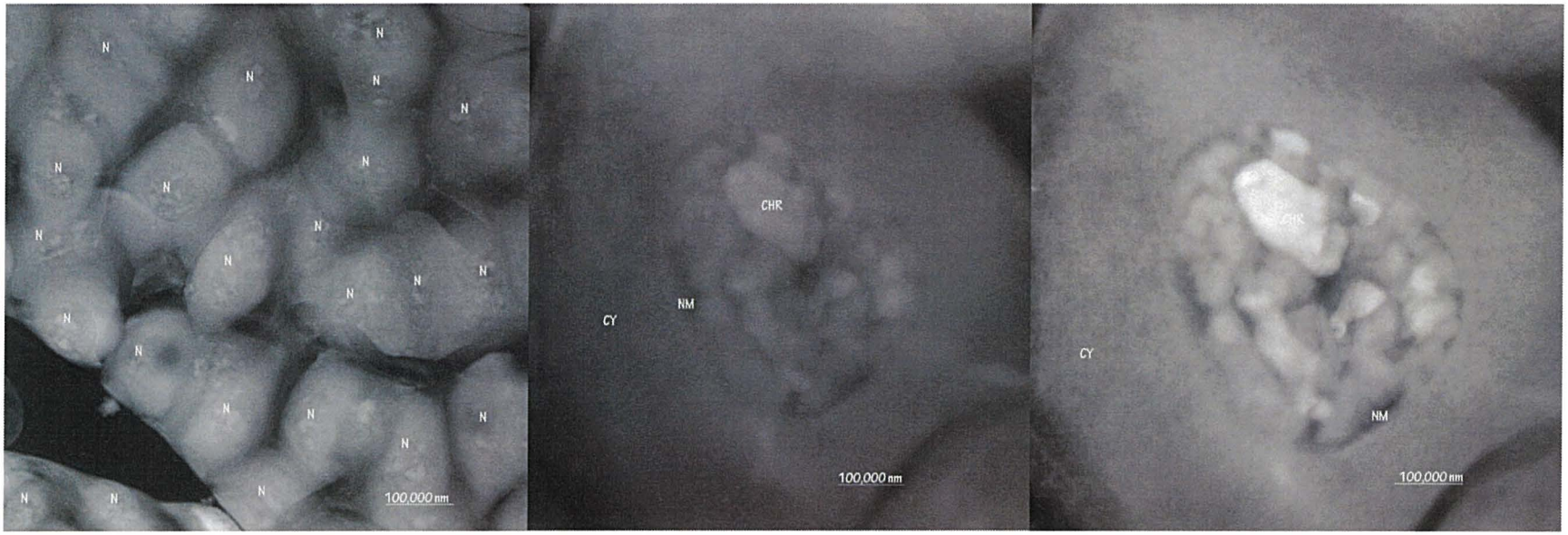
The following results show the changes in the nuclei of the cells of salivary glands of the active and wandering stages of larvae of the blow fly *Calliphora vicina* using PI as the nucleic acids dye and view with a confocal microscope, Figure 7.1a-f shows the cells for each individual day at three different magnifications and Figure 7.2 shows the changes over time.



10X 40X 63X

Day zero, end of feeding stage.

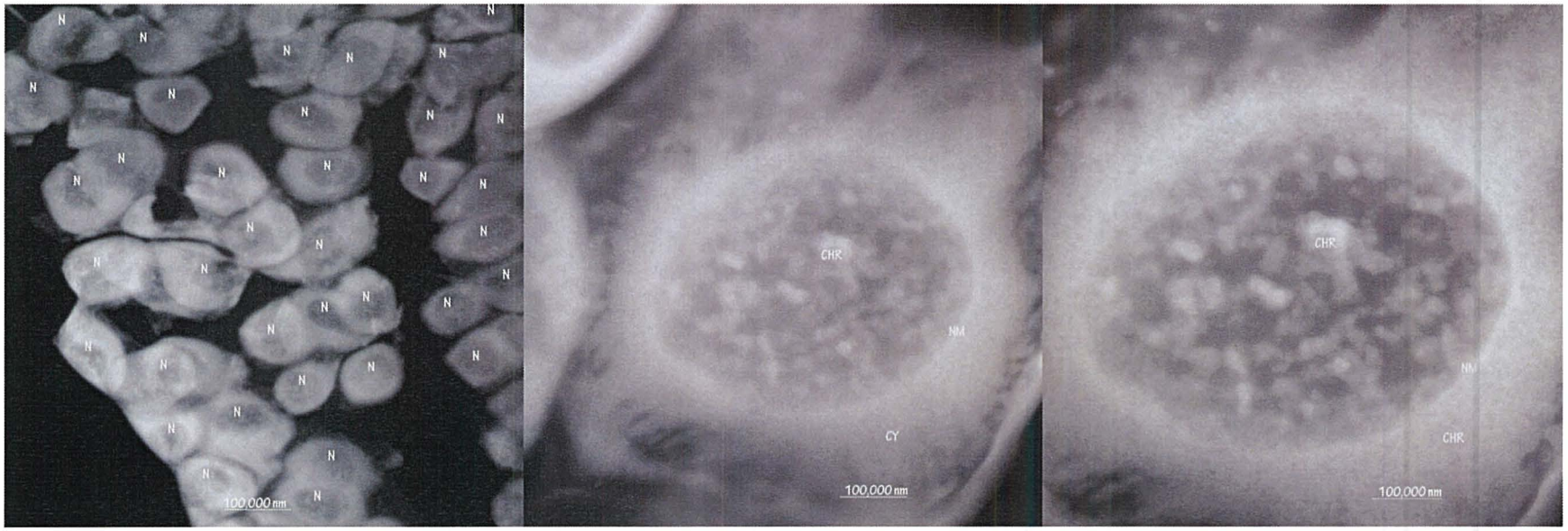
Figure 7.1a: Salivary gland cells of larvae showing the nucleus (N), chromosomes (CHR), nuclear membrane (NM), and cytoplasm (CY); stained with propidium iodide (PI), viewed using Confocal Laser Scanning Microscope.



10X 40X 63X

Day one of wandering stage.

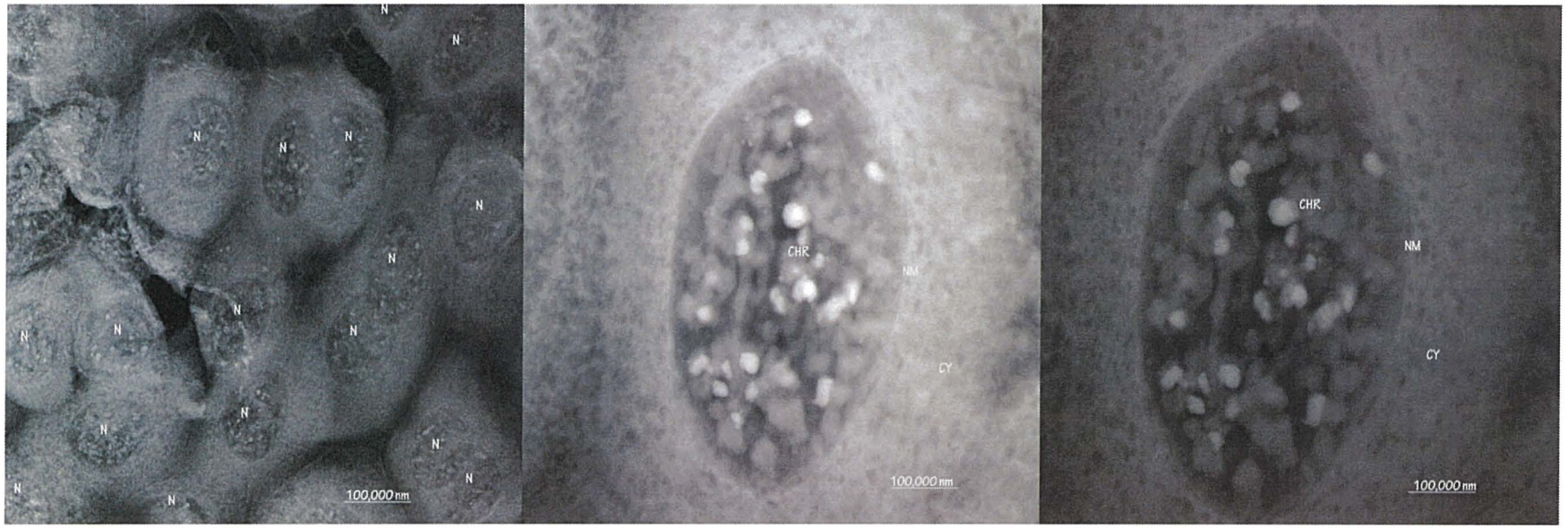
Figure 7.1b: Salivary gland cells of larvae showing the nucleus (N), chromosomes (CHR), nuclear membrane (NM), and cytoplasm (CY); stained with propidium iodide (PI), viewed using Confocal Laser Scanning Microscope.



10X 40X 63X

Day two of wandering stage.

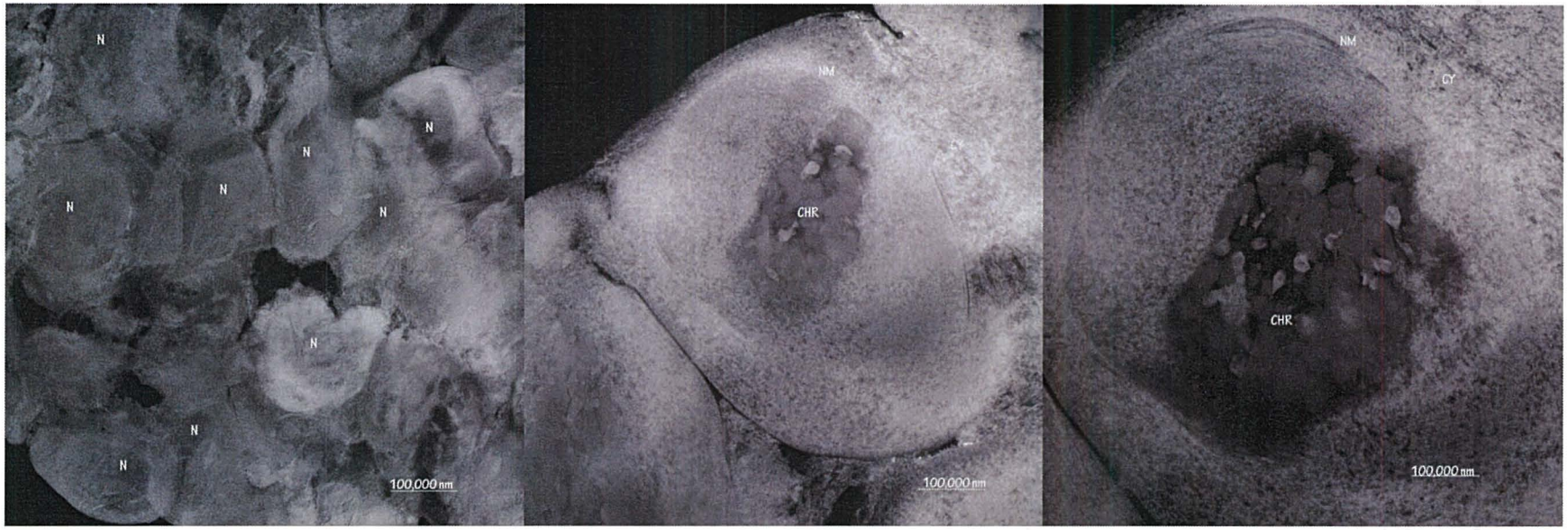
Figure 7.1c: Salivary gland cells of larvae showing the nucleus (N), chromosomes (CHR), nuclear membrane (NM), and cytoplasm (CY); stained with propidium iodide (PI), viewed using Confocal Laser Scanning Microscope.



10X 40X 63X

Day three of wandering stage.

Figure 7.1d: Salivary gland cells of larvae showing the nucleus (N), chromosomes (CHR), nuclear membrane (NM), and cytoplasm (CY); stained with propidium iodide (PI), viewed using Confocal Laser Scanning Microscope.



10X 40X 63X

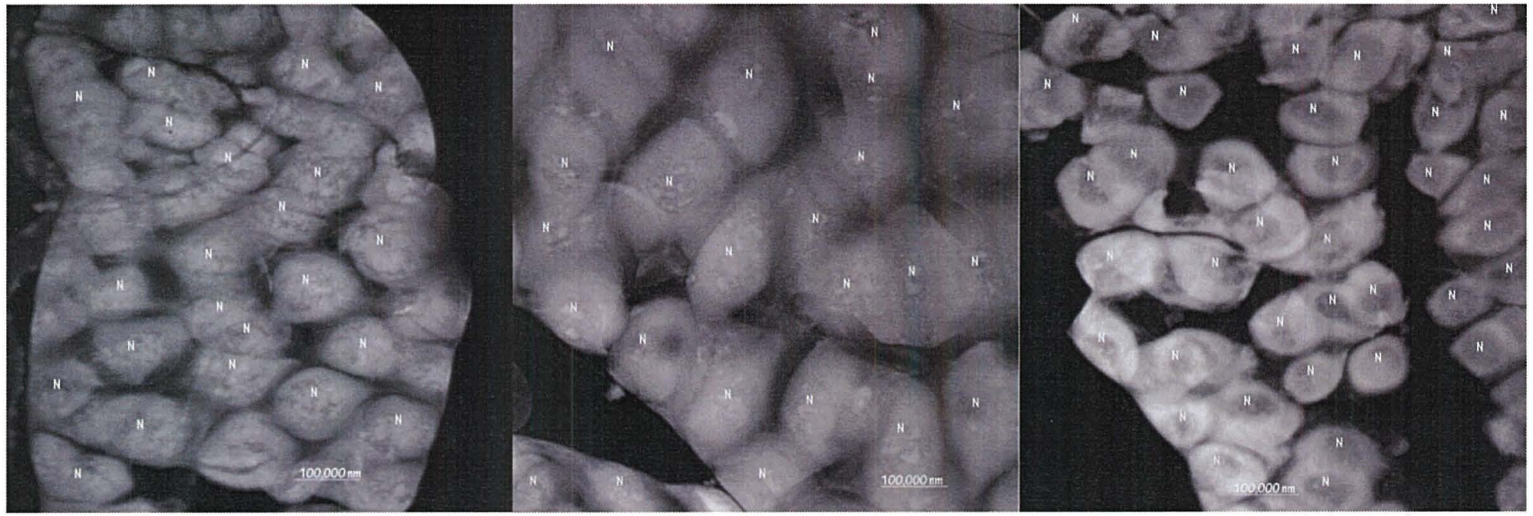
Day one of pupal stage.

Figure 7.1e: Salivary gland cells of pupae showing the nucleus (N), chromosomes (CHR), nuclear membrane (NM), and cytoplasm (CY); stained with propidium iodide (PI), viewed using Confocal Laser Scanning Microscope.

10X 40X 63X

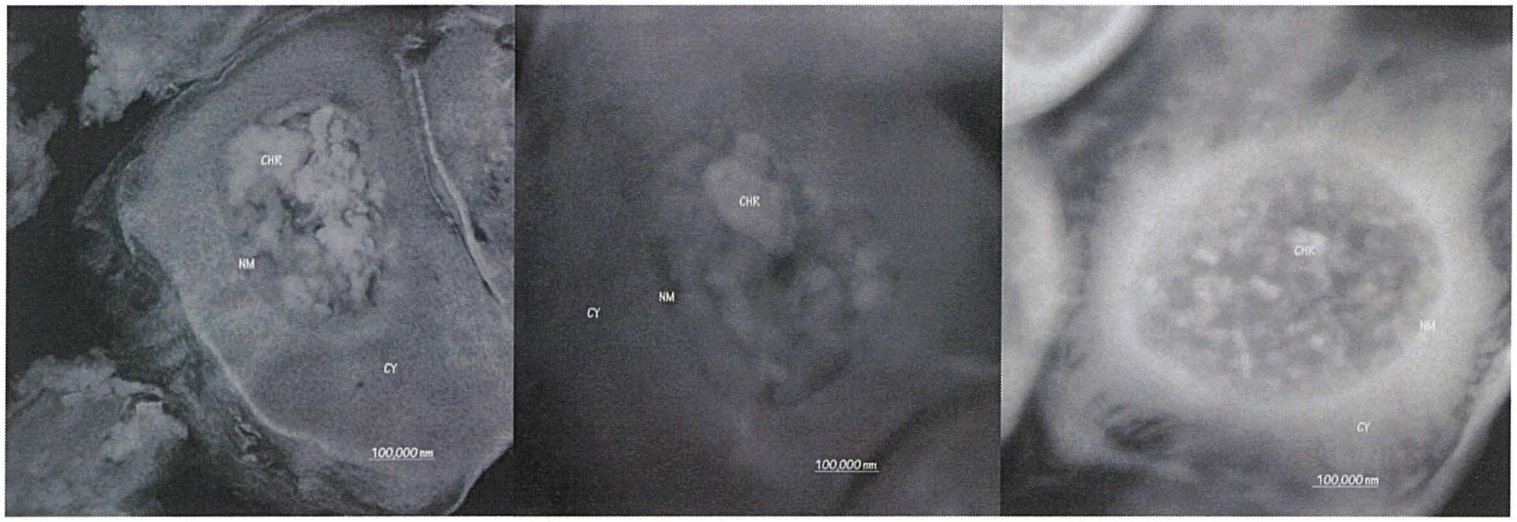
Day two of pupal stage.

Figure 7.1f: Salivary gland cells of pupae showing the nucleus (N), chromosomes (CHR), nuclear membrane (NM), and cytoplasm (CY); stained with propidium iodide (PI), viewed using Confocal Laser Scanning Microscope.

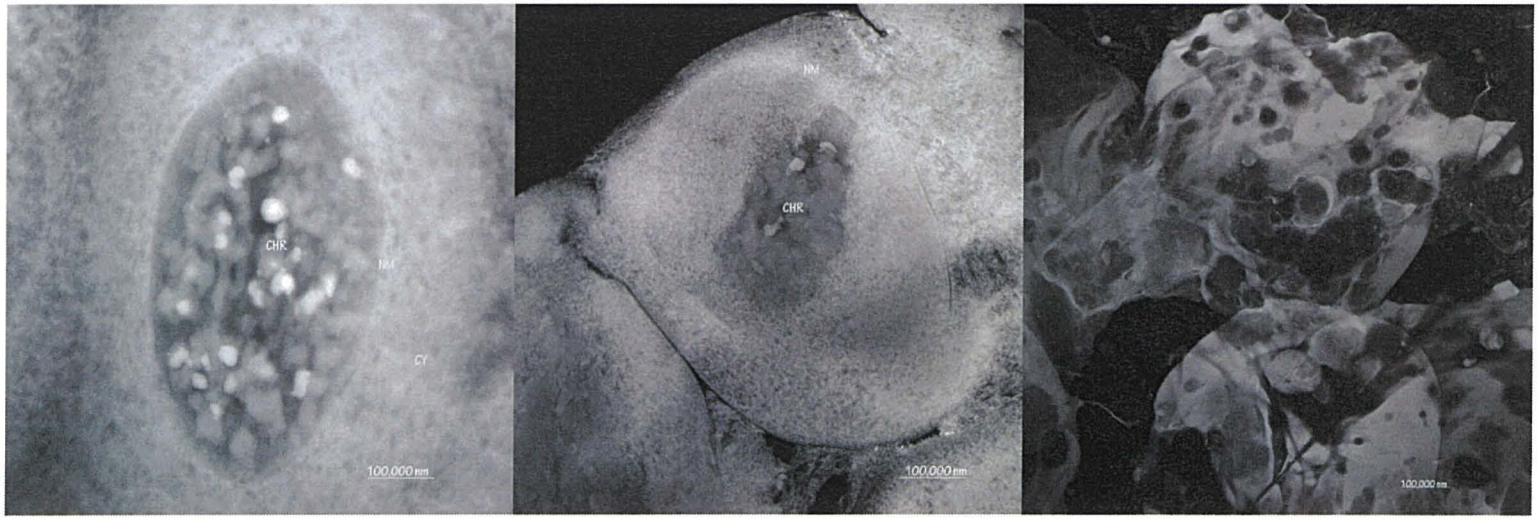


10X Day zero, feeding day one, wandering day two, wandering

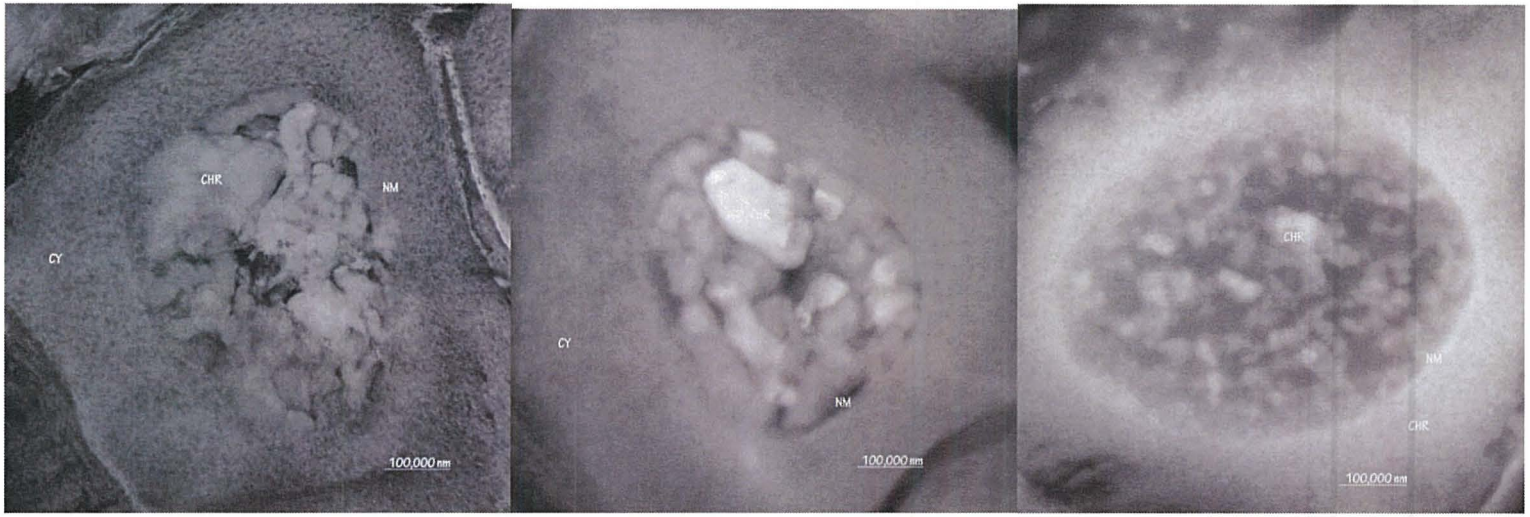
Fiigure 7.2a day three, wandering day one, pupa day two pupa



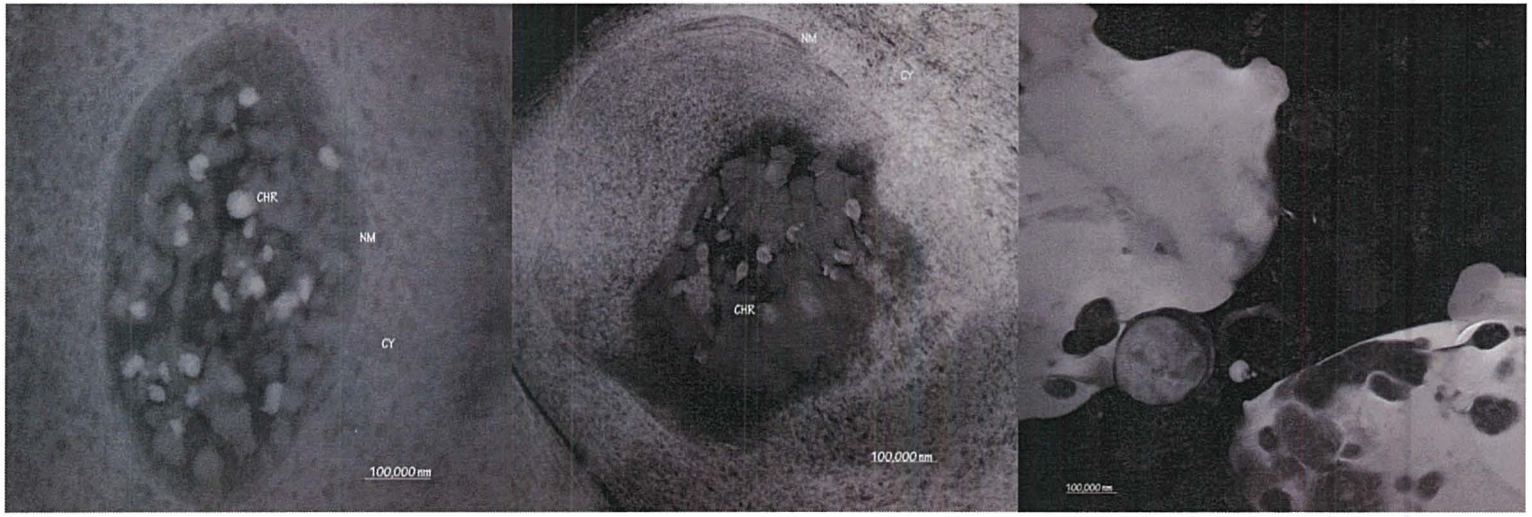
40X Day zero, feeding day one, wandering day two, wandering



Fiigure 7.2b day three, wandering day one, pupa day two pupa



63X Day zero, feeding day one, wandering day two, wandering



Fiigure 7.2c day three, wandering day one, pupa day two pupa

The PI stained cells of the salivary glands were clearly seen with a 10X objective, showing the different cells with their nucleus at the centre, Figure 7.1. At day zero the chromosomes were highly condensed and covered fully the entire nucleus. The margination was throughout the nucleus.

The first day of the wandering stage showed the chromosomes still highly condensed, but with more visible shapes of the chromosomes. The margination of the chromatin was evenly distributed throughout the nucleus. No banding pattern was observed at this stage.

The second day of the wandering stage showed the chromosomes more condensed, showing the shape of the chromosomes with their banding pattern.

For the third day of the wandering stage showed signs of degeneration and some of the nuclei had started budding. At this stage also the chromosomes are clearly seen but there is sign of them losing shape and colour as compared to the previous day.The space occupied by chromosomes within the nucleus
has been reduced at this stage, that is to say that marginalization was not even.

The first day of the pupal stage showed the outlines of the cells but nuclei could not been much, it can only be seen as a dark region in the cell and even the cells themselves have lost their integrity. But viewed at higher magnifications, the nucleus were seen clearly with its chromosomes in it, but have lost shape and colour. And the space they occupy had reduced as compared with the third day wandering larvae stage. And the condensation of the chromosomes has increased greatly.

The second day of the pupal stage, the salivary glands showed the cells with patches, no proper shape and the cells had lost their integrity. At higher magnification some of nuclei showedphagocytic processes.

7.5 Discussion

The salivary glands of the larvae of blow fly *Calliphora vicina* are one of the organs that need replacement as larvae transit into the pupal stage. The chromosomes at the active feeding stage of the larvae, set as day zero,have no banding pattern and look

diffused. The diffused nature could be as a result of the chromatid conjugation which when disturbed in only one chromosome of the set. The polytene chromosome does loose it banding pattern completely as a result and look diffused, given the so called pompon-like chromosome (Zhimulev and Koryokov, 2009).

The nucleus of the cell of the salivary glands of the larvae on the first day of its wandering stage showed no degeneration of nuclear material. For the nuclear materials looked in tacked with no sign of any alteration in it shape or form. The white portions in the nucleus were an indication of how active those DNA regions were. And could be the genes responsible for the synthesis of enzymes that were required for apoptosis as not yet active (Martelli *et al.*, 2001).

The nuclear materials at the second day the larvae wandering stage showed the banding pattern of the polytene chromosomes with a clear view of the nucleoplasm (Zhimulev *et al.*, 1976; Zhimulev and Koryokov, 2009). The distinction in the arrangement of the chromatins marked that this stage could be the best for study of polytene chromosomes in nuclei of cells in salivary glands of blow fly *Calliphora vicina*. The puffing nature indicates active genes at this stage. There is no clear indication of degeneration but the extreme end space of the nucleus has no nuclear material as were observed in the previous days.

On the third day, budding was observed indicating that the degeneration of the nucleus started on this day.Earnshaw (1995) indicated that chromatin budding is one of the typical morphological changes that occur when apoptosis start setting in. The nucleus viewed at higher magnification showed the white patches are very bright, condensation started, which is a sign of the collapsing of the chromatin. This could be the stage where the executioner caspases are activated and subsequently cleave distinct cellular proteins as PARP, lamin etc., leading to the characteristic morphological changes just observed (Gaggero *et al.*, 2004; Martinon and Tschopp, 2004).

On the first day of pupa, the stained salivary gland viewed at low magnification, no cell was very visible except for some dark corners. But when viewed at higher magnifications, the nuclei seen were shrink and the chromatin in it had lost its integrity

completely, looking highly condensed and without form and banding pattern. It was observed that morphological changes typical of apoptosis consist of a collapse of chromatin (that, most cases had to do with striking crescent or half-moon shape) contrary to nuclear periphery, later with progressive condensation of the chromatin and shrinkage of the entire nucleus (Earnshaw, 1995).

Levy and Bautz (1985) observed at twenty-seven hours after pupariation phagocytic process in the salivary glands of *Calliphora vicina*. Although this process occured with the entire cell and that was what they worked on to check the type of programmed cell death. Herein the results a picture was captured showing phagocytosis. After this stage, twenty hours later, a pupa was picked and dissected, no salivary glands were found.

Several other researches have been taken place to study apoptosis in other organism's salivary glands, such as *Drosophila arizonae* and *Drosophila mulleri*(Lanella *et al.*, 2008) and*Apis mellifera*(Silva-Zacarin *et al.*, 2007).

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Chapter Eight

Conclusions

The ageing of larvae of blow flies or flesh flies, if blow flies are not present, is the bread and butter of forensic entomology for estimating the minimum post mortem interval. Blow flies arrive first of human corpses and animal carcasses, they lay eggs with little delay. The eggs hatch into first instar larvae, then after a certain period, these first instar larvae moult subsequently into second and third instar larvae. While the larvae feed on the dead body, they increase in size, most prominently, they increase in length. This increase is directly proportional to the time the larvae have spent on the body, which means the larval length can serve as parameter for calculating the age of the larvae. The increase in length is also dependent on the ambient temperature. After the three stages of feeding larvae, two more stages of the blow fly development are spent on the dead body, the wandering stage and the pupal stage. In these two stages, the relationship between length and age no longer holds and therefore these stages have not been used for forensic estimations of the post mortem time. However, these two stages are longer than the three preceding stages of larval development. Gaining access to the wandering stage and the pupal stage for post mortem time interval estimations would be very important for forensic entomology. This has been achieved in this thesis.

The age of pupae of the blow fly *Calliphrao vicina* can reliably be estimated without the need for extensive instrumentation, molecular procedures or the use of colour by applying a method that is exceptionally fast. The simple dissection of the yellow body from the metamorphosing midgut structure allows an estimate of the pupal age with a resolution of 40 °D using seven landmarks based on morphological changes in the degeneration pattern of the larval midgut inside the pupa.

For the first time the reliability of an easily applicable pupal ageing system has been successfully tested and confirmed with the help of volunteers.

Ageing blow fly pupae based on morphological changes of external characters of the pharate developing adults is a simpler method than using the yellow body of the transforming midgut. It is also the simplest method currently available.

The intrinsic error in this procedure is around 5 % in terms of ADD or over the range of pupal development ± 10-20 °D. Variation in *C. vicina* isolates and operator

might add an extrinsic error of around 10 % or ± 20-40 °D. New methods for ageing pupae should include a blind evaluation in the original lab and comparison blind comparison between different laboratories.

Using more characters and a wider range of degradation (15 characters with 43 points) than Richards *et al.* (2012) (4 stages), Zajac and Amendt (2012) (10 characters with 18 points), and Defilippo *et al.* (2013) (5 stages with 6 landmarks), a practically linear relationship between accumulated points and age is achieved over the time period of pupal development, which is lacking in the systems of the aforementioned publications.

The minimum temperature of *C. vicina* has been for the first time established under laboratory conditions.

A new, lower minimum temperature has been established for *C. vicina* for survival under diapaus. This is the first study that also includes arrest under diapause.

The yellow body formed around the larval midgut contains the bacteria of the larval midgut and lyses the larval midgut epithelium as well as the bacteria in it to produces a sterile environment that allows the formation of the adult midgut. The current hypothesis that the function of the yellow body serves to rescue nutrients by lysing the larval midgut is rejected. The remains of the yellow body are eventually discarded by the adult as a meconium.

The first larval instar of *C. vicina* depends on bacteria as nutrition. Specific *Sphingomonas* species are associated with the egg surface and consumed by the first instar larvae. The importance of *Sphingomonas* species as possibly the sole food source of first instar larvae is still controversial. The midgut microbiome changed considerably between first and second instars.

Sphingomonas species associated with eggs are stable transmitted from mother to offspring and are independent of the substratum into which eggs are deposited. This means that blow flies carry permanent symbiotic bacteria and *C. vicina* carry specific endosymbiotic *Sphingomonas* bacteria. This is the first report of endosymbiotic *Sphingomona* bacteria.

The endosymbiotic bacteria require a reservoir for these bacteria on or in the adult female fly. *Sphingomonas* species could not be detected on the surface, which lead to the hypothesis the adult fly might have an internal provision to house *Sphingomonas* species; a rectum ampulla might be such a location for bacteriome.

The nuclei of the salivary glands of *C. vicina* larvae show sufficient change during the three days of the wandering stage to allow the ageing of the wandering stage. This is the first investigating in ageing the wandering stage based on morphology.

Defilippo F, Bonilauri P and Dottori M (2013) Effect of temperature on six different developmental landmarks within the pupal stage of the forensically important blowfly *Calliphora vicina* (Robineau-Desvoidy) (Diptera: Calliphoridae). *Journal of Forensic Sciences* 58: 1554-1557.

Richards CS, Simonsen TJ, Abel RL, Hall MJR, Schwyn DA and Wicklein M (2012) Virtual forensic entomology: Improving estimates of minimum post-mortem interval with 3D micro-computed tomography. *Forensic Science International* 220: 251-264.

Zajac BK and Amendt J (2012) Bestimmung des Alters forensisch relevanter Fliegenpuppen. Morphologische und histologische Methoden [Age estimation of forensically important blowfly pupae. Morphological and histological methods]. *Rechtsmedizin* 22: 456-465.