DEVELOPMENTAL AND GENE EXPRESSION CHANGES DURING INTRA-PUPARIAL DEVELOPMENT IN *CHRYSOMYA ALBICEPS* (DIPTERA: CALLIPHORIDAE).

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Declaration

I, *Lucinda van der Westhuizen*, declare that the dissertation / thesis hereby handed in for the qualification *Magister Scientiae* Forensic Science Interdisciplinary at the University of the Free State, is my own independent work and that I have not previously submitted the same work for a qualification at / in another University / faculty.

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Abstract

Criminal investigations are dependent on postmortem interval estimations (PMI) to solve crimes by determining when a homicide occurred. Time of death is estimated using several components, including pathology; however, a pathologist can only determine time of death up to 72 hours postmortem. Therefore, during and beyond this period, entomological evidence is very valuable as the age of the eldest immature fly stage available is used to determine the time of death. In forensic investigations, it is important to have validated growth information at hand for entomological samples, involving all developmental stages, to determine the age of the samples collected.

In forensic investigations, blowflies (Diptera: Calliphoridae) are important as primary colonizers of cadavers, especially in exposed areas, and current age estimation methods of entomological samples are based on morphological changes of the immature stages. Larvae, often encountered at crime scenes, already have established age estimation methods and preservation protocols. However, pupal developmental and preservation studies are in the minority. The main reason for the lack of data is based on the difficulty of working with pupal samples due to the puparium which conceals morphological changes. Considering the importance of the pupal stage, additional techniques such as molecular analysis are required to augment techniques to improve age estimation of the pupal stage.

This dissertation reports on a study that followed a multidisciplinary approach to age estimation of *Chrysomya albiceps* (blowfly) pupae, which are abundant at crime scenes due to their predatory nature. Investigating the morphological changes occurring within the puparium and the possibility to link this to differential gene expressions changes. Two methods for age estimation were therefore developed: external morphology changes and temporal gene expression changes. In addition, different preservation protocols were tested to determine the most effective preservative dependant on the type of analysis.

Validated collection and preservation methods are not readily available for pupal samples and the use of unvalidated collection and preservation methods ultimately hinders age estimation. Laboratory colonies of *C. albiceps*, reared from wild samples, collected at the University of the Free State, were utilized for pupal sample collection during three trials to obtain optimum

number of samples. Three preservation protocols (pierced before hot water kill (HWK) or not pierced before HWK and placing in 70% ethanol or 10% buffered formalin) for morphological examination were tested on 580 – 590 pupae aiming to retain the native morphology. For nucleic acid integrity, to conduct age estimation by gene expression, 78 pupae were subjected to liquid nitrogen as a killing method and samples were preserved at -80˚C. After morphological and molecular examination of pupae the following preservation method is proposed: for morphological examination pupae are pierced prior to HWK and then stored in 70% ethanol, for long- and short-term preservation as ethanol is the best for retaining native morphology; and for gene expression analysis, pupae are immediately subjected to liquid nitrogen and stored at -80˚C.

In addition to previously identified markers, we proposed four new landmarks and based on these results, age estimation can be refined up to 96 hours at 26˚C. The temporal gene expression levels based on 78 pupal samples were quantified using qRT-PCR. It emerged that *actin*, which was previously used as a reference and target gene, cannot be used as a reference gene due to the changes in expression levels during pupal development and that *RpS17, 18S* and *Rp49* are the best reference genes for normalization.

This study made a valuable contribution to the forensic science field in criminal investigations by validating current suggested reference genes, analysing previously identified target genes and identifying new morphological markers which can be used as a baseline for future *C. albiceps* studies.

Key words: Blowflies, postmortem interval, pupal development, preservation, morphological markers, gene expression changes, liquid nitrogen, 70% ethanol, HWK.

Abbreviations

ΔΔCt	Comparative Cycle threshold
Ct	Cycle threshold
20E	20-Hydroxyecdysone
18S	Ribosomal protein S18
28S	Ribosomal protein S28
ace	Acetylcholine esterase
an	Antennae
as	Anterior spiracles
APF	After puparium formation
bcd	Bicoid
CODIS	Combined DNA Index System
ce	Compound eyes
cps	cephalopharyngeal skeleton
cs	Chitin synthase
cDNA	Complimentary Deoxyribonucleic acid
Ct	Cycle threshold
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNASE	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenediaminetetraacetic
ecr	Ecdysone receptor
FA	Formaldehyde
FBI	Federal Bureau of Investigation
fl	Flagellum

GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
gDNA	Genomic Deoxyribonucleic acid
GST1	Glutathione S-transferase-1
hsp60	Heat shock protein 60
hsp90	Heat shock protein 90
HWK	Hot water killing
JH	Juvenile Hormone
l	Legs
lbi	Labium
lbl	Labellum
lsp-2	Larval serum protein
MgCl2	Magnesium Chloride
mp	Mouthparts
ms	Mesothoracic scutellums
NDNAD	National DNA Database
NPE	Non pierced ethanol
pa	Palpus
PC	Pupal cuticle
PCR	Polymerase Chain Reaction
PE	Pierced ethanol
PIA	Period of insect activity
PMI	Postmortem interval
PTTH	Prothoracicotropic hormone
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
rh	Respiratory horns
rop-1	Resistance to organophosphate 1

Rp49	Ribosomal protein 49
RpS17	Ribosomal protein 17
RPLPO	Large ribosomal protein
rt	Respiratory trunk
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
sg	Spiracle gills
sll	Slalom
STR	Short Tandem-Repeat
t	Tarsi
Trp	Transient receptor potential
UFS	University of the Free State
USA	United States of America
usp	Ultraspiracle
w	White gene
w	Wings
wa	Wing apex
wv	wing vein

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Chapter 1: LITERATURE REVIEW

1.1 Introduction

Forensic science is a ‘mixed science’ (Houck and Siegel 2010) where a varied range of scientific principles and techniques are applied in criminal investigations (Evett *et al*. 2000; Tilstone *et al*. 2006; Houck & Siegel 2010; Roux *et al*. 2012). In the pursuit of justice, it is vital to rely on “hard sciences” where fundamentals of science were tested for its forensic application by performing controlled experiments with a high degree of accuracy. Science is generally unbiased and due to the principles of scientific rigor, inherently undisputable (Kiely 2001).

Forensic science is the most misunderstood among all the scientific disciplines, primarily because it is difficult to define (Inman and Rudin 2000; Tilstone *et al*. 2006). It encompasses all fields of science from various areas of the humanities to the formal and natural science disciplines. Several different study areas are associated with forensic work, namely criminalistics, pathology, anthropology, odontology, engineering, toxicology, and behavioural sciences (Reeder 1999; Roux *et al*. 2009; Houck and Siegel 2010). Forensic entomology, specifically medico-criminal entomology, where insects aid in criminal investigations, also forms part of the forensic study areas (Greenberg & Kunich 2002). Forensic Genetics is used as an investigative tool for the judicial process to examine physical evidence containing genetic material (this includes, but is not limited to, blood, hairs, and bodily fluids) collected at crime scenes to identify the source from which it originated. Homicides, suicides, and cases associated with death investigations, where the body is exposed to the environment, can make use of both forensic entomology and forensic genetics. These two seemingly diverse fields can be used in combination to provide stronger and more reliable forensic data.

For this study we combined aspects of both entomology and genetics to answer the forensic question related to the estimation of a post-mortem interval (PMI) based on insect evidence found on a crime scene. In this chapter we will explain the history of forensic entomology and forensic genetics; explore how insect evidence are used to determine a PMI and how genetics boost entomology in terms of a PMI estimation.

1.2 History of forensic entomology and forensic genetics

Forensic entomology is not a new science. Apparently, the Chinese were the first to use insects as evidence in crime scene investigations. In the 13th century, Sung Tz'u was the first to document a forensic entomology case (as cited by Greenberg and Kunich (2002)). In his training manual, '*The washing away of wrongs*', he relates to a murder investigation that was conducted in a rural agricultural village where insect evidence was used to solve the mysterious murder of a local farmer. At that point in time, it was believed that maggots arise spontaneously, and even today, a part of the human population still firmly believes in this phenomenon. From the 1660s, forensic entomology, as a science, evolved further. This development was propelled by three major research movements conducted by different pioneers.

A) Francesco Redi (1668) was the first to show that maggots are not generated spontaneously. He used the flesh of several different animal species to demonstrate that maggots arise due to eggs that were deposited by flies.

B) From 1700 to the 1800's, medicocriminal entomology experienced steady, but significant growth. During that period, different physicians from Germany and France performed mass exhumations of graves. As their focus was initially directed to the fauna of the grave, flies were then not yet linked to murders. Orfila and Lesueur (1848) observed different types of arthropods associated with buried corpses, in order to feed. However, at that stage, they only recognised that maggots are essential for decomposition (as cited by Benecke (2001); Greenberg & Kunich (2002)). This marked the second movement in entomology when the groundwork for a system of insect classification was established, providing a value for identification and succession studies of insects associated with decomposing bodies.

C) In the mid-1800's, interest quickly shifted toward insect types, their life cycles and what they could reveal about crimes. In 1855, Bergeret reported the first forensic entomology case using insect succession and life-cycles to generate a PMI (as cited

by Greenberg and Kunich (2002)). According to Gennard (2007), this case aided in establishing forensic entomology as a criminal investigative tool.

The first systematic study to be conducted in forensic entomology, which also falls under the third movement, was done by Jean Pierre Megnin in the late 1800's. He linked the stages of decomposition to specific insects, consequently establishing the science of forensic entomology. He studied and documented the predictable patterns of insect colonisation in cadavers for years until he published his most important book, '*La Fauna des Cadavres*' in 1894. In this book, Megnin proposed eight insect succession waves for exposed bodies and only two for buried corpses. This difference was attributed to the fact that concealed bodies are not susceptible to the same series of insect colonisation. According to Benecke (2001), this information became the foundation for time-of-death estimations performed by modern-day forensic entomologists.

Forensic entomology methodologies apply to suicides, homicides and untimely deaths. The most noteworthy characteristic about flies is their sense of smell. Insects can colonise a corpse within minutes regardless of the cause of death. The most frequent question directed to a forensic entomologist during an investigation is "When did death occur?" and insects can provide a broader timeline towards answering this question. Understanding the biology of different insect species is crucial for establishing a time-of-death. Therefore, accurate identification of insect species forms the basis for all valid applications of forensic entomology (Greenberg & Kunich 2001; Amendt *et al*. 2007). Advances in molecular genetics and entomology enable insect species identification by targeting DNA variation at a much faster rate. Such advances also provide an alternative method to analyse minute amounts of DNA from the gut content of maggots and identify the host fed on (Wells *et al*. 2001). This improved the standards and knowledge applied in forensic sciences.

Forensic science, as a whole, has a fascinating history. It is centralised around work conducted by individual scientific pioneers, rather than by a systematised group that was publicly funded (Kiely 2001). The direction of forensic genetics was mainly influenced by the increase in crime in the 1970's. Since then, significant changes in science, legal and social entities affected the criminal justice system as well as forensic science. This, in turn, dramatically changed the law-science interphase (Peterson & Leggett 2007). Significant progress in forensics was mainly due to advances in different fields of science during the

Industrial Revolution. It was a time, not identified by absolute advances in science, but more a period where science was applied to real-life problems (Tilstone *et al*. 2006).

Prior to the incorporation of molecular biology in forensic genetics, identification of suspects, as well as victims, was initially conducted by means of blood group markers (Martin *et al*. 2001). In the early 1900's, Karl Landsteiner demonstrated that there are different types of human blood and his research led to the establishment of the ABO blood typing system (Landsteiner 1901). However, this system was still not specific enough because blood group markers are similar among different individuals (Phillips 2008). Advances in human genetic research drove the evolution of forensic genetics and the 1980's were known for scientific innovations. For example, the use of restriction fragment length polymorphism (RFLP) was proposed in the early 1980's, although it did not hold the potential for imminent distinctiveness amongst individuals (Martin *et al*. 2001; Peterson & Leggett 2007).

Forensic DNA technology was born when Sir Alec Jeffreys developed the first DNA typing method in 1984. He laid the foundation for forensic genetics when he studied human DNA diversities. A new type of marker was discovered when he noted that there were different repeating non-coding areas in human DNA that he called 'minisatellites'. The sequences at such regions might be the same for individuals; however, the number of times it is repeated differs amongst different individuals (Armour & Jeffreys 1992). This phenomenon is referred to as a 'genetic fingerprint' and was eventually used in forensic laboratories in less than two years after discovery to identify suspects by profiling their unique minisatellite makeup. The first case solved with the use of genetic fingerprinting was in 1986 when Colin Pitchfork was convicted for the rape and murder of two children (Aronson 2005; Peterson & Leggett 2007). One major setback regarding the use of minisatellites during the 1980's was that large amounts of high molecular weight DNA were required in order to create a profile (Martin *et al*. 2001). The latter limitation of minisatellites prompted the advance seen in DNA fingerprinting techniques in the following years.

DNA fingerprinting had a modest development rate over the years until the invention and introduction of the polymerase chain reaction (PCR) by Kary Mullis in the early 1980's (as cited by Saiki *et al*. (1986) and Martin *et al.* (2001)). This allowed selective amplification of any preferred section of DNA. Shortly after the introduction of PCR, an alternative DNA marker termed short tandem-repeat (STR) was developed. STR's are genetically similar to

minisatellites; however, the repeated DNA region is much shorter in size (Edwards *et al*. 1991). These markers are easily amplified and better suited for amplification of degraded DNA often encountered at crime scenes (Jeffreys 2005; Phillips 2008). A perfect example of the successful application of STR markers was during the investigation led by Erika Hagelberg (as cited by Jeffreys *et al*. 1992). She analysed nanograms of degraded DNA from skeletal remains in 1990 and solved a major war-crime investigation. One of the deceased victims, Josef Mengele, was identified through comparisons of DNA profiles obtained from his son and wife. Within five years, STR typing superseded less sensitive DNA typing methods and dominated the forensic field (Jeffreys 2005; Zagorski 2006).

Typing systems were improved through developments in human genetics and, before long, multiplex PCR systems were introduced to type multiple STR's simultaneously. Large numbers of DNA databases were created soon after, containing multiple STR profiles of criminals and individuals suspected of crimes (Jeffreys 2005; Phillips 2008). The Combined DNA Index System (CODIS) was initiated as a pilot project in 1990, which involved 14 local and state laboratories in the United States of America (USA). In 1994, the DNA Identification Act authorised the Federal Bureau of Investigation (FBI) in the USA to fully implement the system (Peterson & Leggett 2007; Phillips 2008). Michael Howard introduced the first integrated national criminal database in the United Kingdom (UK) in 1995, which is called the National DNA Database (NDNAD). This is still the largest despite the initiation of several databases by different countries (Jeffreys 2005).

Forensic scientists are, however, still actively seeking new advances in technologies to improve forensic investigations. One approach is that of combining genetics and entomology. The use of genetics in entomology was introduced in the 1990s. Insect species identification through the use of molecular genetics was the first documented combination of genetics and entomology (Sperling *et al*. 1994). The study done by Sperling and colleagues in 1994 serves as an example of molecular methods being applied in the identification of immature and adult stages of blowflies. Through this, an avenue was opened for more rapid identification of insect specimens found at crime scenes. These pioneers also laid the foundation for the importance of preservation of insect evidence for DNA analysis (Gennard 2007). Scientists started to focus on gut analysis of insects to clarify if blowfly larvae can be used to detect host DNA (Campobasso *et al*. 2005). Another progression regarding the use of genetics in

forensic entomology was gene expression analysis to improve age estimations (Ames *et al*. 2006, Zehner *et al*. 2006, Foran 2007; Tarone & Foran 2011).

1.3 Forensic entomology and the utility of insect evidence

Knowledge about the distribution, behaviour and biology of insects can be applied in legal investigations (Steven & Wall 2001; Amendt *et al*. 2007). Lord and Stevenson (1986) recognised three categories of forensic entomology, namely, urban, stored product and medico-legal. 'Urban entomology' relates to situations where insects are associated with the human environment and these include bed bug, cockroach and termite infestations. Insects contaminating food products, where segments or the entire insect is found in food products are dealt with under 'stored product entomology' (Gennard 2007; Byrd & Castner 2010; Brown 2012). 'Medico-legal or medicocriminal entomology' is where evidence is drawn from insects collected during legal investigations relating to violent crimes affecting humans and wildlife (Gennard 2007; Singh & Sharma 2008; Byrd & Castner 2010).

Insects make out a large part of complete evidence sampled at a death-related crime scene, and therefore, entomologists are typically consulted at such incidents (Amendt *et al*. 2007). Questions posed to entomologists in such scenarios contribute by providing information on where, how, when and under which conditions a person potentially perished or a crime was committed. To be precise, the geographical area of the crime can be determined, the season, whether the victim had a drug problem, trauma sites on the body and if sexual assault followed (Introna *et al*. 2001; Amendt *et al*. 2007; Gennard 2007). The latter questions are less frequently posed, whereas estimating the time-of-death or PMI by examining collected insects is more frequently requested. Answering this question can provide an indication of when the crime occurred, and this estimate can be provided over a time period of hours, weeks or even years (Amendt *et al*. 2004; Gennard 2007; Singh & Sharma 2008; Byrd & Castner 2010).

1.3.1 PMI estimations based on insect evidence

Death investigations are typically carried out by a team of experts from diverse disciplines ranging from anthropologists, pathologists, and forensic entomologists. In the first 48 – 72hrs

postmortem, time of death is calculated by the pathologist (medical examiner), via the use of natural processes associated with a decomposing body (Campobasso & Introna 2001; Kelly 2006; Richards 2007). Estimations by the pathologist become increasingly difficult when the body is further along the decomposition stages, when there are no typical changes in the soft tissue. Consequently, beyond this point, entomological evidence becomes more valuable for determining a more precise PMI (Campobasso & Introna 2001; Amendt *et al*. 2007; Pohjoismaki *et al*. 2010; Ramos-Pastrana *et al*. 2014).

Two approaches can be followed to provide a time-of-death interval using insect evidence: insect succession and developmental data (Byrd & Castner 2010). Species identification and its life cycle form the baseline for both approaches.

1.3.2 Insect succession

A series of insects colonise a corpse during decomposition, a process that can be characterised into several stages. Each decomposition stage is associated with a specific group of arthropods and their arrival is seen as a predictable sequence (ecological succession). Some fly species have a cosmopolitan distribution, whereas others are restricted to specific areas (Lopes de Carvalho & Linhares 2001; Richards *et al*. 2008; Voss *et al*. 2011; Davies & Harvey 2012; Defilippo *et al*. 2013). Carcass decomposers can be divided into two groups, namely those that consume soft tissues (Diptera) and those exploiting the hair and skin material (Coleoptera) (Kelly 2006; Gennard 2007; Byrd & Castner 2010). Flies and beetles are considered the most common insects to be found at or on a corpse. Diptera species in the families Calliphoridae (blowflies), Sarcophagidae (flesh flies) and Muscidae (house flies) are usually present during the initial stages of decomposition (Louw & Van der Linde 1993; Amendt *et al*. 2004; Kelly 2006; Singh & Sharma 2008). Additional families from Diptera that have been noted for colonising carrion include Phoridae (coffin flies) and Piophilidae (cheese skippers) (Kelly 2006).

Insect succession and the rate of decomposition are influenced by several biotic and abiotic factors, namely geographical location, season, habitat, climatic condition, exposure, and the physical condition of the remains (Voss *et al*. 2011). Differences in succession patterns in various zoogeographical zones as well as variation in life cycles of different forensically

important species are essential aspects of forensic entomology. When the insect succession pattern is known for a particular geographical area under a set of circumstances, PMI estimation can be conducted (Byrd & Castner 2010).

PMI estimations based on insect succession concentrates on the eldest species present. Therefore, a broader estimation will be provided and can vary from three weeks to months especially if beetles are also found at the scene.

1.3.3 Developmental data

Small insects, including blowflies, are poikilotherms. Their developmental rate is highly temperature-dependent, and therefore, the focus of developmental data is on the duration of each developmental stage (Kelly 2006; Byrd & Castner 2010). PMI estimation using developmental data is usually conducted on colonisers that arrive first at a cadaver, namely blow and flesh flies, which can serve as accurate biological clocks in determining the time of death (Amendt *et al*. 2004). Such estimation can be done to the closest day, even hour, if the collected data is accurate and thorough.

A few hours after death, eggs are deposited primarily in the orifices of the cadaver, in high numbers up to a few thousand. The timeframe in which these flies locate and colonise a body is mainly influenced by the accessibility of the body. The progeny of initial colonisers will complete their immature development lifecycle on the body itself (Greenberg & Kunich 2002; Amendt *et al*. 2004). There are three developmental stages that immature blowflies pass through: egg, larval, and pupa before adult eclosion. Egg development is the most rapid in comparison to the entire life cycle and occupy 3 – 7% of development (Greenberg & Kunich 2002; Amendt *et al*. 2004). Determining the age of blowfly eggs and pupae using morphological data can be challenging. Therefore, eggs are typically reared to adult form (Figure 1.1) and back-calculation of PMI is conducted (Ames *et al*. 2006; Zehner *et al*. 2006; Tarone *et al*. 2007; Brown 2012; Chua & Chong 2012).

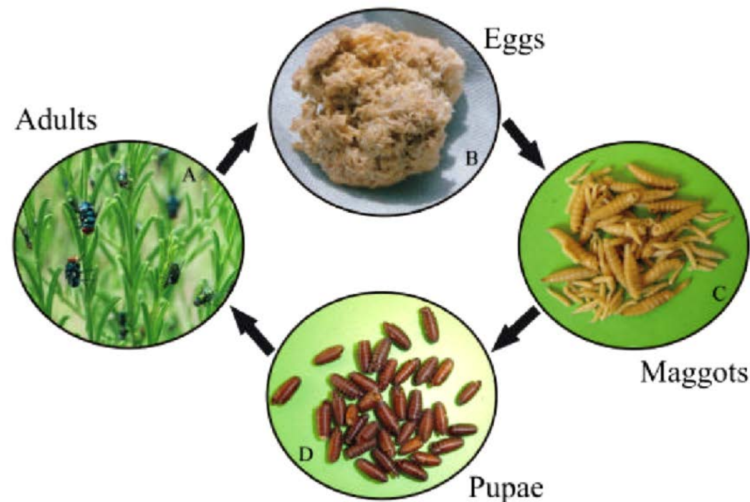

Figure 1.1: Life cycle of the blowfly (Diptera: Calliphoridae) (Kelly 2006).

The larval stage includes three sub-developmental phases: first, second, and third instars. If, and when the larvae reach their maximum weight, the post-feeding stage is reached, and this is also known as the 'wandering phase'. During this stage, larvae migrate away from the food source to seek a suitable dry location for pupation (Greenberg & Kunich 2002). The entire larval stages occupy 25 – 57% of the total developmental time of flies. Larvae of blowflies are used for PMI calculation purposes due to their high abundance during the first stages of decomposition (Richards 2007). The immature stages of flies occupy almost 67% (two-thirds) of the entire life-cycle, and knowledge of the duration of every stage can be used in PMI determination. After collection at a crime scene, the preservation, packaging and transportation of specimens is extremely important to ensure that the samples are not damaged or contaminated, and that species are correctly identified. After identification, the developmental stages are compared to published information on infantile fly growth to determine when eggs were deposited. The latter results in the calculation of the period of insect activity (PIA) being done (Grassberger & Reiter 2001; Amendt *et al*. 2004; Tarone *et al*. 2007; Shiravi *et al*. 2011).

Larval age is determined by using length, width and crop content. An empty crop will correlate with this phase because larvae migrate away from the food source as soon as the wandering phase is reached. If the length of the larvae is known, it can be compared to published curves of development. For example, the isomorphen and isomegalen diagrams, in correlation with temperature, can aid in age determination. Other characteristics that can aid

larval age estimation include using slits in the posterior spiracles to differentiate stages from one another (Grassberger & Reiter 2001; Amendt *et al*. 2007; Richards *et al*. 2008; Brink 2009; Brown 2012).

The pupal stage represents the midway point between the larval and adult forms. It is defined as a resting stage where most of the conspicuous changes take place within the puparia (Karandikar & Ranade 1964). The occupation percentage of the pupal stage differs among species and is longer in some blowflies such as *Calliphora vicina,* where it constitutes approximately 60% of the entire life-cycle (Greenberg & Kunich 2002). Overall, it covers more than 40% of the total immature developmental stages and, during this period, the larvae will undergo several changes in preparation for adult emergence (Gaudry *et al*. 2006; Zehner *et al*. 2006; Davies & Harvey 2012; Defilippo *et al*. 2013).

Frankel and Bhaskaran (1973) discussed concepts and aspects of intra-puparial development (morphology) and metamorphic (cellular changes) events occurring during pupariation and pupation. Part of their discussion dealt with the incorrect use of terminology in several published articles relating to pupation and pupariation. The terminology used to describe the pupal period was recently revisited by Martin-Vega *et al*. (2016). The revision of terminology was meant to clarify that intra-puparial development refers to the entire pupal period, which includes the pre-pupa, pupa, and the developing pharate adult. Pupation was sometimes erroneously described as the process of puparium formation, where hardening and darkening of the puparium occurs (Karandikar & Ranade 1964; Cepeda-Palacios & Scholl 2000). A more precise version of events is that pupariation refers to the formation of the puparium from the third instar larval cuticle initiated by the moulting process, in preparation for pupation, which is the formation of the pupa and consequently the pharate adult (Greenberg & Kunich 2002; Martin-Vega *et al*. 2016).

Greenberg and Kunich (2002) eloquently referred to the pupal stage as a 'black box', as most research focuses on larval development. A black box is a complex system or device that can be viewed in terms of its inputs and outputs without in-depth knowledge of its internal workings. The puparium, which offers a protective casing for the pupae, represents this box (Figure 1.2). Within this box, larva (the input) undergo a radical transformation into adult flies (the output).

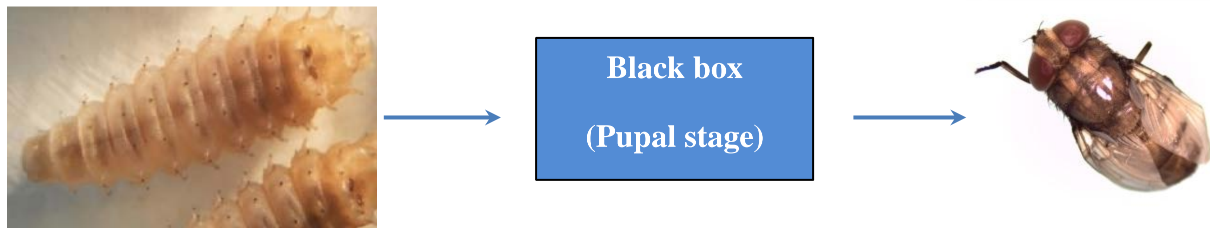

Figure 1.2: Black box concept representing the pupal stage of all flies

1.3.4 Using pupae for PMI estimation

Studies on pupal development are considerably limited in forensic entomology (Karabey & Sert 2014). However, there have been numerous cases where pupae were located on clothes, decaying flesh or hair (as cited by Nuorteva *et al*. (1974) and Greenberg and Kunich (2002)). Such pupae will then represent the eldest form present on which PMI estimation should be conducted.

Traditional methods for determining the age of pupae concentrates on the puparium colour change (Greenberg & Kunich 2002), which can be seen as the third instar larval cuticle that contracts and undertakes a barrel-shape during pupation (Karandikar & Ranade 1964). The main setback of this technique is that darkening of the puparium from creamy white to dark brown, or nearly black, can only be used for the first ten hours after pupariation. Second, there is no published data on the colouration rates of different species (Greenberg & Kunich 2002; Brown 2012). Unlike larvae, the pupal stage has no visible size change, and therefore, length measurements are not useful for age estimation. Consequently, pupae are only classified as relatively "young" or "old", resulting in a broader PMI estimate (Brown 2012). Dissection and examination of the pupa is often required to identify landmarks that correlate with age (Greenberg & Kunich 2002; Gaudry *et al*. 2006). Reference data on morphological development is essential toward understanding the transformation procedure from larvae to adult within the puparium and consequently to use these markers for age estimation.

Three fundamental studies laid the foundation for morphological research on fly pupae. First, Agrell and Lundquist (1973) divided the pupal stage into three phases: histolytic and histogenetic changes along with differentiation. Second, later in 1983, Finell and Jarvilehto studied pupal development at a species level and distinguished additional stages: histolysis,

juvenile and histogenetic stage, then immature and mature stages, and differentiation (Finell & Jarvilehto 1983). Third, Greenberg and Kunich (2002) published the first article on pupal developmental stages and duration at different temperatures. In their study, Greenberg and Kunich distinguished 11 stages of the *Phormia regina* pupal period at 22˚C and 29˚C. Their main conclusion was that, as with larvae, the pupal stage is also highly temperature-dependant, and even the same generation may have variable pupation and hatching times (Finell & Jarvilehto 1983).

Since 2012, several morphological and histological studies have been conducted on forensically important fly species to determine the pupal developmental changes and their duration at different temperatures. Notable examples include *Calliphora vicina* and *Lucilia sericata* (Brown 2012; Davies & Harvey 2012; Karabey & Sert 2012; Zajac & Amendt 2012; Defilippo *et al*. 2013), *Chrysomya albiceps* and *Chrysomya rufifacies* (Pujol-Luz & Barros-Cordeiro 2012; Hu *et al.* 2019). Initially, research on pupal development in members of Diptera focused on the physiology of individual organs and systems in various species of the Drosophilae (Poodry & Schneiderman 1970; Voas & Rebay 2004; Kojima 2004) and Calliphoridae (Finell & Jarvilehto 1983; Crossley 1965) families (Table 1.1). This increased the possibility to obtain a more precise PMI estimation by using morphological changes during the pupal phase.

Table 1.1: Fly characteristics studied by several authors for the pupal stage.

Characteristics	**Species**	**Authors**
Compound eyes	*Calliphora erythrocephala* *Drosophila* *Musca domestica*	Finell & Jarvilehto 1983 Voas & Rebay 2004 Hewitt 1907
Abdominal muscles	*Calliphora erythrocephala*	Crossley 1965
Wings	*Drosophila* *Musca domestica*	Wehman 1969 Hewitt 1907
Legs	*Drosophila* *Musca domestica*	Kojima 2004 Poodry & Schneiderman 1970 Hewitt 1907
Leg sensory organs	*Phormia regina*	Lakes-Harlan et al. 1991

		Lakes & Pollack 1990

Another approach that is currently under investigation is that of differential gene expression using molecular techniques (Ames *et al*. 2006; Zehner *et al.* 2006; Tarone & Foran 2011). Gene expression regulates the development of specific traits throughout insect growth, hence, providing an independent data source for blowfly age estimation.

The controlling mechanism behind the switch between different stages (larval-pupal and pupal-adult) is still relatively unknown and poorly studied (Konopova & Jindra 2008). It can be one of several stimuli received by the insect, such as nervous stimuli conveyed to the skin by the nervous system or particular hormones that are secreted by the frontal part of the pupae and transported by the blood (Fraenkel 1935). During the larval-pupal and pupal-adult switches, several changes occur within the larvae to accomplish complete adult structures (Bainbridge & Bownes 1981; Gates 2002). Numerous hormones are known to influence this transformation by regulating gene expression.

1.4 The forensic application of genetics in terms of insect development

1.4.1 Hormones and development

Countless proteins are temporarily needed as tissues and organisms develop. Consequently, genes that encode these proteins are activated and inactivated as required. Initially, gene regulation throughout development has been studied in *Drosophila melanogaster* (Arbeitman *et al*. 2002). Subsequently, the *Drosophila* model can be used for the identification of candidate genes in blowflies for further studies with an *a priori* expectation of informative regulation. Such comparisons are possible due to similarities in genetic and physiological processes in members of Drosophilidae and Calliphoridae (Arbeitman *et al*. 2002; Stolc *et al*. 2004; Tarone *et al*. 2007; Tarone & Foran 2011).

Moulting and metamorphism are controlled by three hormones, which can be loosely referred to as the ‘brain’ behind all development, and these include the prothoracicotropic hormone

(PTTH), juvenile hormone (JH) and ecdysteroids (Riddiford *et al*. 2003; Nation 2008). The primary hormone responsible for inducing and regulating metamorphism is ecdysone. In turn, the gene product responsible for inducing ecdysone further activates a hierarchy of early and late response genes that are differentially controlled throughout pupal development (Gates 2002, Zhou *et al*. 2004; Gaudry *et al*. 2006; Boehme *et al*. 2013). During the initiation of metamorphosis, the hormone 20-hydroxyecdysone (20E) peaks and prepupal formation begins to mark the formation of the puparium. After puparium formation, another spike in 20E production appears (Figure 1.3), and thus, the transition from prepupal to pupal occurs, resulting in the initiation of adult development (Thummel 1995; Gates 2002; Nation 2008). The epidermis of the individual becomes pupally devoted and larval-specific genes are no longer expressed. Total transformation in form and function through opposing developmental pathways are established simultaneously in response to the production of 20E. Seemingly, ecdysone regulates the development of almost every postembryonic tissue in insects (Fisk & Thummel 1996; Gates 2002; Beckstead *et al*. 2005; Flatt *et al*. 2005; Warren *et al*. 2006; Nation 2008; Riddiford *et al*. 2010). Nevertheless, the regulation of development by the steroid hormone signalling activity remains rudimentary. Therefore, that ecdysone regulates developmental changes throughout the entire life-cycle of flies makes this gene a perfect candidate for age estimation studies in insects.

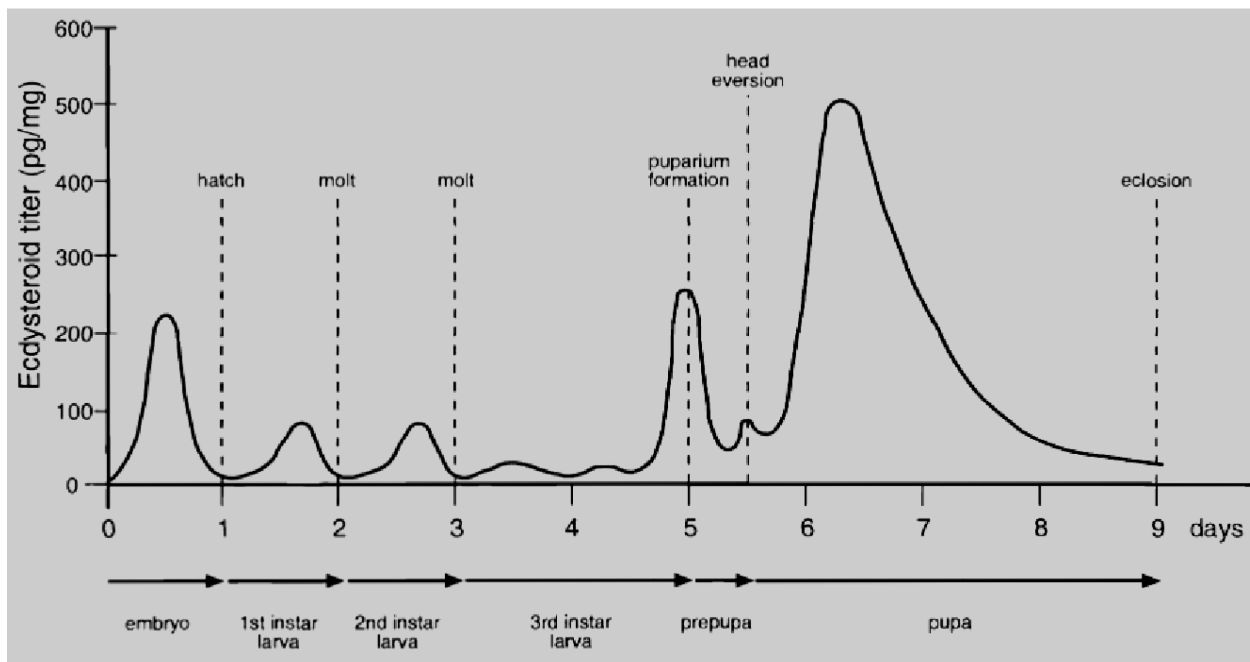

Figure 1.3: Six pulses of ecdysone during the major development periods of *Drosophila melanogaster*. Head eversion occurs at around 5.5 days, and thus, representing the onset of

pupal development and the highest level of ecdysone in the insect body. Dotted lines specify the main transitional stages (Gates 2002).

1.4.2 Differential gene expression

Extensive studies on the levels of gene expression changes have been conducted in the past, aiming to understand the biological mechanism behind the transformation in form and function (Fraenkel 1935; Bainbridge & Bownes 1981; Fisk & Thummel 1996; Beckstead *et al*. 2005; Foran 2007). To date, however, there is limited data regarding gene expression for the pupal stage. Nonetheless, Ames *et al*. (2006), Foran (2007; 2011) as well as Brown (2012) have suggested that it is possible to correlate insect age with specific gene expression changes within each developmental stage.

Zehner and colleagues first attempted to estimate the age of pupae using molecular tools in 2006 on *Lucilia sericata* (Zehner *et al.* 2006). The study only focused on three time points during pupal development: 48h, 96h and 144h. Ames *et al*. (2006) also attempted to determine pupal age in *Calliphora vicina* by analysing gene expression during the same three time points as Zehner *et al*. (2006). Although both studies could not conclusively determine pupal age through gene expression analysis, they provided some evidence that there may be a correlation between gene expression and age and recommended further investigations. Tarone *et al*. (2007) studied gene expression changes during the embryonic period of *Lucilia sericata*. Total RNA was extracted from egg masses and the expression levels of three genes, bicoid (*bcd)*, slalom (*sll)* and chitin synthase (*cs)* were analysed by comparing them against reference genes (ribosomal protein 18S (*18S*), ribosomal protein 49 (*Rp49*), *actin*). Reference genes, previously called housekeeping genes, are expected to be expressed constantly throughout development due to the fact that they are necessary for basic cell survival (Pfaffl 2001). Tarone and associates concluded that even though the egg stage lasts for only one to nine hours, it can be divided into smaller periods, allowing for a more precise age estimation (Tarone *et al*. 2007).

In 2011, Tarone and Foran further attempted to improve on the previous study by including the later stages of fly development in the form of larvae and pupae (Tarone & Foran 2011). Expression patterns of fly development genes including *sll*, heat shock protein 60 (*hsp60*) and

hsp90, resistance to organophosphate 1 (*rop-1*), *cs*, acetylcholine esterase (*ace*), ecdysone receptor (*ecr*), ultraspiracle (*usp*) and white (*w*) were evaluated. All these genes were expressed differentially throughout development. In addition, morphologically similar feeding and post-feeding larvae could also be differentiated by seven of these genes (*cs*, *ecr*, *hsp60* and *90*, *rop-1*, *usp* and *w*). As ecdysone is responsible for the switch between feeding and post-feeding larvae, thus initiating metamorphosis, it is unsurprising that the *ecr* gene exhibited changing expression patterns during this period. Tarone & Foran (2011), resolved that other developmentally informative genes in blowflies could be identified, which would help improve age estimation analysis.

Gene expression patterns, specifically during the pupal stage of *Calliphora vicina*, were explored by Brown (2012) and Boehme *et al*. (2013). Brown (2012) concluded that expression patterns of *ecr*, larval serum protein (*lsp-2*) and transient receptor potential (*Trp*) genes accurately determined insect age relative to external morphological changes. Boehme *et al*. (2013), on the other hand, studied expression patterns of four genes (*15_2*, *2014192*, *actin* and *arylphorin receptor*), two of which were newly identified genes (*15_2* and *2014192*), at three different rearing temperature (15˚C, 20˚C, 25˚C). They found that the expression pattern of *15_2* increased to its maximum at day 2 of pupal development whereafter it steadily decreased. The genes *2014192* and *actin* had similar expression patterns whereby their levels were initially constant but increased after 50% of pupal development. In addition, all genes studied excluding the *arylphorin receptor* gene were significantly affected by rearing temperature: faster development; faster gene expression progression. The genes studied by Brown (2012) and Boehme *et al*. (2013) ultimately showed potential as molecular markers for age estimation due to the expression differences noted between early and late phases of pupal development.

Currently, studies focussing on selecting and validating reference genes for use in different blowfly species are limited. Cardoso *et al*. (2014), for example, compared expression patterns of ten candidate reference genes in larvae and adult flies of *Cochliomyia hominivorax*, *Cochliomyia mecallaria* and *Chrysomya albiceps*. They found that the expression behaviour of *actin*, glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*) and *Rp49* were promising since their expression levels were most stable in different tissues. Another study by Bagnall and Kotze (2010), on the Australian sheep blowfly *Lucilia cuprina*, analysed 11 genes during all life stages and confirmed that *18S*, ribosomal protein 28S (*28S*), glutathione S-transferase-

1 (*GST1*), β-tubulin and the large ribosomal protein (*RPLPO*) are suitable reference genes for this specific fly species. The analysis of gene expression and validation of reference genes is, however, still ongoing and this methodology has great potential to improve PMI estimations and broaden the field for age estimations using pupae.

1.5 Aim and Objectives

With this study we aim to contribute to the determination of a developmental model based PMI estimate in the case where intact *Chrysomya albiceps* puparia are found on a crime scene.

Objectives are as follow:

1. Determine the most effective preservation protocols for puparia that will ensure successful downstream analysis based on external morphological and genetic landmarks expressed over the intra-puparial period.

2. The identification of robust morphological landmarks denoting the age of pupae along the intra-puparial developmental timeline.

3. The identification of differential gene expression during prepupal, pupal and early adult development correlates with time at a certain point during insect development.

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Chapter 2: MATERIALS & METHODS

2.1 Study site

Adult flies, eggs and larvae of wild *Chrysomya albiceps* were recovered from pig carcasses placed on the west campus of the University of the Free State (UFS), Bloemfontein, 29˚06'02.80" S 26˚10'20.95" E. The carcasses were placed in an open field inside steel cages to prevent disturbance from animals in the area. Carcasses were monitored on a daily basis over a period of one month for fly activity and sample collection.

Ethical approval (UFS-ESD2021/0274) was granted by the Environmental and Biosafety research ethics committee (Appendix 1).

2.2 Sampling and breeding protocols

2.2.1 Collection and handling of wild *Chrysomya albiceps* samples

Wild-caught specimens were transferred to the insectarium at the Department of Zoology and Entomology of the UFS. Larvae of *C. albiceps* were identified using a standard key and reared under a constant temperature of 26˚C (± 2˚C correction factor) with a day/night cycle of 12:12 until pupal formation. Prior to emergence, pupae were transferred to smaller containers consisting of sawdust and these were placed in bugdorms until adult emergence.

2.2.2 Establishment of laboratory colonies

All *C. albiceps* colonies were reared under controlled laboratory conditions as described above. A breeding program was established by providing the colonies with a constant supply of water and granulated sugar. To initiate ovary maturation and also serve as an oviposition medium, 200g chicken liver was placed into cages holding reared colonies. After oviposition, eggs were collected from the cages, divided equally and reared in large separate plastic containers to control for overcrowding. The containers were half-filled with sawdust, covered with foil, moist paper towels and mesh lids. Chicken livers were placed on paper towels and

replaced every second day to prevent fluid accumulation from liver putrefaction. Fresh liver was reintroduced until the post-feeding larval stages were reached. The post-feeding stage became evident when larvae started to migrate away from the food source, thus 'wandering larvae' that go searching for a suitable dry area to initiate pupation.

Wandering larvae were transferred to larger containers containing only sawdust. As soon as the pupal stage was reached, pupae were retrieved and transferred to smaller containers within the bugdorms. Granulated sugar and water were supplied to emerging adult flies in the containers and replenished as necessary. The breeding program was repeated when new colonies were needed.

2.2.3 Sample collection for morphological analysis

At the commencement of the trial, cages were checked daily for newly laid eggs (Figure 2.1). As soon as egg masses were observed, they were reserved for rearing and placed in separate containers to control for overcrowding. The rearing procedure was conducted in the same manner as described above with the growth buckets containing sawdust, moist paper towel and chicken liver, that served as a feeding medium. The emerging larvae were allowed to continue development in the containers until the post-feeding larval stage was reached.

Figure 2.1: Egg masses collected from fly cages.

Third instar larvae were checked every two to four hours to identify and capture newly formed post-feeding larvae in the wandering phase. During this stage, larvae prepare themselves for pupal formation by burrowing into the sawdust. Larvae in the wandering phase were placed in separate containers with sawdust (Figure 2.2) to control for overcrowding. The wandering phase larvae were checked every four hours for newly formed pre-pupae.

Figure 2.2: Post-feeding larvae were divided in separate plastic containers to avoid overcrowding of individuals.

Various sub-stages of post-feeding larval development were noted. Early phase represented larvae that contracted their head and tail when picked up but continued movement immediately after they were placed back into the sawdust. The intermediate phase is when the larvae stay contracted in a c-shape form for a few seconds before continuing movement. Late wandering larvae were signified by larvae that formed a c-shape and remained in this form for longer than a few seconds (Figure 2.3). Wandering larvae were monitored closely until newly formed pre-pupae were observed.

Figure 2.3: Wandering phase is identified by a c-shape formation when larvae are pressed and picked up.

Pre-pupae, which are the interphase between post-feeding larvae and pupae, were white in colour, immobile and had a soft puparium. This phase was categorised as 0-hour and marked the initiation of puparium formation, as contraction of the larvae is perpetual (Figure 2.4).

Figure 2.4: White puparium formation indicating the commencement of pupal phase.

Typically, wandering individuals do not reach the pre-pupal phase at the exact same time. Therefore, containers with relocated post-feeding larvae were monitored every two to four hours in order to recover all newly formed pre-pupae. On every occurrence, an average of 80 – 100 pre-pupae were retrieved from different containers. These pre-pupae were again placed in different containers (based on the time of collection) filled with sawdust to use for further pupal development sampling and marked as follows: first pre-pupae batch sampled; second

pre-pupae batch sampled etc. A separate inventory of the collection time for all the 0-hour pre-pupae was also kept. This allowed for sufficient samples to be collected from all the conducted experiments (Figure 2.5).

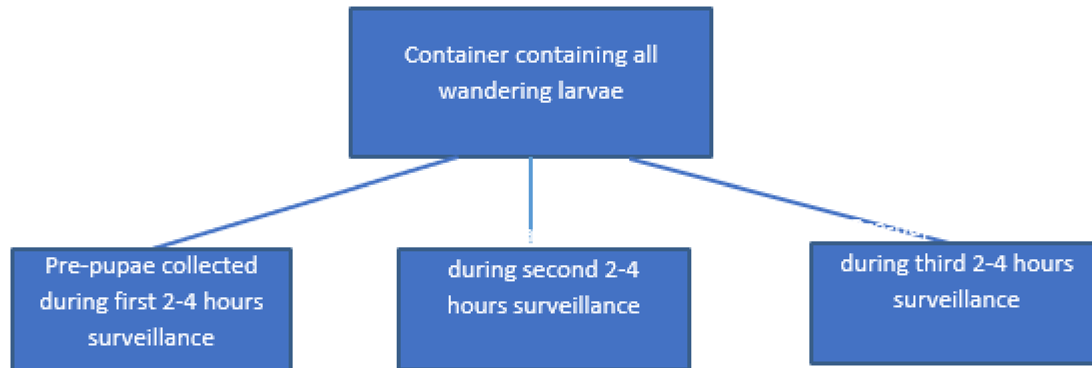

Figure 2.5: The different pre-pupae batches obtained from post-feeding larvae at 2 - 4 hour intervals.

Boiling water was used to kill 10 - 15 pre-pupae after which the individuals were placed in a preservation solution. The objective for using this killing method was first to arrest development and solidify the liquefied state that is typical of early pupal development. Harvesting pre-pupae at this point was considered to be time point 0 hours. Following that, pupae were retrieved every eight hours, killed and preserved similarly as the 0 hour until adult eclosion. This sample harvesting experiment (referred to in later chapters as trial 1, 2 and 3) was performed concurrently in triplicates to collect biological and technical replicates. The main aim of the trial was to sample specimens at fixed time periods for analysis. In addition, a trial testing preservation method was also conducted. Each of the trials produced enough larvae for the testing of preservation methods as well as morphological tracking.

Chapter 3 will detail the methodology used to test for the best preservation method, whereas Chapter 4 will contain information regarding specifics of the morphological study.

2.3 Collection, preservation and RNA extraction for molecular analysis

2.3.1 Collection and preservation of samples for RNA extraction

Pupae were collected as explained above. However, upon collection, the pupae were immediately frozen in liquid nitrogen to halt development and preserve the samples. These samples were transported in liquid nitrogen to the Department of Genetics at the UFS and stored at -80˚C until RNA extraction.

2.3.2 Total RNA extraction for molecular analysis

Total RNA was extracted from 78 pupae using the Trizol method as explained below (each time point was extracted in duplicate).

2.3.3 RNA extraction Protocol

All hardware was sterilised by steaming in an autoclave to eliminate contamination and RNases. Pupae for each time point were added to the mortars separately along with liquid nitrogen and ground to a fine powder. Powdered material was transferred to clean tubes that were pre-chilled in liquid nitrogen to prevent the sample from thawing. All samples were standardised to a weight of 40 mg, to which 500µl of Trizol was added. For samples below 40 mg, only 250µl of Trizol was added. Samples were incubated at 15 - 30˚C for five min in the presence of Trizol reagent.

Thereafter, 100µl of chloroform was added and the tubes were shaken vigorously by hand. The samples were incubated once again at 15 – 30˚C for two to three minutes. The samples were then centrifuged at no more than 12 000 x g for 20 minutes at 4˚C. The upper aqueous layer was transferred to a new eppendorf tube after centrifugation and the RNA was precipitated by the addition of 250µl isopropyl alcohol. Samples containing the RNA were incubated at 15 - 30˚C for 10 minutes before centrifugation at 12 000 x g for 15 minutes at 4˚C. The supernatant was removed from all the samples and the RNA pellet (a white, gel-like pellet on the side of the tube) were washed once with 75% ethanol. Samples were mixed by vortexing and centrifuged for the last time at 7 500 x g for five minutes at 4˚C. The supernatant was discarded and the RNA pellet was air-dried for two to three minutes. The RNA pellets were dissolved in 40 µl DEPC water and incubated at 55 - 60˚C for ten minutes.

Samples were either stored at -20˚C for immediate use or at -80˚C for long term storage. The quantity and quality of samples were determined immediately after extraction.

2.3.4 Quantification of extracted RNA

After RNA extraction, samples were assessed for quantity as well as the purity of the extracted RNA using the NanoDrop® spectrophotometer, ND-1000[1]. The quality of RNA was determined by measuring the absorption ratio at 260 nm - 280 nm. For RNA, a ratio of ~1.8 is generally accepted as "pure", whereas anything below that value was considered an indication of contaminants still present in the sample. After treating the RNA samples with DEPC water, the standardised concentration was 0.25ng/µl. The remaining total RNA was stored at -80˚C after extraction.

2.3.5 DNase treatment

After RNA extraction, traces of DNA may still be present in the sample; therefore treating the samples with a DNA digesting enzyme limits this possibility. Samples were therefore first treated with DNase I to eliminate gDNA contamination.

While tubes were kept on ice, 2 µl of RNA was added along with 1 µl of 10X Reaction buffer with $MgCl_2$, 5 µl of DEPC and 1 µl of DNase I. The mixture was briefly vortexed, centrifugated and incubated at 37˚C for 30 min. Then, 1 µl of 25 mM EDTA was added to the samples before incubation at 65˚C for 10 min.

2.3.6 Agarose gel electrophoresis

Following DNase treatment of the samples, agarose gel electrophoresis was performed to establish that the samples were not degraded, that no DNA was present and that a sufficient quantity of RNA was extracted. Degradation can be identified by the presence of smears and DNA can be detected by the presence of a thick, bright band towards the bottom of the gel. To ensure that the total RNA is intact, two bands should be visible on the gel. It will have a sharp 28S and 18S rRNA band where the 28S is twice as intense as the 18S (2:1 ratio). In

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[1] NanoDrop is a registered trademark of NanoDrop Technologies, Inc.,

flies, these two bands appear as one as the 28S band migrates to the position of the 18S band due to the heating step during extraction (Winnebeck *et al*. 2010).

A 1.2% agarose gel was prepared by adding 10 ml 10X FA gel buffer and 90 ml DEPC treated water to 1.2 g agarose. The mixture was heated until the agarose was melted. It was cooled down to 65˚C, whereafter 1.8 ml formaldehyde (FA) was added. The gel mixture was poured into a gel tray and left to set. Prior to sample loading, the gel was equilibrated by placing it in 1X FA gel running buffer for 30 min.

A loading buffer was prepared by mixing 750 µl 5X RNA loading buffer with 3.5 µl GelRed^{TM}. After preparation of the loading buffer, 1.5 µl of this loading buffer was added to 4.5µl of the RNA sample and incubated at 65˚C for 3 – 5 min. The RNA mixture was loaded into the wells of the gel and ran for 50 min at 400V and 90A. Afterwards, the gels were visualised under a UV light and photos were taken on the G-box gel documentation system using the GeneSnap software.

2.3.7 Reverse transcription

First strand cDNA was synthesised from RNA by adding the following to a PCR tube while the tube was on ice: 2 µl of total RNA, 1 µl oligo(dt) and 9.5 µl DEPC-treated water. The mixture was vortexed and pulse centrifuged before incubation at 70˚C for 5 min. All the incubation steps were done using the G-Storm thermal cycler. The tubes were again placed on ice and 5X reaction buffer, 0.5µl RiboLock^{TM} Ribonuclease Inhibitor and 2µl dNTP mix were added to the tubes. The mixture was again vortexed and pulse centrifuged before it was incubated at 37˚C for 5 min. Thereafter 1µl of RevertAid^{TM} M-MuLV reverse transcriptas was added. The mixture was incubated for 1 hour at 42˚C along with an additional heating step of 10 min at 70˚C to stop the reaction. cDNA samples were diluted by adding 1 µl of the sample to 49µl DEPC treated water to obtain a 0.02 concentration of cDNA. All the cDNA samples were stored at -20˚C for use in further molecular analysis.

2.3.8 Selection of reference genes and primer design

Previous studies on other insects were referred to select candidate reference genes (Tarone *et al*. 2007; Bagnall & Kotze 2010; Tarone & Foran 2011; Cardoso *et al*. 2014). The genes were selected from studies that focused on either validating the expression stability of the genes over a range of different samples or in functional studies where it was used as endogenous genes. The genes *GAPDH, Rp49* and *RpS17* were chosen from a validation study focussing on *Lucilia sericata* and *Chrysomya albiceps.* These genes were seen as good candidates due to the identified constant expression pattern (Cardoso *et al*. 2014).

One additional reference gene, namely *18S* rRNA, was also considered for use as a reference gene. The sequence of this gene was acquired from the NCBI GenBank database (AF322425). The partial sequence of *L. sericata* was aligned against orthologs from eight different insect species, namely: *Calliphora nigribarbis*, *Cochliomyia macellaria*, *Neobellieria bullata*, *Musca* sp., *Musca domestica*, *Pseudogonia rufifrons*, *Drosophila persimilis* and *Drosophila simulans*. This was done using the tblastx algorithms to search for possible orthologs of *18S*. A global alignment was performed with the nine sequences using the ClustalX program. Conserved regions were identified among the aligned sequences after which primer pairs were designed using Primer Designer (v4.20, Scientific and Educational software).

2.3.9 Selection of target genes

Ecr and *actin* were identified as possible target genes based on previously conducted studies (Tarone & Foran 2011; Brown 2012; Boehme *et al*. 2013), even though *actin* was used as a reference gene by several studies (Tarone *et al*. 2007; Bagnall and Kotze 2010; Cardoso *et al*. 2014).

2.3.10 Amplification of selected reference genes

Gradient PCR was performed using synthesised cDNA obtained from samples collected during Trial 3. This was done in order to determine the optimal annealing temperature of the different primers for the PCR reaction. The following reagents were added to a PCR tube while the tube was on ice: 2 µl synthesised cDNA, 5 µl 1X KAPA2G Robust HotStart Readymix, 0.3 µl of 0.5 uM primer (forward and reverse) and 2.4 µl of nuclease free water in

a total volume of 10µl. This was performed in a G-Storm PCR system (Somerton Biotech, UK). Once optimisation was completed and the optimal annealing temperature determined this was used for conventional PCR prior to sequencing.

Sequencing was conducted using the ABI 3130 to confirm that the gene sequence corresponded to the referenced articles as well as the pre-designed gene sequence for *18S*. Lastly, qRT-PCR was performed to determine the expression patterns of the selected candidate genes.

2.3.11 Quantitative real-time PCR

Two biological replicas were used for 13 samples and four technical replicates were performed per locus. In total, 520 samples were analysed on a real-time PCR machine (QuantStudio 5, Thermo Fisher Scientific) using 96-well plates. A melt curve analysis was also performed with every experiment to determine the presence of primer dimers. The following reagents were added: 5 µl 1X Kapa, 0.2 µl 0.2 uM forward primer, 0.2 µl 0.2 uM reverse primer, 2 µl cDNA and 2.6 µl PCR grade water, to a total volume of 10µl.

After all the plates were completed the data were analysed using the DataAssist v3.01 software program (Applied Biosystem) that utilises the comparative Ct (ΔΔCt) method.

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Chapter 3: EFFECT OF DIFFERENT PRESERVATIVES ON THE INTRA-PUPARIAL DEVELOPMENT STAGES OF *CHRYSOMYA ALBICEPS*

Abstract

Several studies investigated different techniques for optimal preservation of immature blowfly stages. However, no standard protocol has been established for the preservation of pupae yet. It is crucial for age estimation studies that the preservation and storage of entomological samples be done correctly. In this study, three different methods were tested: pierced ethanol, non-pierced ethanol and non-pierced 10% buffered formalin. For morphological examination of fly pupae, 70% ethanol seems to be requisite for short- and long-term preservation periods. All characteristics required for age estimation during this study were perfectly preserved in 70% ethanol.

Key words: preservation, standard protocol, fly pupae, age estimation, 70% ethanol, formalin.

3.1 Introduction

A quality-assured, methodical approach must be in place to collect, preserve, package, and transport forensic samples. This not only guarantees a chain of custody, but also prevents contamination and destruction of evidence (Catts 1992; Campobasso & Introna 2001; Adams & Hall 2003; Amendt *et al*. 2007; Day & Wallman 2008). It is also true for entomological specimens, and prior to analysing entomological samples in the laboratory, effective collection and preservation procedures are critical to ensure that species identification or age determination is conducted accurately (Day & Wallman 2008; Brown *et al*. 2012). That is because inappropriate handling of entomological samples, mainly due to inadequate sampling protocols, may directly affect the accuracy of downstream analysis.

Preservation protocols are essential for age estimations (Adams & Hall 2003; Amendt *et al*. 2007; Day & Wallman 2008; Singh & Sharma 2008; Brown 2012; Brown *et al*. 2012) since the preservative used can alter morphological markers used for estimating insect age. The collection of entomological specimens from a crime scene is not always prioritised or carried

out by entomologically trained individuals. Currently, pupae collected at crime scenes are either kept alive until arrival at the laboratory (Brown 2012) or placed directly into preservation fluid. Both methodologies can potentially influence age estimations. In the case of live samples, development is allowed to continue until review. A plethora of unaccounted conditions may present themselves from the time of collection and throughout the period of transporting samples to the laboratory and during development if allowed to continue in the storage facility. Estimating age without knowing all conditions that influence development may lead to inaccuracies.

Standards and guidelines for collection and preservation of eggs, larvae and adult flies have been established by numerous researchers (Adams & Hall 2003; Amendt *et al*. 2007; Day & Wallman 2008; Byrd *et al*. 2010; Amendt *et al*. 2011; Richards *et al*. 2013). However, there are fewer guidelines on the preservation of pupae (Brown 2012; Brown *et al*. 2012). Despite several studies on pupal development, preservation techniques were only explored by Brown (2012). Brown *et al*. (2012) suggested in their pupal preservation study that pupae should be pierced prior to hot water killing (HWK) and then placed in ethanol. Piercing the chitinous puparium is essential as it will allow the preservative to fully access the pupal tissues (Brown *et al*. 2012). HWK plays an essential role in the preparation of entomological specimens for analysis. Hot water halts development, allowing for accurate age estimation to be conducted. This method is recommended by several authors who studied larval and pupal sampling methods, yet there are still debates regarding the optimal water temperature that should be used (Adams & Hall 2003; Amendt *et al*. 2007; Day & Wallman 2008; Richards *et al*. 2013; Karabey & Sert 2014). Considering collection at crime scenes, such methods are either impractical, e.g. HWK, or the chemicals required are not easily available (Day & Wallman 2008).

The aim for this chapter was to find an easier executable alternative to the protocol proposed by Brown *et al.* (2012) for puparia and pupae preservation to mitigate for the logistical constraints experienced by crime scene technicians.

3.2 Materials and methods

Different preservation treatments were used to examine their effects on the puparium and characteristic age markers of the pupae, including discolouration. This experiment was conducted in triplicate, per set of conditions (thus, three trials: A, B, C), for statistical purposes.

3.2.1 Sampling

Adults, larvae and eggs of *Chrysomya albiceps* were sampled from the western side of the University of the Free State (UFS) campus, Bloemfontein, South Africa. Specimens were transported to the insectarium where second and third generation colonies were established under a constant environmental regime of 26˚C (± 2˚C correction factor) at a day/night cycle of 12:12. Additional information regarding colony establishment was described in Chapter 2.

Larvae were monitored until the wandering phase was reached. The pre-pupal period (formation of white puparium) was designated as stage 0-hour. Specimens were sampled at 8-hour intervals until adult emergence. Between 580 and 590 pupae were selected over the course of the three trials. Pupae were killed by placing them in boiling water, i.e., HWK method, for 30 – 60 seconds to halt development.

3.2.2 Preservation techniques

The preservation liquids tested after HWK were 70% ethanol (with samples pierced or not pierced) and 10% buffered formalin. Different preservation schemes were tested to determine a method that would be better at conserving the developmental markers associated with the developing pupae and puparia. For specimens preserved in 70% ethanol after HWK, the puparium was pierced once at the position of the three body segments (head, thorax, abdomen). Care was taken to only pierce the puparium and not through the entire pupa. Samples placed in formalin were not pierced due to the rapid penetration characteristics of formalin (Fox *et al*. 1985; Cox *et al*. 2005; Buesa 2008). Treatments were designated as follow:

- Treatment 1: Pierced, ethanol (PE)

- Treatment 2: Non-pierced, ethanol (NPE)
- Treatment 3: Non-pierced, 10% buffered formalin.

Furthermore, all samples from treatment 3, that were initially stored in formalin, were subsequently transferred to 70% ethanol. This was done to limit the dehydration caused by formalin. Samples were transferred at different times:

- Trial A specimens were transferred to ethanol within 3 days.
- Trial B specimens were transferred to ethanol at seven days
- Trial C specimens were transferred to ethanol at two weeks,

Two weeks after the completion of a trial, the pupae were removed from the puparium and returned to their corresponding preservatives.

3.2.3 Morphological examination

As a control for puparium discolouration, some of the 0 hour pupae were photographed immediately after sampling, before the specimens were placed in the preservatives. All evaluations regarding the effects of various preservative treatments on developing pupae were examined and compared 30 days after the trials were completed. External morphological examinations were performed using a Leica EZ4 StereoZoom® HD dissection microscope (Leica Microsystems, Wetzlar, Germany).

3.3 Results

3.3.1 Effects of preservatives on the puparium

At the onset of the pupal developmental phase, the puparium had a distinct creamy, white colour that turned brown to reddish-brown within eight to ten hours, as pupal development progressed. The creamy, white colouration of the puparium of 0 hour pre-pupae stored in ethanol, whether the specimens were pierced or not, was severely affected. Discolouration was noted at several locations on the puparium (Figure 3.1). The puparium of some specimens already exhibited a dark-brown colour, which is usually only evident a few hours after pupal formation. Specimens that were not pierced (Figure 3.1 a and b) discoloured faster

(i.e. within a day of preservation) and this effect was noted more on the anterior ventral side. Samples that were pierced (Figure 3.1 c and d) turned brown within two days after placement in ethanol. This discolouration was not limited to specific points on the puparium, but instead was evenly spread over the entire pupae. Furthermore, some pierced puparia collapsed from its native rounded form.

Figure 3.1: Brownish discolouration of pupae stored in 70% ethanol. (**a** - **b**) Pupae that were not pierced before the samples were placed in ethanol. (**c** - **d**) Pupae that were pierced before the samples were placed in ethanol.

Formalin-fixated specimens (Figure 3.2) examined at two- and five days post-transference into 70% ethanol, had the same soft texture and white, creamy colouration compared to the same age specimens examined immediately after collection.

Figure 3.2 a (dorsal) – b (ventral): 0 hour pupae stored in 10% buffered formalin two and five days before transfer to 70% ethanol retained the creamy white colour and soft texture of the puparium when examined at two- and five days.

3.3.2 Effects of preservatives on the pupae

When assessing morphological characteristics that may assist in age estimation of specimens, it is essential that no structural damage occurs, and native colour and size be retained.

Whereas 10% formalin resulted in better preserved specimens for the early-stage-pupae (i.e., 8-hour APF specimens), the 70% ethanol (whether it was pierced or not-pierced) preserved specimens better for all subsequent hours after pupariation.

During the early stages (8 hour APF) of pupal development, removing the pupa from the puparium was difficult (Figure 3.3 a – h) for those stored in 70% ethanol. Only 20% of the 8 hour APF pupae that were stored in 70% ethanol from the start, whether pierced or not pierced, could be successfully removed. In some instances, only the posterior or anterior (Figure 3.3 g - h) part of the pupa could be successfully removed. The pupa appeared brittle and dehydrated (Figure 3.3 e – h). However, where a portion of the pupa could be removed, its morphology was discernible (Figure 3.3 c and d). Specimens that were pierced prior to placement in ethanol were the most difficult to remove from the puparium as they were more dehydrated and brittle than non-pierced specimens (Figure 3.3). Furthermore, some of the ethanol preserved 8 hour APF pupae had a slight yellow-brownish discolouration.

The early stage (8 hour APF) pupa specimens initially stored in 10% buffered formalin retained a native white, creamy colour and its morphological integrity was largely maintained (Figure 3.3 f). Specimens could not be successfully removed from the puparium 30% of the time (Figure 3.4 a) however, the remaining pupae were perfectly preserved, easily removed, relatively soft and no discolouration was noted, as indicated in Figure 3.4 b. However, the duration specimens were kept in the 10% formalin preservative influenced the integrity of the pupa, whereby attempted removal resulted in partial damage to the pupa.

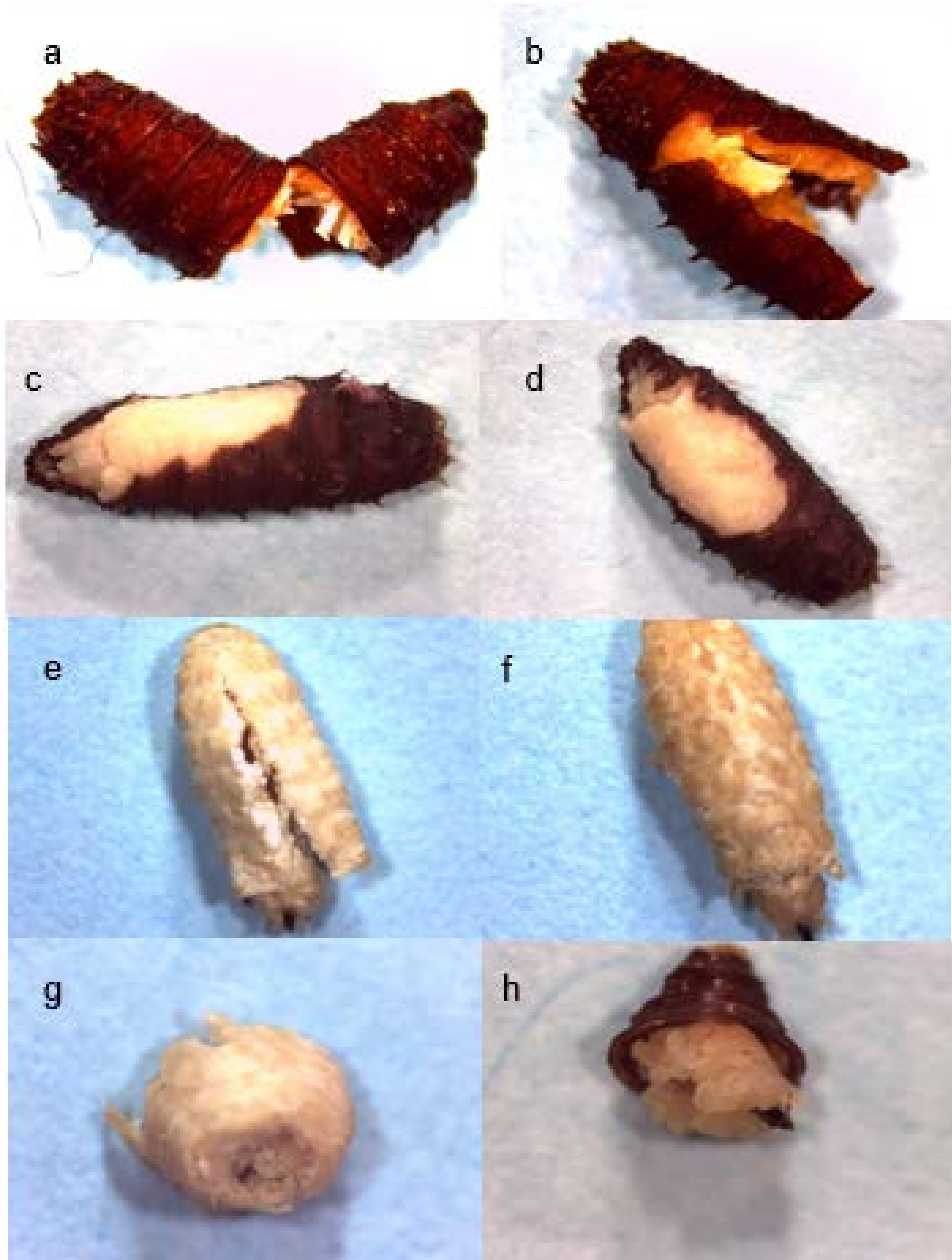

Figure 3.3: (**a** – **d**): Pupal cuticle wall still attached to the inside of the puparium of pupae stored in 70% ethanol resulting in difficulty removing the puparium. (**e, g, h**): Highly dehydrated pupae stored in 70% ethanol, if puparium is successfully removed only parts of the pupae can be visualised. (**f**): pupae sored in 10% buffered formalin that could successfully be removed.

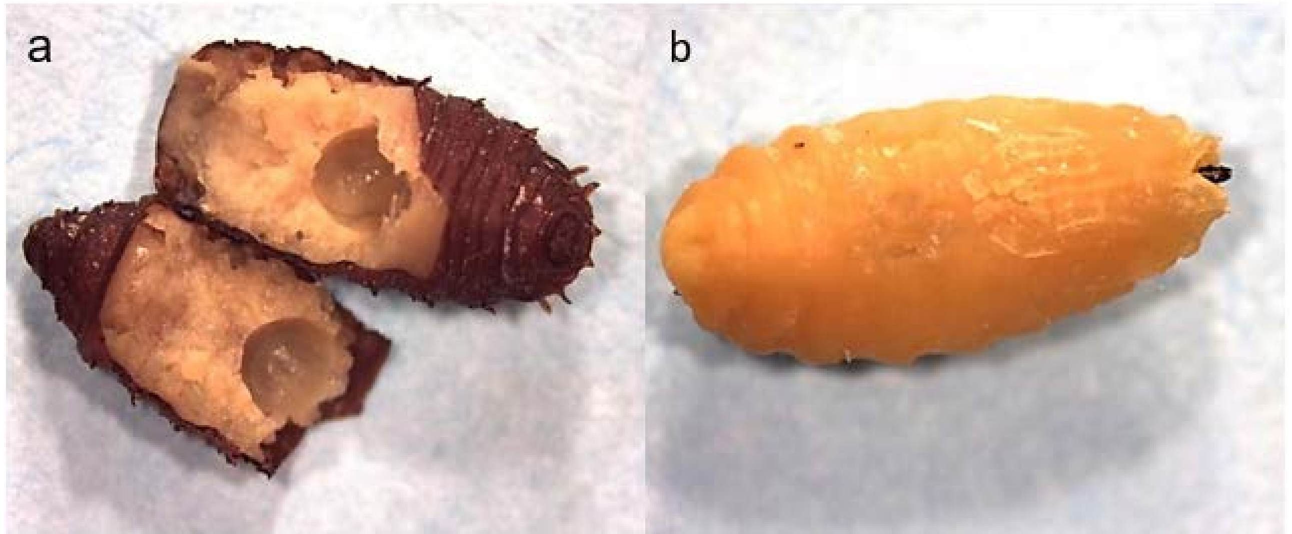

Figure 3.4: a – The cuticle of 8 hour pupae, stored in 10% buffered formalin, still firmly attached to the puparium. b – Pupae that were successfully removed from the puparium.

Pupae older than 8 hours, stored in 70% ethanol, were generally better preserved compared to samples first fixated in 10% buffered formalin. Only 36% of all the ethanol-fixated samples (pierced and not pierced) were dehydrated, fragile and had an off-white discolouration. This was mainly noted in pierced ethanol samples from the 24 hour time period (Figure 3.5 a - b) compared to the rest of the ethanol-fixated specimens (Figure 3.5 c - e). All pupae initially fixated in formalin, older than 8 hours, were dehydrated and discoloured, with the severity of discolouration increasing as pupal development progressed. Samples fixated in formalin for a longer period were more affected than samples stored for less than a week before transferring them from formalin to ethanol (Figure 3.6 a - b). The pupal cuticle membranes for samples from Trial C were severely dehydrated and discoloured to such an extent that identification of morphological features was problematic (Figure 3.6 c - d). Such misidentification was observed in almost all samples.

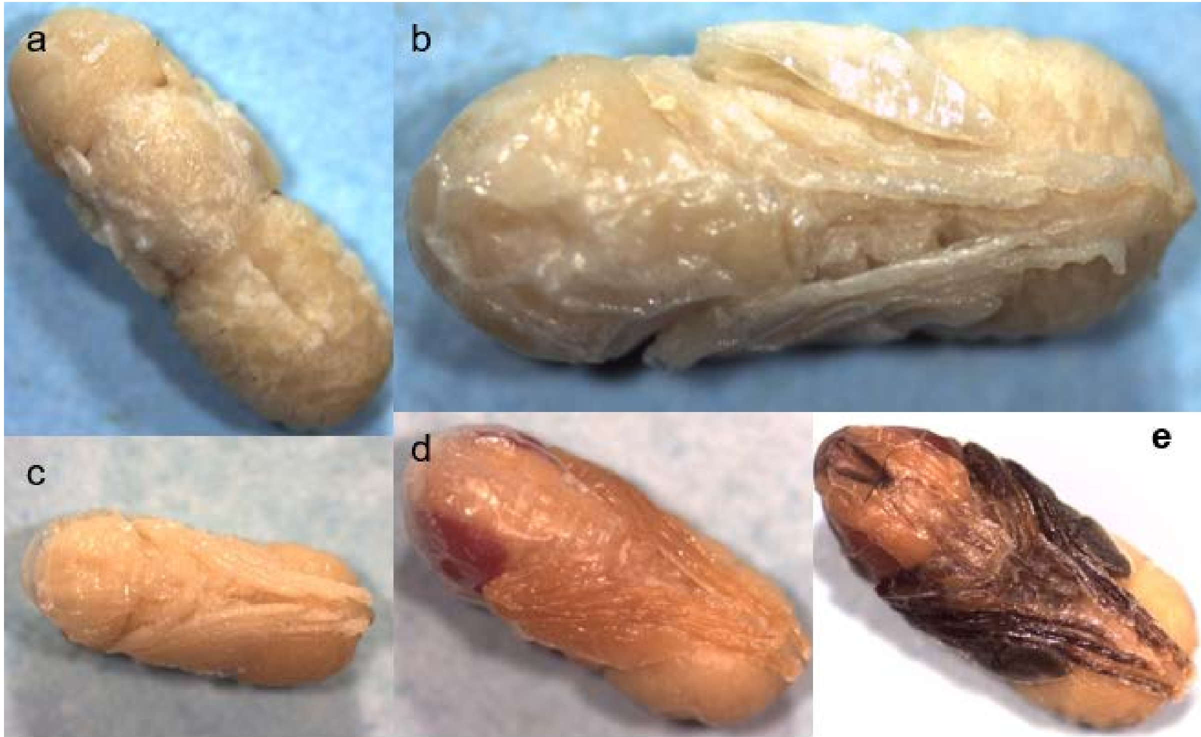

Figure 3.5: (**a** – **b**): A portion of the samples stored in ethanol was dehydrated and had a whitish discoloration. (**c** – **e**): Rest of the samples were perfectly preserved.

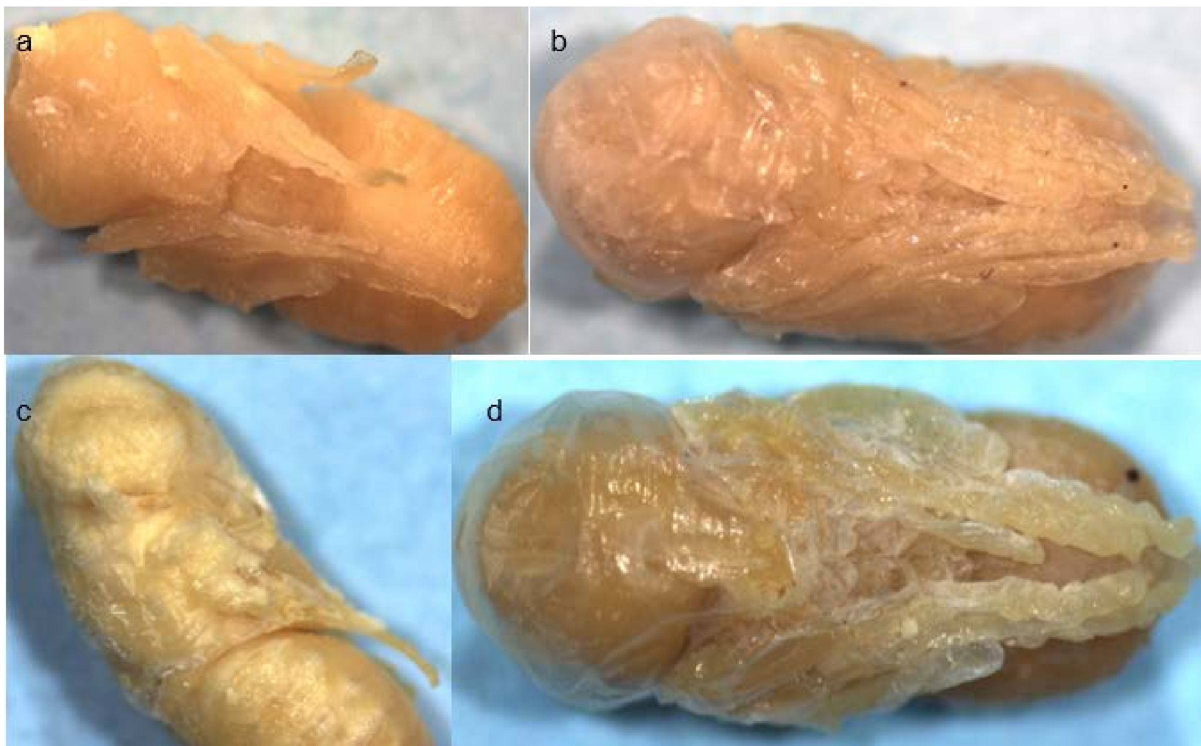

Figure 3.6: Comparing samples of the same age stored in formalin before transferring to ethanol at different times. (**a** - **b**) Pupae samples 16 and 32 hours transferred to ethanol within a week; (**c** - **d**) Pupae samples 16 and 32 hours transferred to ethanol at two weeks.

During the pupal period, a membranous structure that surrounded the entire pupa was noted. This cuticle membrane was first evident from around 24 hours after pupariation. The effect of preservatives on this cuticle membrane was evident for pupae fixated in both ethanol and 10% buffered formalin. The membrane for most pupae fixated in ethanol (pierced or not pierced) from 16 – 58 hours were reasonably well preserved. From 64 hours onwards, the cuticle membrane appeared wrinkled, and in several instances, appeared as if the preservative fluid was trapped under the membrane (Figure 3.7 a) in specimens that were pierced and fixated in ethanol. The membrane appeared more wrinkled in specimens that were initially fixed in formalin (Figure 3.7 b).

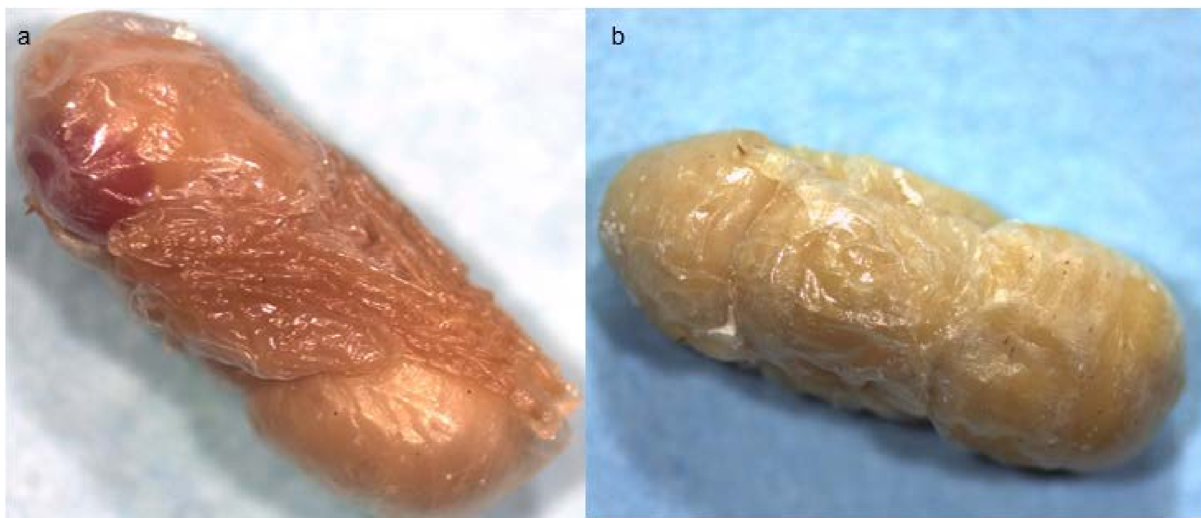

Figure 3.7: Clear differentiation between the membrane and the pupae. **a)**: 64-hour individuals stored in 70% ethanol; **b)**: 64-hour Individuals stored in 10% buffered formalin.

As pupal development progressed, specimens that were initially fixated in formalin took on a dark-yellowish colouration compared to the creamy-white ethanol preserved specimens (Figure 3.8 a - d). This was especially noticeable on the head, thorax, and abdomen areas of the pupae. After 48 hours, specimens initially fixated in formalin were more dehydrated, and the observation landmark morphological features was challenging (Figure 3.8 a – b). Leg and wing development was easily recognisable in almost all specimens, irrespective of the preservative. However, the identification of eye colouration, antennae and mouthpart development were not easy to evaluate in formalin preserved specimens due to discolouration (Figure 3.8 e - h). Pupae stored in ethanol had native colouration of eyes, antennae, and mouthparts (Figure 3.8 g – h). Figure 3.9 is a comparison between pupal samples stored in 70% ethanol vs formalin of the exact same age (56 hours APF); pupae that were first stored

in formalin had no eye colouration on the anterior-dorsal side of the head compared to the samples fixated in 70% ethanol (Figure 3.9 a – b).

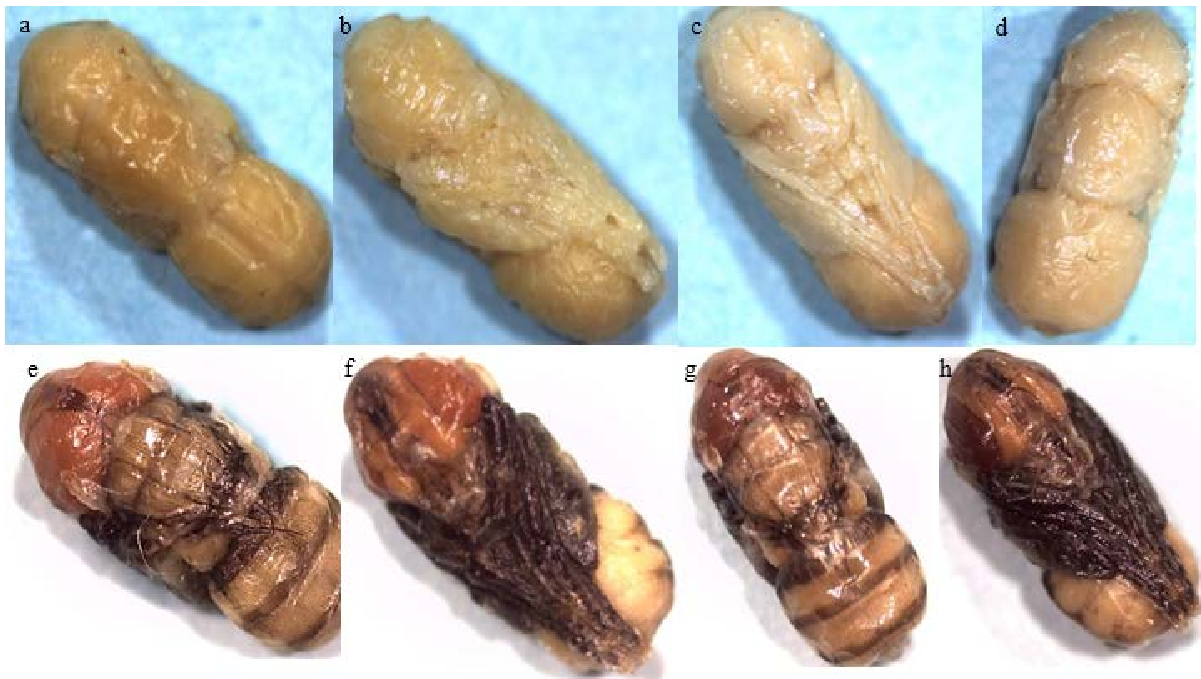

Figure 3.8: Pupae first stored in formalin before transferred to 70% ethanol had a yellowish discoloration (**a** – **b**, **e** - **f**) in comparison with samples stored in ethanol which showed native coloration (**c** – **d**, **g** - **h**).

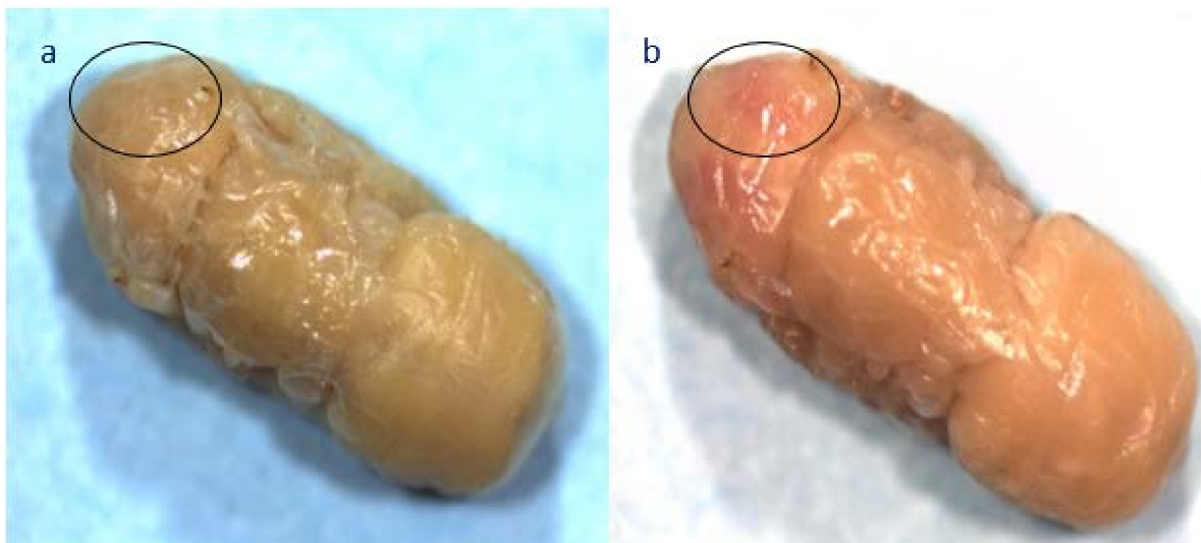

Figure 3.9: Discolouration of 56 hour pupae preserved in 10% buffered formalin suppressed native coloration of pupae (**a**) in comparison with pupae stored in 70% ethanol (**b**).

3.4 Discussion

Postmortem estimation using insect evidence can be determined utilising several methodologies; the most widely used methods are based on morphological characteristics of forensically important fly species. It is therefore important that age-related morphological markers are retained in puparia and pupa collected from a crime scene.

Puparium discolouration

Age estimation using characteristics of the puparium has been disregarded over the years. The main reason is that after 24 hours, the colour remains dark-brown due to sclerotization (Greenberg & Kunich 2002; Brown 2012). Discolouration of the native puparium colour in 0 hours APF was observed in those specimens preserved in 70% ethanol. However, when stored in 10% buffered formalin, the native colouration was retained for almost a week, even after transferring to 70% ethanol.

Puparium removal

Critical to the examination of morphological characteristics is the intact removal of the pupae from the puparia. Eight hours after the formation of the white pupa, the puparium was already hardened, darkish-brown and the development of adult structures was imminent. However, the puparium still forms part of the pupa at this stage as it is formed from the integument of the third instar larvae (Dennel 1947). Only after several hours a moulting process took place separating the pupa from the puparium. Despite the preservative used, it was difficult to remove the pupa from the puparium and the fragile pupal tissue tended to pull away with the puparium during the early stages after pupariation. Several researchers were able to successfully remove pupae from the puparia (Brown *et al*. 2012, Karabey and Sert 2014), and Adams and Hall (2003) at the early stages post pupariation, however the success rate of the protocols followed were not clearly stated. One main difference between previous studies and the current study was that puparia were pierced prior to HWK in previous studies as opposed to it being pierced after they were HWK in the current study. Removing the pupae from the puparium was not an issue for any subsequent stages after pupariation for any of the preservation media used.

Pupae discolouration

Discolouration of the pupae is one of the major concerns when preserving a sample, as it influences accurate age calculations negatively. Ethanol and formalin had very different effects on intra-developmental markers at the various stages of the pupal phase. Samples that were first HWK and then stored in 70% ethanol, whether pierced or not, were perfectly preserved and showed almost no signs of discolouration with the exception of one or two samples. However, these samples also showed a degree of putrefaction, possibly due to the slow penetration of the killing liquid.

Certain types of preservatives such as formalin appear to have a dehydrating effect, especially in later stages of pupal development when pupae are naturally highly sclerotised. The resulting brittleness makes it challenging to examine the pupae for morphological markers, pertaining to the age estimation of a specimen. When examination too soft or brittle pupae, it can result in the destruction of tissues as it is challenging to handle such samples. During the early stages of pupal development, the pupae are mainly comprised of fat tissue, thus reasonably soft. Specimens from the eight-hour interval that were first stored in formalin were perfectly preserved and firm despite the duration in formalin before transferring to ethanol. Even though the membrane surrounding the pupa appeared dehydrated as pupal development progressed, the pupae themselves were never too soft or brittle to be examined morphologically. Ethanol also had a dehydrating effect on the specimens, though not to the same degree as formalin. Furthermore, some researchers (Amendt *et al*. 2007; Buesa 2008; Amendt *et al*. 2011) suggest that formalin should not be considered as a preservative as it can affect human health and safety and cause DNA degradation of samples.

An interesting feature about formalin fixation is that it causes a yellow discolouration of samples, specifically in pupae 16-hour to 64-hour after pupariation. This was noted in most specimens examined despite the fixation duration in formalin prior to transferring to 70% ethanol. Formalin is known to penetrate tissue at a high rate depending on the density of the samples. However, it fixates slowly and can take up to 48 hours, depending on the temperature at which it is fixed. It also interacts directly with proteins by cross-linkages, and it may be too successful and although it prevents autolysis (Fox *et al*. 1985; Cox *et al*. 2005; Buesa 2008) it increased turgor to such a point that pupae were brittle and hard. This effect resulted in extreme difficulty to examine any morphological changes that occurred in later post-pupariation specimens.

Another disadvantage associated with formalin and 70% ethanol fixation is the shrinkage of tissue (Fox *et al*. 1985; Brown *et al*. 2012). However, this was not observed in the specimens of *Chrysomya albiceps* evaluated as part of this study.

3.5 Conclusion

According to Adams and Hall (2003) different species react differently to various preservation liquids and techniques, emphasising the importance of additional preservation studies that focus on the morphological effect on each forensically important species. This will assist in establishing easily executable standard guidelines for the collection and preservation of pupal samples found at a crime scene.

Considering the importance of entomological specimens and the need for accurate age estimations, it is essential to ensure that specimens are sampled, preserved, and stored correctly. Current pupae collection techniques, used at crime scenes, suggest that specimens should be kept alive for rearing or stored under cool conditions until examination (Amendt *et al*. 2007). Even though this collection method is easily executable, it is inadequate and not an acceptable preservation technique for pupae sine specimens is not retained in the exact state it was found at sampling time and this could affect age estimation in pupae.

The aim of chapter was to find an easier executable alternative to the protocol proposed by Brown *et al.* (2012) for puparia and pupae preservation. Brown *et al*. (2012) proposed piercing the puparium, using hot water to halt development, to limit enzymatic degradation of the pupa and to solidify the content of the puparia followed by storage in 80% ethanol at -20ºC for external morphology analyses. In this study on *C. albiceps* pupae, it was demonstrated that different preservation liquids and techniques have various effects on pupal morphology. Formalin is the best preservative for pre-pupae (0 hour) to retain the creamy white colour as well as conserving the true-to life coloration of the puparium. Unfortunately, it was not successful for the bulk of the pupal timeframe landmarks conservation. Ethanol (70%) was found to be overall the best preservative for the remaining timeframes post-pupariation. As shown in this study, piercing causes the samples to become more dehydrated, especially when stored at a minimum period of a month before analysis. Ideally, samples will

be analysed immediately when received at the laboratory; however, this is not always possible. Considering the timeline before samples are analysed, piercing of samples impedes correct age estimation. Taking all of this into consideration, the following is suggested when collecting *C. albiceps* puparia from a crime scene: HWK, followed by preservation of unpierced pupae in 70% ethanol. Since the coloration of the puparium is of limited use in determining the age of the pupal stage, it is more important to use a method that will conserve morphological characteristics of the pupa for determining age of the specimens.

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Chapter 4: EVALUATION OF EXTERNAL MORPHOLOGICAL MARKERS FOR THE INTRA-PUPARIAL DEVELOPMENT OF *CHRYSOMYA ALBICEPS* (DIPTERA: CALLIPHORIDAE)

Abstract

Chrysomya albiceps (Diptera: Calliphoridae) are prevalent during early stages of decomposition. In various criminal cases that include insects, pupae can also be found. Various authors attempted to correlate time to metamorphic events to improve post mortem interval (PMI) estimation by focussing on the major morphological characteristics in *Lucilia sericata*, *Drosophila melanogaster*, *Calliphora erythrocephala*, *Calliphora vicina* and *Phormia regina*. Therefore, this study was done to improve current trends on the chronological events that occur during the pupal period in *C. albiceps* in South Africa, to improve morphological landmarks. In total four new morphological markers were observed (calypters, respiratory gills, pretarsi development and growth of a dull green hue on the thorax) in addition to the already identified markers.

Key words: *Chrysomya albiceps*, pupae, PMI, morphological landmarks.

4.1 Introduction

The pupal period, which covers a large percentage of the total development time (Greenberg & Kunich 2002), is important in PMI developmental studies. Unfortunately, the relatively simplistic developmental markers (instar stage, weight, and length) that are used to determine the age of larvae cannot be applied to the pupal period (Grassberger & Reiter 2001; Greenberg & Kunch 2002; Amendt *et al*. 2004). During the pupal period, development can be tracked to a certain extend by noting the changes to the puparium i.e., a change in colour (white puparium to dark brown) and the eversion of the respiratory horns (Amendt *et al*. 2004). Unfortunately, these markers are only useful for a very narrow timeframe at the beginning of the pupal period however, the change the pupa undergoes provides much more in terms of developmental markers (Robertson 1936; Frankel and Bhaskaran 1973;

Bainbridge & Bownes 1981; Truman & Riddiford 2002; Zehner *et al*. 2006; Davies & Harvey 2012; Pujol-Luz & Barros-Cordeiro 2012).

Many researchers (Cepeda-Palacios & Sholl 2000; Zehner *et al*. 2006; Brown 2012; Davies & Harvey 2012; Pujol-Luz & Barros-Cordeiro 2012; Defilippo *et al*. 2013; Karabey & Sert 2014) worked towards narrowing the broad developmental model-based PMI estimation spectrum, by detailing intra-puparial development for a wide range of forensically important calliphorid fly species. However, many of the developmental markers proposed are not easy to use because it is not always visible, and some are open to misinterpretation due to its qualitative nature. One of the aims of this study was to review the current identified morphological markers for ease of use by general entomologists and to investigate if additional markers could be identified for *Chrysomya albiceps* in our region.

Chrysomya albiceps is a consistent carrion ecosystem species in South Africa (Louw & Van der Linde 1993; Braack 1981). The aggressive cannibalistic nature of *C. albiceps* larvae in a maggot mass, results in them being the dominant species present in a carrion ecosystem. Research on the intra-puparial development of *C. albiceps* was still outstanding at the time this study was embarked upon. Subsequently, a few more articles on the intra-puparial development of the species emanated from the South American continent providing a relevant baseline to compare our results against.

4.2 Materials and Methods

A detailed account of colony establishment and the rearing of larvae until the time of sampling the target life stage (pupae) is given in Chapter 2. Conditions in the insectarium were maintained at 26˚C (± 2˚C) with a day / night cycle of 12:12.

Boiling water was used to arrest development and to ensure solidification of the contents of the puparia. The pre-pupae were designated as 0 hours; pupae were subsequently sampled every eight hours. Ten to fifteen individuals were sampled per time interval and stored in 70% ethanol. Pupae were removed from the puparium and stored in 70% ethanol until analysis.

External morphology of the pupae was examined using a Leica EZ4 StereoZoom® HD dissection microscope. The morphological conclusions drawn by Pujol-Luz and Barros-Cordeiro (2012) and Karabey and Sert (2014) were used as a baseline to track and define morphological developmental changes of pupae in this study.

Disclaimer: We did not analyse the landmarks in terms of its morphometrics, therefore no scalebars are indicated in the images presented.

4.3 Results

Pupariation

The contraction of the post-feeding larvae is permanent. The post-feeding larvae became more immobile as the larval cuticle becomes more sclerotized.

0 Hours

At the onset of pupariation, the immobile creamy-white puparial structure (Figure 4.1 a) was pliable. The overall form of the post-feeding larva was retained but the first three segments were retracted. As sclerotization of the puparium progressed, the creamy-white colour changed to dark brown (Figure 4.1 b) within a few hours. At this point in time, it was impossible to remove the pre-pupa from the puparium.

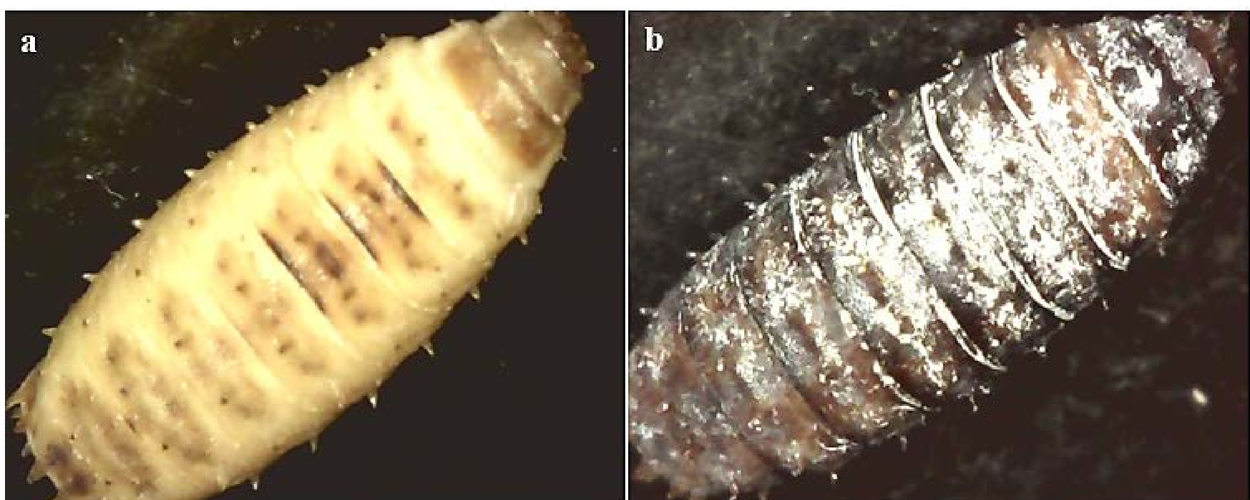


Figure 4.1 *Chrysomya albiceps* puparia at (**a**) 0 hours, creamy-white puparium. (**b**) dark-brown puparium after few hours.

8 Hours

At the completion of pupariation, successful removal of the pupa from the puparium was possible. The pupa was white at this stage.

At the beginning of this stage, the head was still concealed (Figure 4.2). The cephalopharyngeal skeleton (Figure 4.2 a) was still attached to the anterior end of the cryptocephalic (hidden head) pupa.

Partial eversion of the legs (Figure 4.2 a) and wings (Figure 4.2 b) of the pupa was evident. The legs reached the posterior margin of the predestined thorax.

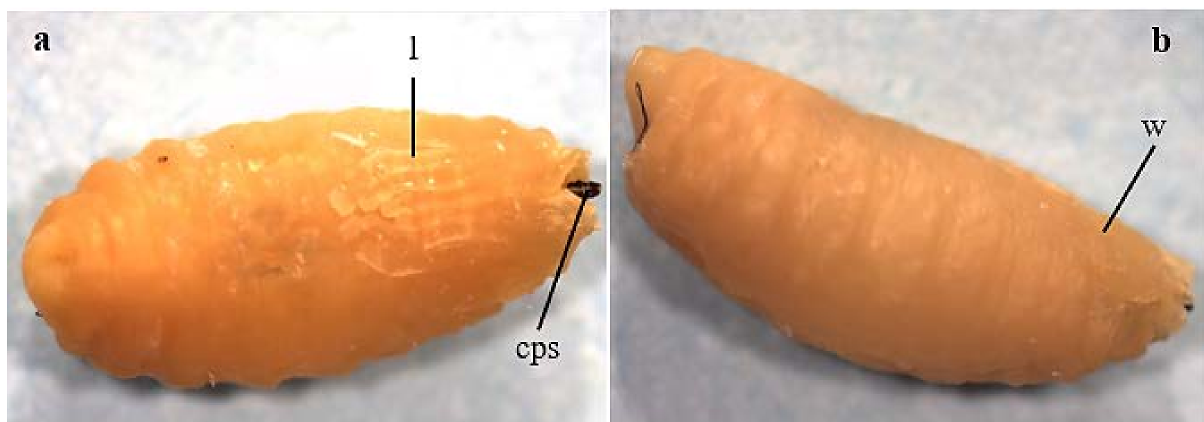


Figure 4.2 *Chrysomya albiceps* cryptocephalic pupa, 8 hours after pupariation. **(a)** Ventral - extend of legs. **(b)** Lateral - undifferentiated wing. **Abbreviations:** cps: cephalopharyngeal skeleton; l: legs; w: wing.

This stage is transient and ends as soon as the evagination of the head occurs. At the onset of the evagination of the head (between 8 - 16 hours), the pupa is defined as a phanerocephalic pupa. At this stage the head, thorax and abdomen differentiation were evident, and the development of several appendages of the head and thorax were set off. As the head was everted, the cephalopharyngeal skeleton was released from the pupa to the inner surface of the puparium.

16 Hours

At this stage the tagmata of the adult form were established; the pupa was a uniform white-yellowish colour (Figure 4.3). The respiratory horns were repositioned from an anterior position in the cryptocephalic pupa to an antero-laterally position in the phanerocephalic

pupa. The respiratory horns were unpigmented (Figure 4.3 b insert) at this stage. This relocation occurred as the head of the developing pupa was pushed outwards. Tube-like, dorsolateral respiratory trunks spanned from an anterior position on the thorax to a position in the proximity of the compound eyes, connecting the pupal respiratory system to the exterior environment via the respiratory horn.

After head eversion, the imaginal appendages of the head became visible. The compound eyes had a cream colouration until 48 hours after pupariation. Antennae, visible as buds, were located ventrolateral on the head. Mouthpart development was evident and was seen as the oral lobe margins being visible (Figure 4.3 a); at first the labellum margin was square.

The legs and wings were membranous and undifferentiated. Whereas the legs at 8 hours after pupariation were restricted to the posterior margin of the thorax, the legs had increased in length, and the metathoracic leg pair were in line with the posterior end of the abdomen (Figure 4.3 a). The outline of the pretarsi of the metathoracic legs were visible (Figure 4.3 a). Bilateral structures were noted at the posterior margin of the thorax (Figure 4.3 b). These structures were clearly visible up to 88 hours after pupariation.

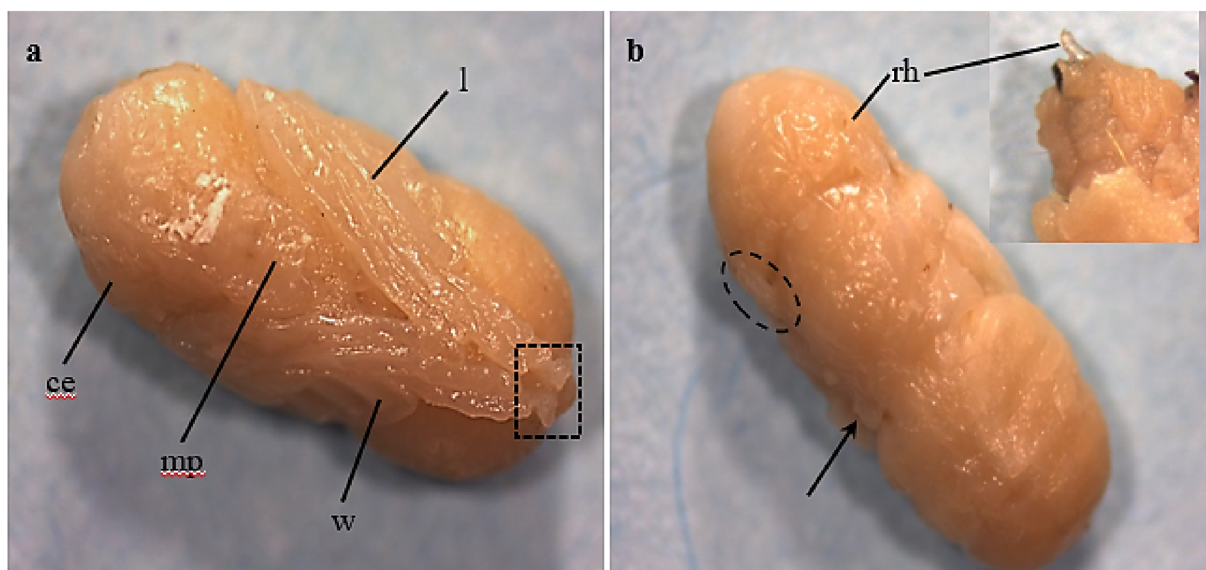


Figure 4.3 *Chrysomya albiceps* phanerocephalic pupa, 16 hours after pupariation. (**a**) Ventral - Head, thorax, and abdomen delineated; Compound eyes same coloration (i.e. cream) as rest of pupa; Outline of mouthpart; Thoracic appendages (wings and legs) extended to posterior margin of pupa; Fishtail shape pretarsi (boxed) of metathoracic legs. (**b**) Dorsal – Pupal respiratory system (in the oval) forms cylindrical projections; All elements of pupal respiratory system unpigmented; Bilateral unidentified structures (arrow) noted at posterior margin of thorax. **Abbreviations:** ce: compound eye; l: leg; mp: mouthpart; rh: respiratory horn; w: wing.

24 Hours

The pupa was slightly darker compared to the pupa from the previous timeframe. Separation of the pupal cuticle from the newly formed adult cuticle was noticed at the anterior region of the head (Figure 4.4 a and b). The respiratory trunks (Figure 4.4 a) appeared to be more inflated compared to the 16 hour form; the respiratory horns were slightly pigmented.

The antennae were located between the undefined compound eyes, in an anterior-medial position on the head (Figure 4.4 b). At this time, the antennae were compact, whitish in colour and displaced (from its lateral position seen at 16 hours post pupariation) to move to a medial position on the head. The proximal aspect of the mouthpart was bi-lobed, in comparison to the distal end which was single lobed. At this point in time, the maxillary palpus was in the shape of a sharp-pointed arrow (Figure 4.4 b); the location of this structure was just underneath the compound eyes.

The pale legs no longer appeared as inflated, transparent, undifferentiated tissue, as leg segmentation was more defined for all leg pairs compared to the 16 hour form. The legs were

surrounded by a shiny membrane. The metathoracic leg pair extended past the abdomen and the mesothoracic leg pair was in line with the last segmental suture of the abdomen. Leg segmentation was evident on the ventral side of the abdomen, with the tarsomeres of the metathoracic leg pair visible (Figure 4.4 c). Due to the pupal cuticle, the fine structural elements of the pretarsus were not clear and it appears as a fishtail shape (Figure 4.4 c), however it was more defined than the 16 hour form. The tibia of the metathoracic leg pair was hidden behind the developing wings. The wings were still undifferentiated. The thoracic unidentified structures were completely formed, overhanging onto the anterior margin of the abdomen (Figure 4.4 a).

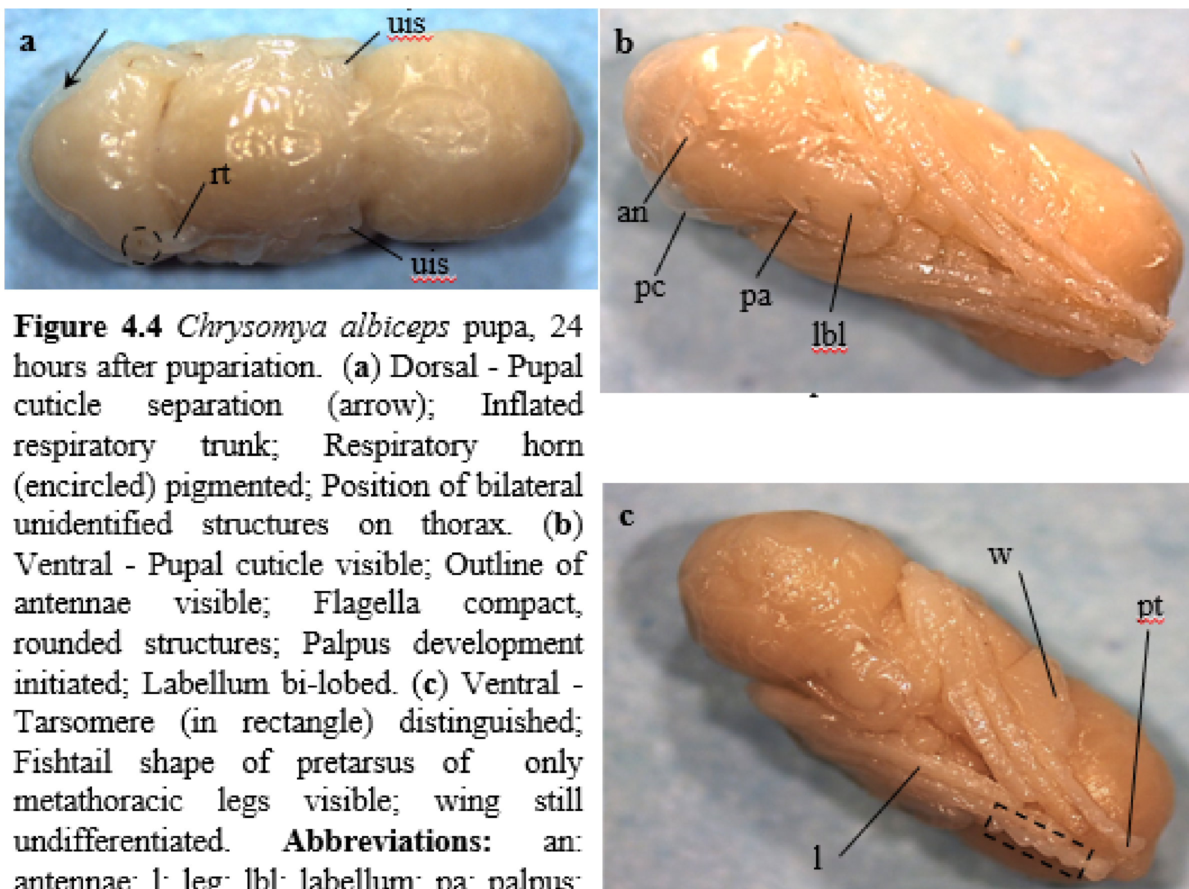


Figure 4.4 *Chrysomya albiceps* pupa, 24 hours after pupariation. (**a**) Dorsal - Pupal cuticle separation (arrow); Inflated respiratory trunk; Respiratory horn (encircled) pigmented; Position of bilateral unidentified structures on thorax. (**b**) Ventral - Pupal cuticle visible; Outline of antennae visible; Flagella compact, rounded structures; Palpus development initiated; Labellum bi-lobed. (**c**) Ventral - Tarsomere (in rectangle) distinguished; Fishtail shape of pretarsus of only metathoracic legs visible; wing still undifferentiated. **Abbreviations:** an: antennae; l: leg; lbl: labellum; pa: palpus; pc: pupal cuticle; rt: respiratory trunk; pt: pretarsus; uis: unidentified structure; w: wing.

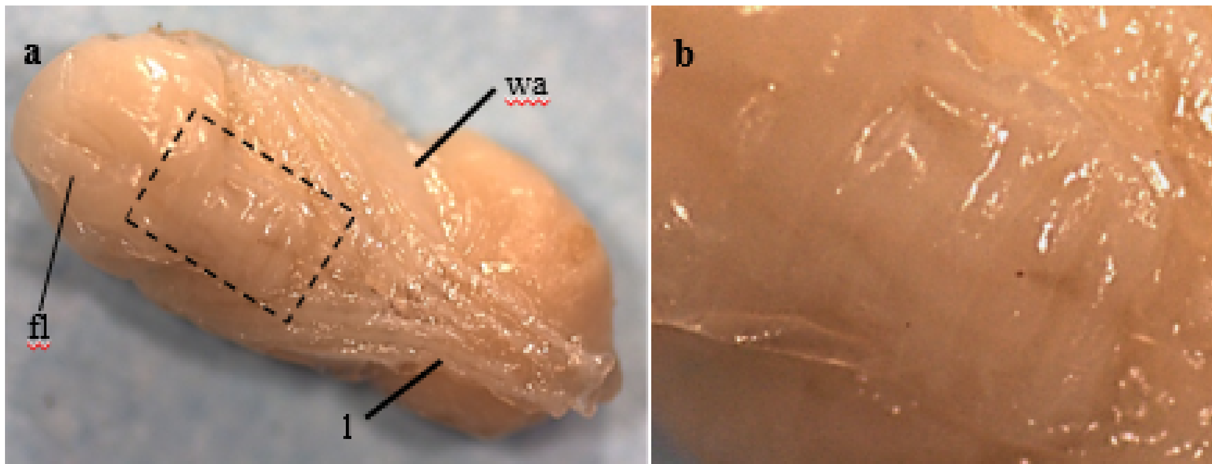
32 Hours

The overall colour of the pupa (Figure 4.5) was similar to that seen during the previous timeframe. The second area of separation of the pupal cuticle was in the area anterior to the abdomen. The spiracle gills of the pupal respiratory system were noticeable due to the

pigmentation of this structure; it was located anteriorly on the thorax, in a position just posterior of the compound eyes (Figure 4.5 c).

The flagellum of the antennae was elongated (compared to the compact, bud-like appearance seen previously) with a sharp pointed end (Figure 4.5 a). The extend of the mouthpart became fixed at this point; further development of this structure manifested as the proximal end turning slightly bi-lobed. Although the pupal cuticle separated from the pupa, the membrane was not ruptured, therefor some structures on the head did not show up adequately in the photographs (Figure 4.5 b). Maxillary palpi development was concurrent with the general mouthpart development. At this stage the labrum was in the form of a blunt arrow.

Due to the accidental tearing of the pupal cuticle at the position of the legs, further visualization of leg segmentation was possible (Figure 4.5 a); the legs were still unpigmented at this stage. Wing veins were not distinguishable yet however, the pale white wing apex outline was visible (Figure 4.5 a).



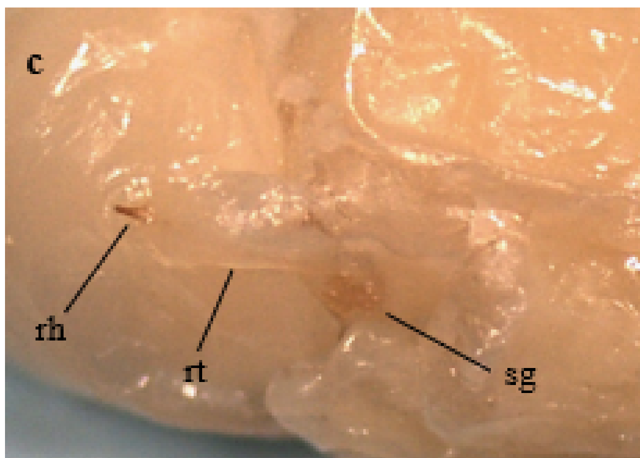


Figure 4.5 *Chrysomya albiceps* pupa, 32 hours after pupariation. (**a**) Ventral view – mouthpart within the rectangle, Visible outline of wing apex; (**b**) Zoomed-in on mouthpart illustrating difficulty observing some aspects due to pupal membrane; (**c**) Detail of respiratory system illustrating pigmentation of spiracular gills and respiratory horn and lack thereof for the respiratory trunk. **Abbreviations:** fl: flagellum; l: leg; rh: respiratory horn; rt: respiratory trunk; sg: spiracular gill; wa: wing apex.

40 Hours

The overall colour of the pupa remained unchanged. Accidental rupturing of the pupal cuticle revealed the morphological details of the head, thorax, abdomen, and the appendages. The respiratory trunks became truncated (Figure 4.6 a) as the development of the compound eyes progressed. The outer margins of the respiratory trunks were translucent, whereas the inside of the respiratory trunks was light-brown, and the respiratory horns were a darker brown (Figure 4.7 a - c). The spiracle gills (predestined anterior spiracle) were more pronounced; closer examination revealed the convoluted nature of this structure (Figure 4.7 a - c).

Details of the antennae were now clearly discernible due to fact that the pupal cuticle was not obscuring it. Compared to the 24 hour pupa, the overall form (Figure 4.6 c) was more

elongated with a clear separation between the two flagella; the tips of the flagella were pointed. The labellum (Figure 4.6 c) took on its adult form, with the proximal portion no longer bi-lobed and its distal end an approximate heart-shape.

The pale wings (Figure 4.6 b) were still largely undifferentiated at this stage of development. Whereas the fishtail shape of the pretarsus of the metathoracic leg was already visible 24 hours after pupariation it was also evident at this stage in the mesothoracic and prothoracic legs. Sutures lines on the thorax and abdominal segmentation were evident but superficial (Figure 4.6 a).

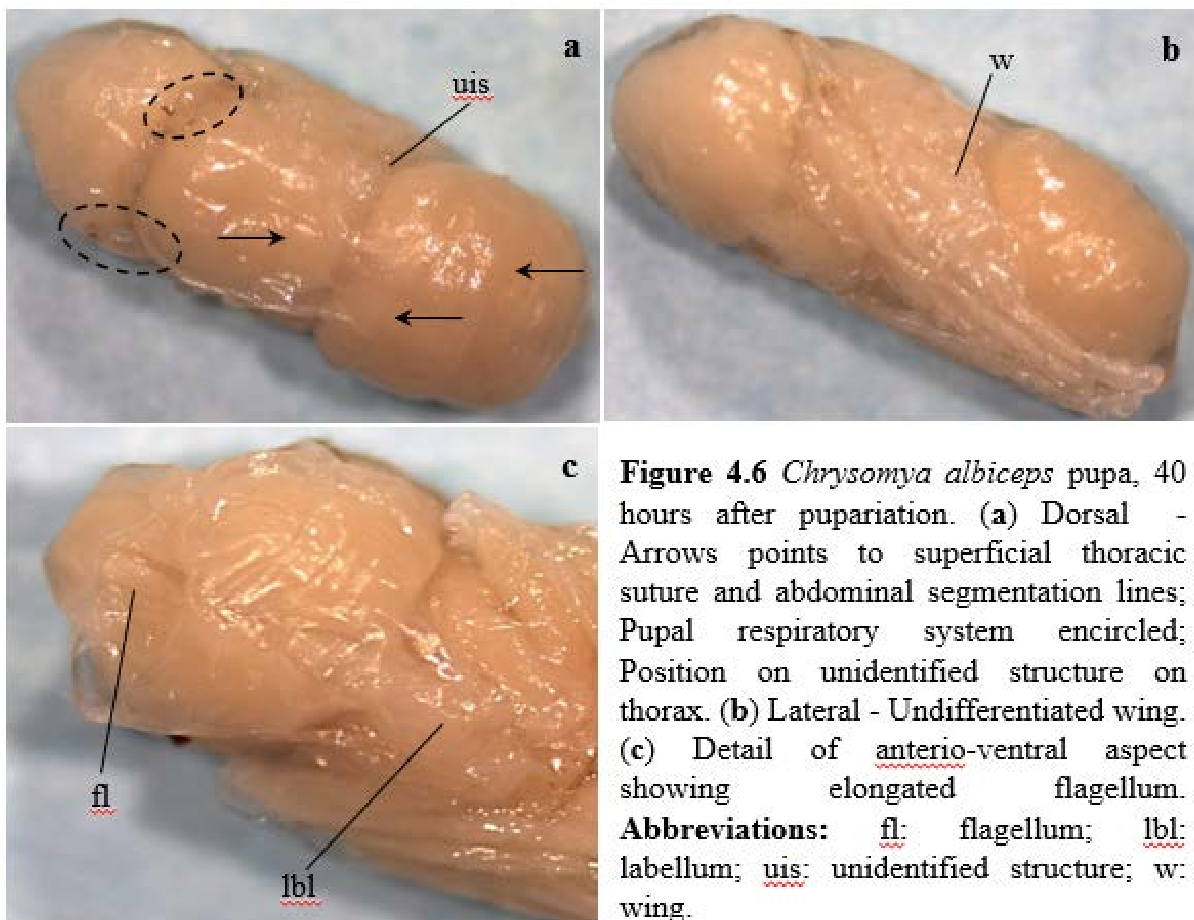


Figure 4.6 *Chrysomya albiceps* pupa, 40 hours after pupariation. (**a**) Dorsal - Arrows points to superficial thoracic suture and abdominal segmentation lines; Pupal respiratory system encircled; Position on unidentified structure on thorax. (**b**) Lateral - Undifferentiated wing. (**c**) Detail of anterio-ventral aspect showing elongated flagellum. **Abbreviations:** fl: flagellum; lbl: labellum; uis: unidentified structure; w: wing.

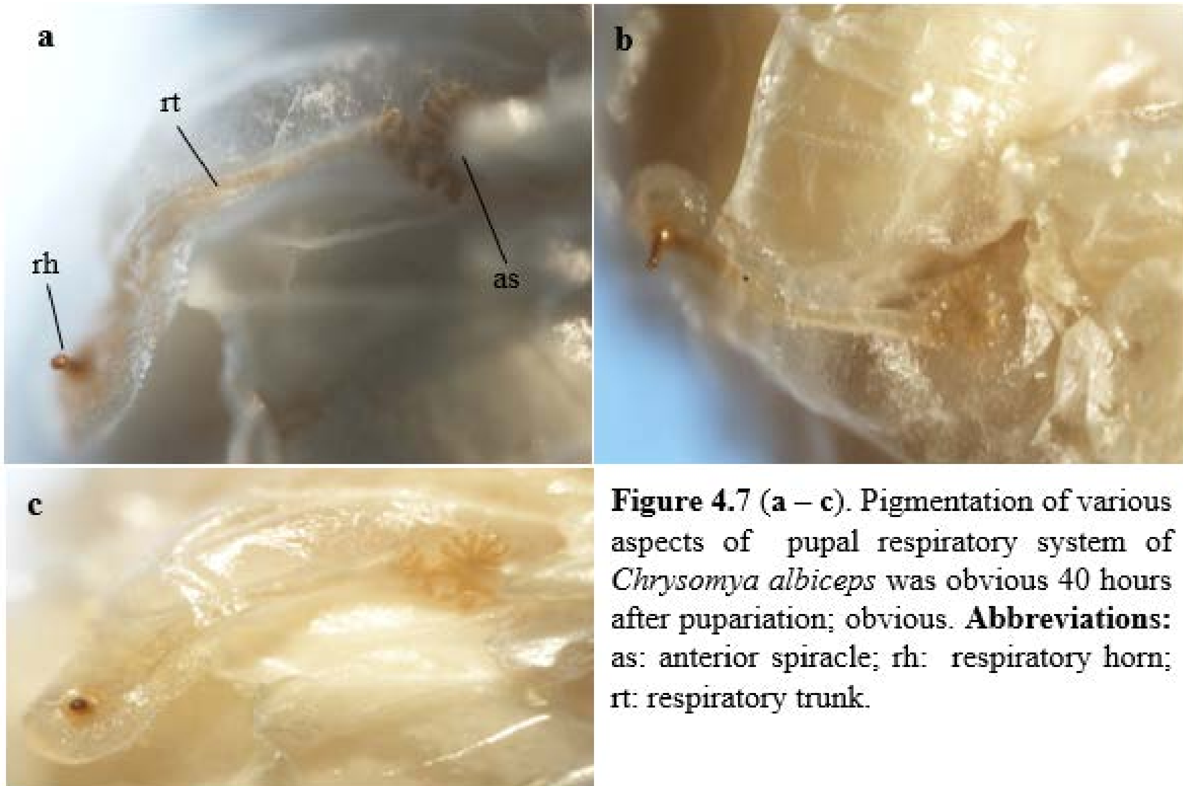


Figure 4.7 (**a – c**). Pigmentation of various aspects of pupal respiratory system of *Chrysomya albiceps* was obvious 40 hours after pupariation; obvious. **Abbreviations:** as: anterior spiracle; rh: respiratory horn; rt: respiratory trunk.

48 Hours

The general colour of the pupa remained unchanged. The pupal cuticle was detached from the entire pharate adult (in Figure 4.9 this loose membrane was clearly seen on the outlines of the pharate adult). As noted with the accidental tearing of the cuticle at previous time stamps the pupal cuticle was delicate and easily ruptured (Figure 4.8) when handled.

The outlines of the compound eyes (still devoid of its eventual red colouring) were visible (Figure 4.8). At this point the ommatidia of the eyes were vaguely visible. The flagella reached its full length, and its distal margins were rounded (the distal ends were more pointed during the previous timeframe). As the mouthpart contracted, the structure became stubby; the margins of the labellum became rounded. The maxillary palpi increased in length as development progressed (Figure 4.8).

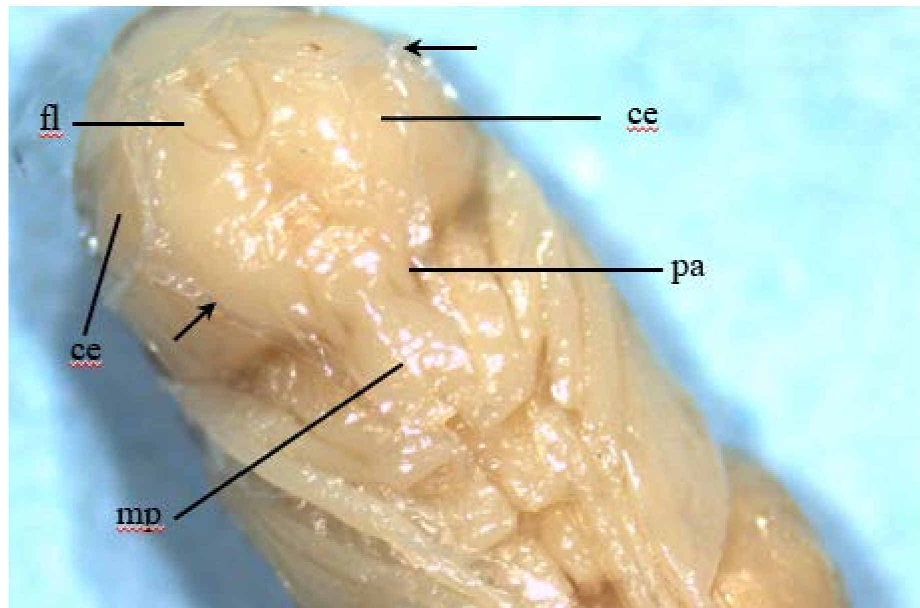


Figure 4.8 *Chrysomya albiceps* pharate adult, 48 hours after pupariation. Compound eyes still devoid of colour; distal ends of flagella rounded; Maxillary palpi increased in length and mouthpart was contracted; Delicacy of pupal membrane evident (arrows). **Abbreviations:** ce: compound eyes; fl: flagellum; mp: mouthpart; pa: palpus.

The anterior spiracles were dark-brown and clearly visible (Figure 4.9 a). The legs were narrow, and segmentation was discernible in all leg pairs. The femur of the prothoracic leg pair was shorter and bulkier than that of the other two pairs of legs. Wings were folded, light-grey and vein differentiation was subtle (Figure 4.9 a); no pigmentation to any aspect of the wings was noted. No further development of the abdominal region was evident at this stage.

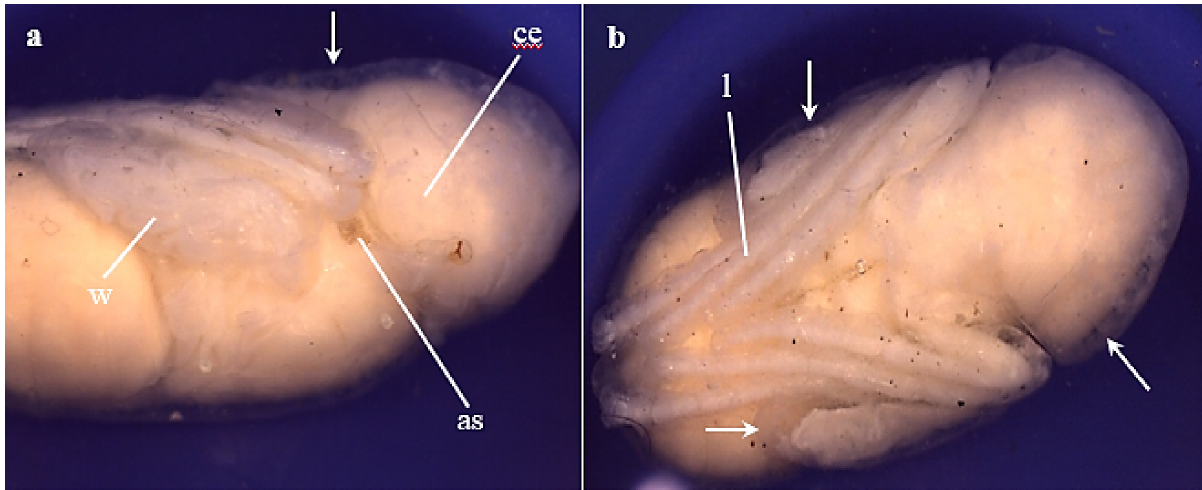


Figure 4.9 *Chrysomya albiceps* pharate adult, 48 hours after pupariation; pupal membrane indicated by arrows. (**a**) Lateral - Wing vein differentiation evident; Anterior spiracles on thorax clearly visible in a position posterior to the compound eye. (**b**) Ventral - Leg segmentation was defined. **Abbreviations:** as: anterior spiracles; ce: compound eye; l: leg; w: wing.

56 Hours

The pharate adult was not of uniform colour anymore, mainly due to eye colour development and pigmentation of the thoracic appendages (Figure 4.10).

The compound eyes were pink at their margins and light pink in the centre (Figure 4.10). At this stage no further development of the antennae and mouthpart were evident.

The unidentified structures were more pronounced (Figure 4.10 b) compared to what was seen previously. The light brown coloration of the legs (Figure 4.10 a) signalled the beginning of pigmentation of these structures; pigmentation was more pronounced anteriorly (Figure 4.10 a). Wing veins were slightly darker pigmented (Figure 4.10 a) compared to the colour seen during the previous timeframe. Segmentation lines on the abdomen appeared to be deeper set (Figure 4.10 b) than what was seen previously.

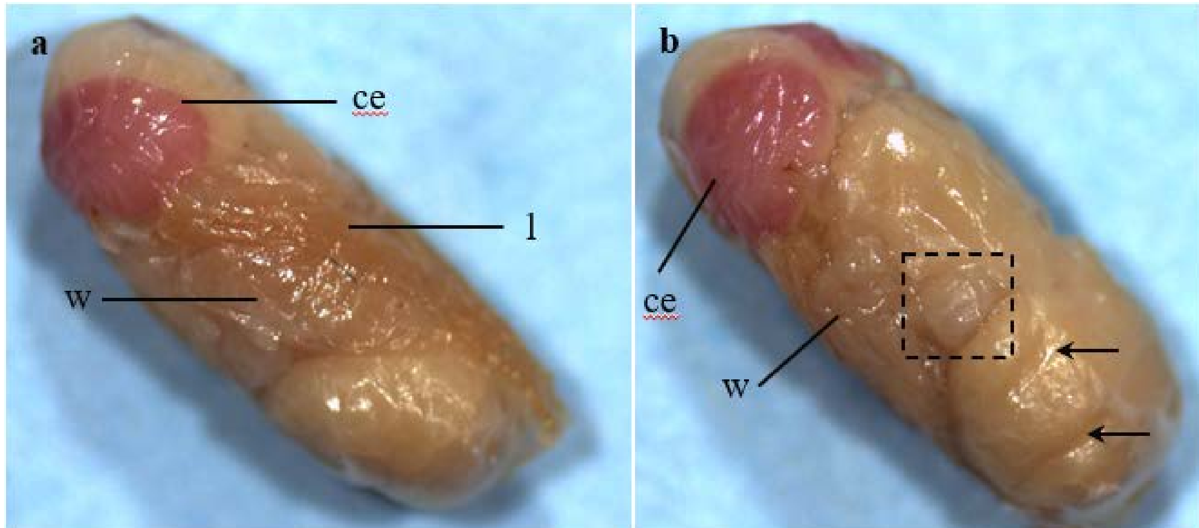


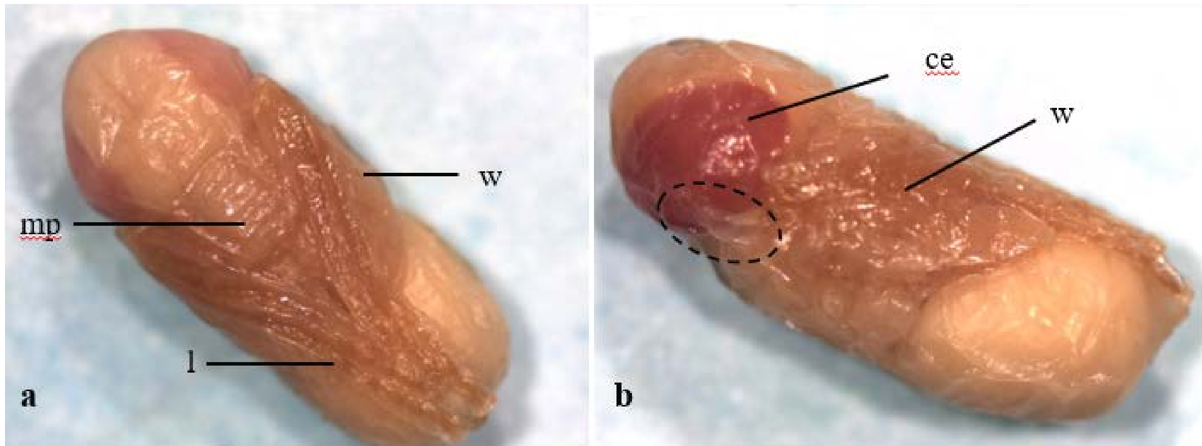
Figure 4.10 *Chrysomya albiceps* pharate adult, 56 hours after pupariation the compound eyes turned pink (slightly darker pink margins). (**a**) Pigmentation of anterior aspects of legs more pronounced; Pigmentation of wing veins noticeable. (**b**) Abdominal segmentation lines (arrows) appeared more defined; Unidentified structures (boxed) more defined. **Abbreviations:** ce: compound eye; l: legs; w: wing.

64 Hours

Except for the further colour development of the compound eyes and the increased pigmentation of the thoracic appendages, the rest of the pharate adult was of a uniform colour albeit slightly darker (Figure 4.11 a and b) than during the previous timeframe. No assessment of the pupal respiratory system could be made due to the pupal membrane obscuring these structures (Figure 4.11 b - c).

The entire compound eye was a uniform colour (Figure 4.11 b) at this stage, opposed to the margins being darker than the central portion during the previous timeframe. The darker margins of the flagellum and scapus signalled maturation of the antenna; aristae were light brown. Mouthpart development (Figure 4.11 a) was similar to that seen at 56 hours after pupariation.

The anterior sections of the thoracic appendages were darker than the posterior aspects of it. (Figure 4.11 a – b). No obvious changes were observed for the abdomen (Figure 4.11 b).



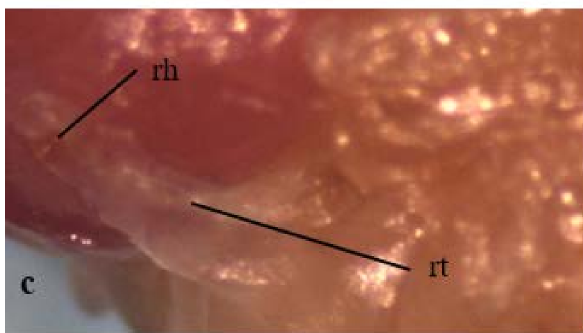


Figure 4.11 *Chrysomya albiceps* pharate adult, 64 hours after pupariation. (**a**) Ventral - Slightly pigmented legs and wings; more pigmented anteriorly. (**b**) Lateral - compound eye uniform color; anterior aspect of wing slightly darker; encircled is the pupal respiratory system. (**c**) details of the respiratory system not clear. **Abbreviations:** ce: compound eye; l: leg; mp: mouthpart; rh: respiratory horn; rt: respiratory trunk; w: wing.

72 Hours

The general colour of the pharate adult (Figure 4.12 a – b), was darker compared to that of the previous timeframe due to increased pigmentation and sclerotization of various structures. The spiracle gills (Figure 4.12 c) were not visible, whereas the appearance of the rest of pupal respiratory system (Figure 4.12 a and c) was similar to that of the previous timeframe.

The compound eyes were dark red. Pigmentation of certain aspects of the antennae were pronounced; the margins of the flagellum darkened, and the aristae were now clearly discernible. Bristles in the area between the compound eyes (Figure 4.12 a) and around the ocelli were pronounced due to increased pigmentation. Dark bristles were also noticeable in the area around the mouthparts (Figure 4.12 b). The central portion of the mouthpart apparatus was light brown.

The true proximal and distal aspects of the legs are difficult to ascertain due it being folded against the body in the puparium. Tanning of the legs will be described in terms of its anterior and posterior region relating to the general body plan. The legs were dark brown, almost

black, at the anterior region of the pharate adult (i.e., in the vicinity below the compound eyes) and brown towards the posterior (Figure 4.12 b). The leg bristles were a light-black tone. The primary wing venation was defined as aspects of the wing darkened. The wing membranes were grey, the costa was sclerotized, and setae were clearly visible on the wing margins. The unidentified bilateral structures were still noticeable on the lateral aspect of the pharate adult (Figure 4.12 a and c). Thoracic bristles (Figure 4.12 a) were pronounced at this stage due to sclerotization of these structures. Furthermore, bristles were also noted on the lateral aspect of the thorax. Fine hairs were noted at various positions on the abdomen (Figure 4.12 a).

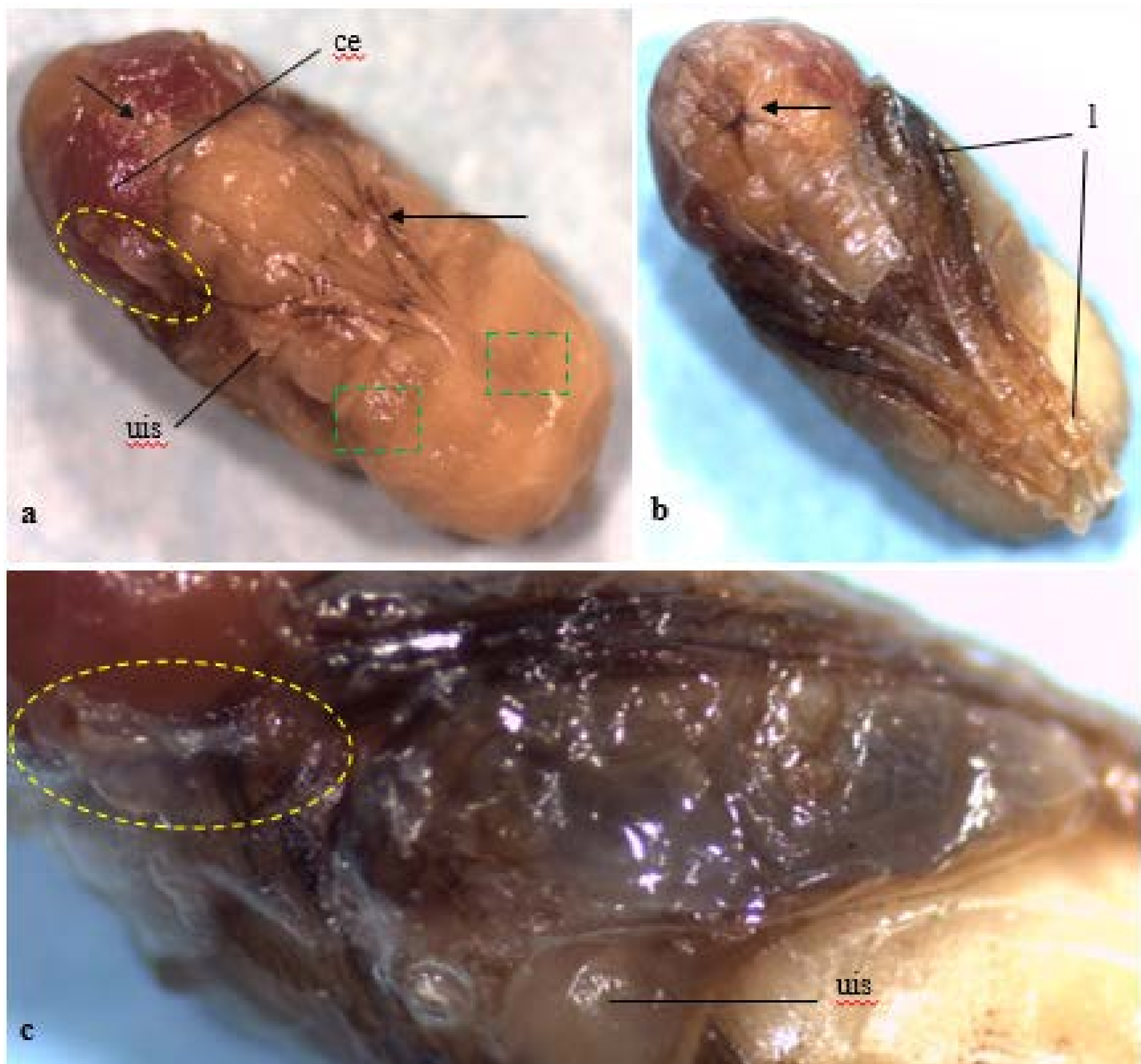


Figure 4.12 *Chrysomya albiceps* pharate adult, 72 hours after pupariation. (**a**) Dorsal – encircled, the pupal respiratory system; dark-red compound eyes; pigmented bristles on the head and thorax indicated with arrows; green rectangles indicate the areas on the abdomen with fine hairs; bilateral unidentified structures clearly visible. (**b**) Ventral - arrow indicate the pigmented bristles around the mouthpart; the differential pigmentation of the legs pronounced. (**c**) Lateral view of the thorax - Encircled, the pupal respiratory system; Unidentified structure pronounced. **Abbreviations:** ce: compound eye; l: legs; uis: unidentified structure.

80 Hours

The pharate adult was darker (Figure 4.13) compared to what it was during the previous timeframe. The pupal respiratory system (Figure 4.13 b) was similar to what was observed during the previous timeframe.

Bristles on the head (Figure 4.13) were fully developed in terms of its pigmentation and length. At this stage the ocelli were completely developed. Aristae were more pigmented compared to the previous timeframe. The frontal lunule and the central portion of the mouthpart were completely pigmented. The oral lobe bristles and hair on the maxillary palpi were prominent due to an increased level of pigmentation.

The legs were completely pigmented (Figure 4.13 a). Due to pigmentation of the wing veins, the wings appeared darker (Figure 4.13 a) compared to the previous time frame. The fine hairs (Figure 4.13 b) at different locations on the thorax and abdomen were more advanced (denser covering of hair) compared to the previous timeframe. These hairs were especially prominent within the sutures of the thorax and abdomen. Pigmentation of the thoracic bristles appeared to be more extensive (Figure 4.13 b) to what was seen previously.

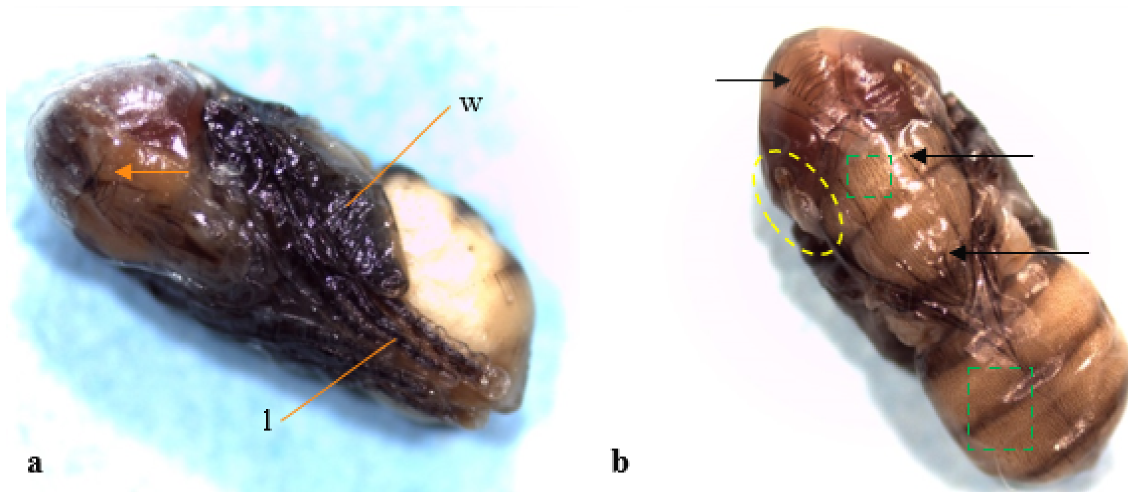


Figure 4.13 *Chrysomya albiceps* pharate adult, 80 hours after pupariation. (**a**) Vento-lateral view – Prominent pigmentation of the oral lobe bristles, the legs and wing veins. (**b**) Dorsal – the pupal respiratory system (encircled) similar to the condition at 72h after pupariation; Arrows points to the prominent bristles in the area between the compound eyes and on the thorax; Green rectangles draw attention to the dense covering of hairs on the thorax and abdomen. **Abbreviations:** - l: legs; w: wing.

88 Hours

The pharate adult (Figure 4.14) appeared very similar to what it was during the previous timeframe. The only change at this stage was the faint blue hue noted on the lateral aspect of the thorax (Figure 4.14 a and b) and the more intense intersegmental banding on the abdomen.

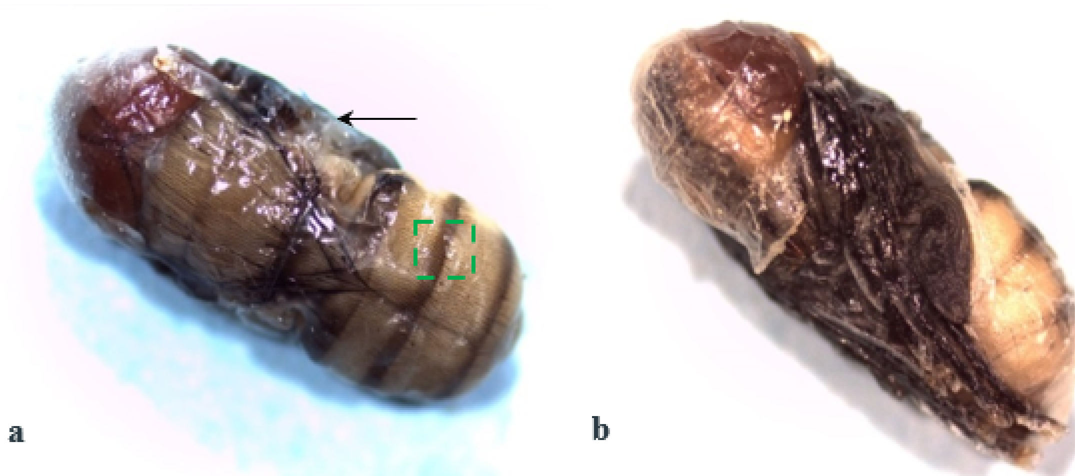


Figure 4.14 *Chrysomya albiceps* pharate adult, 88 hours after pupariation. Dorsal (**a**) and ventral (**b**) views of the pharate adult; Generally, it is similar to the 80h-after-pupariation pharate adult; the only change at this stage is the faint blue hue (arrow) noted on the lateral aspect of the thorax and the more pronounced intersegmental banding (boxed) of the abdomen.

96 – 104 Hours

The adult fly was completely formed (Figure 4.15 a). At this stage the fly was ready to emerge from the puparium. The newly emerged adult (Figure 4.15 b) was not able to fly immediately, and full adult coloration of the fly was not developed. The metallic green colour (Figure 4.15 c) develops within a few hours after emergence.

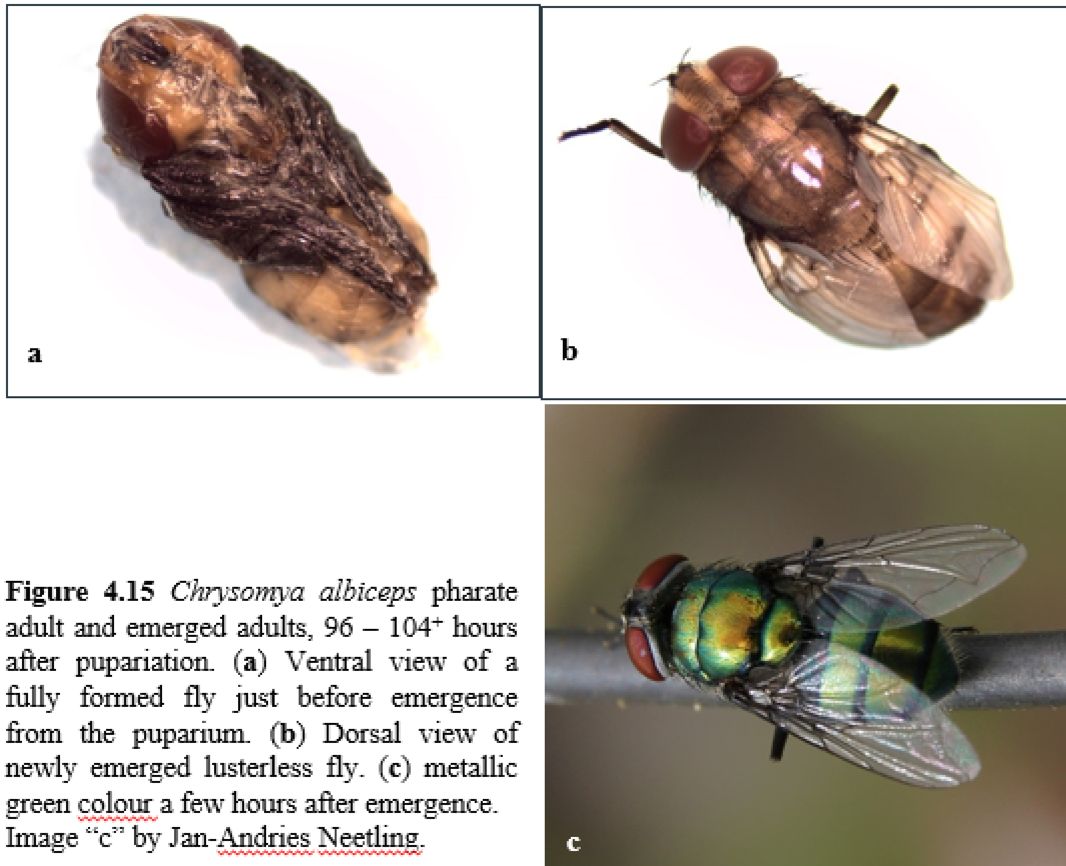


Figure 4.15 *Chrysomya albiceps* pharate adult and emerged adults, 96 – 104^{+} hours after pupariation. (**a**) Ventral view of a fully formed fly just before emergence from the puparium. (**b**) Dorsal view of newly emerged lusterless fly. (**c**) metallic green colour a few hours after emergence. Image "c" by Jan-Andries Neetling.

4.4 Discussion

Using insect evidence to determine a post-mortem interval is one of the major applications in Forensic Entomology. The pupal period occupies a large percentage of the immature life cycle, and it is therefore important to optimize the post-mortem clock for this timeframe. This research was conducted to identify robust intra-puparial developmental landmarks for *Chrysomya albiceps* pupae examined during this study.

When evaluating the body of work on the intra-puparial period, it was important to distinguish between the concept "stage" versus "landmark". We interpreted that a "stage" was a cluster of morphological transformations within a specific timeframe and that a "landmark" was a morphological feature that can be used along a developmental timeline continuum to track change. In this evaluation, we identified those external morphological landmarks of which the transformation was distinct, i.e., robust intra-puparial landmarks. Some of these landmarks were proposed by various researchers previously. We contributed to the body of work on intra-puparial development by proposing the use of four additional

landmarks (Table 4.1) i.e., the calypters, the transformation of the pupal respiratory system and the adult anterior spiracles, development of the pretarsi seen as a fishtail shape at the distal end of the legs and the development of a dull green hue on the lateral aspects of the thorax. Although photograph of some of these additional landmarks were noted in the literature on intra-puparial development we reviewed (Pujol-Luz and Barros-Cordeiro 2012; Greenberg & Kunich 2002; Karabey and Sert 2013; Salazar-Sousa *et al*. 2018), these landmarks were not described as landmarks of interest by these researchers. Furthermore, we noted distinct changes to certain previously identified landmarks (Table 4.1); for instance, the developmental changes to legs are extensively used at a landmark in previous work (Pujol-Luz & Barros-Cordeiro 2012; Karabey and Sert 2013), however, the pigmentation development in legs first seen at the anterior aspect of the organism, before expanding to the whole leg was not reported as such previously.

Development was tracked in terms of hours after pupariation (formation of the puparium), and collections were made at 8-hour intervals. In Table 4.2 the most pertinent morphological landmarks where transformations were distinct are logged and will be discussed in subsequent paragraphs. It was important to use landmarks that will render consistent results when analysed i.e., would independent analysts come to the same conclusion using these landmarks when analysing pupae? To achieve this, landmark changes involving qualitative measures were eliminated.

Table 4.1: The developmental stages of *Chrysomya albiceps* pupae at 26˚C. Newly identified markers are indicated by an asterisk (*) next to the hour and underlined under comments.

Time / Hours at 26˚C	**Comments**
0h	Pre-pupae Impossible to remove the pre-pupa from the puparium without causing major damage Overall form of the third instar larvae is retained, but the first three segments are retracted. White puparium – Increasing pigmentation of puparium
8h	Cryptocephalic pupa (hidden head) Pupa can be removed from the puparium. Pupa uniform in colour. Segments of the third larva form are still evident. Cephalopharyngeal skeleton embedded in the anterior end of the pupa Partial eversion of the legs, i.e., legs not past the thorax

	Wings undifferentiated, i.e., uniform colour and texture; Extend of wings not past the posterior aspect of the thorax
*16h	Phanerocephalic pupa (head visible) Cephalopharyngeal skeleton not embedded in pupa, plastered to the inside of the puparium Tagmata of the adult form established, i.e., not in the larval form anymore Respiratory horns in antero-lateral position; Respiratory trunks evident Compound eyes a uniform cream colouration Antennae undifferentiated, i.e., as buds; In ventral-lateral position on head Margins of the mouthpart visible; the structure is largely undifferentiated Metathoracic legs extended to the posterior end of the abdomen; Legs membranous, largely undefined structures, Beginnings of segmentation of one of the leg pairs; Fishtail shape of the pretarsus seen in one of the leg pairs Wings, slightly overhanging the posterior margin of the thorax. Calypters noted as bilateral structures at the posterio-lateral margin of the thorax
*24h	Antennae positional move – in an anterio-medial position compared to the anterio-lateral position in the 16h form Separation of pupal cuticle from adult cuticle at the head Mouthpart differentiation seen as maxillary palpi development Leg segmentation more pronounced; Tarsomere of the metathoracic legs were clearly demarcated; Fishtail shape of pretarsus still only observed for the metathoracic leg pair, however it appears to be more defined although the fine structural elements is not clear
*32h	Antennae - whereas the flagellum was bud-like previously, it is elongated at this stage. Spiracle gills of pupal respiratory system visible. Structure was possibly formed earlier, but it is only evident at this stage due to pigmentation;
*40h	Inside margin of respiratory trunk sclerotised (at the previous timestamp no pigmentation was noted) Pretarsi of meso- and prothoracic legs also now visible Seen as a fishtail shape Thoracic sutures and abdominal segmentation visible Unidentified structure on thorax
*48h	Pharate adult Leg segmentation discernible for all 3 leg pairs Femur of prothoracic legs shorter and bulkier than that of other leg pairs Wing vein differentiation apparent
*56h	Leg sclerotization -more pronounced anteriorly Compound eyes develop red colouring, seen as a darker shade at the margins of the eye and a lighter shade in the middle Unidentified structure more defined
*64h	Aristae noticeable, possibility developed earlier, but only visible at this stage due to pigmentation being laid down Margins of the flagellum and the scape is darker than the rest of the structures Entire compound eye a uniform red colour
*72h	Bristles on the head distinct, bristles were possibly formed earlier, but it is apparent due to pigmentation Leg bristles discernible - possible developed earlier, but it noticeable at this stage due to tanning Wing colour development - wings appear darker possibly due to pigmentation of

	some wing veins and due to micro-bristles pigmentation development Abdominal hair noticeable - hair probably developed earlier, but is clearly visible at this stage due to pigmentation of these structures
*80h	Maxillary palpi bristles prominent - possible developed earlier, but due to increased pigmentation it is clearly visible Pigmentation of the entire leg - whereas leg pigmentation was ununiform previously, the entire leg is pigmented at this stage. Prominent hairs clustering in thoracic sutures and intersegmental abdominal area, hairs seem more pigmented and denser on the thorax and abdomen.
*88h	<u>Subtle blue hue to lateral aspect of the thorax</u>
96h	Adult Emergence– No visible chance seen compared to the previous stage

During pupariation, the puparium is formed from the cuticle of the post-feeding larva. The organism is neither a pupa nor a post-feeding larva at this stage and is referred to as the pre-pupa (Fraenkel & Bhaskaran 1973). At this stage the puparium was still tightly attached to the pre-pupa until completion of the first apolytic event. Larval-pupal apolysis is initiated during the formation process of the puparium. This process was completed when the pre-pupa (0 hour), was transformed into the cryptocephalic pupa (8 hour). At the onset of the cryptocephalic pupal phase the puparium colour was brown, and the pupa was enclosed in its own cuticle, i.e., the pupa was no longer attached to the puparium. Due to this the pupa could be successfully removed from the puparium and be analysed.

During the cryptocephalic phase (8 hours after pupariation) the body form was still that of the post-feeding larva; the head of the pupa remained concealed, and leg and wing eversion was evident at this stage. Karabey and Sert (2014), with their study on *Lucilia sericata*, reported that the legs were seen as buds in the cryptocephalic pupa. It was noted that leg and wing development was further along in the cryptocephalic *C. albiceps* pupa we examined i.e., the extend of the legs spanned about a third across the length of the cryptocephalic pupa and the wings about a quarter across its length. Brown *et al*. (2012) classified the legs as short at this stage of development. The morphological markers of the cryptocephalic phase noted in our study correlates with the study done by Ma *et al*. (2015) on *Chrysomya rufifacies* and by Barros-Cordeiro *et al.* (2016) on *Lucilia cuprina* and *Cochliomyia macellaria*.

Sixteen hours after pupariation, the pupal head was everted, and the adult tagmata were delineated. It was also at this stage that the bilateral structures (Figure 4.16 a) on the posterior margin on the thorax were noticed. These structures were clearly visible when the pupa was viewed from the dorsal aspect. Initially, we could not determine what these structures were. Although these structures were seen in the images presented for other studies on intra-puparial development, it was not discussed or identified. We postulate that these structures are the calypters. We base our assertion on the position of the calypters as seen in adult *C. albiceps* flies (Figure 4.16 b). The calypter development was not reported in any morphological study since the 1900's (Hewitt 1907). The landmarks unique at 16 hours after pupariation were: (i) undifferentiated antennal buds located in a ventro-lateral position on the head (ii) the undifferentiated and unpigmented mouthpart, (iii) unpigmented respiratory horns and (iv) the pretarsi of only the metathoracic leg pair were developed.

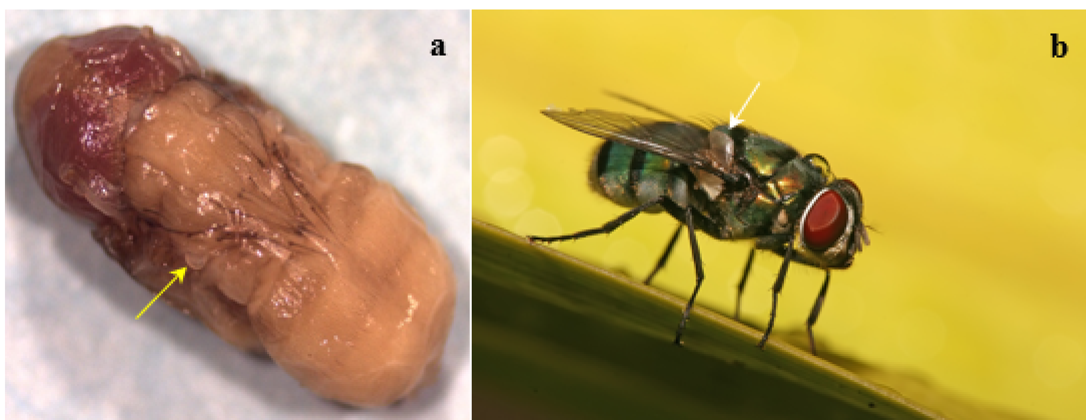


Figure 4.16 *Chrysomya albiceps* adult before and after emergence from the puparium. (**a**) The bilateral unidentified structures (yellow arrow) noted during the development of the pupa, the structure as seen in the pharate adult at 72 hours after pupariation correspond to (**b**) the location of the calypters (white arrow) in emerged adult flies. Image "b" is a Wikimedia Commons image by Muhammad Mahdi Karim.

At 24 hours after pupariation, legs were segmented (not seen at the previous timestamp). Furthermore, the segmented legs were not pigmented; leg pigmentation developed at a subsequent point in time.

At 32 hours after pupariation pigmented spiracle gills were evident. These structures develop over time and at the end of pupation it will function as the anterior thoracic spiracles of the adult. In addition to this structure, the unpigmented nature of the internal margin of the

respiratory truck defines this time frame. The internal margin of the respiratory trunks becomes sclerotized as development progressed. Additional to these features (or in conjunction of it) the absence of pretarsi development in the meso- and prothoracic legs as well as the absence of thoracic suture and abdominal segmentation lines can be considered to define this stage.

40 Hours after pupariation, thoracic suture line and abdominal segmentation line became evident. It was noted that these lines became more prominent in subsequent stages, however this change is qualitative in nature. Therefore, in conjunction of this feature (thoracic suture and abdominal segmentation lines) for the 40 hour stage the nature of the bristles of the head and thorax should be considered. At this point of time these bristles might be present, but it is not discernible because it was unpigmented.

By 48 hours after pupariation complete pupal cuticle separation was noted at the margins of the pupae; this event signals a change from the phanerocephalic pupa into the pharate adult phase. This phase has the longest duration and comprises almost 52% of the total intra-pupal development. It is usually sub-divided according to eye colour change by previous researchers in the field of intra-puparial development. Eye colour changes were clearly explained by Pujol-Luz & Barros-Cordeiro (2012) as well as by Ramos-Pastrano *et al*. (2017). Furthermore, some researchers, put forward the approach that the pharate adult phase can be broken down further according to bristle and hair development, as well as pigmentation and coloration of several structures (wings and legs). The question is how this relates to the question posed in relation to one of our aims, i.e. Are the changes to the landmark robust enough for practical use? Colour change of the compound eyes were not used to track development because of the qualitative nature of this measure. However, use was made regarding the uniformity of the colour of the compound eye. A similar approach was followed regarding pigmentation of structures. Defining incremental pigmentation was not used to track development due to its qualitative nature; whether a structure was uniformly pigmented or not was used as an alternative.

48 Hours after pupariation, all legs were segmented, but the pigmentation across the length of these legs were not uniform as it is seen in subsequent timeframes. Furthermore, wing venation can be differentiated (as can be seen in subsequent stages), however, the pigmentation of the wing veins was not evident at this stage.

At 56 hours after pupariation, the colouring of the compound eye served as a landmark. Whereas the compound eyes were devoid of colour during the previous stages, a pink coloration was established at this stage. However, this stage can be distinguished from subsequent stages since the pink coloration was not uniform, i.e., the outer margins were darker than the central portion of a compound eye. Some researchers used compound eye coloration in terms of intensity over time. They circumvent the qualitative nature of this by employing standardised colour charts to quantifies this. We found a more practical approach to eye coloration to be in terms of the uniformity of colour of the compound eyes.

64 Hours after pupariation the entire eye is an unform pink colour, and it will remain as such for all stages subsequently. What differentiate this stage from subsequent stages is that the bristles on the head and thorax were not noticeable at this stage due to its unpigmented nature.

The 72 hours after pupariation stage is defined in that the bristles of the maxillary palpi were not developed (or pigmented), whereas it was pigmented in subsequent stages. What differentiate this stage from the previous stage (64 hour) is that the head and thoracic bristles were pigmented. To be concise; the 74 hour pupae were recognised by the pigmented head and thoracic bristles whereas the bristles on maxillary palpi were not pigmented.

From 80 hour after pupariation the changes were subtle qualitative changes. However, if you consider a feature that only becomes visible from 88 hours onwards, this stage can be defined by the absence of this landmark. At 88 hours after pupariation a green tinge to the lateral aspect of the thorax was noted. It is a feature that is very subtle and the practical use of this debatable.

Robust landmarks between the 88 hour and 96 hours after pupariation could not be determined. Various other researchers mention the prominence of the ptilinum just before emergence (correlating to our 96 hour form); unfortunately, this feature was not observed during this study.

4.5 Conclusion

The aim of this chapter was to identify robust morphological landmarks specific to each time frame (stage). Observations were made at eight-hour intervals and the morphological landmarks defined were distinct enough to be used for stage distinction of almost all the stages; unfortunately, no distinguishing landmarks were established for the last two stages of the pupal period.

Some researchers used fine structural changes to track developments, i.e., Karabey & Sert (2014) and Flissak & Moura (2018) referenced the abdominal stigma and as a landmark, whereas Karabey & Sert (2014) also noted when the ommatidia became evident. Unfortunately, these structural elements were not observed in this study, possibly due to the limitations of the microscope used.

Brown (2012) suggested that at periods where changes of external features do not allow for distinction, using histology to track internal metamorphic development can fill the gap. The possibility of using histology to distinguish between the last two stages should be considered for future studies.

Future research should be invested to fine-tune the intra-puparial clock by scoping for developmental landmarks over shorter observation windows. Taking the lead from Brown (2012) it would be optimal to also do histological analysis when scoping for landmarks over shorter time intervals as a measure to track development. Using a similar approach as suggested in the previous paragraphs, the pupariation period can also benefit from the effort to finetune the clock as this stage lasts almost 36 hours.

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Chapter 5: GENE EXPRESSION CHANGES THROUGHOUT THE PUPAL DEVELOPMENTAL STAGE OF *CHRYSOMYA ALBICEPS* TO DETERMINE POSTMORTEM INTERVAL

Abstract

Insects are key components in postmortem interval (PMI) estimation as their developmental patterns in combination with morphological changes are used to estimate time of death. To narrow the time of death estimation, morphological changes during pupal growth should be considered in combination with other sophisticated molecular techniques such as gene expression changes, as methods for insect age determination. The study aimed to validate reference genes for use in normalization as well as investigating the potential of *ecr* as target gene. A set of five candidate reference genes (*GAPDH, actin, RpS17, Rp49*) were selected based on their use in previous studies and one additional designed primer set for the *18S* gene for qRT-PCR normalization and validation. The validation study proved the effectiveness of the reference genes for normalization based on the stability values obtained for *RpS17, Rp49* and *18S*. *Actin*, showed high levels of variance across the pupal stage and should be considered as a candidate gene marker, along with *ecr*, for pupal development.

Key words: PMI estimation, gene expression changes, reference genes, validation, qRT-PCR, candidate gene, *ecr*.

5.1 Introduction

Time of death or postmortem interval (PMI) is essential in criminal investigations for determining when a victim was murdered, or accidental death occurred. This estimate is initially calculated using the decomposition changes of the corpse (fresh, bloat, active, advanced decay and dry) in combination with postmortem characteristics called livor, algor and rigor mortis (Vass 2001). The body's postmortem changes, during the initial 72 hours after death, may yield inaccurate PMI estimations. However, entomological evidence present during, and beyond this 72-hour period provides a good alternative method, and potentially more accurate tool for PMI estimation (Kelly 2006; Tarone & Foran 2011).

Insect evidence, especially blowflies, are key evidence in criminal murder or death investigations, as they are attracted to the aroma of a corpse minutes after death and provide the most accurate tool for determining the time of death. Their developmental pattern/time can be utilized to determine the PMI (Kelly 2006). Published developmental data or succession patterns are methods used to assess the development time of insects associated with a cadaver (Greenberg & Kunich 2002).

Investigators can use the age during various stages of evidentiary fly development to estimate their initial time of colonization (Greenberg & Kunich 2002; Foran 2007). Larval development stages are preferred as time of death indicators because their length and weight, all of which changes significantly over time, can be easily determined (Ames *et al*. 2006; Boehme *et al*. 2013). Determining the age of blowfly pupae is somewhat more difficult because their lengths and weights remain relatively constant during this stage. Morphologically, it is possible to determine the age, but the puparium first needs to be removed with great precision to reveal the hidden informative features. This process is, however, very laborious (Byrd & Castner 2010). Published developmental data, for use in PMI estimations, for this stage are not as abundant (Greenburg & Kunich 2002).

Pupal morphological studies using forensically important fly species are still ongoing to establish a validated baseline for age estimation (Cepeda-Palacios & Sholl 2000; Ames *et al*. 2006; Brown 2012; Pujol-Luz & Barros-Cordeiro 2012; Defilippo *et al*. 2013; Karabey & Sert 2013; Salar-Souza *et al*. 2018). Due to the importance of the pupal stage in PMI estimations (Byrd and Castner 2009; Davies & Harvey 2012), it is imperative that additional techniques be investigated to determine pupal age more efficiently and accurately using a validated process. A technique that shows great promise for augmenting morphological analysis for age determination is gene expression analysis (Zehner *et al*. 2006; Tarone *et al*. 2007). During development, a hierarchy of genes regulate the transformation from larvae to pupa and pupa to adult fly (Arbeitman *et al*. 2002). Gene expression changes have been extensively studied in *Drosophila melanogaster,* with overall gene expression through the entire development process having been conducted (Thummel 1995; Arbeitman *et al*. 2002). These studies provided a good foundation for studying similar changes in alternative insect species.

Seemingly, expression levels of various pupal development genes, can be linked to specific morphological markers at specific time points, and this, may help in improving age determination efforts in flies (Ames *et al*. 2006, Zehner *et al*. 2006, Tarone & Foran 2011, Brown 2012, Boehme *et al.* 2013). Several studies were conducted on the expression analysis of different genes that regulate body structure development, but such genes remained expressed far beyond the time when the actual morphological marker can be seen (Couso *et al*. 1995; Boquet *et al*. 2000; Brennan *et al*. 2001; Zhou & Riddiford 2002). Considering the overall complexity of gene regulation in any organism, it is important to remember that morphological changes often occur later than the initiation of gene expression, yet, the change in gene expression levels may still correlate with developmental progress.

To accurately determine the expression levels of genes of interest, a set of validated reference genes are required for normalization. Reference genes, which were previously referred to as "housekeeping genes", should ideally be consistently expressed at a constant rate in all cell types throughout development, as they are required for basic cell survival (Thellin *et al*. 1999). Such genes are typically well-suited for use as normalization factors in quantitative real-time PCR (qRT-PCR) experiments (Thellin *et al*. 1999; Pfaffl 2001; Dorak 2007; Cardoso *et al*. 2014). The expression patterns of reference genes should be constant across different samples, despite experimental conditions, to be classified as adequate for normalization of differently expressed genes (Vandesompele *et al*. 2002).

Tarone *et al*. (2007) applied this method, qRT-PCR, to a shorter developmental period, eggs of *Lucilia sericata.* They found a significant potential to decrease the time estimation for precise age determination by comparing gene expression changes to reference genes. Several researchers (Tarone & Foran 2011; Brown 2012; Boehme *et al*. 2013) expanded on this approach, using different species, and found that target genes, which are differentially expressed throughout development, can determine the age of the pupae when compared to consistently expressed genes (Tarone *et al*. 2007). However, currently the range of possible candidate genes are limited.

Selecting target genes for gene expression studies in forensic entomology studies can be quite challenging. First, the target gene should control a specific developmental feature at a specific point in time. Second, the target gene should either be expressed at a very high or low level during development. At the start of insect development, a candidate gene should

have a high expression level that decreases drastically as development progresses, and therefore, acting as an age specific marker (Pfaffl 2001). A few expression analysis studies conducted in blowflies used the ecdysone receptor (*ecr*) gene as a candidate for age determination due to its association with development (Tarone & Foran 2011; Brown 2012). *Ecr* is directly induced by the steroid hormone ecdysone (20-hydroxyecdysone) and is known as a major control gene for changes in form and function of insects. It regulates downstream hormonal expression, which assists in the transition from larvae to pupae and pupae to adult (Thummel 1995). Therefore, this gene shows potential as a target gene for aging pupae due to the expression variability during intra-puparial development.

Differential gene expression during insect growth offers enormous potential to improve age estimation (Ames *et al*. 2006; Zehner *et al*. 2006; Tarone *et al*. 2007; Tarone & Foran 2011; Boehme *et al*. 2012; Cardoso *et al*. 2014). Aging using gene expression has already been applied to eggs and pupae of several Calliphoridae species of importance in forensic investigations (Ames *et al*. 2006; Zehner *et al*. 2006; Foran 2007; Tarone *et al*. 2007; Tarone & Foran 2011; Boehme *et al*. 2013; Brown *et al*. 2014). *Chrysomya albiceps* tends to be a dominant taxon around dead bodies at murder or death scenes due to its carnivorous nature, yet few qRT-PCR studies have been tested on this particular species.

The current study aimed to identify and validate selected genes for use as references in examining differential expression of candidate targets during development in the blowfly, *C. albiceps*. Specifically, two target genes, *ecr* and *actin*, was tested in the current study. *Ecr* was previously identified as a good candidate gene, but *actin* appears to be useful as either a reference or target gene in some studies. Our results showed that *ecr* and *actin* would be beneficial as a candidate gene.

5.2 Materials and Methods

Sampling of pupae was conducted as described in Chapter 2.

5.2.1 RNA extraction for molecular analysis

Samples were collected at 8-hour intervals until adult emergence. After collection, each sample was immediately subjected to liquid nitrogen and stored at -80˚C until analysis. RNA was extracted from 78 pupae using the Trizol method (Ambion, by *life* technologies) and each time point was extracted in duplicate.

Gel electrophoresis was performed through 1.2% formaldehyde agarose gels, to determine the quality and integrity of the extracted RNA. RNA isolates were quantified using the NanoDrop® spectrophotometer, ND-1000[2]. DNase treatment was applied to the RNA samples. Then, each RNA isolate was standardized by adding 2 µl of total RNA to 8µl DEPC-treated water. Finally, first-strand cDNA was synthesized using RevertAid™ M-MuLV reverse transcriptase and all the cDNA samples were diluted to a 1:10 ratio (1 µl cDNA: 10 µl DEPC) for qRT-PCR.

5.2.2 Reference and target gene selection

Putative reference genes *GAPDH*, *Rp49*, *actin* and *RpS17* (Table 5.1) were chosen from a previous study on *Lucilia sericata* and *Chrysomya albiceps* (Cardoso *et al*. 2014). The genes showed stable expression patterns in different fly tissues. The *18S* rRNA gene was selected as an additional reference gene to be tested in this study. Initially, a partial sequence of the *18S* rRNA gene from *Lucilia* sericata was retrieved and searched against the NCBI NR database (AF322425) using the tblastx algorithms to find orthologs from other insects. This search returned sequences from eight different insect species, namely: *Calliphora nigribarbis*, *Cochliomyia macellaria*, *Neobellieria bullata*, *Musca sp*., *Musca domestica*, *Pseudogonia rufifrons*, *Drosophila persimilis* and *Drosophila simulans*, Then, a global multiple sequence alignment was performed with the nine sequences through the ClustalX program. Conserved regions were identified among the aligned sequences and used to design a pair of primers. The pairs were designed using the software package Primer Designer4. *Ecr* and *actin* were identified as possible target genes based on previously conducted studies (Tarone & Foran 2011; Brown 2012; Boehme *et al*. 2013), even though *actin* was used as a reference gene in several studies (Tarone *et al*. 2007; Bagnall and Kotze 2010; Cardoso *et al*. 2014).

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[2] NanoDrop is a registered trademark of NanoDrop Technologies, Inc.,

5.2.3 Amplification of selected reference genes

A temperature gradient was set up for all primer sets to determine optimum annealing temperature. The annealing temperatures ranged from 55˚C to 60˚C with a 2˚C increment rate. The PCR reaction contained 2µl synthesized cDNA, 0.2µl each of 0.3uM forward and 0.3uM reverse primers, 5 µl 1X KAPA ROBUST 2G readymix and nuclease free water. The thermal cycling conditions included one cycle at 95˚C for 3min, 35 cycles at 95˚C for 15 sec, gradient at 55˚C to 60˚C and 72˚C for 1 min. Once the optimal annealing temperature was determined this was used for conventional PCR prior to sequencing.

Sequencing was conducted by adding 1µl big dye to 0.3µl of 0.3 forward primer, 2µl 5X sequencing buffer, 2µl template cDNA and 4.7µl nuclease free water. Thermal cycling conditions of an initial step at 96˚C for 1 min, 25 cycles at 96˚C for 10 sec, 50˚C for 5 sec and extension at 60˚C for 4 mins; were used to confirm that the gene sequence corresponded to the referenced articles as well as the pre-designed gene sequence for *18S*.

5.2.4 qRT-PCR

Each qRT-PCR reaction contained 2µl cDNA, 0.2µl each of forward and reverse primers, 5µl of KAPA SYBR® FAST and 2.6µl PCR Grade water in a total volume of 10µl. All samples were analysed on a QuantStudio 5 (Applied Biosystem) using 96-well plates. For these reactions, two biological replicates were prepared with four technical replicates per time interval. In total, 432 reactions were performed including several negative control (NTC) reactions. The cycling conditions included one cycle at 95˚C for 5 min, with 40 cycles at 95˚C for 10 sec, 60˚C for 1 min, with an extension at 72˚C for 15 sec; along with a product dissociation stage at 0.3˚C included after 40 cycles to obtain a dissociation curve. Melt curve analysis was performed to ensure that each primer pair was specific and produced a single peak. Data were analysed using the DataAssist v3.01 software program (Applied Biosystem) that utilizes the comparative delta-delta Ct (ΔΔCt) method (Livak and Schmittgen 2001).

5.3 Results

5.3.1 Total RNA extraction

RNA integrity was evaluated by including a standard 1kb molecular weight marker during agarose gel electrophoresis. The absence of smearing on the gel indicated good quality RNA samples. It was also evident that the 28S band was not visible only the 18S band. High intensity bands were observed for *18S* with an average size of 800bp however, overall the integrity and the yield of the RNA was good (Figure 5.1). All samples had RNA quantities that were within the range of 400 – 1000 ng/µl after extraction. Nanodrop® spectrophotometry of all extracted sampled showed that protein contamination was absent as indicated by a consistently obtained value of 2.0 at the 260/280 ratio.

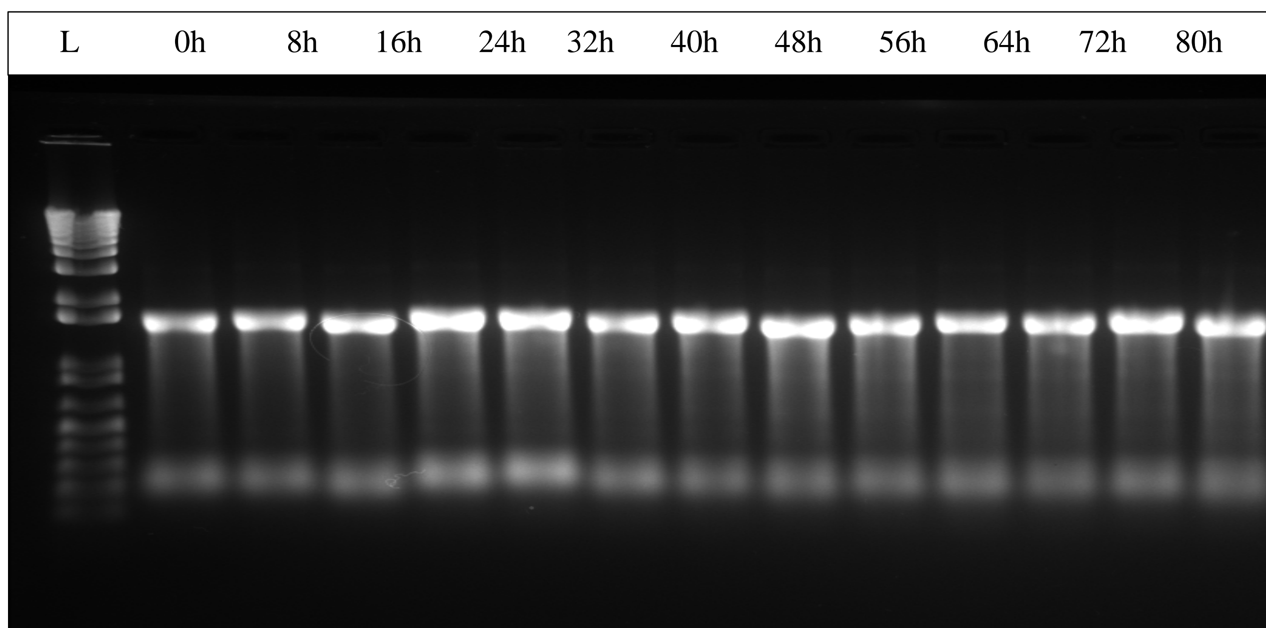

Figure 5.1: Gel electrophoresis of a representative sample of the total RNA extractions analysed by gel electrophoresis. Lane L denotes the 1kb molecular weight marker. Different pupal age time point is indicated above each gel lane.

5.3.2 Primer design and PCR optimisation

The primer sequences of all candidate reference genes and the corresponding amplicon sizes are indicated in Table 5.1. *Rp49, RpS17, 18S* and *actin* gene primers showed positive

amplification in all samples using conventional PCR, at an annealing temperature of 60˚C (Figure 5.2). *GAPDH* did not amplify in any of the samples or at any annealing temperature; therefore, the gene was disregarded from further investigation.

Table 5.1: Candidate reference genes, as taken from Cardoso *et al*. 2014, including primer sequences for 18S.

Symbol	Gene name	Primer Sequence (5' – 3')	Amplicon Size (bp)
Actin	Actin 5C	F: GCCATGTATGTTGCCATCC	158
		R: CGACCAGCCAAATCCAAAC	
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase	F: GTCAGTGACACCCACTCCTC	128
		R: TTGATCAAGTCGATGACACG	
RpS17	Ribosomal protein 17	F: TCGTGTAAGAACCAAGAC	100
		R: GCAGATACGCTTGTTG	
Rp49	Ribosomal protein L32	F: GCACCAAGCACTTCATCC	169
		R: AGTGGGAAGCATGTGACG	
18S	Ribosomal protein S18	F: AGTGCTCTTCATCGAGTGTTGTT	150
		R: CTCGCGTCGTAATACTAATGCC	

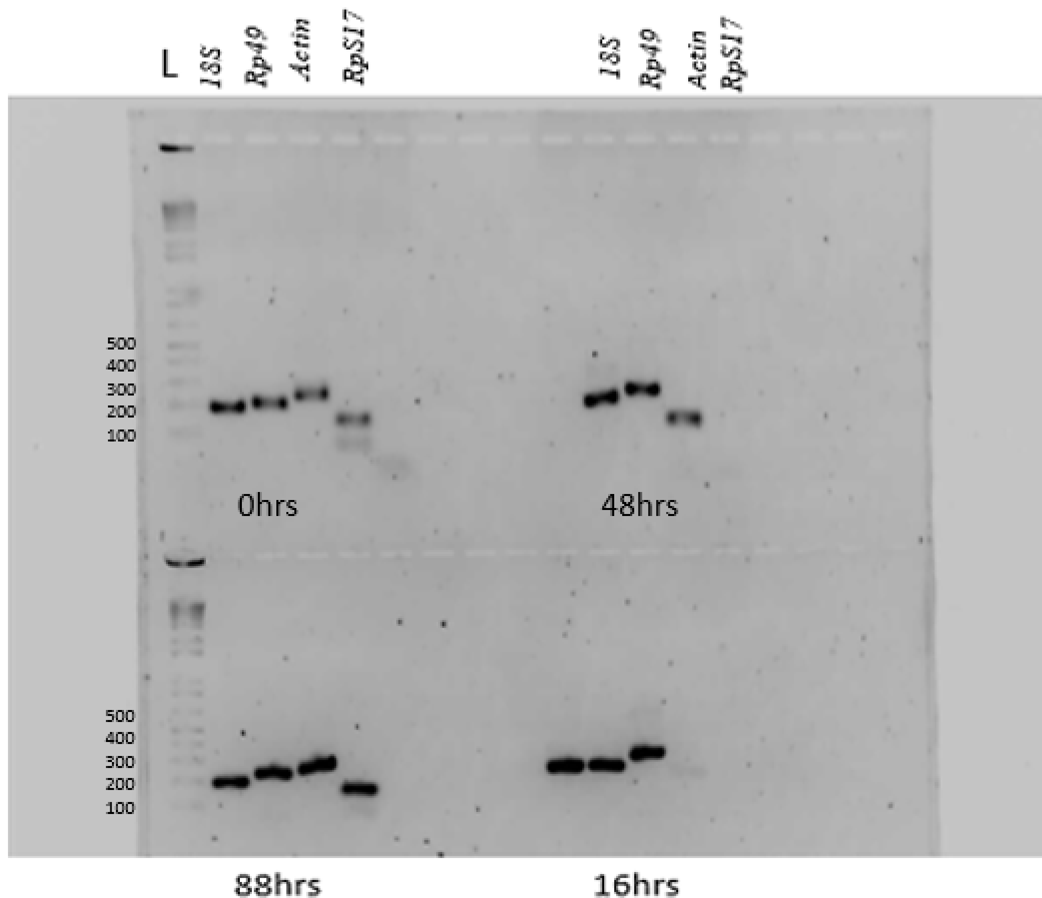

Figure 5.2: Amplification of selected reference genes using conventional PCR.

All four reference genes (*actin*, *RpS17, Rp47* and *18S*) were first selected as candidate control to identify the most suitable reference gene or genes for normalization. This analysis was done using the reference gene validation feature (termed 'endogenous control analysis') in the software package DataAssist. The endogenous control analysis showed that the stability score for *Rp49* and *RpS17* were 1.09, whereas *18S* and *actin* yielded scores of 1.55 and 2.36, respectively (Table 5.2). The 0 hour sample was used as a calibrator sample as it represents the starting point of the pupal period. Based on the results from DataAssist, *Rp49*, *18S* and *RpS17* showed the lowest expression variability within all samples when biological replicates were pooled together. Pooling the data of all time stages for each reference gene showed high variability in the expression pattern for *actin*, with the Ct value ranging from 16.94 – 24.39. *Rp49, RpS17* and *18S* had the lowest variance across all the tested time points (Figure 5.3). Therefore, we adopted *Rp49, RpS17* and *18S* genes as references in the current study. *Actin*, on the other hand, was excluded as a reference due to its variability, but considered as a candidate as per previous studies (Tarone & Foran 2011; Brown 2012; Boehme *et al*. 2013).

Table 5.2: Stability values of selected reference genes after endogenous control analysis.

Gene	Stability value
Actin	2.3648
18S	1.5512
Rp49	1.0992
RpS17	1.0919

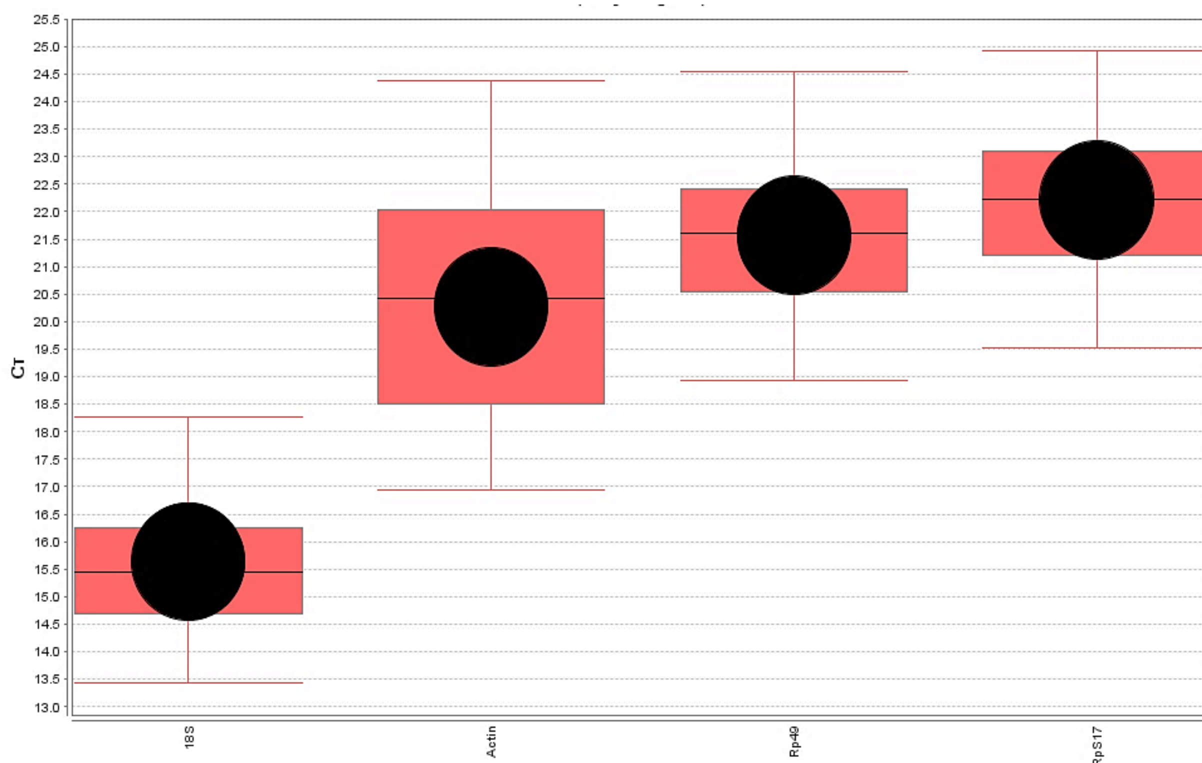

Figure 5.3: Maximum and minimum Ct expression values for the selected candidate reference genes. Data for all the time stages were pooled for each gene. The middle line of each box indicates the median. *Rp49, RpS17* and *18S* had the lowest difference between the maximum and minimum Ct expression values compared to *actin*.

Unfortunately, the *ecr* gene was disregarded as a target gene due to primer dimers that were observed after qRT-PCR were performed. As indicated by the melt curve analysis in figure 5.4, two peaks can be seen when ideally only one peak should be produced.

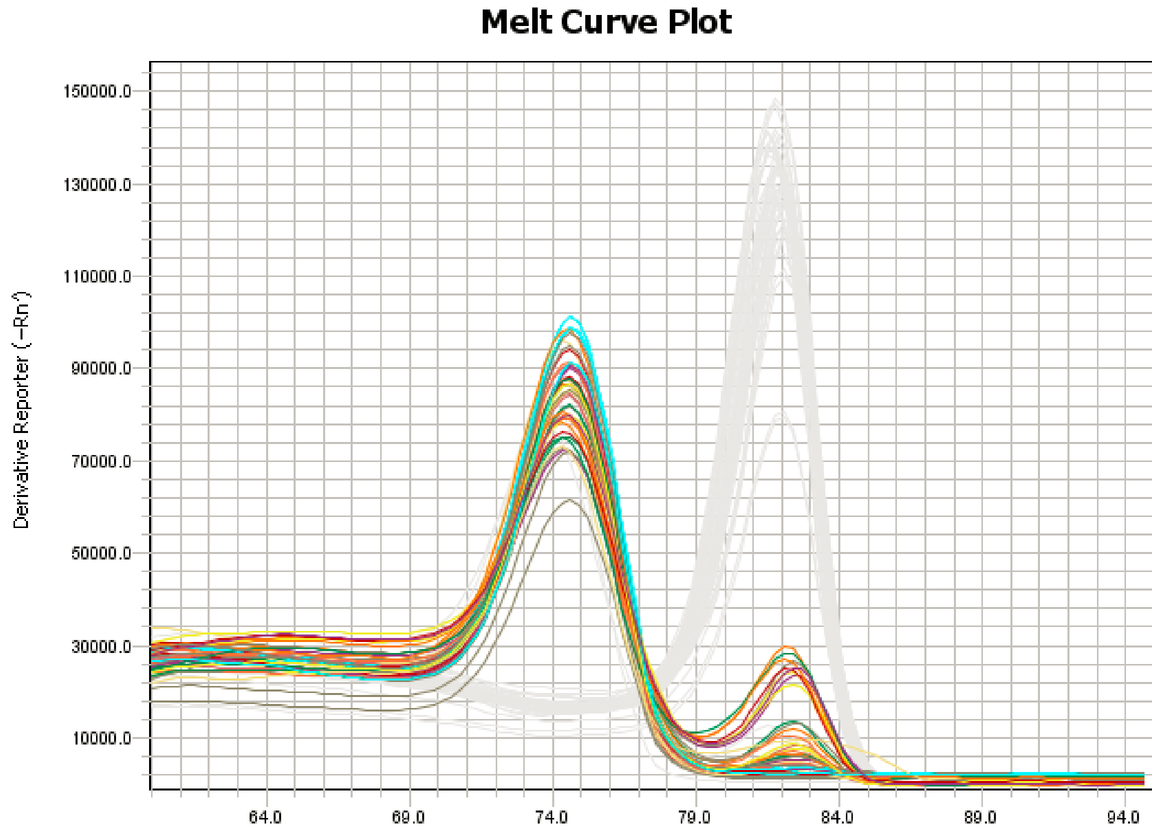

Figure 5.4: Melt curve analysis of *ecr* after performing real-time PCR.

5.3.3 qRT-PCR results

In this study, five previously identified reference genes were selected and analysed for use in normalizing gene expression in *Chrysomya albiceps*. Due to the variability in expression pattern *actin* was selected as a target gene. The expression pattern of *18S* (18.3) were the highest while *RpS17* (24.9), *Rp49* (24.5) and *actin* (24.4) were expressed at lower levels in all samples. The average Ct and fold change values of *actin* can be seen in table 5.3, lower Ct value shows higher expression which correlates to the fold change. The expression of *actin* showed a slight increase at 8 hours and then a slight decrease at 16 hours. From 32 hours a steady increase was seen up to 64 hours, where after a sharp increase was observed from a fold change of 7.986 to 53.073 at 88 hours (Figure 5.5).

Table 5.3: Average expression pattern of *actin* during pupal development of *C. albiceps*.

Gene	Pupal developmental hour	Average CT	Fold change (FC)
	0 Hr	22.6128	1
	08 Hr	20.4821	1.1922
	16 Hr	22.4921	1.0367
	24 Hr	23.411	1.0025
	32 Hr	20.8761	1.2771
	40 Hr	20.7398	1.4127
	48 Hr	22.0103	1.6046
	56 Hr	20.7866	3.0244
	64 Hr	18.6678	7.9864
	72 Hr	17.7583	17.5239
	80 Hr	18.446	52.2144
	88 Hr	17.3569	53.0734
Actin	96 Hr	17.867	40.3711

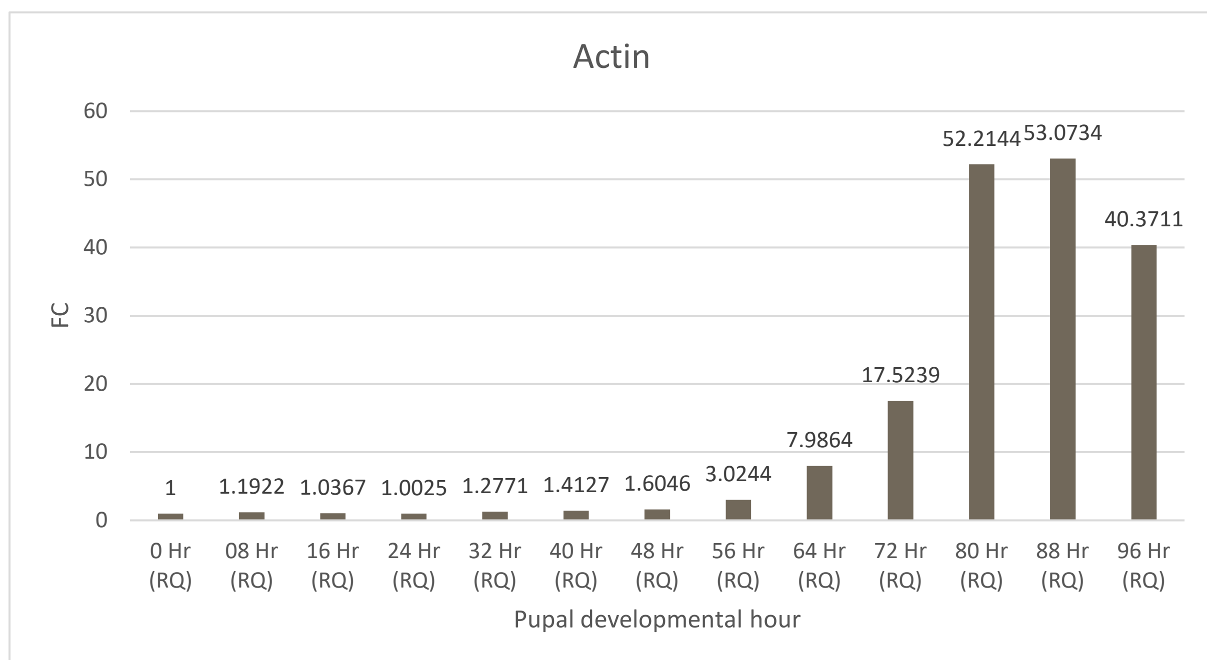

Figure 5.5: The expression changes of *actin* throughout pupal development. A slight fluctuation can be seen between 0- and 56-hrs. From 32 hours a steady increase was seen up to 64 hours where after a sharp increase was observed from a fold change of 7.986 to 53.073 at 88 hours.

5.4 Discussion

qRT-PCR was performed using first-strand cDNA synthesised from RNA extracted from 78 pupae. The aim was to confirm if pupal age can be determined using preselected target genes, normalized by validated reference genes, during development in the blowfly, *C. albiceps*.

Good quality RNA is crucial for expression analysis. According to Winnebeck *et al*. (2009), insect RNA will appear as if it is degraded because the 28S band migrates down to the 18S band due to the heating step that is added in the extraction step. This is the main cause of the high intensity band that can be observed and should be considered when working with insects.

Age estimation of pupal samples, using gene expression changes, are still ongoing in a variety of Diptera species (Ames *et al*. 2006; Zehner *et al.* 2006; Tarone & Foran 2011; Brown 2012; Boehme *et al*. 2013; Wang *et al*. 2015). However, the research to date on the set of genes to use for normalization of gene expression analysis, across all species, are conflicting (Tarone *et al*. 2007; Boehme *et al*. 2013; Cardoso *et al*. 2014). *Actin* and *ecr* were initially identified as target genes, but then *actin* was subsequently included as one of the potential reference genes (*RpS17, Rp49* and *18S*) whose suitability in qRT-PCR studies was tested in tissues of *C. albiceps*.

Reference gene validation is not only important to identify commonly stable expressed genes for insects, but also for normalization of differentially expressed genes. Since there is limited information available regarding the validation of reference genes in fly species (Bagnall & Kotze 2010; Brown 2012; Cardoso *et al*. 2014) this study aimed to validate already identified and confirmed genes for future use. Reference gene primers were selected from an already published study (Cardoso *et al*. 2014). The primer sequences provided for *GAPDH*, however, did not amplify the fragment as expected despite the use of several PCR protocols. This is likely due to the temperature differences between the forward (Tm = 64.5˚C) and reverse (Tm = 58.35˚C) primers. Ideally, primers used for qRT-PCR experiments should have the features: Tm = 60 ±1˚C, length of 18 – 25 bases and a GC content between 40 and 60% (Udvardi *et al.* 2008). We were able to confirm during this study that *18S*, *Rp49* and *RpS17* genes can be used as references for normalizing the expression levels of target genes in

Chrysomya albiceps pupae. Therefore, future qRT-PCR studies on *C. albiceps* can utilise these genes for gene expression analysis.

For *actin,* however, it was found that this gene is not a suitable reference gene in the current study. Our findings contradict those by Cardoso *et al*. (2014), who resolved that *actin* would suffice as a reference gene in *Chrysomya albiceps*. After normalization of *actin* to the reference genes validated in this study it was noted that the expression patterns were not constant. This is mainly due to the involvement of *actin* in the formation of the cytoskeleton (Beckmann 2004), and it is no surprise that the expression levels of this gene are highly increased after 64 hours as the sclerotization increased. The sample size used by Cardoso *et al.* (2014) should also be taken into consideration as a larger dataset are likely to reveal additional information that would not be easily detectable in small datasets.

Even though *ecr* expression patterns were not able to be investigated during this study it still holds potential as a broad age estimation marker due to change in the expression levels throughout pupal development. A*ctin*, however, fluctuated so little during the initial 32 hours that it almost seemed stable during the first half of the pupal development and then the expression levels rapidly increased to its maximum at 88 hours. To remedy the primer dimer issues encountered for *ecr* we recommend the design of new primers.

Accurate age estimation of blowflies for the purpose of postmortem interval estimation is essential in forensic criminal cases. Due to conflicting information available in current gene expression research in blowflies, this study contributes to support already validated reference genes for future use and confirmation that *actin* is more suitable as target gene.

5.5 Conclusion

Gene expression analysis, not only for the pupal stage but for all immature fly stages, can provide vital information. The standard technique is also easily applied and can be incorporated into everyday forensic laboratories. During immature blowfly development the already defined entomological guidelines can be used, but where the morphological markers are limited gene expression can be used.

It is clear, not only from this study but several others, that more in depth research is required across all fly species to determine a set of genes that can be used as normalization genes and candidate target genes.

Currently, the validation of genes suggested to be references is in the minority and ultimately hinder accurate normalization of gene expression analysis. Based on the results of the current study, *RpS17, 18S* and *Rp49* would be the best suitable set of reference genes for data normalization in *C. albiceps* in South Africa. *Actin* would not be a suitable reference gene due to the high variance observed among sample at different stages of development. Therefore, the validation of additional reference genes remains essential. Our study also implied that *actin* regulates specific developmental features as it was expressed more after 50% of intra-puparial development. Thus, *actin* should be considered as a candidate gene marker for pupal development.

*Ec*r still holds enormous potential as a possible candidate gene because several studies already proved that the expression levels vary, in different species. It is also essential to identify and target additional gene possibilities that are known to regulate specific morphological markers. Age estimation and ultimately PMI estimation can be improved by the more genes identified.

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Chapter 6: CONCLUDING REMARKS

The intra-puparial stage is essential in post mortem interval (PMI) estimation due to the length of this stage. It has been reported that this stage occupies more than 50% of the total immature development time. As *Chrysomya albiceps* is a dominant immature life stage at crime scenes, due to their predatory nature, and limited research has been done using this species, it was our species of choice to research. We aimed to contribute to pupal developmental studies for *C. albiceps* in South Africa, as this research has not been done before in this region. Pujol-Luz & Barros-Cordeiro (2012) reported that the intra-puparial development time for *C. albiceps* from Santa Maria, Brazil were 90 hours, compared to Salazar-Souza *et al*. (2018) who reported on intra-puparial development time for *C. albiceps* from Rio de Janeiro, Brazil to be 99 hours. During this study the intra-puparial development timeline was 104 hours. These experiments were performed under similar temperature regimes. Brown (2012) noted in her comparative analysis of intra-puparial timelines the reality of inter-specific differences due to location.

In light of what was explained in the above paragraph, it is important to continue and broaden the research to obtain a more accurate timeline for the pupal development stage. Considering the large percentage, it is essential to identify what exactly happens during this period to improve age estimation in blow flies. In order to narrow the PMI estimation spectrum, that is currently available for pupal specimens, using a wider range of forensically important fly species. This study, however, should not be used for PMI timeline determination in *C. albiceps* in South Africa but rather to establish landmarks for intra-puparial development for *C. albiceps*. Therefore, we examined external morphological landmarks and also gene expression landmarks for this species in South Africa.

Before establishing morphological or gene expression landmarks, optimal killing and preservation methods were first explored for both types of analysis. To ensure accurate age estimations of pupae it was important to kill the specimens once found, to stop development, and place in a preservative that will not damage the integrity or quality of the sample. Perfect killing and preservation are not always possible, especially at crime scenes and it is vital to use the right chemicals. When sampling at a crime scenes the majority of entomological samples are normally transported alive until analysis or placed directly into ethanol which

results in inaccurate age estimations. When at crime scenes our suggestion is that 50% of samples should be collected for morphological analysis and 50% for gene expression analysis.

For morphological examination, hot water killing, placing the specimens un-pierced in 70% ethanol, was the best protocol to conserve the true-to-life colour and form of the pupae. Formalin as a preservative showed enormous potential, especially during early stages of pupal development (o-hours and 8-hours), however this chemical is not readily available in everyday crime scene kits used by entomologists. To make sure that samples are adequately killed and preserved for molecular analysis and gene expression the following methods are suggested: killing by liquid nitrogen and preserved at -80˚C until analysis.

In order to establish morphological landmarks several articles were reviewed; however, some of the mentioned landmarks are not easy to access. Biggest identification landmarks were the calypters, respiratory gills and tarsi, which were not used previously or described in detailed.

The prospect of using gene expression changes, in combination with entomological data, to determine the age of forensically important fly species, in all life stages, have gained the attention of several researchers around the world. As shown in this study gene expression changes can be used when there is no visible change in morphological markers and vice versa. This study supports the use of *actin* as a target gene and not as a reference gene due to the variability in expression. During the early stages of pupal development *actin* showed a slight variation in expression where morphologically the changes were significant. Between 88 and 96 hours there are no significant changes in the morphology of *C. albiceps* however, when reviewing the expression levels of *actin*, the expression value decreased by almost 10% compared to 88 hours.

The importance of additional comprehensive pupal developmental studies, especially in a wider range of fly species, are evident from the results shown in this study. From preservation, when collected at a crime scene, identification of morphological changes and the gleaming possibility of using molecular techniques to map gene expression changes to morphological characteristics for age determination.

Citations

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Appendix 1

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UFS·UV
RESEARCH DEVELOPMENT
NAVORSINGSONTWIKKELING

Environment & Biosafety Research Ethics Committee

11-Nov-2021

Dear **Miss Lucinda Van Der Westhuizen**

Project Title: **Morhological and gene expression changes during intra-puparial development in Chrysomya albiceps (Diptera: Calliphoridae)**

Department: **Genetics Department (Bloemfontein Campus)**

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Environment & Biosafety Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2021/0274/21**

Please note the following:

1. **This ethical clearance is valid for two years from the issuance of this letter.**
2. **If the research takes longer than two years to complete, please submit a Continuation Report to the Ethics Committee before ethical clearance expires.**
3. **If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an Amendment.**
4. **When the research is concluded, please submit a Final Report to the Ethics Committee.**

Approved

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Environment & Biosafety Research Ethics Committee
University of the Free State

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