FORENSIC ENTOMOLOGY ON THE GAUTENG HIGHVELD

Allison Elizabeth Gilbert

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master in Science.

Johannesburg, 2014

DECLARATION

I, Allison Elizabeth Gilbert, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

.....

A.E. Gilbert

On this the 16th day of MAY, 2014.

DEDICATION

To my Mum and Dad, brothers and Aunty Marj.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

PUBLICATIONS

Gilbert, A.E., Kelly, J.A., Koekemoer, L.L., Coetzee, M. (2014) Decomposition of a carcass in Summer and Winter in Gauteng. Journal of the South African Veterinary Association. IN REVIEW.

ORAL PRESENTATIONS

Gilbert, A.E., Spillings, B.L., Koekemoer, L.L., Coetzee, M. Pilot study on Forensic Entomology on the Gauteng Highveld. Inqaba Biotechnology's 2nd Annual African DNA Forensics Conference. Pretoria, South Africa. 2010.

Gilbert, A.E., Spillings, B.L., Koekemoer, L.L., Coetzee, M. Forensic Entomology on the Gauteng Highveld. Entomological Society of Southern Africa XVII Conference. Bloemfontein, South Africa. 2011.

Gilbert, A.E., Spillings, B.L., Koekemoer, L.L., Coetzee, M. Multiplex PCR for easy identification of forensically important blowflies on the Gauteng Highveld. Entomological Society of Southern Africa XVIII Conference. Potchefstroom, South Africa. 2013.

POSTERS

Gilbert, A.E., Spillings, B.L., Koekemoer, L.L., Coetzee, M. Forensic Entomology on the Gauteng Highveld, South Africa. International Congress of Entomology XXIV Conference. Daegu, Korea. 2012.

Gilbert, A.E., Spillings, B.L., Koekemoer, L.L., Coetzee, M. Forensic Entomology on the Gauteng Highveld, South Africa. Faculty of Health Sciences Research Day 2012. Johannesburg, South Africa.

ABSTRACT

Forensic Entomology utilises arthropods in legal investigations that involve death, neglect and abuse of humans and animals and even civil cases like insurance claims. This study aimed to make general observations on the decomposition of a pig carcass (*Sus scofa* Linneaus) in relation to recorded temperatures of the carcass and the surrounding site during both summer and winter on the Gauteng Highveld. The study also aimed to identify the dominant blowfly species occurring in the region. Six species were identified: *Calliphora vicina, Chrysomya marginalis, Ch. albiceps, Ch. chloropyga, Lucilia sericata* and *L. cuprina.* The cephaloskeleton, anal spiracles and anterior spiracles were dissected from the first, second and third larval instars of the flies to isolate the key features currently used in morphological identifications. The ITS2 region was investigated for the development of a multiplex PCR method to identify these species. The multiplex PCR method did not include *Chrysomya albiceps* but does successfully differentiate between the other five commonly occurring blowflies.

ACKNOWLEDGEMENTS

I wish to thank my supervisors Prof. Maureen Coetzee and Prof. Lizette Koekemoer for all their guidance and support through my Masters studies. I wish to thank my collaborator Dr. Janine Kelly for all her input, guidance and support.

I wish to thank the South African Veterinary Foundation for funding as well as the NRF for funding via the DST/NRF Research Chair initiative via Prof. Coetzee.
I offer the deepest thanks to the Melville Koppies Nature Reserve Committee for allowing me access to the koppies for my project and the South African Weather Service for providing data.

I thank all the staff and students at the Vector Control Reference Laboratory especially Dr. Belinda Spillings for training in the DNA work and Miss. Shüné Oliver for all her guidance.

I thank my parents and family for all their support and encouragement. A warm thanks to Julian Birkett and Kelvin Eyre for all their assistance, and a special thanks to the late Inspector Wayne Hutchison for all his encouragement when I was finding my feet and for pointing me in the right direction towards my career.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1. An overview of forensic entomology

1.1. Literature Review

1.1.1. General Forensic Entomology

Forensic entomology is the study of arthropods that forms part of legal investigations (Amendt *et al.*, 2007, 2011). The insects provide information about the crime scene and possibly events around the crime. Insects have been noted as being important in crime-related investigation cases and have been traced back to historical cases long before modern research validated their use (Catts and Haskell, 1990). One of the most commonly known historical cases dates back to 1247 in China where an investigator used the occurrence of flies that were attracted to the residues of blood on a sickle to find both the murder weapon and the murderer (McKnight, 1981). From the 1600's onwards researchers, forensic pathologists and entomologists, have expanded the field of forensic entomology and documented the insects associated with corpses (Smith, 1986), the order they arrive and occur on a corpse (Payne, 1965) and their uses in investigations. By the late 1900s forensic entomology had been established as a branch of science that is valid and widely used in modern crime investigations (Smith, 1986; Hall, 1990; Amendt *et al.*, 2007; Hall and Huntington, 2010).

Forensic entomology is most commonly used to estimate the post mortem interval (PMI) (Payne, 1965; Hall and Huntington, 2010). The post mortem interval estimates the time that has passed between death occurring (Figure 1) and the discovery of the remains. In other words, it helps to estimate the window period in which death could have occurred (Bass, 1984; Archer, 2004; Clark *et al.*, 2006; VanLaerhoven, 2008; Wells and Lamotte, 2010; Villet, 2011). The potential accuracy of the PMI estimations decrease as the time

after death increases (Wells and Lamotte, 2010). Entomological evidence can narrow the time span and thus increase the accuracy of the PMI estimations (Wells and Lamotte, 2010, Villet, 2011). To produce these entomological estimations, all environmental and physical findings are taken into account: the state of the corpse, any other faunal evidence and factors such as temperature and rainfall (Hanski, 1987; Catts and Goff, 1992; Anderson, 2010).

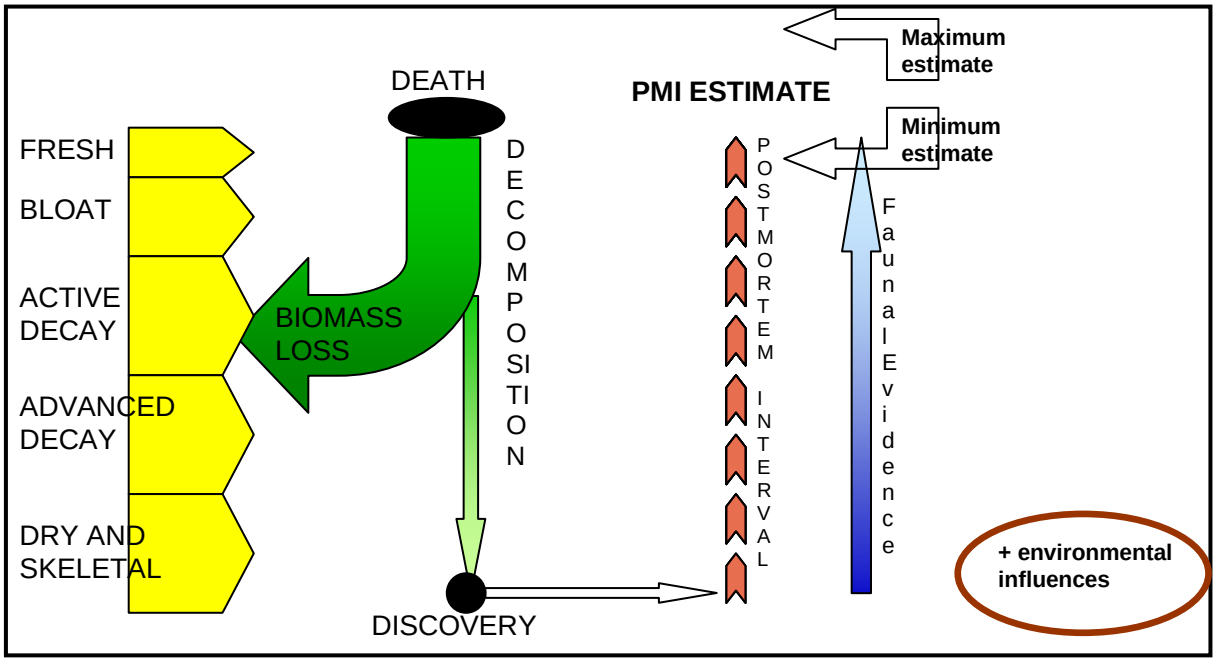


Figure 1.1. Diagrammatic representation of the decomposition of a carcass and the interaction between the faunal evidence, the environmental influences and the calculation of the post mortem interval estimates (adapted from Catts, 1990). This diagrammatic representation shows how after death the body loses biomass as it progresses through the different stages of decomposition (shown on the left side of the diagram in yellow and green). Upon discovery of the body the post mortem interval can be estimated. The PMI is estimated using the faunal evidence and the effects of the environmental influences on both

the fauna and the decomposition. This estimate creates a window period, bound by the minimum and maximum estimates, in which death could have occurred.

Necrophagous (carrion) insects are attracted to dead bodies and are facultatively dependant on the decaying corpse for survival (Payne, 1965; Braack, 1987). Payne (1965) described how after death there is a definite ecological succession of arthropods associated with the decomposing carcass. This has been confirmed by many studies conducted since then (Smith, 1986; Hall, 1990; Bass, 1996; Byrd and Castner, 2010; Villet, 2011). As with all organic material, as a corpse or carcass decays it goes through a set biological breakdown process (Bass, 1996; Carter, *et al*., 2007). Each stage of decay is characterised by a specific group of insects that are specialised to survive in the specific conditions of a decomposing carcass and the order in which they arrive on a body is termed the succession (Payne, 1965). This succession is composed of waves of insects, the first being Diptera (flies), which are attracted to the carcass soon after death and through the early stages of decay. The later stages of decay attract more Coleoptera (beetles) that remain until the carcass has been reduced to hair and bones (Payne, 1965). There is a small margin of overlap of the two insect groups on a carcass as some adult beetle species arrive in the early stages of decomposition (Smith, 1986; Braack, 1987; Catts, 1990; Bass, 1996; Midgley, 2007 thesis; Ridgeway, *et al.*, 2014). The rate of development of insects on a carcass is greatly influenced by various external factors such as the physical location of a carcass (is it in the sun or shade, inside or outside a building), changes in environmental temperatures (Anderson, 2010), changes in humidity (Carter *et al*., 2007), coverings over the body (Kelly, *et al*., 2011), burning of bodies (Lord, 1990) and even the presence of chemicals and drugs (Higley and Haskell, 1990).

Diptera are termed the primary decomposers on a corpse or carcass, the most common being species belonging to the families Calliphoridae (blow flies), Sarcophagidae (flesh flies) and Muscidae (house flies). The most important of these flies are the Calliphoridae as adult flies have been noted on a corpse within an hour of death, which aids in creating a narrow PMI estimate of the time of death (Braack, 1987; Bass, 1996; Bourel *et al.*, 2003). The maggots are also present on the corpse through many of the stages of decomposition (Payne, 1965; Smith, 1986; Lord, 1990; Anderson, 2010) and are often the main insects used to determine the PMI. Flies undergo a holometabolous metamorphosis as the larval forms (maggots) are different in appearance and life style to the adult forms (flies) (Zumpt, 1965). The larval stages are often difficult to identify to species on general morphology and the main characteristics used are the structure of their anal spiracles and their cephaloskeleton (Zumpt, 1965; Castner, 2010). While there are morphological keys available to identify the larvae, it requires considerable skill to use these keys accurately (Hall, 1948; Zumpt, 1965; Prins, 1982; Halloway, 1991; Castner, 2010). Adult flies are easier to identify by morphology, therefore most samples taken for forensic entomological investigations are reared through to adults before being identified (Catts, 1990; Castner, 2010). Modern research has moved towards molecular techniques as tools for the identification of forensically important insects especially the Diptera (Sperling *et al.*, 1994; Harvey, *et al.*, 2003; Ratcliffe, *et al.*, 2003; Williams and Villet, 2013).

Forensically important Coleoptera generally appear during the later stages of decomposition, even though some are present in the early stage. These insects also undergo a holometabolous metamorphosis (Castner, 2010). The larvae (grubs) and adults (beetles) are generally very distinctive but few have been described in full (Castner, 2010). The most dominant families of beetles are the Silphidae (Carrion beetles) and Dermestidae

(Skin beetles) (Smith, 1986; Braak, 1987; Catts, 1990; Kelly *et al.*, 2009; Byrd and Castner, 2009). The Coleoptera are mainly used to determine the PMI in corpses and carcasses that are in the late stages of decomposition when the beetle populations are dominant, even though there are some beetles that are also present in the earlier stages of decomposition (Braack, 1987; Anderson, 2001; Midgley, 2007).

There are often other organisms that occur on decomposing bodies. These species may include predators, parasites or scavengers that visit the carcass to prey on the insects living off the remains (Smith, 1986; Putman, 1983) Other organisms that visit corpses and carcasses to feed also need to be identified. Forensic entomologists need to be able to identify the organisms and assess the damage they have done to the corpse or carcass as well as any damage done to the other insects associated with the corpse or carcass (Bryd and Castner, 2010). These other organisms range from mites, ants, true bugs, birds and mice to larger scavengers such as cats, dogs and wildlife (Prins, 1983; Braak, 1986; Byrd and Castner, 2009).

Forensic entomology is not only limited to death investigations. Other uses include investigations of forensically related insects in cases of child neglect, child abuse (Benecke and Leggig, 2001; Beneke, 2010) as well as neglect and abuse of the elderly (Lord, 1990; Benecke *et al.*, 2004; Beneke, 2010). An example of the use of insects in a child neglect case was outlined by Goff *et al.* (1991) where the age of the maggots found in a child's diaper were used as evidence against the parent for neglecting and abandoning the child.

1.1.2 Myiasis

Myiasis is the invasion of living tissue by fly larvae even though some of the flies live off both dead and living tissue. Some of the forensically important fly families (Calliphoridae and Sarcophagidae) are known to be myiasis causing flies in both animals and humans. Some flies are opportunistic and others are 'true myiaisis' flies and their life cycles are dependent on the larval stages in the living tissue of a host (Stevens, 2003), examples of these types of flies are the botflies and warble flies of the family Oestridae. An animal example is the screw worm fly that causes huge tissue damage in infected cattle and sheep (Zumpt, 1965; Otranto and Stevens, 2002; Stevens, 2003) and some of the *Chrysomya* and *Lucilia* species are great pests in the farming industry especially those of the sheep farms as they cause flystrike (Halloway, 1991). *Lucilia sericata* (a green-bottle blowfly), on the other hand is a medically important insect as it is used in maggot debridement therapy (Stevens, 2003; Williams *et al.*, 2008). Maggot debridement therapy is the use of sterile, laboratory reared maggots to clean the necrotic tissue around severe wounds.

Forensic entomologists have also been involved in cases of wildlife poaching (Anderson, 1999; Watson and Carlton, 2003; Watson and Carlton, 2005) as well as neglect and maltreatment cases concerning animals (Erlandsson and Munro, 2007). There has also been work done on naturally occurring myiasis in pets and domesticated animals (Anderson and Huison, 2004).

1.1.3. Insect lifecycles

1.1.3.1. Flies

After the eggs hatch the larvae progress through three instar stages, moulting between each stage. The third instar larvae then pupate and develop into adults which later emerge from

the pupal casings (Fig. 1.2). The time taken to complete the life cycle varies with each species of fly and can be affected by numerous factors, most importantly temperature (Clark *et al.* 2006; Donovan *et al.*, 2006; Ireland and Turner, 2006; Villet, *et al.*, 2010).

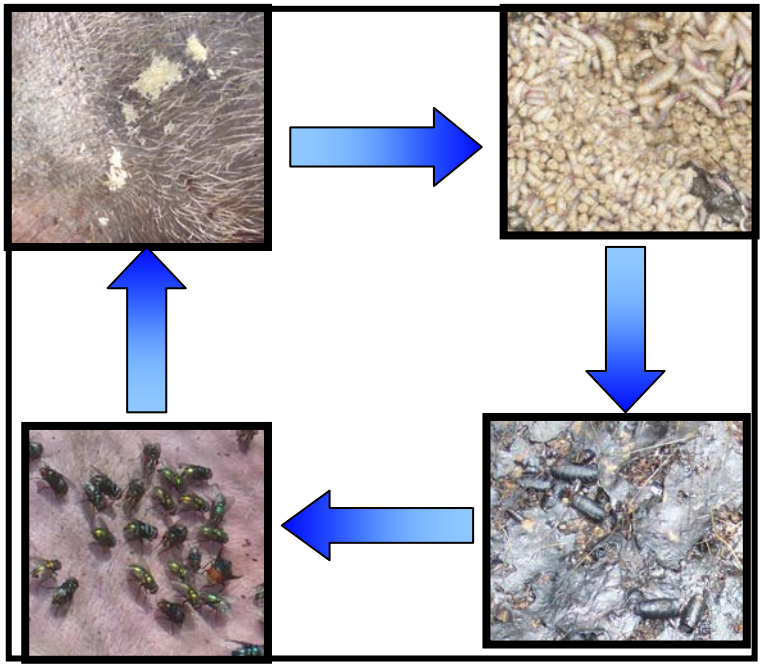


Figure 1.2. Diagram showing the life cycle of a fly. Moving clockwise from the top left, eggs, larvae, pupae and adult flies.

The PMI is calculated using entomological data gathered at the crime scene to estimate backwards the time that death could have occurred. Most of the forensically important and recognised fly species have undergone intensive laboratory studies and growth curves created where their development (age and size) has been recorded over time at different temperatures (Villet, 2007; Richards and Villet, 2008; Richards, *et al.*, 2008; Richards, *et al.*, 2009; Wells and Lamotte, 2010; Nederegger *et al.*, 2010; Richards, *et al.*, 2013). An investigator will take the sizes and ages of samples received from a scene and estimate their ages from these growth rate data records and thus be able to estimate how long the flies had been present at the scene, creating an upper limit for the estimated time of death as death had to have occurred before the first fly could deposit eggs (Wells and Lamotte,

2010). The investigator also takes into account the condition of the body (decompositional stage), the location and other aspects directly affecting the body and what insects are present at the scene. The nearest available environmental data (usually from a weather recording station) also needs to be taken into account as it provides the temperature data for the time that the insects were developing, thus allowing fluctuations in temperature (Wells and Lamotte, 2010) to be taken into account during the calculation of the PMI estimates.

The developmental rate of the fly species is fundamental in the calculation of the PMI as well as general characteristics, specialised adaptations and behaviours. It is generally accepted that most of the Calliphoridae do not oviposit at night except a small selection of species that will oviposit on very small animal carcasses at night (Greenburg, 1990). Wells and King (2001) showed that in some species the female can hold an egg in the vagina that develops before the rest of the egg batch which results in maggots of different ages being present. Many aspects such as these are specific to species and need to be taken into account during investigations thus making detailed knowledge of the species involved vital (Erzinçlioglu, 1990).

1.1.3.2. Beetles.

Coleoptera are important insects in the later stages of decomposition with the most common being the Dermestidae and Silphidae (Byrd and Castner, 2010). The beetles are holometabolous with the larval forms being morphologically different in appearance from the adults. The larvae and adults feed on the dryer sections of corpses and carcasses and thus dominate the late stages of decomposition (Boucher, 1997; Byrd and Castner, 2010;

Midgley, *et al.*, 2010). The larvae can vary in the number of instars before pupating to become adults (Boucher, 1997). The adult and larval stages are very easily recognised.

1.1.4. Molecular studies

Correct and swift identification of any organism is vital during a forensic investigation (Byrd *et al.*, 2010) Molecular DNA techniques are commonly used as tools to aid in the correct identification of people, animals, plants and insects (Harvey *et al.*, 2003; Dalton and Kotze, 2011). Due to the complexity involved with identifying the larval stages of flies, molecular based identification of specimens is widespread among forensic entomologists (Ratcliffe *et al.*, 2003; Harvey, *et al.*, 2008; Wells and Stevens, 2008, 2010; Debry, *et al.*, 2013; Stamper, *et al.*, 2013). Molecular identifications are generally avoid observer bias and can use severely damaged specimens (Smith and Baker, 2008; Wells and Stevens, 2008). Molecular biology of insects is currently used to aid in taxonomic studies as well as phylogenetic and population studies (Otranto and Stevens, 2002; Kutty, *et al.*, 2008; Kutty, *et al.*,2010; Marinho, *et al.*, 2012; Singh, *et al.*, 2013).

Within the molecular work on insects there is a preference in current research to focus on the cytochrome c oxidase I (COI) and II (COII) regions of the DNA even though many other regions have been isolated and worked on (Harvey *et al.*, 2003; Smith and Baker, 2008; Wells and Stevens, 2008). Another simple and commonly used molecular technique for the identification of insects is the isolation of the internal transcribed spacer regions (ITS) (Koekemoer, *et al.*, 2002; Ratcliffe, *et al.*, 2003). The COI, COII and ITS regions have all successfully been used to differentiate between closely related insect species (Koekemoer, *et al.*, 2002; Ratcliffe, *et al.*, 2003).

1.2. Rationale for research

Within South Africa there have been many studies carried out in the northern areas (Kruger National Park), central areas and the Eastern and Western Cape (Braack, 1981; Williams and Villet, 2006; Kelly *et al.*, 2009; Kelly *et al.*, 2011). There has been some work done around the Gauteng Highveld area with Zumpt (1965) noting flies that were common on carrion. Meskin (1980) noted observations on the morphological, biological and ecological aspects of the most common Calliphoridae in the region and Pellatt (1996, Honours Dissertation) recorded the insects visiting a dog carcass during winter on the Gauteng Higheld. There have also been more recent studies done in the central regions of South Africa (outputs from students based at the University of the Free State). There have been cases based on the Gauteng Highveld where entomologists have been consulted in police investigations (pers. comm. Prof. Richard Hunt, Prof. Maureen Coetzee, Dr. Mervyn Mansell) but with none of the entomologists being specialised in the field of forensics. However, there is very little recently published data available for the Gauteng Highveld, which differs ecologically from the other sites, thus studies based in this area needed to be done.

1.3. Aims

This dissertation addresses the following aims:

1.3.1.) To observe the trends of decomposition of a carcass during the summer and winter months of the Gauteng Highveld.

1.3.2.) To document the morphological features used in identification of the forensically important Calliphoridae occurring on the Gauteng Highveld.

1.3.3.) To create a molecular identification tool of the forensically important Calliphoridae occurring on the Gauteng Highveld.

Chapter 2 - Decomposition of pig carcasses and the corresponding temperatures in summer and winter

2.1. Introduction

Decomposition is defined as the breaking down of cells of an organism. This process as defined by Saukko and Knight (2004) is a "mixed process ranging from autolysis of individual cells by internal chemical breakdown to autolysis from liberated enzymes, and external processes introduced by bacteria and fungi from the intestine and outer environment". Decomposition is affected by many different factors which can influence the rates or speed at which it can occur. These factors are mainly environmental and include temperatures, rainfall and the overall seasonal patterns of the area (Hanski, 1987; Catts and Goff, 1992; Pinheiro, 2006; Sharanowski *et al.,* 2008; Anderson, 2009; Bucheli *et al.,* 2009; Kelly *et al.,* 2009). Decomposition of a carcass consists of many changes over time and can be categorised into different stages (Hewadikaran and Goff, 1991; Bass, 1996). Some studies group these into six distinguished stages: fresh; bloat; active decay; advanced decay; dry remains and skeletal remains, with the latter two sometimes grouped together to make five stages (Payne, 1965; Schoenly, 1992; Bass, 1996; Kelly *et al.,* 2009; Matuszewski *et al.,* 2010; Kelly *et al.,* 2011).

There are general descriptions of the different stages of decay but these are variable and influenced by many external circumstantial factors (Schoenly, 1992; Saukko and Knight, 2004; Kelly *et al.,* 2009). However, a major factor is seasonality, with differences in temperature, rainfall and insect populations (Bass, 1996; Archer, 2004) that can greatly affect the rate of decomposition and can even disrupt and change these rates of decay (Carter *et al.*, 2007).

Temperature is a vital factor to be taken into consideration by forensic entomologists (Archer, 2004). The most common use of insects (predominately the maggots) found on a human corpse or animal carcass is the calculation of the estimated PMI (Archer, 2004; Clark *et al.*, 2006; VanLaerhoven, 2008). The PMI estimates the time that has passed between death occurring and the discovery of the remains. These estimates are calculated using the larval instar stage and the size of the maggots as an indication of their age (Ireland and Turner, 2006). This is done using growth rate data to determine the sizes of the maggots at different temperatures and then correlated with information collected from the nearest national weather recording station to the scene of the investigation (Clark *et al.*, 2006; Ireland and Turner, 2006; VanLaerhoven, 2008).

In South Africa, there have been a number of studies done on decomposition and environmental factors. These studies have been carried out in the northern areas (Kruger National Park), central areas and the Eastern and Western Cape (Braack, 1981; Williams and Villet, 2006; Kelly *et al.*, 2009; Kelly *et al.*, 2011). However, there is very little recently published literature is available for the Gauteng Highveld, which differs ecologically from the other sites.

This study aimed to identify broad trends of decomposition of a pig carcass and the associated internal and external changes in temperature during the summer and winter months on the Gauteng Highveld.

2.2. Methods and Materials

Pig carcasses (*Sus scofa* Linneaus) were used as accepted substitutes for human bodies (Catts and Goff, 1992). A single carcass was used per season to observe the trends. Both of the pigs were females and weighed between 80 kg to 90 kg to represent a fully grown human adult sized body. They were humanely killed with a bolt gun and wrapped in plastic and transported to the study site. Animal ethics was approved by the University of the Witwatersrand's Animal Ethics Committee (Clearance number: 2010/22/01). After each trial was completed the carcass remains were disposed of by incineration by Envirocin. Only one carcass was used per season as the carcass was merely used to ensure the collection of forensically relevant flies. The carcasses were left undisturbed for the span of the trials and were not weighed or lifted until removal.

The study was carried out in an enclosed part of the Melville Koppies Nature Reserve (co-ordinates: -26,1709; 28,0029) and was an undeveloped open area with restricted access (Fig. 2.1). The area is located within a Johannesburg suburban area that has large areas of open land with savannah and grassland vegetation. The site is characterised by long grasses, small shrubs and a few trees with small animals and birds (Fig. 2.2). The carcasses were placed inside a metal mesh cage (Fig. 2.2) to stop scavenging by rodents and small animals. The grid of the cage was mesh (diameter of approximately 25mm), which allowed the free movement of all fluids, gasses and invertebrates.

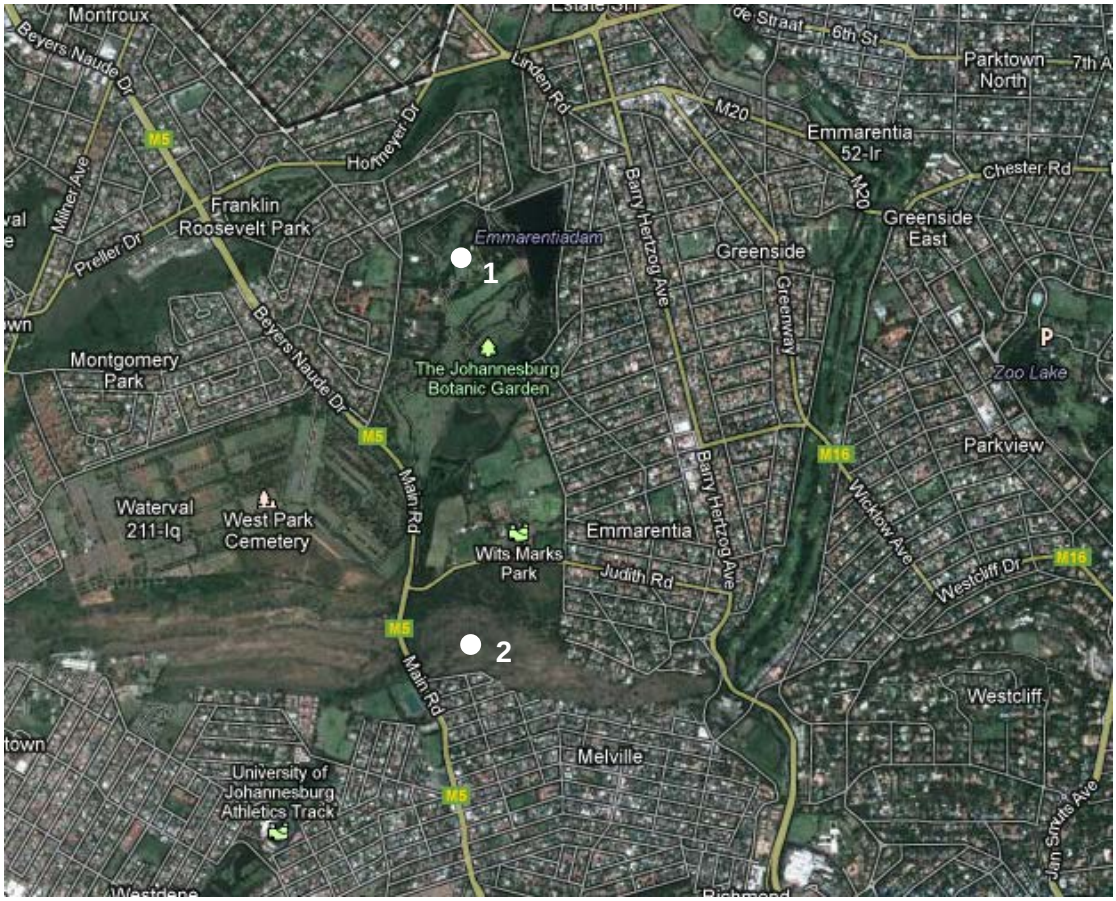


Figure 2.1. Google map showing the location of the study site (2) on Melville Koppies and the nearest South African Weather Station recording site (1) in the Johannesburg Botanical Garden.

Figure 2.2. The study site for the summer trial (A) and the winter trial (B). The winter site was approximately 15 metres from the summer location at the site.

The Gauteng region has summer and winters that are longer than autumn and spring (South African Weather Service, pers. comm.) with summer and winter spanning approximately four months each. The range of the seasons were selected based on temperature averages from the previous ten years and summer and winter were selected to cover the four months with the highest and lowest averages respectively. The summer trial ran from mid November 2010 to mid March 2011 and the winter trial from mid May 2011 to mid September 2012.

ColdChain ThermoDynamics ™ ibuttons (Fairbridge Technologies ™, South Africa) were used to record the internal and external temperatures of the carcass. The internal ibutton was inserted into the abdomen via a small incision. The external temperatures were recorded by placing an ibutton next to the carcass on the floor of the cage where it would receive morning sun. The ibuttons recorded the temperature every 60 minutes. The daily and weekly averages were calculated and used for analysis. Temperature and relative humidity were recorded at a distance of approximately 1.5 metres from the carcass to provide recordings of the ambient conditions at the site.

The daily temperatures and rainfall were obtained from the nearest South African Weather Service station at the Johannesburg Botanical Gardens (station number 0475879 0, co-ordinates -26.1500; 28.0000), which is approximately two kilometres away from the study site as (Fig. 2.1).

The pig carcasses were observed every day at midday for the first three weeks and every second day for the remainder of the trial. Due to the slow rate of decomposition the winter trial was extended and the site was visited once a week from the twentieth week onwards.

The carcasses were not moved during the trials. At each observation, photographs were taken and the state of the carcass recorded. Both carcasses were left until they were in the final, dry stage of decomposition.

The ibutton data were downloaded using the ColdChain ThermoDynamics Software ™ of Fairbridge Technologies ™ and captured and prepared for analysis using Microsoft Excel (2003). A t-test was used to statistically compare all samples recoded. All statistical analyses were done using Statistica 8.0 (Statsoft, Inc. ©).

2.3. Results

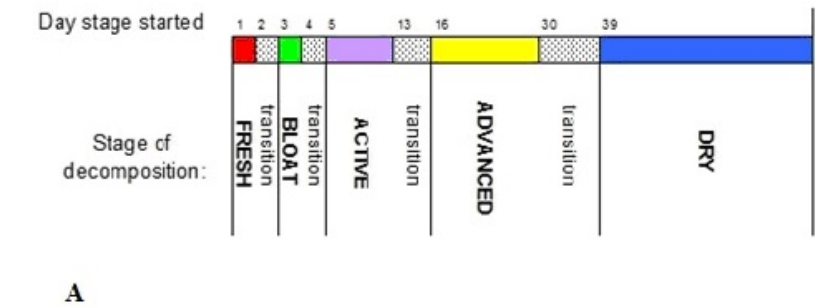
Decomposition stages

In the summer trial the carcass decomposed with each stage having similar characteristics to those described by others (Braack, 1981; Kelly *et al*., 2006; Pinheiro, 2006). There was a transitional stage between the stages as the carcass progressed into the next stage (Fig. 2.3A). In summary, in the fresh stage the body was still soft and the limbs could be moved. The bloated stage resulted in the carcass bloating and being firm and hard to the touch (Fig. 2.4, Appendix C). The end of the bloated stage resulted in the carcass starting to change colour and a weak odour was noticed. The active decay stage was noticeable as the carcass deflated and there was an active presence of insects (maggots). This stage resulted in a very large amount of the tissue and flesh being removed and there was a very strong odour of decay. In the advanced decay stage, there was very little flesh left on the carcass and it had started to become dry with sections of the skeleton being exposed. The dry stage was completed within the season and the only remains were the carcass' skeleton with a few small areas of dry skin and hair remaining (Fig. 2.4, Appendix C). In the summer

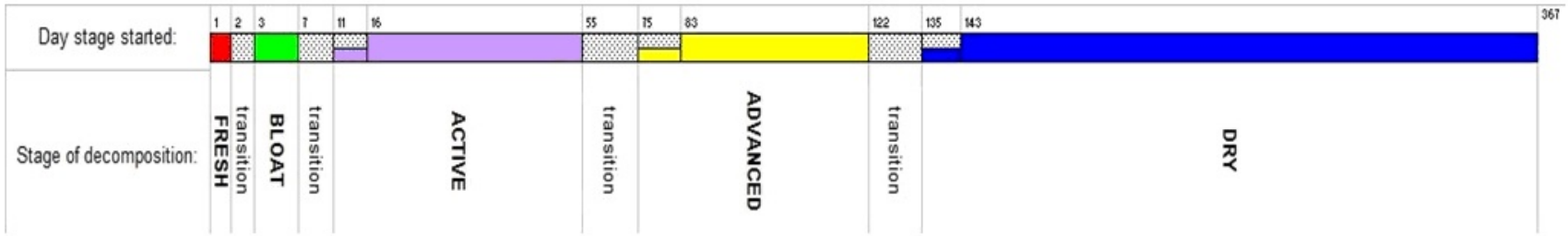
period it took approximately 16 weeks to reach the final stage of decomposition and spanned the full season range (Fig. 2.3A, Appendix C).

The winter trial, however, spanned 12 months. The carcass was then removed due to time constraints and before decomposition reached full skeletal stage. The same characteristics as above were noted but over a longer time span (Fig. 2.3B, Appendix C). The active decay stage was hard to identify as being initiated because insect activity was restricted to the areas beneath and inside the carcass. As time progressed, the transition stage leading into the advanced decay stage was also hard to define as most insect activity was under the carcass and it was difficult to assess when most of the maggots had migrated away from the area. By the end of the active decay stage the carcass had sections of its head and thorax that had started to dry out and had sections of a solid pale mass of adipocere.

Adipocere is “caused by the post mortem hydrolysis and hydrogenation of adipose tissue” (Saukko and Knight, 2004) and develops a white-ish yellow substance that is soft to the touch (Forbes *et al.,* 2002; Carter, *et al.,* 2007; Uberlaker and Zarenko, 2011). As the process moved into the advanced decay stage more of the carcass developed adipocere and all activity, both insect and decomposition, slowed drastically. By the second half of the trial, most of the carcass was adipocere with small sections of hair and dry skin. At the end of the full year after the start of the trial, sections of the adipocere had dried out and there were sections where it had clumped and was crumbly to the touch. The thorax and abdomen still appeared to be a more solid area of the carcass and had larger deposits of adipocere and large areas that still had sections of dry skin and hair.



A



B

Figure 2.3. Decomposition stages of the (A) summer carcass and (B) winter carcass.

Each stage of decomposition is represented by a different colour. The Fresh stage is represented by red; Bloat stage by green; Active stage by purple; Advanced stage by yellow and the Dry stage by blue. There is a transitional section between the stages which is represented by a black and white stippled block.

Recorded Temperatures

Summer trial

The external temperatures tracked the internal temperatures with the minimum temperatures consistently lower than the internal temperatures and the maximum temperatures consistently higher (Figure. 2.4). There was an inconsistency during weeks one and two, where the internal temperatures were higher than the surrounding temperatures and this coincided with the period when the maggot activity was at its highest and the carcass was in the active decay stage. The temperatures were all significantly different (t-test: $p < 0.005$; $n = 51$; d.f.= 50).

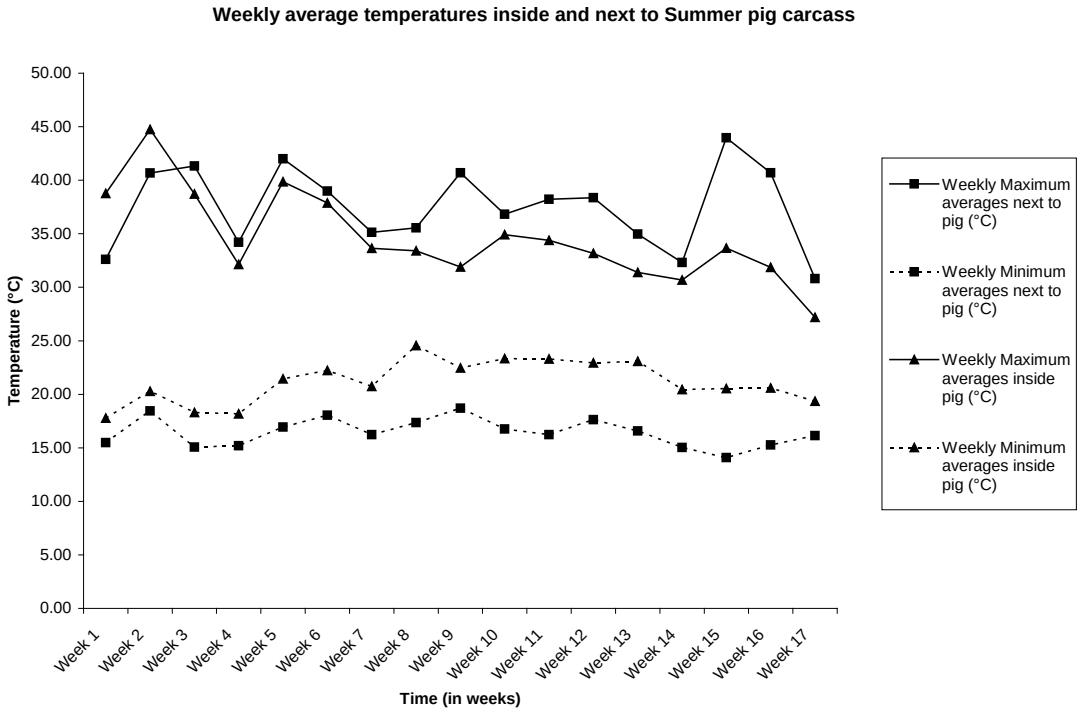


Figure 2.4. Weekly average temperatures inside and next to the carcass for the summer trial.

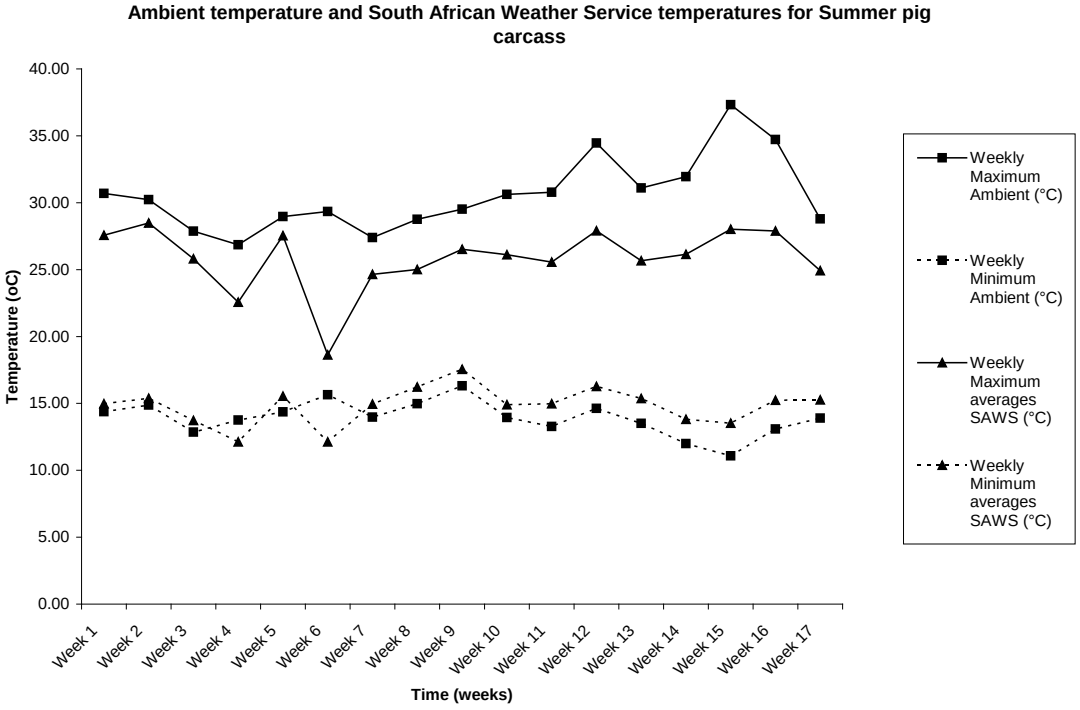


Figure 2.5. Weekly average temperatures from the South African Weather service and the ambient temperatures from around the study site for the summer trial.

The temperatures of the ambient temperatures track the SAWS temperatures (Fig. 2.5), with the minimum SAWS temperatures consistently higher than the ambient temperatures and the maximum SAWS temperatures consistently lower than the ambient temperatures. There is an inconsistency between week five and seven where the maximum and minimum SAWS temperatures both showed a deep dip and the temperatures fell below the maximum and minimum ambient temperature recordings. This correlated with a time of high rainfall and the area around the carcass was wet for a number of days and insect activity slowed down during this time. The temperatures recorded from the area around the carcass and the SAWS recorders showed more fluctuations (Fig. 2.5, 2.6) in the temperatures occurring nearer the carcass when compared to those from the SAWS. The difference of the means were shown to be significantly different (t-test: $p. < 0.005$; n= 34; d.f= 33).

The internal carcass temperatures and those recorded from the immediate area next to the carcass showed more fluctuation than those of the SAWS (Fig. 2.6).

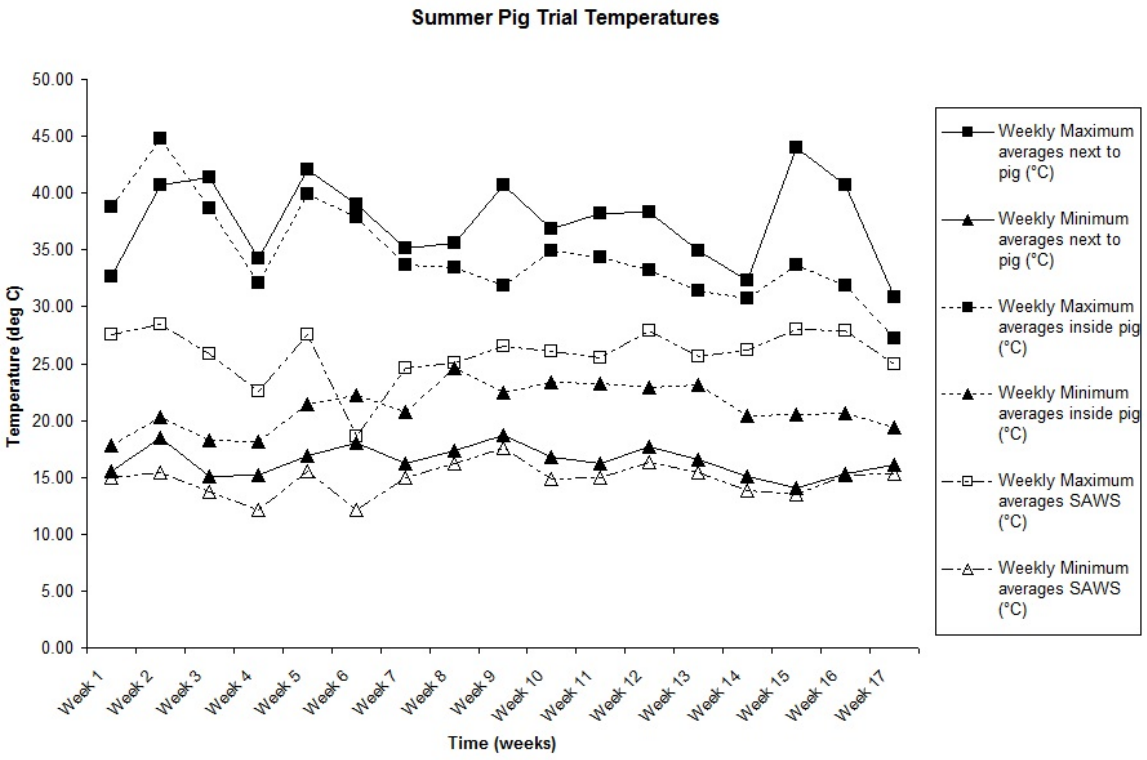


Figure 2.6. Weekly average temperatures recorded inside, next to the carcass and from the South African Weather Service for the summer trial.

The average minimum temperatures inside the summer carcass had smaller range fluctuations than those recorded outside of the carcass as the mass of the carcass helped in insulating the effects of the outside temperatures as well as the insect activity creating its own heat.

There was rainfall occurring throughout the trial and the relative humidity was high as expected since the trials were done in an area that receives summer rainfall (Fig. 2.7).

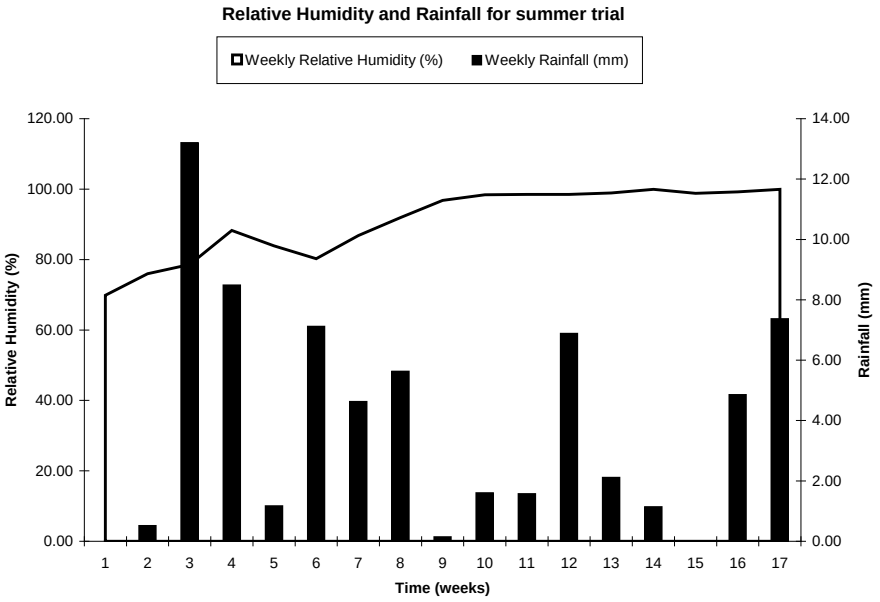


Figure 2.7. The relative humidity recorded at the study site and the rainfall from the South African Weather Service for the summer trial.

Winter Trial

The temperatures recorded from inside the winter carcass and just outside and next to the carcass showed that there was less variation in the temperatures occurring inside the carcass when compared to those immediately outside the carcass (Fig. 2.8). There is hardly ever a difference in the temperatures of more than a few degrees and these times when the temperatures differed correlated to the active decay stage when the carcass had the most insect activity. The difference of the means were shown to not be significantly different (t-test: p= 0.2158; n= 102; d.f= 101).

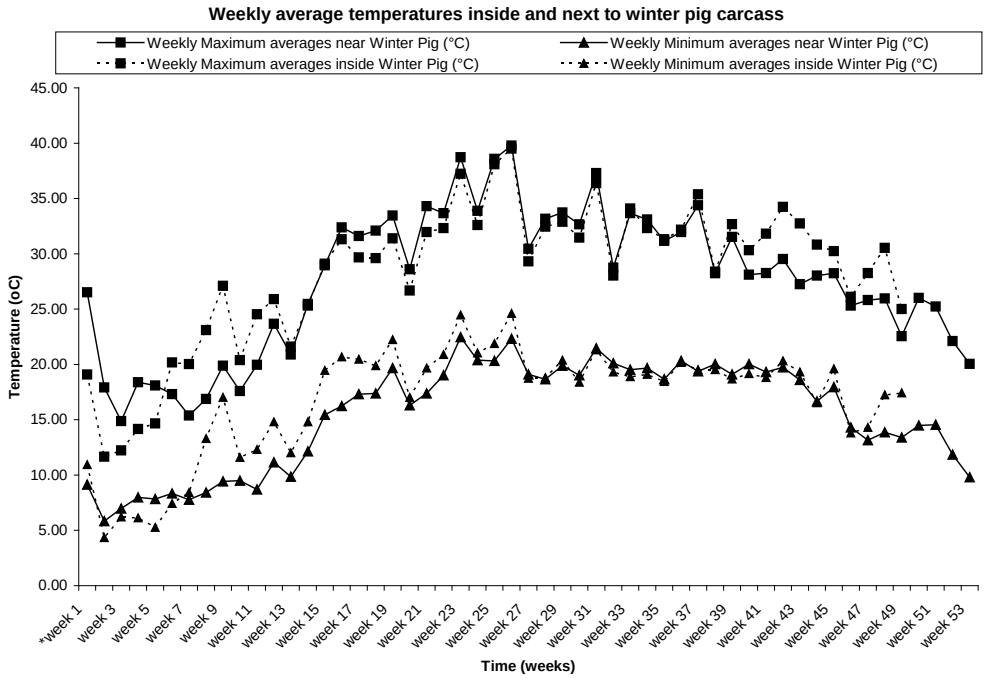


Figure 2.8. Weekly average temperatures inside and next to the carcass for the winter trial.

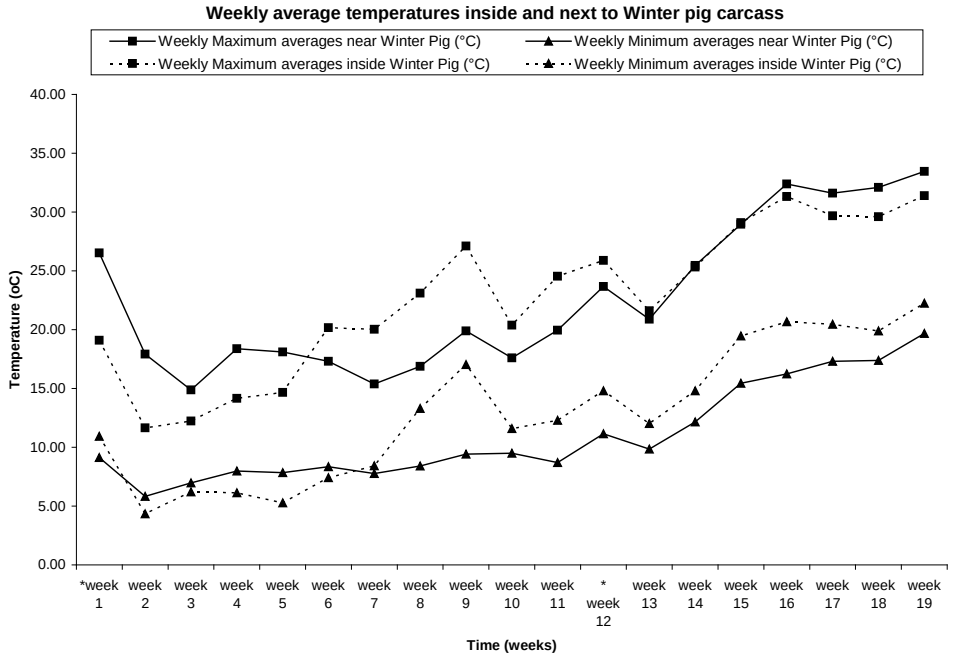


Figure 2.9. Weekly average temperatures inside and next to the carcass for the 'true winter' time of the winter trial.

When comparing difference of means for the winter time only (Fig. 2.9) the data were found to still not be significantly different (t-test: p= 0.089; n= 34; d.f = 33). The temperatures recorded from SAWS and the surrounding area of the carcass showed that there was less fluctuation in the minimum temperatures but more fluctuation in the maximum temperatures (Fig. 2.10). The difference of the means were shown to be significantly different (t-test: p< 0.005; n= 102; d.f= 101). When comparing difference of means for the winter time only (Fig. 2.11) the data was found to still be significant (t-test: p< 0.005; n= 34; d.f = 33).

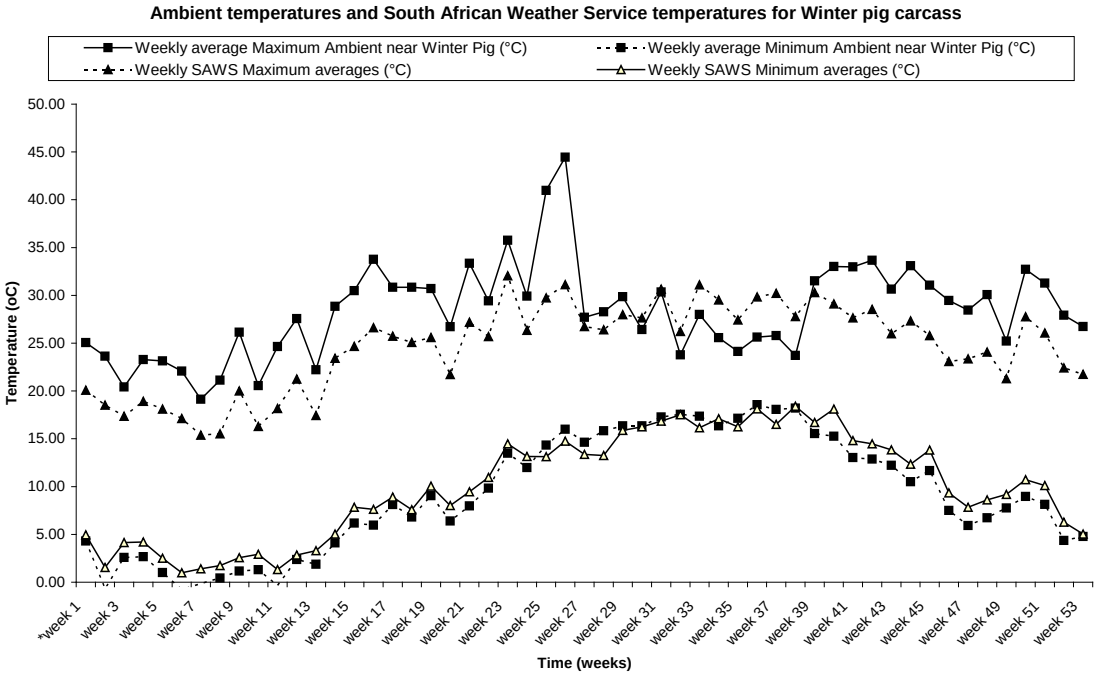


Figure 2.10. Weekly average temperatures from the South African Weather service and the ambient temperatures from around the study site for the summer trial.

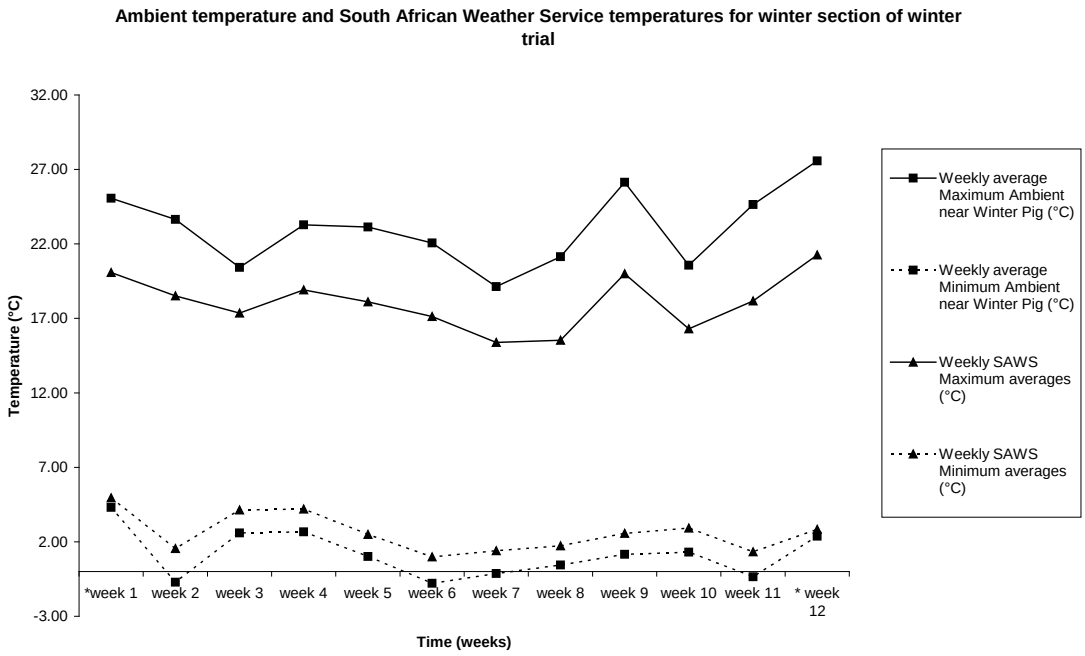


Figure 2.11. Weekly average temperatures from the South African Weather service and the ambient temperatures from around the study site for the 'true winter' time of the trial

The difference between the means showed that the temperatures at the site of the carcass, both inside and outside, were significantly different (t-test: $p < 0.005$; n= 159; d.f= 158) when compared to the South African Weather Service temperatures (Fig. 2.12). When comparing difference of means for the winter time only (Fig. 2.13), the data were found to still be significantly different (t-test: $p < 0.005$; n= 57; d.f = 56).

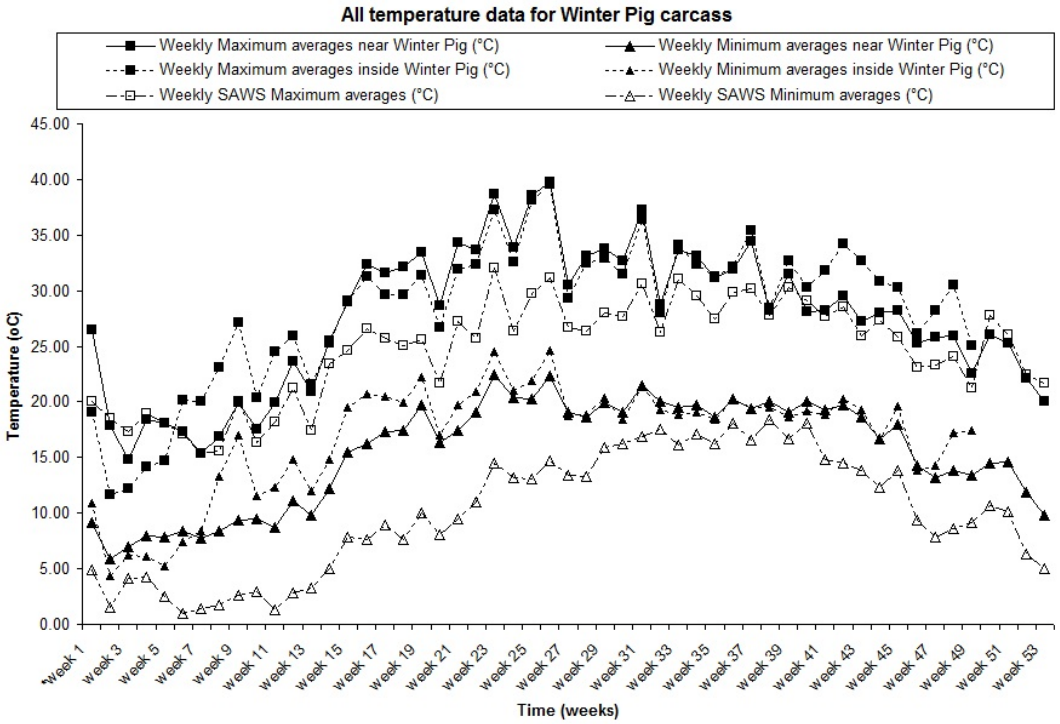


Figure 2.12. Weekly average temperatures throughout the winter trial beginning mid-May 2011.

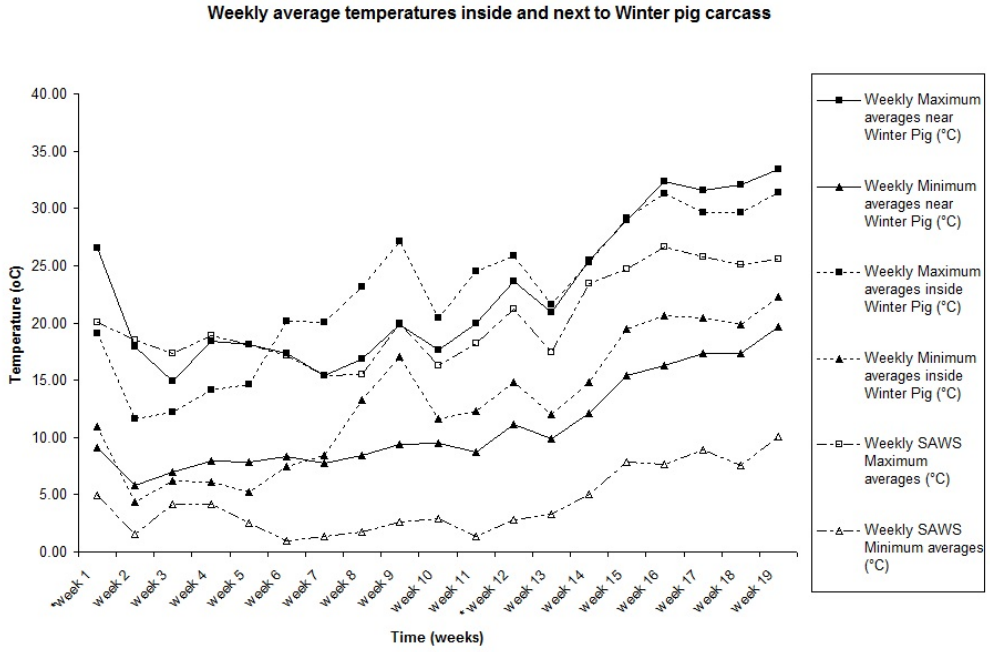


Figure 2.13. Weekly average temperatures for the winter months of the winter trial, mid-May to mid-September 2011.

There was very little rain during the colder part of the winter trial (Fig. 2.14). This can be seen in the first section of the trial with the only rainfall being due to a cold front passing over the region at the time. The relative humidity was lower during the winter times and peaked during spring and summer as did the rainfall.

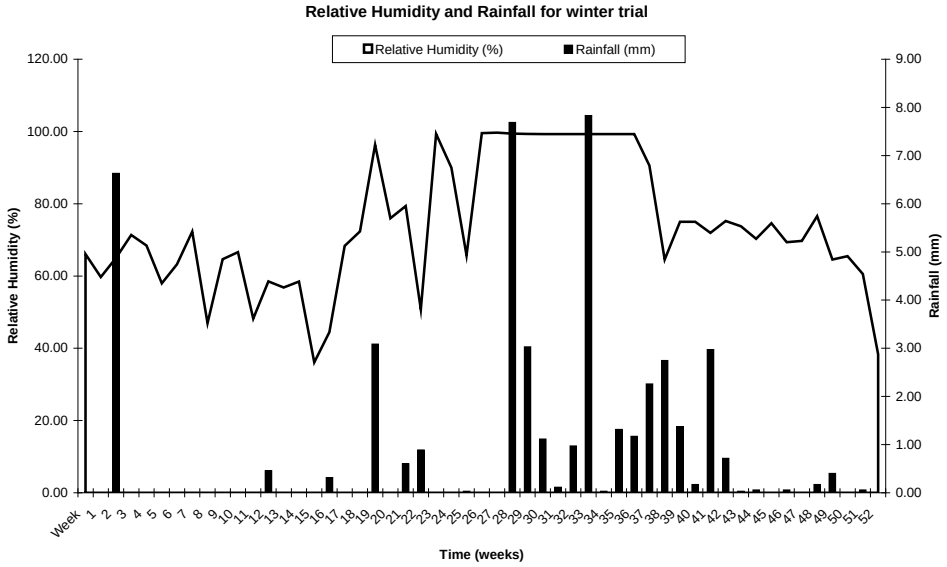


Figure 2.14. The relative humidity recorded at the study site and the rainfall from the South African Weather Service for the winter trial.

2.4. Discussion and Conclusions

The total time taken for the carcass of the summer trial to decompose was much shorter than the winter trial as one would expect and as has been shown by other studies (Bass, 1996). The summer trial showed that on the Gauteng Highveld it is possible for a carcass representing a fully grown human adult size body to be reduced

to skeletal remains within 59 days and the decomposition rates tend to be similar to those described by others with all the characteristics of each stage occurring.

The winter carcass was only removed after it had been at the study site for one year. At the time of removal it still had approximately one eighth of its apparent body size present. The winter carcass took longer to move through the early stages of decay due to the colder temperatures and it slowed drastically over time due to the formation of adipocere which was not investigated further due to the carcass being used for other purposes and their decomposition states merely observational. The carcass was exposed for extended times to cold temperatures (daily minimum average of approximately $5^{o}C$ and a maximum average of approximately $14^{o}C$) and dry conditions at the start of the trial. The exposure to water in June 2011 due to the cold front correlated with the formation of masses of adipocere (Carter, *et al.* 2007; Ubelaker and Zarenko, 2011; Saukko and Knight, 2004).

The temperatures recorded inside and outside the carcass differed from the temperatures received from the National Weather service. This could be a impact when estimations or predictions of the post-mortem interval are done using SAWS temperatures that differ from temperatures at the crime scene (Niederegger *et al.*, 2010). There may be fluctuations and an increase in temperature produced by the maggot mass as well as smaller fluctuations occurring at the crime scene such as the scene being shaded or in direct sunlight (VanLaerhoven, 2008; Niederegger *et al.*, 2010; Villet, 2011). If there are other temperature factors at play that have large influences on the development of the maggots, these may influence the outcomes of the PMI estimates. The applicability of these estimates to a crime scene needs to take

this temperature variation in consideration (Clark *et al.* 2006; Ireland and Turner, 2006; VanLaerhoven, 2008; Niederegger *et al.*, 2010; Villet, *et al.*, 2010; Villet, 2011)

The significant differences occurring between the temperatures recorded inside and outside the summer carcass were expected. These were probably due to the mass of the carcass helping to insulate the internal recorder thus maintaining temperatures that did not fluctuate as much as the external temperatures. Decomposition results in the production of heat together with the maggot masses producing and regulating their own heat (Turner and Howard, 1992; Slone and Gruner, 2007). The internal temperatures were expected to become the same as surrounding temperatures as decomposition progressed as eventually there would be a stage where there was no carcass to provide insulation.

There was an expected significant difference between the SAWS information and that recorded at the carcass. This was expected because of the slight difference in altitude plus the SAWS station is next to a body of water (Emmarentia Dam which has a surface area of approximately 0.09km^2) and in an open land area (the Johannesburg Botanical Gardens which is approximately 1.25km^2 in area), while the study site was on a small "koppie" surrounded by long wild grasses, trees and shrubs.

There were unexpected peaks of the temperature inside the carcass and especially the external temperatures (recorded next to the carcass) which were very distinct during the last three weeks of the summer trial (Fig. 2.4). This is possibly due to the metal of the cage heating up and affecting the ibutton that was placed next to the carcass.

There was also a peak in the internal temperatures, though not as great, and also probably due to the natural heating of the cage and the skeletal remains resulting in the ibutton which was situated amongst the bones, being buffered and therefore exposed to more heat. A similar development was seen in the winter trial (Figs 2.12, 2.13) where the internal and external temperatures started to climb above the SAWS temperatures at about week six. As the trial continued the range of the maximum temperatures narrowed but the carcass temperatures remained higher than the others. This is also believed to be due to the heating of the metal cage as well as the volume of adipocere and dry tissue of the carcass heating and insulating the internal ibutton.

This study provides information that can be used as a baseline for future work on the decomposition of a carcass on the Gauteng Highveld. It has potential for both human and animal-related cases (Anderson, 1999). The results from the summer trial showed similar rates of decomposition to those from the Kruger National Park (Braack, 1981) as both studies focussed on larger carcasses, areas of high summer rainfall and the carcasses progressed rapidly though the initial stages of decomposition. The formation of adipocere during the winter trial was an unexpected result which needs further investigation.

Chapter 3 - Morphological aspects of the larval stages of the dominant blowflies on the Gauteng Highveld

3.1. Introduction

Flies are generally the first insects to arrive on a carcass (Bourel *et al.*, 2003) and are thus very important in forensic investigations. There are three families that are primarily involved and they are the Calliphoridae (blow flies), Sarcophagidae (flesh flies) and Muscidae (house flies). The most important of these flies are the Calliphoridae as they arrive soon after death and they complete their life cycles on the corpse or carcass (Smith, 1986; Byrd and Tomberlin, 2010).

The family Calliphoridae includes the genera *Chrysomya*, *Calliphora* and *Lucilia* all of which are found on carcasses in Southern Africa (Zumpt, 1965; Meskin, 1986; Kelly, 2006; Villet, 2011). The species within these genera are generally widespread throughout the country with some having smaller restricted distributions (Braack, 1981; Braack, 1987; Williams, 2003; Richards, *et al.*, 2009a). *Chrysomya albiceps* occurs across the country whereas *Ch. chloropyga* is limited to the more temperate regions of South Africa (Richards, *et al.*, 2009a, b, c).

Meskin (1991) described the eggs of some of the forensically important species occurring in South Africa. Many studies have described the morphology and composed identification keys for these flies (Zumpt, 1965; Prins, 1982; Holloway, 1991; Szpila and Villet, 2011). Molecular techniques have also been used for species identification to supplement the morphological identifications (Harvey *et al.*, 2003,

2008; Tourle *et al.*, 2009). The morphology of the larval stages of flies generally use external characteristics as well as the structure of the cephaloskeleton, anal spiracles and anterior spiracles.

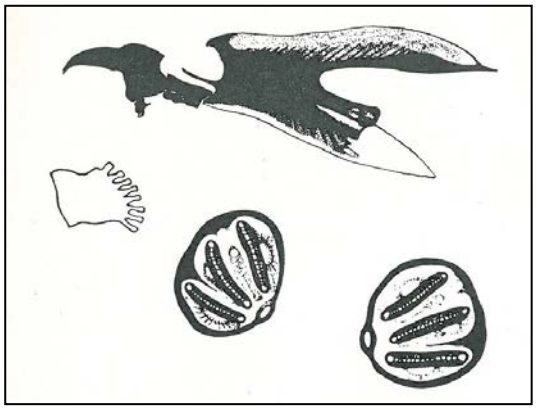


Figure 3.1. Graphic representation of the key features used in maggot morphology. The picture shows those of a third instar *Lucilia sericata* - from Zumpt (1965).

Figure 3.1 shows the cephaloskeleton (top), the anterior spiracle which is very distinctive with its finger like lobes (left) and the pair of anal spiracles mirroring each other (bottom and right). The outer darkened regions that encase the spiracle are the peritremal rings, the three slits are easily differentiated and the smaller round section at the 'bottom' is enclosed by the peritremal rings and is called the button (Zumpt, 1965).

A clear understanding and knowledge of these life cycles is required when determining the Post Mortem Interval as the PMI is determined based on the species-specific time taken for the insects to have developed to the stage currently being looked at. Thus an accurate identification of a species is vital (Harvey *et al.*, 2003). There have been a number of studies done globally that have updated old

morphological keys (Zumpt, 1965) and created new keys to the fly species (Holloway, 1991; Szpila and Villet, 2011). The larval stages are more difficult to identify even with morphological keys and the adults are still most commonly used for identifications. However, even with the current trends leaning towards molecular identification techniques (Wells and Stevens, 2008; 2010) it is still necessary to first identify the flies using morphological characteristics before carrying out molecular techniques.

3.2. Aim and objective

This study aimed to generate a list and picture dataset of the distinguishing morphological features of the three larval instars of the dominant Calliphoridae species occurring on the Gauteng Highveld to add to the already established datasets that exist. Focus was on the key features currently used for larval identification: the cephaloskeleton, anal spiracles and anterior spiracles.

3.3. Methods and Materials

All specimens used for this study were collected as either larvae or adults from the pig carcasses used in Chapter 2.

The maggots that were collected were bred through to adulthood before being identified. All flies were identified using the keys of Zumpt (1965) and Holloway (1991). The flies were then all bred through for another generation from which a few larvae were killed at six hourly intervals. The smaller larvae were placed directly into

a 80% ethanol solution while the larger larvae were placed in boiling water for ten seconds to kill them and then transferred into a 80% ethanol solution (Zumpt, 1965).

The larvae were then dissected and pictures taken of the anal spiracles, cephaloskeleton and anterior spiracles of each larva representing each of the three larval stages. The dissections were carried out using an Olympus SZX7 dissecting microscope and the photographs were taken using an Olympus BX50 compound microscope equipped with a CC12 Soft Imaging System camera and using the analysis LS Research software. All dissections were viewed at 40x; 100x and 400x magnification.

To dissect out the various features, the last segment and front section of the maggots were cut off and placed onto a slide for dissection (pers comm.. R. Hunt). The anal spiracles were cut out of the last segment and the tissues and tubules teased away from the back of the section. The section was then placed on a slide in a drop of PBS under a cover slip and photographed. The anterior spiracles were treated in a similar manner to the anal spiracles. The cephaloskeleton was gently pulled out from the front section and the surrounding tissues teased away with dissecting needles. The mouth hook was then placed in a drop of PBS on a slide and photographed. The first instar cephaloskeleton was not removed from the surrounding section as the whole segment was placed under a cover slip before being viewed under the microscope.

3.4. Results and Discussion

Six species of blowflies were identified as being the most dominant on the Gauteng Highveld. These were *Calliphora vicina, Chrysomya marginalis, Ch. albiceps, Ch. chloropyga, Lucilia sericata* and *L. cuprina. Chrysomya albiceps* occurred only in summer and was the dominant fly in summer. *Calliphora vicina* only occurred in winter and was the dominant winter species.

Lucilia cuprina could not be successfully bred through in the insectary and thus no larval dissections were done on this species.

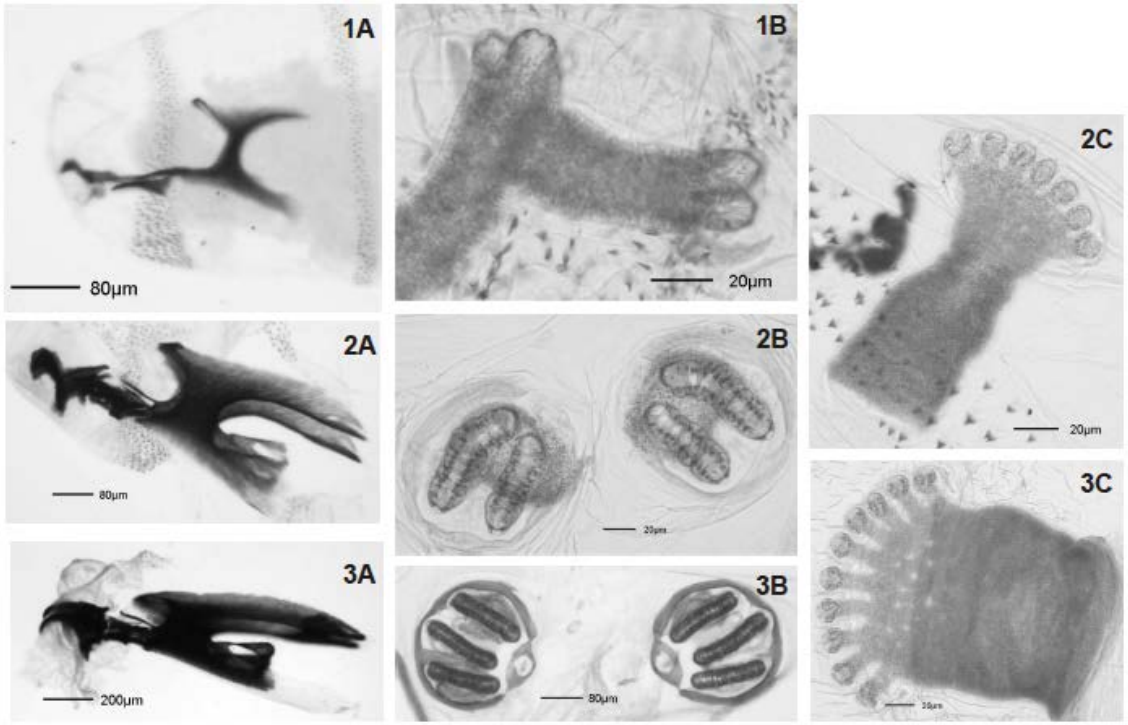


Figure 3.2. The cephaloskeleton, anal spiracles and anterior spiracles of *Calliphora vicina*.

(1A) cephaloskeleton of first instar, (1B) anal spiracles of first instar, (2A) cephaloskeleton of second instar, (2B) anal spiracles of second instar, (2C) anterior spiracle of second instar, (3A) cephaloskeleton of third instar, (3B) anal spiracles of third instar, (3C) anterior spiracle of second instar.

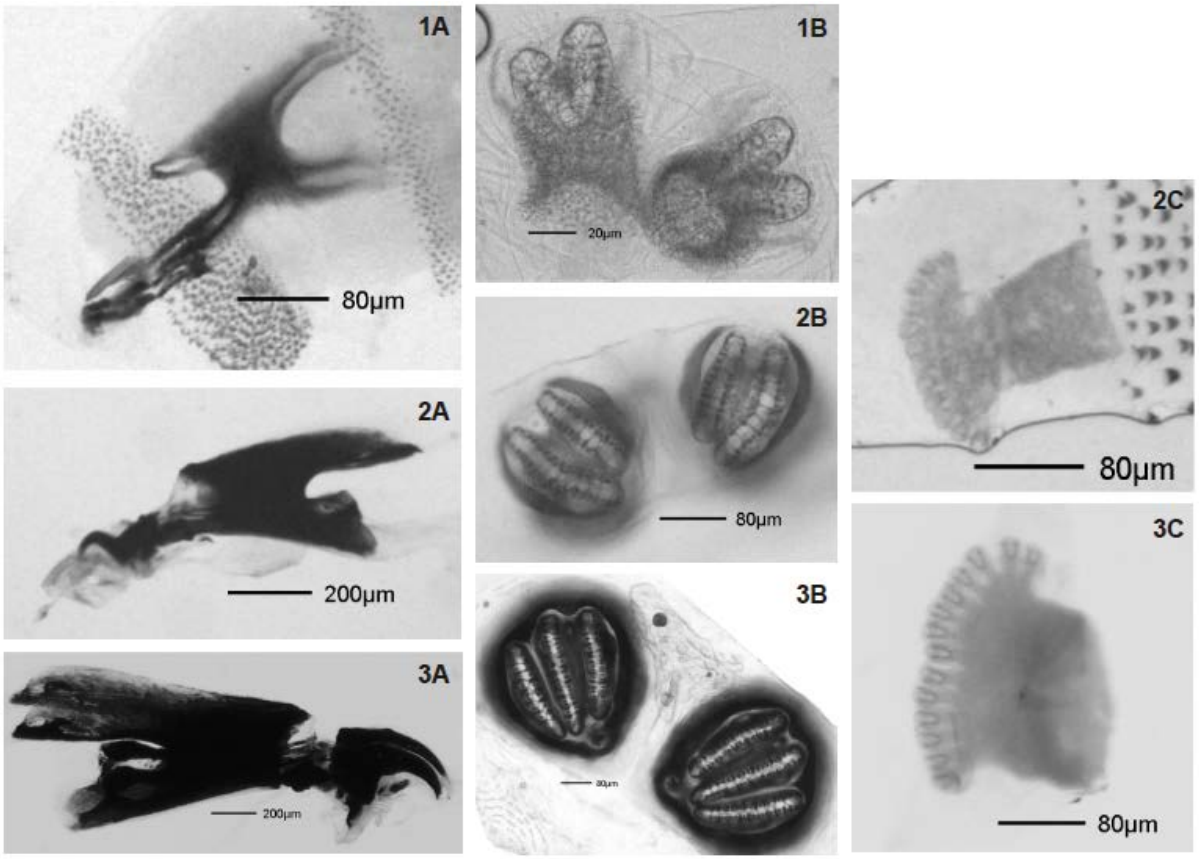


Figure 3.3. The cephaloskeleton, anal spiracles and anterior spiracles of *Chrysomya marginalis*. (1A) cephaloskeleton of first instar, (1B) anal spiracles of first instar, (2A) cephaloskeleton of second instar, (2B) anal spiracles of second instar, (2C) anterior spiracle of second instar, (3A) cephaloskeleton of third instar, (3B) anal spiracles of third instar, (3C) anterior spiracle of second instar.

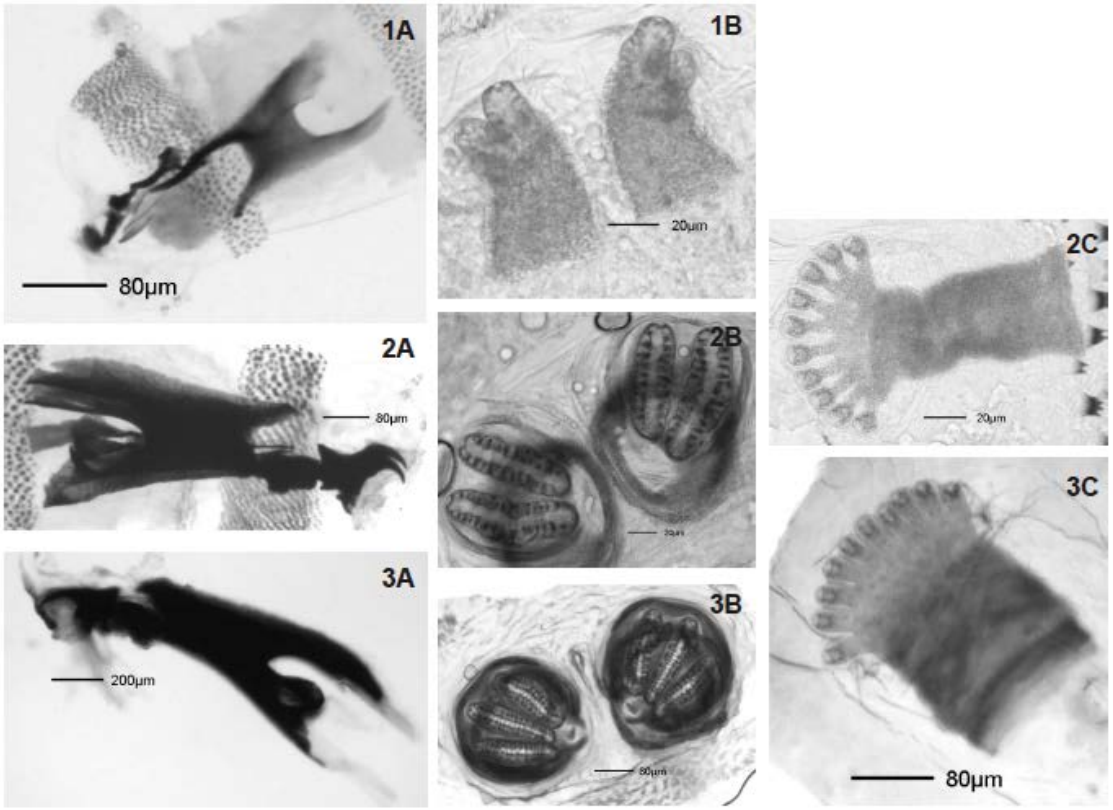


Figure 3.4. The cephaloskeleton, anal spiracles and anterior spiracles of *Chrysomya albiceps.* (1A) cephaloskeleton of first instar, (1B) anal spiracles of first instar, (2A) cephaloskeleton of second instar, (2B) anal spiracles of second instar, (2C) anterior spiracle of second instar, (3A) cephaloskeleton of third instar, (3B) anal spiracles of third instar, (3C) anterior spiracle of second instar.

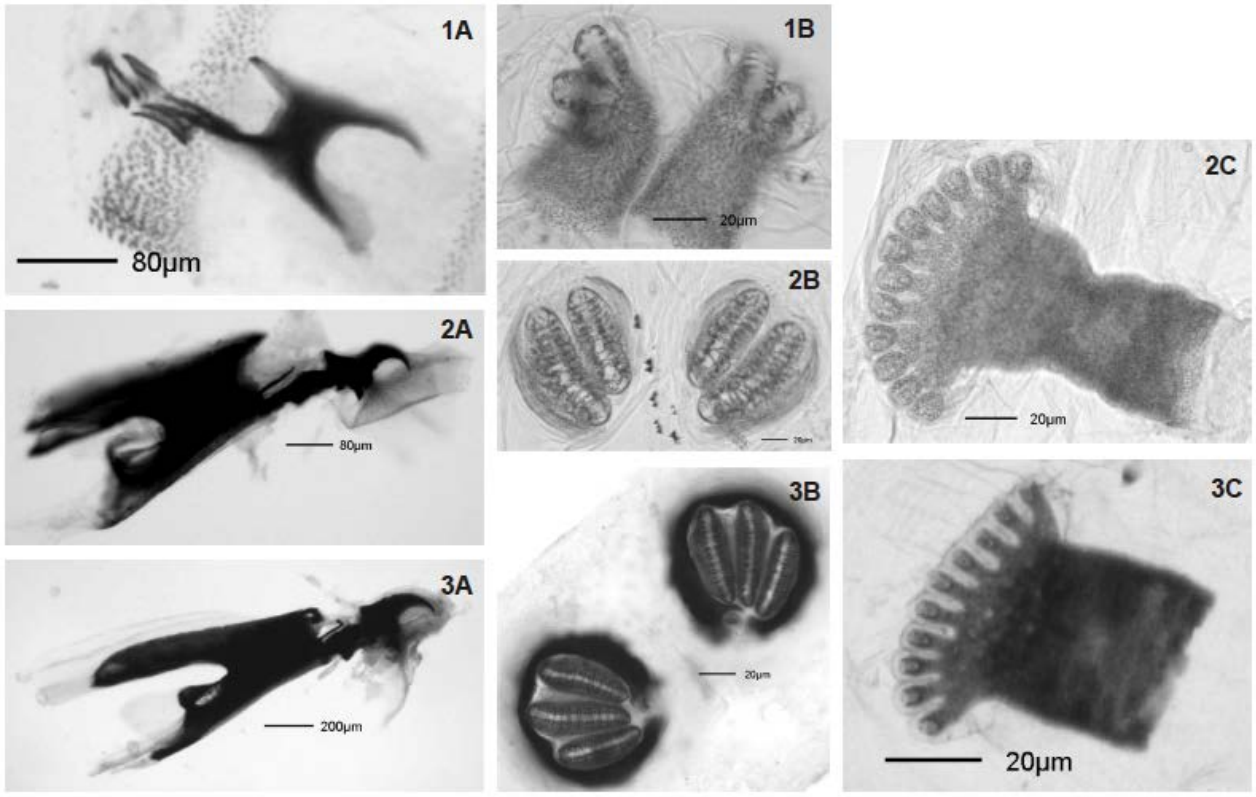


Figure 3.5. The cephaloskeleton, anal spiracles and anterior spiracles of *Chrysomya chloropyga*.

(1A) cephaloskeleton of first instar, (1B) anal spiracles of first instar, (2A) cephaloskeleton of second instar, (2B) anal spiracles of second instar, (2C) anterior spiracle of second instar, (3A) cephaloskeleton of third instar, (3B) anal spiracles of third instar, (3C) anterior spiracle of second instar.

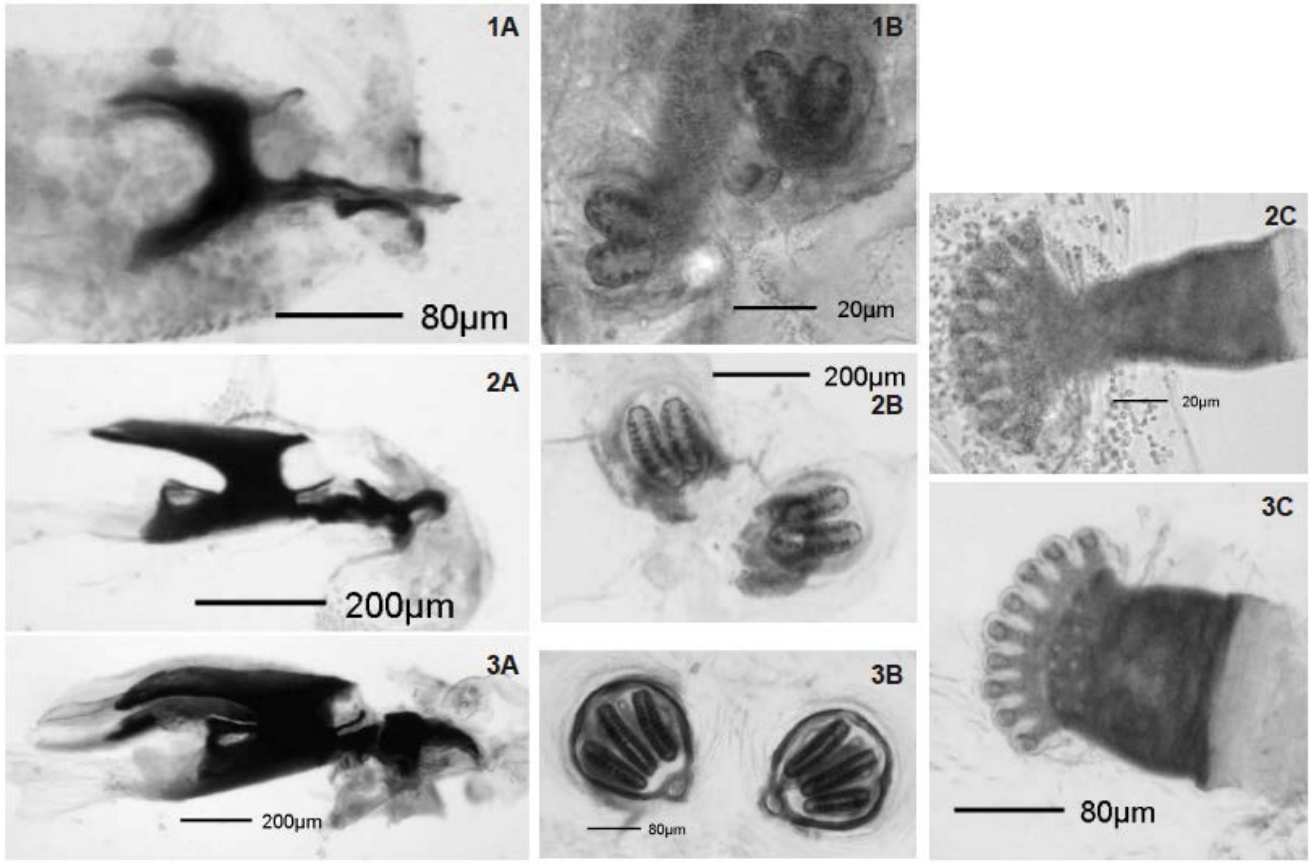


Figure 3.6. The cephaloskeleton, anal spiracles and anterior spiracles of *Lucilia sericata*.(1A) cephaloskeleton of first instar, (1B) anal spiracles of first instar, (2A) cephaloskeleton of second instar, (2B) anal spiracles of second instar, (2C) anterior spiracle of second instar, (3A) cephaloskeleton of third instar, (3B) anal spiracles of third instar, (3C) anterior spiracle of second instar.

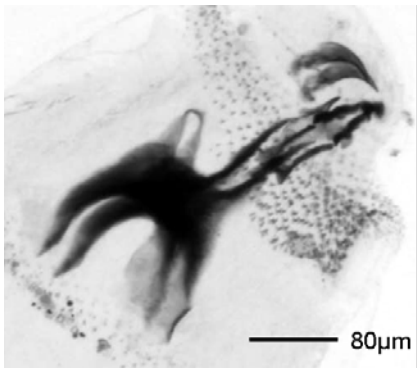


Figure 3.7. Transition from first instar to second instar with the mouth hooks of the second instar cephaloskeleton clearly visible forming above those of the first.

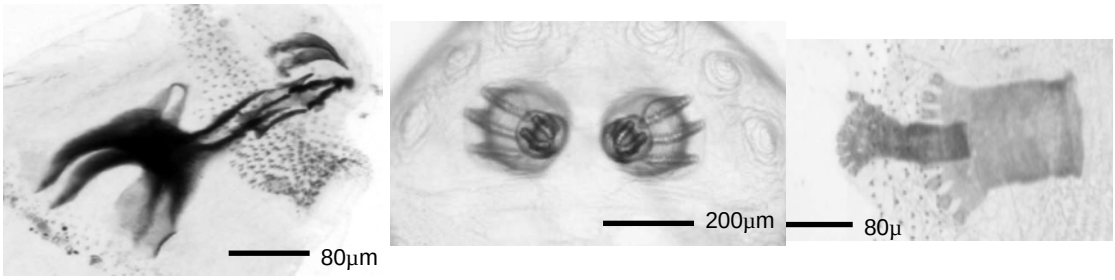


Figure 3.8. Transition from second to third instar showing the cephaloskeleton (left) with the third instar mouth hooks developing above those of the second instar, the third instar anal (middle) and anterior (right) spiracles clearly visible below the second instar.

Each species has a cephaloskeleton and anal spiracles that show features that are species-specific. The first instars have simple anal spiracles comprised of two circular holes, the second instars develop spiracles with two slits and the third instars contain three slits (Zumpt, 1965) as seen in 3B of figures 3.2, 3.3, 3.4, 3.5 and 3.6.

The anterior spiracles are only visible from the second instar onwards as they begin to take on the standard club-shaped appearance (Zumpt, 1965) with the number of lobes varying between the second and third instar stages.

As shown by Szpila and Villet (2011) the first instar anal spiracles cannot be used for species identifications. However, the mouth hooks shown here provide enough specific characteristics to have potential for species identification.

The second instar characteristics for all five species are more defined and can be used to identify the maggots to species level. The anal spiracles are still not distinct enough for species identification but do show some characteristics of the cephaloskeleton that hint towards the species such as those of *Chrysomya marginalis* (Fig. 3.3.). The cephaloskeleton for the second larval stage of each species is distinct enough to be used as a morphological identification tool (Zumpt, 1965; Prins, 1982; Queiroz *et al.*, 1997; Szpilla and Villet, 2011). The transitional stages are very distinct and can aid in the identification of the species (Figs. 3.7, 3.8).

The third instar characteristics are very distinct and all display distinct species-specific aspects of the cephaloskeleton and anal spiracles. The anterior spiracles may differ in number of lobes when compared to those of the second instar but the numbers still fall within a range already identified in earlier morphological work (Zumpt, 1965). The cephaloskeleton is at its most defined with all the smaller sections making up the structure as a whole and easily distinguishable for species identification.

The cephaloskeleton is well described for all species and is the main component of larval morphology used in species identification due to the specificity of the characteristics of each species (Zumpt, 1965; Szpilla and Villet, 2011). These cephaloskeletons, however small, have enough species-specific characteristics for morphological keys to be produced (Queiroz *et al.,* 1997; Szpila and Villet, 2011) thus the isolation of the cephaloskeletons on all three instars of these species of flies occurring on the Gauteng Highveld is helpful and has potential for future applications such as a taxonomic key.

Chapter 4. Isolation of the ITS2 region of the blowflies and the creation of a species specific multiplex PCR

4.1. Introduction

There are various molecular-based methods used for insect species identification (Ratcliffe *et al.*, 2003). Scott *et al.* (1993) outlines the ribosomal RNA genes (rDNA) as being a good diagnostic tool to identify closely-related malaria mosquito species of the *Anopheles gambiae* complex. The rDNA region consists of a number of tandem repeats. One rDNA transcription unit is made up of three coding regions (the 18S, 28S and 5.8S genes) which are separated by non-coding regions, the ITS1 and ITS2 respectively (Fig. 4.1) (Adams, *et al.*, 1992). The non-coding regions have been shown to be different between closely related insect species (Adams *et al.*, 1992; Koekemoer *et al.*, 2002). One of these variable regions is the internal transcribed spacer regions (ITS) (Song, *et al*., 2008; Wells and Stevens, 2008). The ITS regions are rDNA structural components of all eukaryotic cells and are situated between coding regions and are relatively species-specific, which allows for easy identification between closely related species (Scott, *et al.*, 1993; Koekemoer *et al.*, 2002).

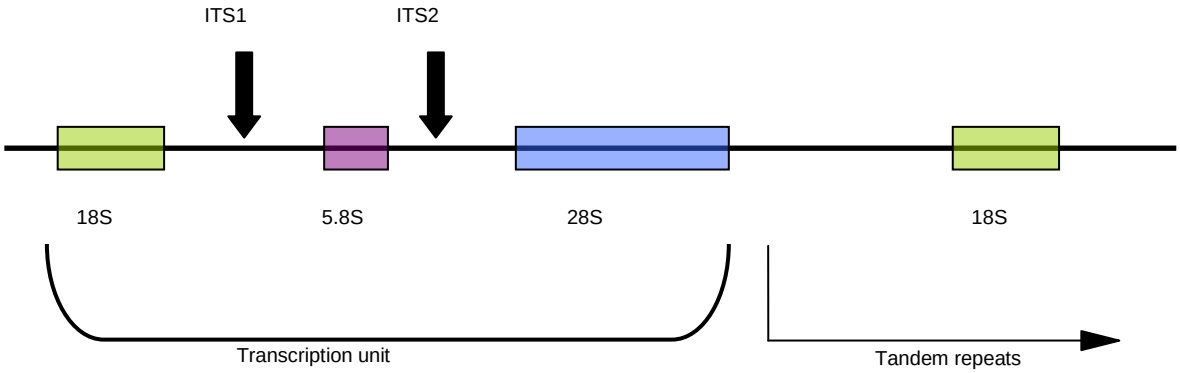


Figure 4.1. Diagrammatic representation of a transcription unit of an insects rDNA. (Adapted from Adams *et al.*, (1992) and Koekemoer (1999)).

Varying molecular based research has been done as a means of identification on many of the Diptera. Basic PCR methods as well as RFLP methods have been used to identify the types of *Drosophila* (Nelson *et al.*, 2008) as well as flies that are general nuisance flies and live stock pests (Ratcliffe, *et al.*, 2003). Much work had been done using molecular methods with general studies being conducted (Sperling, *et al.*, 1994), genetic relationship studies (Harvey, *et al.*, 2001) and cross-continental studies done on the populations of both Australian and southern African blowfly species (Harvey, *et al.*, 2003, 2008).

Within the field of forensic entomology much work has been done on the flies that are considered forensically important focusing on identification methods based on the ITS regions and the mitochondrial regions (COI and COII) (Ratcliffe, *et al.*, 2003). Given that the ITS2 regions have proved useful in diagnostics for identifying other insects (Scott *et al.*, 1993; Koekemoer *et al.*, 2002; Marelli *et al.*, 2006), there is potential for this ITS2 region to be used in the identification of forensically important flies (Nelson, *et al.*, 2008; Song *et al.*, 2008; Ferreira, *et al.*, 2011).

4.2.) Aim and objective

This study aimed to create a molecular identification tool of the forensically important Calliphoridae occurring on the Gauteng Highveld.

- Focussing on the ITS2 region of the DNA and creating a multiplex PCR.

4.3.) Methods and Materials

4.3.1. DNA extractions

Salt precipitation method

All biological material was collected from the pig carcasses placed on Melville Koppies. All flies were collected and identified using the keys outlined by Zumpt (1965) and Holloway (1991). Samples were also taken from maggots by slicing off a piece of their cuticle.

Genomic DNA was extracted from a wing or leg of a fly using the Collins *et al.* (1987) protocol: a salt precipitation method as detailed in the appendix.

4.3.2. Amplification of ITS2 region

Song et al. (2008) primers

The ITS2 region was amplified using the methods outlined by Song *et al.* (2008) as outlined in the appendix. A set of primers of those designed by Song *et al*, (2008) were synthesised by Inqaba Biotechnical Industries (Pretoria, South Africa).

The primers were as follows:

Forward primer: 5'- TGC TTG GAC TAC ATA TGG TTG A -3'

Reverse primer: 5’ - GTA GTC CCA TAT GAG TTG AGG TT -3’

The PCR reaction consisted of 4 U of *Taq* DNA polymerase; 250µM of each dNTP; 2mM $MgCl_2$; 10µM of each primer; 50ng of template in a final volume of 25µl. The PCR cycling conditions were an initial denaturation at 95^{o}C for five minutes, thirty cycles of denaturation at 94 ^{o}C for one minute, annealing at 52 ^{o}C for one minute, extension at 72 ^{o}C for one minute. A final extension at 72 ^{o}C for 10 minutes was used to ensure extension of completed fragments.

The procedure was optimised by varying annealing temperatures and $MgCl_2$ concentration, while keeping cycle number, time, primer and template concentration constant. . The amplicons were electophoresed on a 2% agarose gel stained with ethidium bromide (0.3µg/ml) and the gel was viewed using a gel documentation system (make and supplier detailed in appendix).

Koekemoer et al. (2002) primers

As the primers described by Song, *et al.* (2008) failed to amplify appropriately the primers were discarded for future use and new primers selected as per those described by Koekemoer *et al.* (2002). The primers were synthesised by Inqaba Biotechnical Industries and reconstituted in doubled-distilled water. The primer sequences were as follows:

Forward primer (ITS2A): 5’ TGTGAACTGCAGGACACAT 3’

Reverse primer (ITS2B) : 5’ ACCCCCTGAATTTAAGCATA 3’

The PCR reaction consisted of 5U of *Taq* DNA polymerase; 250µM of each dNTP; 1.5mM $MgCl_2$; 2.5µM of each primer; 50ng of template in a final volume of 25µl.

The PCR cycling conditions were an initial denaturation at 94^{o}C for two minutes, 40 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 50^{o}C for 30 seconds, extension at 72^{o}C for 40 seconds, and a final extension at 72^{o}C for ten minutes. Optimisation was done on the annealing temperature (50^{o}C to 60^{o}C) within the 40 cycles as well as the concentration of $MgCl_2$ (1.5mM to 3.5mM). The products were electophoresed on a 1.5% low melt agarose gel stained with ethidium bromide (0.3µg/ml) for easy removal of the amplified regions for sequencing and the gel was viewed using a gel documentation system.

The PCR product was extracted from the gel segment using the Phenol:Chloroform extraction method (Sambrook, *et al.* 1989). The sample was placed in a 10:1 mixture of Tris Cl and EDTA (pH 8.6) and incubated at 65^{o}C for five minutes. The sample was then extracted three times, first with phenol, secondly with phenol:chloroform (1:1) and then with chloroform, with the aqueous phase being removed and placed in a new tube between each stage. Ammonium acetate (8M), 100% ethanol (2x volume at 4ºC for ten minutes) was used to precipitate the DNA. The aqueous phase was then washed with one volume 70% ethanol (at 4ºC). The DNA fragment was re-suspended in 30µl TE buffer.

4.3.3. Sequencing

The targeted fragments/ amplicons were selected and excised from the gel.
These fragments were sequenced by Inqaba Biotechnical Industries using direct sequencing. PCR amplicons were sequenced on an ABI 3130XL Sequencer using the standard cycle sequencing programmes recommended for the system and used by Inqaba Biotechnical Industries. The primers used for the amplification of the product

were used in the sequencing reactions, one with the forward primer to produce the forward sequence and one with the reverse primer to produce the reverse sequence. The resulting sequence data was viewed using Finch TV and analysed using DNAStar Lasergene 10 Core Suite software package (DNASTAR, Inc., USA).

4.3.4. Species-specific primer design

Lucilia cuprina was the only species where only five samples were sequenced due to the low numbers available, the other species all had a minimum of ten samples selected from approximately fifty samples with the cleanest and most accurate sequences being selected to create species-specific primers.

All sequence data (in section 4.3) was viewed and analysed using (DNAStar). Each sequence was aligned to the online sequence database (National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.gov/Blast.cgi) to confirm the identity of the target fragment. All sequences were manually reviewed in editSeq (DNAStar). A consensus sequences per species was constructed (using DNAStar) and aligned with a species specific consensus sequence to be able to obtain one combined consensus sequence to be used to design species-specific primer pairs

All consensus sequences were aligned using megAlign (DNAStar) and a Clustal W alignment to allow for species-specific nucleotide differences between the species to be identified. Six species (*C. vicina, Ch. marginalis, Ch. albiceps, Ch. Chloropyga, L. sericata, L. cuprina*) were analysed. Regions of the species-specific primers were

selected based on the maximum number of nucleotide differences as well as the position of these nucleotides, preferably on the 5' -end of the target sequences.

The selected species-specific primer regions were further analysed using NetPrimer (www.premierbiosoft.com/netprimer) and the following criteria recorded: Primer melting temperature (TM); C:G ratio; number of dimers, hairpins, palindromes and repeats. The best resulting region was selected as the species-specific primer and synthesised by Inqaba Biotechnical Industries. The resulting primers were diluted with distilled water to a 2.5µM working stock solution for the PCR to be optimised.

Table 4.1 list of the reference sequences and their accession numbers from the NCBI database.

Species	**Accession number**
Calliphora vicina	EF560178
Chrysomya marginalis	None available on NCBI for ITS2 region
Chrysomya albiceps	EF560172.1
Chrysomya chloropyga	None available on NCBI for ITS2 region
Lucilia sericata	EF560187.1
Lucilia cuprina	EF061805.1

4.3.5. Multiplex PCR and validation

The reaction mixture of the PCR using the species-specific primers was maintained as per the protocol used for the ITS2A and ITS2B primer protocol. The reaction contained 5U of *Taq* DNA polymerase; 250µM of each dNTP; 1.5mM $MgCl_2$; 2.5µM

of each primer; 50ng of template in a final volume of 25µl. The PCR cycling conditions were an initial denaturation at 94^{o}C for two minutes, 40 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 50^{o}C for 30 seconds, extension at 72^{o}C for 40 seconds, and a final extension at 72^{o}C for ten minutes.
Optimisation was done on the annealing temperature (50^{o}C to 60^{o}C) within the 40 cycles as well as the concentration of $MgCl_2$ (1.5mM to 3.5mM). All PCR products were electrophoresed on a 2.5% agarose gel stained with ethidium bromide (0.3µg/ml). The blind test was performed to validate the results.

4.4.) Results and Discussion

4.4.1. DNA extractions

All genomic DNA was re suspended in TE buffer and stored at -20^{o}C. The extraction yielded DNA of an average of 50 - 100 ng/µl.

4.4.2. Amplification of ITS2 region

As seen in figure 4.2 the resulting PCR product did not result in fragment sizes that varied distinctly in base pair numbers on the targeted fly species. Optimisation was done using a temperature gradient between 52^{o}C to 66^{o}C and $MgCl_2$ concentration gradients of 1.5mM to 3.5mM per reaction tube.

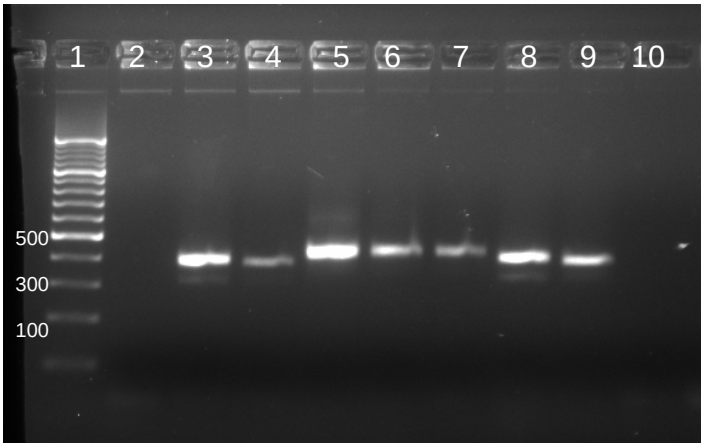


Figure 4.2. Electrophoretogram of PCR results using the Song, *et al.* primers. Lane 1: molecular ladder (100bp O'Range Ruler, Fermentas). Lane 2: no DNA control. Lane 3: Sarcophagidae spp 1, Lane 4: *Lucilia* spp 1, Lane 5: Muscidae spp 1, Lane 6: *Chrysomya* spp 1, Lane 7: Muscidae spp maggot, Lane 8: Sarcophagidae, Lane 9: *Lucilia* spp maggot, Lane 10: blank.

Due to the Song *et al.* (2008) primers not resulting in the fragments with a high variation in base pair numbers between the selected fly species, and there being multiple fragments/ amplicons for the samples (Figure 4.3), the primer set was discarded and new primers were investigated.

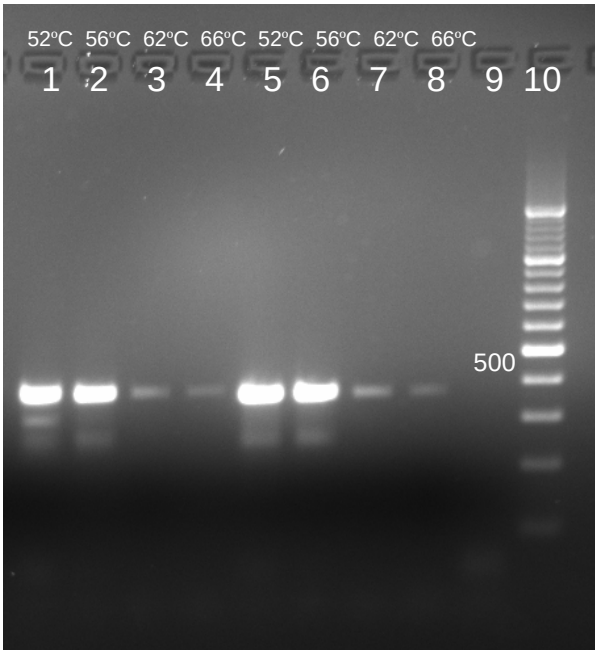


Figure 4.3. Electrophoretogram showing amplicons after the temperature gradient using the Song *et al.* (2008) primers. Lane 1 and 5 represent the 52°C, lanes 2 and 6 the 56°C, lanes 3 and 7 the 62°C and lanes 4 and 8 the 66°C. Lanes 1 to 8 are *Lucilia* spp. Lane 10: molecular ladder (100bp O'Range Ruler, Fermentas) and Lane 9: no DNA control.

The primers designed by Koekemoer *et al.* (2002) were optimised for the flies used in the experiments with both the temperatures and salt concentrations being modified in an aim to optimise for the amplification of a single amplicon and to reduce the number of non-specific amplicons. The ideal conditions found for the primer set to isolate the ITS2 region of the flies was an initial denaturation at 94 °C for two minutes followed by forty cycles of denaturation at 94 °C for thirty seconds, annealing at 52 °C for thirty seconds, extension at 72 °C for forty seconds, and a final extension at 72 °C for 10 minutes. Figure 4.4 shows the PCR products after electrophoresis of these optimised conditions.

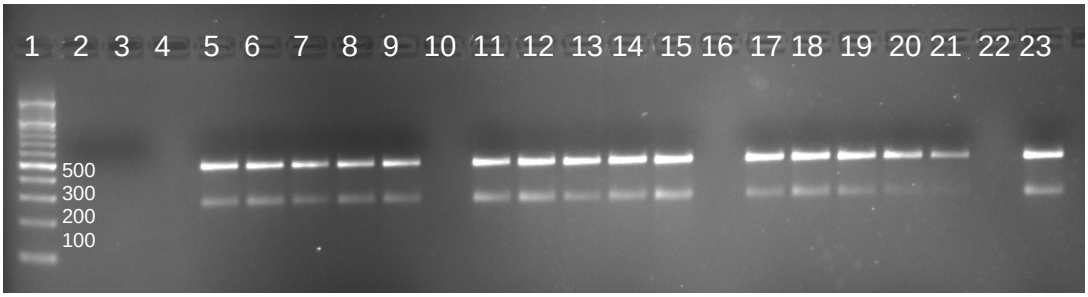


Figure 4.4. Electrophoretogram showing the two resulting fragments/ amplicons that were occurring for all samples using the optimised Koekemoer *et al.* (2002) PCR. Lane 1: molecular ladder (100bp O'Range Ruler, Fermentas); Lane 2: negative control, Lane 3: no DNA control; Lanes 5 to 9: *Lucilia sericata*, Lanes 11 to 15: *Lucilia cuprina*, Lanes 17 to 21 and 23: unidentified *Lucilia* spp. Lanes 10, 16 and 22: blank.

All PCR products resulted in two fragments at ~500 base pairs (bp) and ~250 bp. From the results shown by Song, *et al.* (2008) and Nelson *et al.* (2008) it is known that the target region of the fly ITS2 is approximately 500 base pairs.

Both fragments were extracted and sequenced. BLAST analysis confirmed that both fragments were part of the ITS2 region. Therefore the second smaller fragment was most likely the result of a secondary binding site within the targeted region thus a larger (~500 bp) and smaller (~250 bp) fragment were being produced as a during the PCR. After BLAST results confirmed the two fragments were from the same species, the smaller fragments were discarded and the remaining work done only using the sequence data from the larger fragments.

4.4.3. Sequencing

All PCR products were sequenced by Inqaba Biotechnical Industries and an alignment created using the consensus strands for each of the targeted species (figure 4.5). A consensus sequence was constructed using the sequencing data obtained and published reference sequence. This resulted in a consensus sequence that was longer than the sequence of the amplicon. The method allowed the binding site of the ITS2A primer to be easily identified.

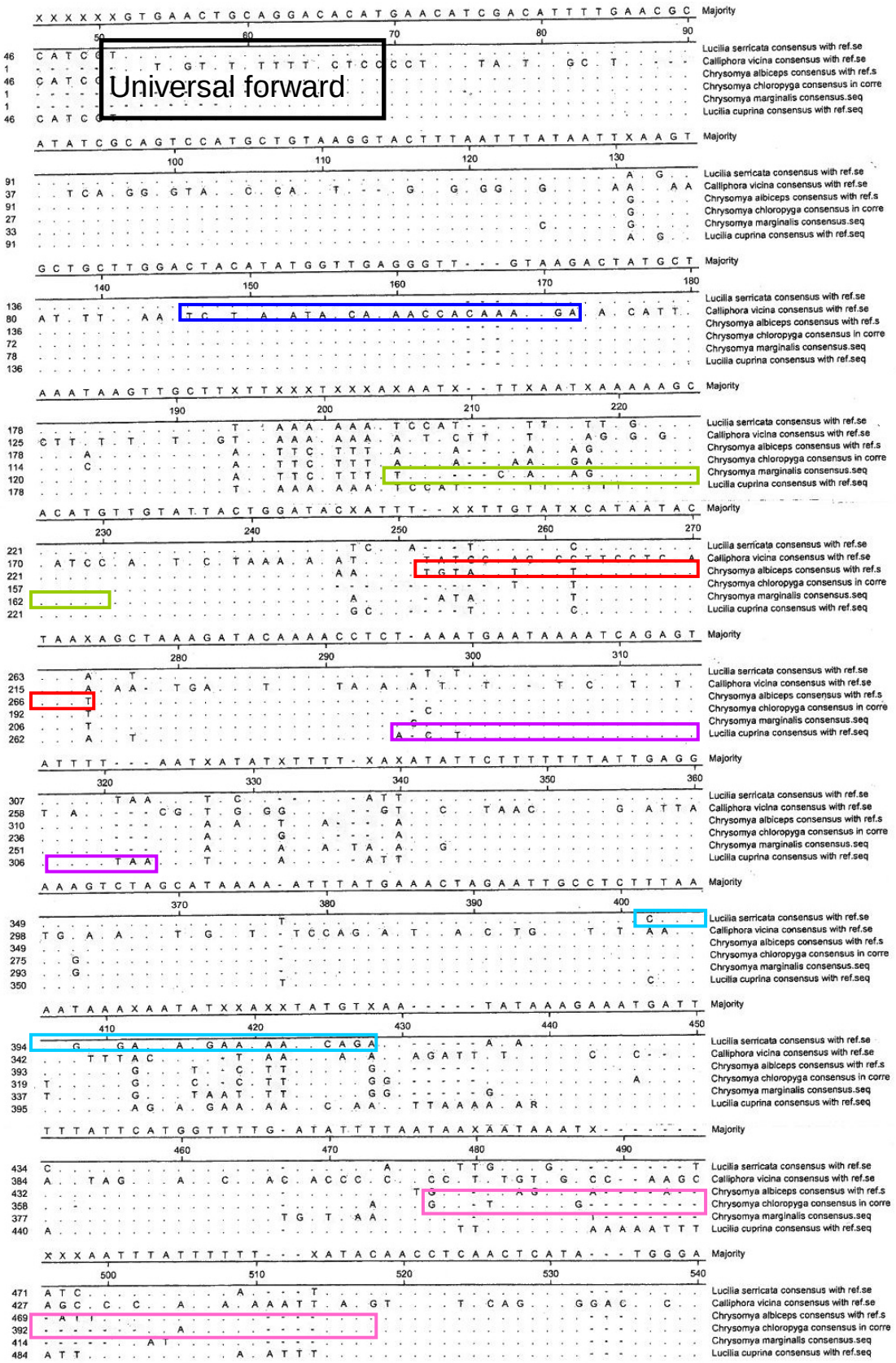


Figure 4.5 Mass alignment of the consensus sequences of each species showing the areas identified where there is the most variation from the consensus sequences for

each species. The universal primer (the original ITS2A) is shown at the beginning of the alignment and the area of greatest variation in base pairs when compared to the consensus for each species is highlighted. The dark blue area indicates the area of highest variation for *C. vicina*, the green section shows the for *Ch. marginalis*; the red area that of *Ch, albiceps*; the purple area that of *L. cuprina*; the pail blue area that of *L. sericata* and the pink area shows that of *Ch. chloropyga*.

4.4.4. Primer design

From the selected regions on the mass alignment primers were designed for each of the species (Table 4.2). The universal primer was maintained as the ITS2A designed by Koekemoer *et al.* (2002) and the species-specific primers were all designed as reverse primers.

Table 4.2. The primer sequences synthesised from the area selected from the alignment and the best results obtained from the NetPrimer analysis. The table shows the size of the expected target fragment for each primer and the critical temperature (Tm) for each of the species-specific created primers.

Primer name (or species name) Primer Set 1	**Primer sequence**	**Size (bp)**	**Tm (ºC)**
Universal primer / forward ITS2A	5’ TGT GAA CTG CAG GAC ACA T 3’		
Calliphora vicina	5’ CTA TTT GTG GTT TTG ATA TTT TAT G 3’	115	54.71

Chrysomya marginalis	5’ CTT TTA GAT TAT AAA AGA AAA TAA GC 3’	161	53.55
Chrysomya albiceps	5’ GAA TAA AAT ACA AAA TTT TAT CC 3’	208	50.29
Chrysomya chloropyga	5’ CTT TAT ATT CTT ATA ATA TCA AAA CC 3’	403	53.55
Lucilia sericata	5’ CTT TTT CTT CTT TTA GAG AGG C 3’	354	57.08
Lucilia cuprina	5’ TAT TCA ATG TGA GGT TTT GTA TCT 3’	245	56.03

Unfortunately this primer set 1, on initial PCR, did not give the expected results with very few of the samples being amplified. After optimisation in the temperature and $MgCl_2$ concentrations, the results (figure 4.6) showed that the best temperature for the PCR was 52^oC with a higher concentration of $MgCl_2$.

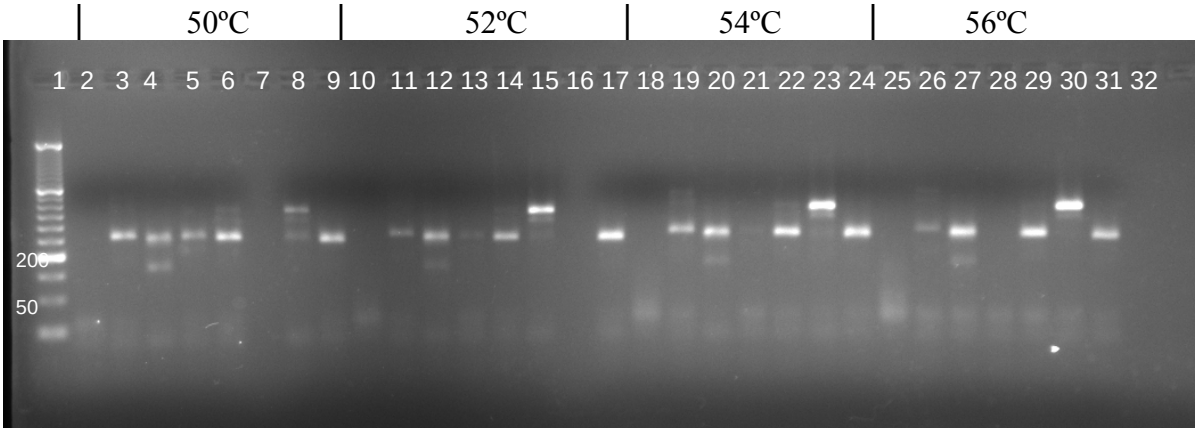


Figure 4.6. The electrophoretogram showing the results of the PCR using the primer set 1. Lane 1: molecular ladder (50bp O'Range Ruler, Fermentas); Lane 2 onwards were loaded negative control, *C. vicina*, *Ch. marginalis*, *Ch. albiceps, L. cuprina, L. sericata* and *Ch.* chloropyga. Lanes 2 – 9 are samples at 50ºC, Lanes 10 – 17 at 52ºC, Lanes 18 – 24 at 54ºC, Lanes 25 – 31 at 56ºC and Lane 32: blank.

Unfortunately this primer set did not give the expected results as even under the conditions that worked best for all the other primers there were still non-specific amplicons on some of the samples and others did not amplify and product. Due to the 3' ends of the reverse primers not being very specific to each species it was possible that the species-specific primers were targeting other regions within the species or even binding to other species. The primers were re-analysed and the 3' ends selected in the targeted area to have higher variation and thus be more species-specific where as the primer set 1 did not have 3' ends that were very specific to each species. The modified primers were synthesised by Inqaba Biotechnical Industries.

Table 4.3. The primer sequences synthesised with a more variable 5' end. The table shows the size of the expected target fragment for each primer and the critical temperature (Tm) for each of the species-specific created primers.

Primer name (or species name) Primer Set 2	**Primer sequence**	**Size (bp)**	**Tm (ºC)**
Universal primer / forward ITS2A	5' TGT GAA CTG CAG GAC ACA T 3'		
Calliphora vicina	5' TCT ATT TGT GGT TTT GAT ATT TTA TGA 3'	119	55.49
Chrysomya marginalis	5' CAT GTG CTT TTT CTT TTA GAT TA 3'	173	53.86
Chrysomya albiceps	5' ATT AGT ATT ATG AAT AAA ATA CA 3'	218	48.51
Chrysomya chloropyga	5' TAA AAA TAC TCT GAT TTT ATT CAA TGT 3'	415	47.76
Lucilia sericata	5' TCT GTA TTT TTC TTT TTC TTC TTT TAG 3'	365	55.49
Lucilia cuprina	5' GTA TAA AAA TTA CTT TAT ATT C 3'	262	53.97

A PCR was then performed using both sets of designed primers, individually with only their target species as well as in their respective multiplex design (Figure 4.7). For individual reactions only two primers were used, the universal forward (ITS2A)

and one species-specific reverse primer (eg. *C. vicina* (primer set 1)). The resulting gel showed that the primer set 1 as a multiplex were not resulting in the correct diagnostic fragments. However, when primers were used from the second set of primers individually the correct size was obtained thus, producing the correct fragment size regardless if it was in multiplex or single primer reactions, this was a result for four of the species as well as for the same four individually. The primer that worked the best for *Ch. chloropyga* was from primer set 1 and the rest of the species primers were taken from primer set 2 for future use (called primer set 3) and development of the multiplex PCR (figure 4.8).

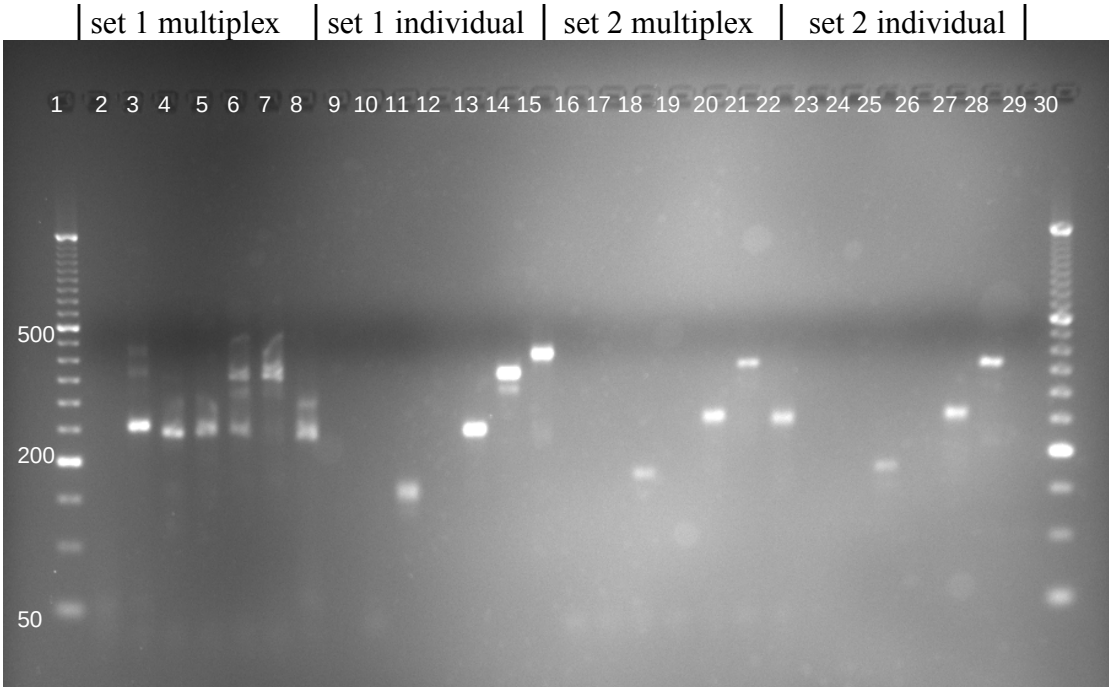


Figure 4.7. The electrophoretogram showing the results of the PCR using the created created primers from set 1 and set 2 run in multiplex form and as individual primers. Lanes 1 and 30: molecular ladder (50bp O'Range Ruler, Fermentas); The remaining wells were all loaded in the following order: negative control, *C. vicina*, *Ch. marginalis*, *Ch. albiceps, L. cuprina, L. sericata* and *Ch. chloropyga.* Lanes 2 to 8

show the samples using the multiplex set 1 primers in a multiplex, Lanes 9 to 15 show the primers form set 1 used individually with each target species, Lanes 16 to 22 show the samples using the multiplex set 2 primers in a multiplex, Lanes 23 to 29 show the primers form set 1 used individually with each target species.

As seen in figures 4.7 and 4.8 there was no amplification from either set of primers for *Ch. albiceps*. Due to time constraints this could not be investigated further and the multiplex PCR was finalised with this species eliminated from further development. The initial calculations (figure 4.5) predicted that *C. vicina* would have a fragment length of 115bp, however, the multiplex trials were amplifying a fragments/ amplicons for the species at approximately 430bp. To confirm that the multiplex was targeting the correct area for the *C. vicina* the PCR was repeated on a 2% low melt agarose gel stained with ethidium bromide (0.3µg/ml) and the resulting fragments/ amplicons excised and extracted (as outlined in section 4.3.1) and sequenced by Inqaba Biotechnical Industries.

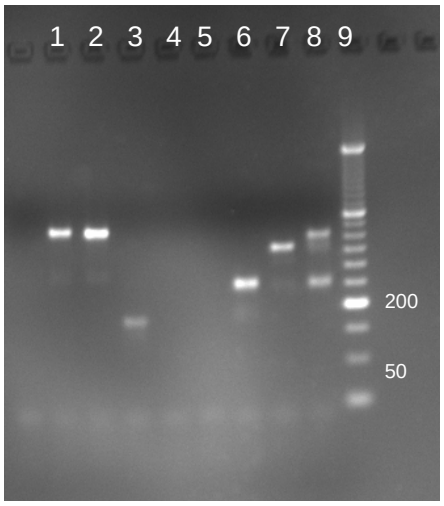


Figure 4.8. The elecrophoretogram showing the results of the multiplex PCR using primer set 3. Lane 1: *C. vicina* Lane 2: *C. vicina*, Lane 3: *Ch. marginalis* , Lane 4:

Ch. albiceps, Lane 5: *Ch. albiceps,* Lane 6: *L. cuprina*, Lane 7: *L. sericata* Lane 8: *Ch. chloropyga,* Lane 9: molecular ladder (50bp O'Range Ruler, Fermentas). The *Ch. chloropyga,* fragment appears to be at the same length as that of *C. vicina* but there is a difference due to the presence of the secondary fragment at ~250bp of *Ch. chloropyga.*

The estimated size of *C. vicina* was not expected to be the largest fragment produced by the mutiplex PCR. The larger amplicons of *C. vicina* was sequenced and resuting BLAST results showed that the region was of the *C. vicina* ITS 2 region. Thus the PCR result of *C. vicina* having a fragment size of ~430bp was a valid, resulting in five primers that were valid to use in a multiplex PCR.

4.4.5. Multiplex PCR and validation

The multiplex primers (Table 4.4) were used and the following conditions for the multiplex with the optimised multiplex PCR reaction consisted of: 2.5 µl 10x reaction buffer (100 mM Tris- HCl (pH 8.3) 500 mM KCl), 1.5 mM MgCl2, 200 µM of each dNTP, 2.5 µM of each primer, 12.3 µl dH2O, 0.2 U of *Taq* DNA polymerase and DNA template (50 - 100 ng/µl) under the cycling conditions given below in table 4.5.

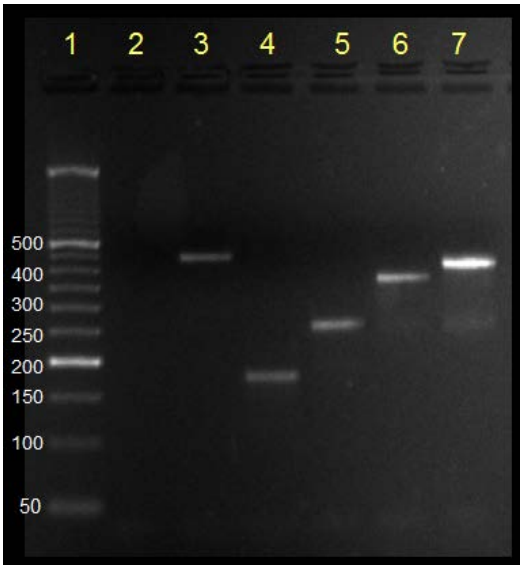


Figure 4.9. Electrophoretogram of the multiplex PCR showing the five species that were amplified. Each species is represented by a fragment of a different size, thus allowing a species specific identification. Lane 1: molecular ladder (50bp O'Range Ruler, Fermentas). Lane 2: no DNA control. Lane 3: *C. vicina* (~450bp). Lane 4: *Ch. marginalis* (~180bp). Lane 5: *L. cuprina* (~260bp). Lane 6: *L. sericata* (~370bp). Lane 7: *Ch. chloropyga* (~320bp).

Table 4.4. The final primers selected and confirmed as those to be used as the multiplex PCR primers for the identification of some of the forensically important blowflies found on the Gauteng highveld.

Primer name (or species name) Primer set 3	**Primer sequence**
Universal primer / forward ITS2A	5' TGT GAA CTG CAG GAC ACA T 3'
Calliphora vicina (2)	5' TCT ATT TGT GGT TTT GAT ATT TTA TGA 3'
Chrysomya marginalis (2)	5' CAT GTG CTT TTT CTT TTA GAT TA 3'

Lucilia cuprina (2)	5’ TAA AAA TAC TCT GAT TTT ATT CAA TGT 3’
Lucilia sericata (2)	5’ TCT GTA TTT TTC TTT TTC TTC TTT TAG 3’
Chrysomya chloropyga (1)	5’ CTT TAT ATT CTT ATA ATA TCA AAA CC 3’

Table 4.5. the cycling conditions for the multiplex PCR

Initial denaturation	94ºC	2 minutes
Cycle x 40	94ºC	30 seconds
	52ºC	30 seconds
	72ºC	40 seconds
Final extention	72ºC	10 minutes

To validate the designed multiplex PCR, a blind test was performed where various samples of the targeted flies as well as other flies were selected. The targeted five species were used as well as *Ch. albiceps,* house flies (Muscidae), flesh flies (Sarcophagidae) and mosquitoes (*Anopheles funestus* - material obtained from the Vector Control Reference Laboratory insectary). All species were identified using morphological keys prior to DNA extraction (Zumpt, 1965, Holloway, 1991) but the house flies and flesh flies were only identified to family level. The *An. funestus* was used to test if the primers would amplify other flies.

The samples were randomly assigned numbers by a third party to ensure that the trial was non-biased and a true blind test of the multiplex PCR. A total of 29 samples was tested, and amplified under the conditions listed in table 4.5

The resulting PCR product was electrophoresed on the gel and scored according to the fragment size and thus a species identification could be made.

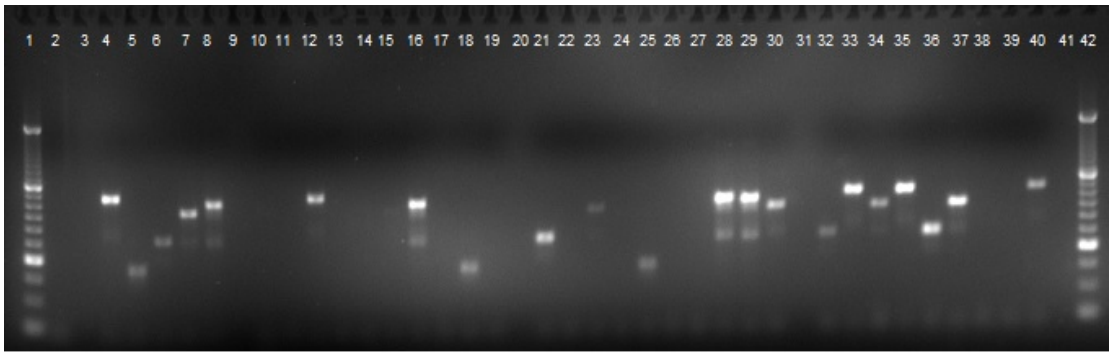


Figure 4.10. The electrophoretogram showing the results of the blind test PCR for the multiplex PCR. The gel contains a 50 bp ladder (lanes 1 and 42) and the control samples (*C. vicina, Ch. marginalis, L. cuprina, L. sericata* and *Ch.* chloropyga) are in lanes 4 to 8. Note the blank wells where no amplification took place during the PCR (lanes 10, 11, 13, 14, 15, 17, 19, 22, 25, 26, 27, 38 and 39). These were the samples of other species not targeted by the multiplex PCR, including *Ch. albiceps* (lanes 13, 24 and 27).

The blind test of the multiplex PCR validates that it will not target other common flies and only amplifies the five out of six species it was designed to. None of the samples were mis-identified and none of the samples of the targeted species failed. The mutiplex results were easy to read off the gel as the 50pb ladder has calibrations that are easy to distinguish. When compared to a routinely-used multiplex PCR for *Anopheles gambiae* it is a procedure that is easy to use and the results are easy to interpret and thus easy to report.

4.5.) General discussion

The ITS2 region is successfully used as a tool in the identification of a number of medicinal plants (Chen *et al.*, 2010) and closely related mosquito species (Koekemoer *et al.* (2002) and forensically important fly species (Song *et al.*, 2008; Nelson *et al.*, 2008; Piao, *et al.*, 2012). Most molecular identifications methods can be costly and involve expensive equipment and skilled personnel in laboratories. Multiplex PCRs are easily achieved in all laboratories and they are cheaper in cost and require little skill in adapting the protocols (Koekemoer *et al.*,2002). Thus this multiplex PCR assay developed for forensically important blowflies has the potential to be used routinely.

Concerns around this multiplex PCR were all related to the extraction methods of the DNA used. The method used is that outlined by Collins *et al.* (1987) which can be a lengthy protocol as it contains an overnight step, unlike the DNA extraction kits which can take 20 minutes in total. Investigations need to be done into the use of DNA extraction kits which will allow for a higher number of samples to be processed in a shorter time. Other concerns that will need future investigation include the possibility of two sets of DNA being extracted from the maggots or adults that have undigested matter in their gut. In this study only a wing, leg or cuticle clipping was used for the DNA extraction but if the whole specimen or other parts of a specimen were to be used there could be second source of DNA being extracted as Wells and Stevens (2010) outlined after successfully extracting human DNA from the gut contents of a maggot recovered from a human corpse. There is also a possibility of

multiple fly DNA samples being extracted from maggots that have predated on other species of maggots.

Due to the high copy number gene (Adams *et al.*, 1992) very little sample material will be needed from the species. There should therefore not be any concern about cross-contamination with other matter such as the gut content of the maggots as it is unlikely a whole maggot will be used in the multiplex PCR. BLAST analysis of the primers used in the multiplex PCR showed that none of the primers targeted any human sequence so there should be no selection of any human matter in the multiplex as well.

The multiplex only targeted five of the six selected species. There is potential to adapt the multiplex to eventually target the selected species as well as expanding the multiplex to include other species and PCR methods such as the restriction fragment length polymorphism (PCR-RFLP) which is already in use to identify some closely related *Chrysomya* species (Nelson, *et al.*, 2008). *Ch. albiceps* species not being amplified will need to be investigated in future studies.

Chapter 5. Concluding Remarks

The field of forensic entomology is growing within the country and there is great potential for future work. The study of carcasses on the Gauteng Highveld identified differences between decomposition across summer and winter. The lengthy time taken for the winter carcass to decompose and the formation of adipocere showed that it is vital for the environmental conditions at a site to be known to aid in predictions. The use of one carcass per season limited the trials but the results showed that other factors such as shade or light conditions should be studied further.

The six most dominant species of forensically important blowflies occurring on the Gauteng Highveld were identified. The apparent seasonal differences between the species warrant additional studies plus the species' abundances change depending on weather conditions. The morphological characteristics isolated from the larval stages of these species, reinforces the research trends in current morphological studies. The isolation of the first instar cephaloskeletons is supported by work done on related species (Queiroz *et al.,* 1997; Szpila and Villet, 2011) and has potential to be used in future work done on creating morphological keys to the larval stages of forensically important blowflies (Prins, 1982; Queiroz *et al.,* 1997).

The fact that one species was not able to be identified in the multiplex PCR means that further studies are needed using different parts of the genome or other methods such as AFLP or RFLP to develop a system that can be easily used for all species which has applications to methods of identification and studies on larger populations of flies (Ratcliffe, *et al.,* 2003; Nelson *et al.,* 2008) as well as phylogenetic studies

such as those done in collaboration with Australia (Harvey, *et al.*, 2003, 2008) and Brazil (Marinho, *et al.*, 2012).

APPENDIX A - animal ethics

AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2010/22/01

APPLICANT: Ms AE Gilbert

SCHOOL: Pathology
DEPARTMENT:
LOCATION:

PROJECT TITLE: **Forensic entomology on the Gauteng Highveld using pig (*Sus Scrofa Linnaeus) carcasses*.**

Number and Species

One 280Kg -100kg *Sus scrofa linnaeus* pig

Approval was given for the use of animals for the project described above at an AESC meeting held on 30.03.2010. This approval remains valid until 30.03.2012

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

None

Signed: _____ (Chairperson, AESC) Date: 16/04/2010

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 16/04/2010

cc: Supervisor:
Director: CAS

Works 2000/lain0015/AESCCert.wps

APPENDIX B - letter of use for study site

Melville Koppies Management Committee
Of the **Friends of Melville Koppies**

125 Third Avenue
Melville 2092

Phone: 011-482-4797
Website: www.mk.org.za

038-274-NPO
PBO 930027329

7 June 2010

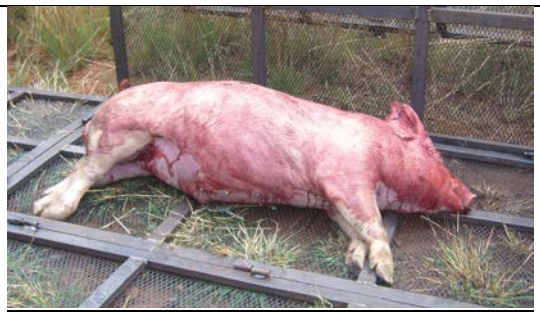
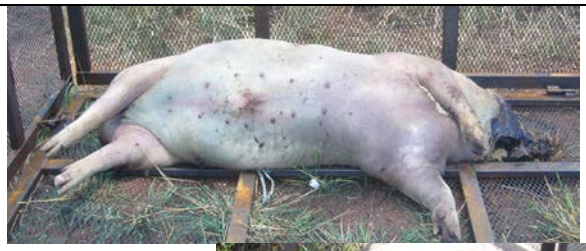
To whom it may concern.

I have given Allison Gilbert permission to use Melville Koppies for field work for her masters project, starting in November 2010. She has sent me all the details of what is involved and I understand them fully.

Wendy Carstens
Chairman of the Melville Koppies Management Committee.

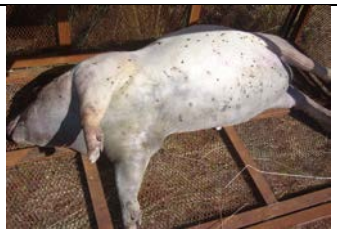
APPENDIX C - pictures of decomposition process of pig carcasses

The decomposition of the summer trial carcass at different days through the trial.

Day 1	
Day 7	 
Day 11	
Day 19	

Day 32	
Day 50	
Day 80	

The decomposition of the winter trial carcass at different days through the trial.

Day 1		Day 143	
Day 7		Day 188	

Day 11		Day 230	
Day 19		Day 268	
Day 32		Day 304	
Day 50		Day 335	
Day 80		Day 367	
Day 110			

APPENDIX D - specialised equipment used

Specialised equipment used for dissections:

Equipment	**Model**	**Supplier**
Dissecting microscope	SZX7	Olympus
Compound microscope	BX50	Olympus
Microscope camera	CC12 Soft Imaging System	Olympus
Camera software	analysisLS Reasearch software	Olympus

Specialised equipment used for viewing electrophoresed gels:

Equipment	**Supplied by**
GeneAmp ® PCR system 2004	Applied biosystems
G:Box Chemi XL gel documentation system	Syngene

Specialised equipment used for removing target fraqgments from electrophoresed gels:

Equipment	**Supplier**	**Catalogue number**
x-tracta gel cutters	LabGadget via AEC-Amersham (PTY) Ltd.	LG202 xtracta

APPENDIX E - Collin's *et al.* (1987) protocol

Protocol for DNA extraction as per Collins *et al.* (1987) adapted appropriately for fly samples

Step1: set heating block to 70ºC.

Place one leg or wing from adult fly into an autoclaved eppie (or sectionof skin if sample is maggot)

Step2: Grind the sample using a pestle, grind in 200µl of grinding buffer.

Step3: Remove pestle and place eppie on heating block for 30 minutes.

Step4: Add 28µl of 8M KAc and mix

Incubate on ice for 30 minutes.

Step 5: Centrifuge for 30 minutes at 13 000rpm

Step 6: Pipette of the liquid and place into a new eppie.

Add 400µl 100% ethanol (cold from the freezer) and mix

Incubate overnight at -20ºC.

Step 7: Centrifuge for 30 minutes at 13 000rpm

Step 8: Pipette off the 100% ethanol. Add 200µl of 70% cold ethanol

Centrifuge for 15 minutes at 13 000rpm

Step 9: Pipette off all liquid.

Air dry eppie overnight on desk with the lid open

Step 10:Re-suspend the pellet in 200µl of 1x TE buffer.

Store in the freezer indefinitely.

Grinding Buffer (Collins *et al.* (1987)

Make up to final volume of 20ml

- 1600µl 1M NaCl

- 1.095g Sucrose

- 2400µl 0.5M EDTA

- 1000µl 10% SDS

- 2ml 1M Tris-Cl (pH 8.6)

8M Potassium Acetate (KAc)

7.85g in 10ml distilled water

TE Buffer (10x stock)

Make up to a volume of 1L (pH 8)

- 100ml 1M tris (pH 8)

- 20ml 0.5M EDTA

APPENDIX F - Reagents

All reagents and buffers made as those outlined in Sambrook, *et al.* 1989)

PBS buffer used in dissections:

Dissolve one tablet in one litre of dH_2O to yield 10mM phosphate buffer, pH 7.4, 140 mM NaCl, 2.7 mM KCl.

Reagents, suppliers and catalogue numbers:

Reagent	**Supplier**	**Catalogue number**
PBS	Merck-calibiochem	524650
Tris-Cl	Merck	SAAR 3063040LP
Acetic Acid	Merck	64-19-7
EDTA	Merck	EO834CI00500
Agarose	Seakem supplied by Whitehead Scientific (Pty) Ltd.	50004
Low melt agarose	Seakem supplied by Whitehead Scientific (Pty) Ltd.	
MgCl	Thermo Scientific	A9001A
Taq	Celtic molecular biosciences	RR001
DNTP's	Takara Bio Inc.	4030
O'range™ 100bp DNA ladder	Fermentas from inqaba Biotechnologies	SM0243

O’range™ 50bp DNA ladder	Fermentas from inqaba Biotechnologies	SM0373
Loading Dye	Thermo Scientific	R0631
Ethidium bromide	Sigma	E1510

REFERENCES

Adams, R.I.P., Knowlers, J.T., Leade, D.P. (1992) The Biochemistry of Nucleic Acids. 11th Edition. Chapman and Hall, Ltd. London.

Amendt, J., Campobasso, C.P., Gaudry, E., Reiter, C., LeBlanc, H.N., Hall, M.J.R. (2007) Best practice in forensic entomology- standards and guidelines. International Journal of Legal Medicine **121**: 90 - 104

Amendt, J., Richards, C.S., Campobasso, C.P., Zehner, R., Hall, M.J.R. (2011) Forensic entomology: applications and limitations. Forensic Science, Medicine and Pathology **7**: 379 - 392

Anderson, G.S. (1991) Wildlife forensic entomology: determining time of death of two illegally killed black bear cubs. Journal of Forensic Science **44**: 856 – 859

Anderson, G.S. (2009) Factors that influence insect succession on carrion. In Forensic Entomology. The Utilisation of Arthropods in Legal Investigations. Second Edition. Eds. Byrd, J.H., Castner, J.L. CRC Press. Florida

Anderson, G.S. and Huits, N.R. (2004) Myiasis in pet animals in British Columbia: The potential of forensic entomology for determining duration of possible neglect. Canadian Veterinary Journal **45:** 993 - 998

Archer, M.S. (2004) Rainfall and temperature effects on the decomposition rate of exposed neonatal remains. Science and Justice. **44**: 35 - 41

Bass, W.M. (1984) The time interval since death. In Human identification: case studies in forensic anthropology. Eds. Rathban T.A, Buikstra J.E. Charles, C. Thomas Publishers. Springfield, Illionois

Bass, W.M. (1996) Outdoor Decomposition Rates in Tennessee. Chapter 12 in Forensic Taphonomy: The postmortem fate of human remains. Ed. Haglund, W.D, Sorg, M.H., CRC Press.

Benecke, M. and Lessig, R. (2001) Child neglect and forensic entomology. Forensic Science International **120**: 155 - 159

Benecke, M., Josephi, E., Zweihoff, R. (2004) Neglect of the elderly: forensic entomology cases and considerations. Forensic Science International **1465:** 195 - 199

Benecke, M. (2010) Cases of neglect involving entomological evidence. In Forensic Entomology. The Utilisation of Arthropods in Legal Investigations. Second Edition. Eds. Byrd, J.H., Castner, J.L. CRC Press. Florida

Boucher, J. (1997). Succession and life traits of carrion-feeding Coleoptera associated with decomposing carcasses in the Central Free State. MSc thesis, University of the Free State, Bloemfontein

Bourel, B., Callet, B., Hedouis, V., Gosset, D. (2003) Flies eggs: a new method for the estimation of short-term post-mortem interval. Forensic Science International **135:** 37 - 34

Braack, L.E.O. (1981) Visitation patterns of principal species of the insect-complex at carcasses in the Kruger National Park. Koedoe **24**:33-49

Braack, L.E.O. (1986) Arthropods associated with carcasses in the northern Kruger National Park. South African Journal of Science **16**: 91 - 98

Braack, L.E.O. (1987) Community dynamics of carrion-attendant arthropods in tropical African woodland. Oecologia **72**: 402 - 409

Bucheli, S.R., Bytheway, J.A., Pustilnik, S.M., Florence, J. (2009) Insect Successional Pattern of a Corpse in Cooler Months of Subtropical Southeastern Texas. Journal of Forensic Science **54**: 452 – 455

Byrd, J.H. and Castner, J.L. (Eds.). (2010) Forensic Entomology. The utility of arthropods in legal investigations. Second Edition CRC Press, Florida

Byrd, J.H., Tomberlin, J.K. (2010) Laboratory Rearing of Forensic Insects. In Byrd, J.H. & Castner, J.L. (Eds.) Forensic entomology. The utility of arthropods in legal investigations Second Edition. CRC Press, Florida

Carter, D.O., Yellowless, D., Tibbett, M. (2007) Cadaver decomposition in terrestrial ecosystems. Naturwissenschaften **94**: 12 - 24

Castner, J.L. (2010) General Entomology and Insect Biology. In Forensic Entomology. The Utilisation of Arthropods in Legal Investigations. Second Edition. Eds. Byrd, J.H., Castner, J.L. CRC Press. Florida

Catts, E.P. (1990) Analyzing entomological data. In: Catts, E.P. & Haskell, N.H. Entomology and death: A procedural guide. Joyce's Print Shop, Clemson. South Carolina

Catts, E.P., Goff, M.L. (1992) Forensic entomology in criminal investigations. Annual Review of Entomology **37**: 253 – 272

Chen, S., Yao, H., Han, J., Liu, Song, J., Shi., L., Zhu, Y., Ma, X., Gao, T., Pang, X., Luo, K., Li, Y., Li, X., Jia, X., Lin, Y., Leon, C. (2010) Validation of the ITS2 Region as a Novel DNA Barcode for Identifying Medicinal Plant Species. PloS One. **5:** 8613 - 8623

Clark, K., Evans, L., Wall, R., (2006) Growth rates of the blowfly, *Lucilia sericata*, on different body tissues. Forensic Science International **156**: 145 - 149

Collins, F.H., Mendez, M.A., Rasmussen, M.D., Mchaffey, P.L., Besanky, N.J., Finerty, (1987) A ribosomal probe differentiates member species of the *Anopheles gambiae* complex. American Journal of Tropical Medicine and Hygiene **37:** 37 - 41

Dalton, D.L., Koetze, A (2011) DNA barcoding as a tool for species identification in the forensic wildlife cases in South Africa. Forensic Science International **207:** 51-54

Debry, R.W., Timm, A.E., Wong, E.S., Stamper, T., Cookman, C., Dahlem, G.A. (2013) DNA-based identification of forensically important *Lucilia* (Diptera: Calliphoridae) in the continental United States. Journal of Forensic Science **58**: 73 - 78

Donovan, S.E., Hall, M.J.R, Turner, B., Moncrieff, C.B. (2006) Larval growth rates of the blowfly, *Calliphora vicina*¸ over a range of temperatures. Medical and Veterinary Entomology **20:** 106 - 114

Erlandsson, M. and Munro, R. (2007) Estimation of the post-mortem interval in beagle dogs. Science and Justice **47:** 150 - 154

Erzinçlioglu, Y.Z. (1990) On the interpretation of maggot evidence in forensic cases. Medicine, Science and the Law **30**: 65 - 66

Ferreira, S., Oliveira, A.R., Farinha, A., Rebelo, M.T., Dias, D. (2011) Forensic entomology: nuclear and mitochondrial markers for Diptera and Coleoptera identification. Forensic Science International: Genetics Supplement Series **3**: e174 - e175

Forbes, S.L., Stuart, B.H., Dent, B.B. (2002) The identification of adipocere in grave soils. Forensic Science International **127**: 225 - 230

Goff, M.L., Charbonneau, S., Sullivan, W. (1991) Presence of faecal material in diapers as a potential source of error in estimations of post-mortem interval using arthropod development rates. Journal of Forensic Science **36:** 1603 - 1606

Greenberg, B. (1990) Nocturnal oviposition behavior of blowflies (Diptera: Calliphoridae). Journal of Medical Entomology **27**:807-810

Hall, D.G. (1948) The Blowflies of North America. The Thomas Say Foundation. Entomological Society of America.

Hall, R.D. (1990) Medicocriminal entomology. In Catts, E.P. & Haskell, N.H. Entomology and death: A procedural guide. Joyce's Print Shop, Inc. Clemson. South Carolina.

Hall, R.D., Huntington, T.E. (2010) Introduction: Perceptions and Status of Forensic Entomology. In Forensic Entomology. The Utilisation of Arthropods in Legal Investigations. 2nd Edition. Eds. Byrd, J.H., Castner, J.L. CRC Press. Florida

Holloway, B.A. (1991) Morphological characters to identify adult *Lucilia sericata* (Meigen, 1826) and *L. cuprina* (Wiedemann, 1830) (Diptera: Calliphoridae) New Zealand Journal of Zoology **18:** 415 - 420

Hanski, I. (1987) Carrion fly community dynamics: patched, seasonality and coexistence. Journal of Economic Entomology **12**: 257 – 266

Harvey, M.L., Mansell, M.W., Villet, M.H., Dadour, I.R. (2003) Molecular identification of some forensically important blowflies of southern Africa and Australia. Medical and Veterinary Entomology **17:** 363 - 369

Harvey, M.L., Gaudieri, S., Villet, M.H., Dadour, I.R. (2008) A global study of forensically significant calliphorids: implications for identification. Forensic Science International **177**: 66 - 76

Hewadikaram, K.A., Goff, M.L. (1991) Effect of Carcass Size on Rate of Decomposition and Arthropod Succession Patterns. American Journal of Forensic Medicine and Pathology **12**: 235 – 240

Higley, L.G. and Haskell, N.H. (2010) Insect development and forensic entomology. In Forensic Entomology. The Utilisation of Arthropods in Legal Investigations. 2nd Edition. Eds. Byrd, J.H., Castner, J.L. CRC Press. Florida

Ireland, S., Turner, B., (2006) The effects of larval crowding and food type on the size and development of the blowfly, *Calliphora vomitoria*. Forensic Science International **159**: 175 - 181

Kelly, J.A., (2006) The influence of clothing, wrapping and physical trauma on carcass decomposition and arthropod succession in central South Africa. PhD Thesis, University of the Free State, Bloemfontein.

Kelly, J.A., van der Linde, T.C., Anderson, G.S. (2009) The influence of clothing and wrapping on carcass decomposition and arthropod succession during the warmer seasons in central South Africa. Journal of Forensic Science **54**: 1105 – 1112

Kelly, J.A., van der Linde, T.C., Anderson, G.S. (2011) The influence of wounds, severe trauma and clothing on carcass decomposition and arthropod succession in South Africa. Canadian Society of Forensic Science Journal **44**: 144 – 157

Koekemoer, L.L. (1999) Systematic studies of the *Anopheles funestus* (Diptera: Culicidae) group in Southern Africa. PhD thesis, University of the Witwatersrand, Johannesburg.

Koekemoer, L. L., Kamau, L., Hunt, R.H., Coetzee, M. (2002) A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. American Journal of Tropical Medicine and Hygiene **6:** 804 - 811

Kutty, S.N., Pape, T., Pont, A., Wiegmann, B.M., Meier, R. (2008) The Muscoidea (Diptera: Calyptratae) are paraphyletic: evidence from four mitochondrial an dfour nuclear genes. Molecular Phylogenetics and Evolution **49**: 639 - 652

Kutty, S.N., Pape, T., Wiegmann, B.M., Meier, R. (2010) Molecular phylogeny of the Calyptratae (Diptera: Cyclorrhapha) with and emphasis on the superfamily Oestroidea and the position of Mystacinobiidae and McAlpine's fly. Systematic entomology **35**: 614 - 635

Lord, W.D. (1990) Case histories of the use of insects in investigations: In: Catts, P.E. & Haskell, N.H. Entomology and death. A procedural guide Joyce's Print Shop, Inc. Clemson. South Carolina.

Marelli, M.T., Floeter-Winter, L.M., Malafronte, R.S., Tadei, W.P., Lourenço-de-Oliveira, R., Flores-Mendoza, C., Marinotti O. (2005) Amazonian malaria vector anopheline relationships interpreted from ITS2 rDNA sequences. Medical and Vetrinary Entomology **19:** 208 218

Marinho, M.A.T., Junqueira, A.C.M., Paulo, D.F., Esposito, M.C., Villet, M.H., Azeredo-Espin, A.M.L. (2012) Molecular phylogenetics of Oestridea (Diptera: Calyptratae) with emphasis on Calliphoridae: insights into the inter-familial relationships and additional evidence for paraphyly among blowflies. Molecular Phylogenetics and Evolution **65**: 840 - 854

Matuszewski, S. Bajerlin, D., Konwerski, S., Szepile, K. (2012) Insect succession and carrion decomposition in selected forests of Central Europe. Part 1: Pattern and rate of decomposition. Forensic Science International **194**: 85 - 93

McKnight, B.E. (1981) The washing away of wrongs: Forensic medicine in

thirteenth-century China. MSc thesis, University of Michigan, Ann Arbor.

Meskin, I. (1980) The guild of necrophagous blowflies (Diptera: Calliphoridae) of the Highveld Region of the Transvaal. MSc thesis, University of the Witwatersrand, Johannesburg.

Meskin, I. (1986) Factors affecting the coexistence of blowflies (Diptera: Calliphoridae) on the Transvaal Highveld, South Africa. South African Journal of Science **82**: 244-250

Meskin, I. (1991) The egg of the Highveld blowfly, *Calliphora croceipalpis* Jaennike (Diptera: Calliphoridae), with a key to the eggs of five other Highveld species. Journal of the Entomological Society of Southern Africa **54**: 185 - 190

Midgley, J.M. (2007) Aspects of the thermal ecology of six species of carcass beetles in South Africa. MSc thesis, Rhodes University, Rhodes.

Midgley, J.M., Richards, C.S., Villet, M.H. (2010) The utility of Coleoptera in forensic investigations. In: Amend, J., Campobasso, C.P., Goff, M.L., Grassberger, M. (Eds). Current concepts in forensic entomology. Heidelberg, Springer

Nelson, L.A., Wallman, J.F., Dowton, M. (2008) Identification of forensically important *Chrysomya* (Diptera: Calliphoridae) species using the second ribosomal internal transcribed spacer (ITS2). Forensic Science International **177:** 238 - 247

Niederegger, S., Pastuschek, J., Mall, G. (2010) Preliminary studies of the influence of fluctuating temperatures on the development of various forensically relevant flies. Forensic Science International **199**: 72 - 78

Otranto, D and Stevens, J.R. (2012) Molecular approaches to the study of myiasis-causing larvae. International Journal of Parasitology **32:** 1345 - 1360

Payne, J.A. (1965) A summer carrion study of the baby pig *Sus scrofa* Linnaeus. Ecology **46**: 592 - 602

Pellatt, N. A. (1996) Carrion study of a large dog (*Canis canis*) during a South African Winter and its application in determining the post mortem interval. BSc Honours research report, University of the Witwatersrand, Johannesburg.

Piao, H.G., Chung, U., Shin, S.E., Ko, K.S., Hwang, J-J. (2012) DNA-based identification of necrophagous fly species using Abdominal-B (Abd-B) homeobox sequence. Korean Journal of Legal Medicine **36**: 74 - 84

Pinheiro, J., (2006) Decay Process of a Cadaver. Ch. 5 in Forensic Anthropology and Medicine: Complementary Science From Recovery to Cause of Death. Eds. Schmitt, A., Cunha, E., and Pinheiro, J. Humana Press Inc. New Jersey

Prins, A.J. (1982) Morphological and biological notes on six South African blow-flies (Diptera, Calliphoridae) and their immature stages. Annals of the South African Museum **90**:201 - 217

Prins, A.J. (1983) Morphological and biological notes on some South African arthropods associated with decaying organic matter. I: Coleoptera, Diplopoda, Arachnida, Crustacea, and Insecta. Annals of the South African Museum **92**: 53 - 112

Queiroz, de Carvalho, M.M, de Mello, R.P. Lima, M.M. (1997) Morphological aspects of the larval instars of *Chrysomya albiceps* (Diptera: Calliphoridae) reared in the laboratory. Memórias do Instituto Oswaldo Cruz **92:** 187 - 196

Ratcliffe, S.T., Webb, D.W., Weinzierl, R.A., Robertson, H.M (2013) PCR-RFLP Identification of Diptera (Calliphoridae, Muscidae and Sarcophagidae), a generally applicable method. Journal of Forensic Sciences **48:** 783-785

Richards, C.S., Villet, M.H. (2008) Factors affecting accuracy and precision of thermal summation models of insect development used to estimate post-mortem intervals. International Journal of Legal Medicine **122**: 401 - 408

Richards, C.S., Paterson, I.D., Villet, M.H. (2008) Estimating the age of immature *Chrysomya albiceps* (Diptera: Calliphoridae), correcting for temperature and geographical latitude. International Journal of Legal Medicine **122**: 271 - 279

Richards, C.S., Williams, K.A., Villet, M.H. (2009a) Predicting geographic distribution of seven blowfly species (Diptera: Calliphoridae) in South Africa. African Entomology **17**: 170 - 182

Richards, C.S., Crous, K.L., Villet, M.H. (2009b) Models of development for the blow fly sister species *Chrysomya chlorpyga* and *C. putoria*. Medical and Veterinary Entomology **23**: 56 - 61

Richards, C.S., Price, B.W., Villet, M.H. (2009c) Thermal ecophysiology of seven carrion-feeding blowflies in Southern Africa. Entomologica Experimentalis et Applicata **131**: 11 - 19

Richards, C.S., Rowlinson, C.C., Cuttiford, L., Grimsley, R., Hall, M.J.R. (2013) Decomposed liver has a significantly adverse [sic] on the development rate of the blowfly *Calliphora vicina*. International Journal of Legal Medicine **127**: 259 - 262

Ridgeway, J.A., Midgley, J.M., Collett, I.J., Villet, M.H. (2014) Advantages of using development models of the carrion beetles *Thanatophilus micans* (Fabricius) and *T. mutilatua* (Castelneau) (Coleoptera: Silphidae) for estimating minimum post mortem intervals, verified with case data. International Journal of Legal Medicine **128**: 207 - 220

Sambrook, S., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning. 2nd Edition. Laboratory Manual. Cold Harbor Laboratory Press, New York

Saukko, P., Knight, B. (2004) Knight's Forensic Pathology. Third Edition. Edward Arnold, Ltd. London

Schoenly, K.G. (1992) A statistical analysis of successional patterns in carrion-arthropod assemblages: implication for forensic entomology and determination of the post-mortem interval. Journal of Forensic Science **37**: 1487 - 1513

Scott, J.A., Brogdon, W.G., Collins, F.H. (1993) Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. American Journal of Tropical Medicine and Hygiene **49:** 520 - 529

Sharanowski, B.J., Walker, E.G., Anderson, G.S. (2008) Insect succession and decomposition patterns on shaded and sunlit carrion in Saskatchewan in three different seasons. Forensic Science International **179**: 219 – 240

Singh, B., Wells, J.D. (2013) Molecular systematics of the Calliphoridae (Diptera: Oestroidea): evidence from one mitochondrial and three nuclear genes. Journal of Medical Entomology **50**: 15 - 23

Slone, D.H., Gruner, S.V. (2007) Thermoregulation in larval aggregations of carrion – feeding blow flies (Diptera: Calliphoridae). Journal of Medical Entomology **44**: 516 – 523

Smith, K.G.V. (1986) A manual of forensic entomology. British Museum (Natural History), London.

Smith, J.A and Baker, N.C. (2008) Molecular genetic identification of forensically important flies from the UK. Forensic Science International: Genetics Supplement Serial 620 - 633

Song, Z., Wang, X., Lianu, G, (2008) Species identification of some common necrophagous flies in Guangdong province, southern China based on the rDNA internal transcribed spacer region 2 (ITS2). Forensic Science International **175:** 17 - 22

Sperling, F.A., Anderson, G.S., Hickey, D.A. (1994) A DNA-based approach to the identification of insect species used for post-mortem interval estimates. Journal of Forensic Sciences **39:** 418 - 427

Stamper, T., Dahlem, G.A., Debry, R.W. (2013) Phylogenetic relationships of flesh flies in the subfamily Sarcophaginae based on three mtDNA fragments (Diptera: sarcophagidae). Systematic entomology **38**: 35 - 44

Szpila, K., Villet, M.H. (2011) Morphology and Identification of First Instars of African Blow Flies (Diptera: Calliphoridae) Commonly of Forensic Importance. Journal of Medical Entomology **48:** 738 - 752

Turner, B., Howard, T. (1992) Metabolic heat generation in dipteran larval aggregations: a consideration for forensic entomology. Medical and Veterinary Entomology **6**: 179 - 181

Tourle, R., Downie, D.A., Villet, M.H. (2009) Flies in the ointment: a morphological and molecular comparison of *Lucilia cuprina* and *Lucilia sericata* (Diptera: Calliphoridae) in south Africa. Medical and Veterinary Entomology **23:** 6 - 14

Ubelaker, D.H., Zarenko, K.M. (2011) Adipocere: What is known after two centuries of research. Forensic Science International **208**: 167 - 172

VanLaerhoven, S.L. (2008) Blind validation of post-mortem interval estimates using developmental rates of blowflies. Forensic Science International **180**: 76 - 80

Villet, M.H., Richards, C.S., Midgley, J.M. (2010) Contemporary precision, bias and accuracy of minimum post-mortem intervals estimated using development of carrion-feeding insects. In: Amend, J., Campobasso, C.P., Goff, M.L., Grassberger, M. (Eds). Current concepts in forensic entomology. Heidelberg, Springer

Villet, M.H., (2011) African Carrion Ecosystems and Their Insect Communities in Relation to Forensic Entomology. Pest Technologies **5**: 1 - 15

Watson, E.J. and Carlton, C.E. (2003) Spring succession of necrophilous insects on wildlife carcasses in Louisiana. Journal of Medical Entomology **40** :338-347.

Watson, E.J. and Carlton, C.E. (2005) Insect succession and decomposition of wildlife carcasses during fall and winter in Louisiana. Journal of Medical Entomology **42**:193-203.

Wells, J.D., King, J. (2001) Incidence of precocious egg development in flies of forensic importance (Calliphoridae). Pan-Pacific Entomologist **77:** 235 - 239

Wells, J.D., and Lamotte, L.R. (2010) Estimating the Post-mortem Interval. In Byrd, J.H. & Castner, J.L. (Eds.) Forensic entomology. The utility of arthropods in legal investigations Second Edition. CRC Press, Florida.

Wells, J.D. and Stevens, (2008) Application of DNA-based Methods in Forensic Entomology. Forensic Entomology DNA. Annual Review of Entomology **53**: 103 - 20.

Wells, J.D. and Stevens, J.R. (2010) Molecular Methods of Forensic Entomology. In Byrd, J.H. & Castner, J.L. (Eds.) Forensic entomology. The utility of arthropods in legal investigations Second Edition. CRC Press, Florida.

Williams, K.A. (2003). Spatial and temporal occurrence of forensically important south African blowflies (Diptera: Calliphoridae). MSc thesis, Rhodes University, Rhodes.

Williams, K.A., Villett, M.H. (2006) A history of southern African research relevant to forensic entomology. South African Journal of Science **102**: 59 – 65

Williams, K.A., Cronje, F.J., Avenant, L., Villet, M.H. (2008) Identifying flies used for maggot debridement therapy. South African Medical Journal **98:** 196 - 199

Williams, K.A., Villet, M.H. (2013) Ancient and modern hybridization between *Lucilia sericata* and *Lucilia cuprina* (Diptera: Calliphoridae) Entomology **110**: 187 - 196

Zumpt, F. (1965) Myiasis in Man and Animals in the Old World. Butterworths, London.