

DNA PROFILING FROM THE CROP CONTENT OF SARCOPHAGIDAE SPP. LARVAE

By

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DECLARATION

I declare that the dissertation/thesis hereby handed in for the qualification *Magister Scientiae* Intermediate Forensic Genetics and Forensic Entomology at the University of the Free State, is my own independent work and that I have not previously submitted the same work for a qualification at/in another University/faculty

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ABBREVIATIONS AND SYMBOLS

| | |
|---------------|------------------------------------------------------------------------------------|
| ANOVA | Analysis of variance |
| a.s. | Anterior spiracles |
| C | Crop |
| Ca | Cardia |
| °C | Degrees Celsius |
| CPS | Cephalopharyngeal skeleton |
| DNA | Deoxyribonucleic acid |
| DNS | Deoksiribonukleïensuur |
| E | Esophagus |
| <i>et al.</i> | And others |
| EtOH | Ethanol |
| GC | Gastric caeca |
| HG | Hindgut |
| LCN | Low copy number |
| M | Mouth |
| MEP | Two month storage in 80% ethanol at 4°C, extracted using phenol/chloroform/isoamyl |
| MEQ | Two month storage in 80% ethanol at 4°C, extracted using QIAmp mini DNA kit |
| MFP | Two month storage at -80°C, extracted using phenol/chloroform/isoamyl |
| MFQ | Two month storage at -80°C, extracted using QIAmp DNA mini kit |
| MG | Midgut |
| µl | Microliters |
| mtDNA | Mitochondrial DNA |
| NA | Not applicable |
| NaCl | Sodium chloride |
| ng/µl | Nanograms per microliter |
| nm | Nanometer |
| PCI | Phenol/chloroform/isoamyl |
| PCR | Polymerase chain reaction |
| pH | Potential of hydrogen |

| | |
|-------|-----------------------------------------------------------------------------------|
| PMI | Post Mortem Interval |
| PSA | Prostate specific antigen |
| r | Rim of spiracular atrium |
| rpm | Revolutions per minute |
| SG | Salivary glands |
| SNP | Single nucleotide polymorphism |
| s.p. | Spinule |
| spp. | Species |
| STR | Short tandem repeat |
| t. | Tubercle |
| unpub | Unpublished |
| WEP | Two week storage in 80% ethanol at 4°C, extracted using phenol/chloroform/isoamyl |
| WEQ | Two week storage in 80% ethanol at 4°C, extracted using QIAmp mini DNA kit |
| WFP | Two week storage at -80°C, extracted using phenol/chloroform/isoamyl |
| WFQ | Two week storage at -80°C, extracted using QIAmp mini DNA kit |

CHAPTER 1

Literature Review

1.1. INTRODUCTION

In forensic entomology, necrophages are generally one of the most important insect groups. These insects appear in a predictable sequence and provides important information regarding the corpse itself such as if the corpse was moved, the Post Mortem Interval (PMI) as well as any manipulation of the corpse (Arnaldos *et al.*, 2005; Amendt *et al.*, 2011).

There are mainly two groups of insects of medicolegal importance namely Calliphoridae and Sarcophagidae, of which the latter was used during this study. The family Sarcophagidae comprises of around 3100 species (Vairo *et al.*, 2015), of which the species of interest in this study was *Sarcophaga cruentata*. A previous study on insect succession in the Free State (Kelly *et al.*, 2011) indicated that members of the family Sarcophagidae, including *S. cruentata*, are present at various stages of decomposition (see Figure 1-1). The family Sarcophagidae is a highly cosmopolitan family and can be found globally living in close proximity to humans (Byrd & Castner, 2010) especially in warmer climates.

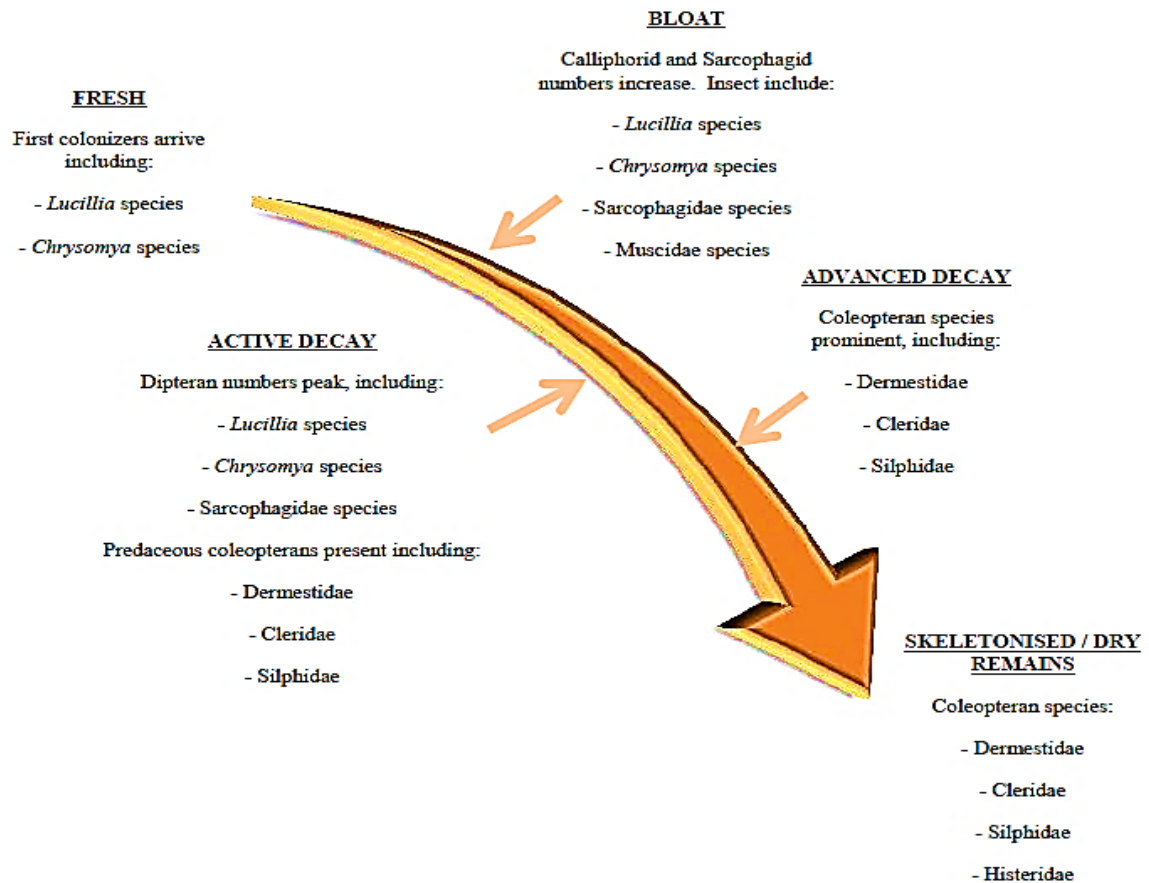


Figure 1-1: Schematic illustration indicating the most prominent insect families present at each stage of decomposition. Figure compiled from information in the work of Kelly *et al.*, (2011).

Insects can provide investigators with essential information that would normally be lost if the body was too degraded for traditional pathological investigational methods, for example providing information on any chemical entities present within the body at the time of death, and PMI estimation. Therefore, it is important to collect and store insect evidence with care to ensure further analyses of the samples are possible. With the integration of deoxyribonucleic acid (DNA)-based analysis methods in forensic entomology, it is essential to define optimal preservation methods to maintain the integrity of the samples for both morphological identification and DNA analysis (Di Luise *et al.*, 2008). Previous studies indicated that it was possible to obtain human or non-human DNA profiles from insect evidence through sensitive mitochondrial DNA (mtDNA) techniques under controlled laboratory conditions (Adams & Hall, 2003; Linville, *et al.*, 2004; Zehner *et al.*, 2004a; Di Luise *et al.*, 2008; Li *et al.*, 2011; Chávez-Briones *et al.*, 2013). It is however still uncertain whether or not the less sensitive, but more informative, short tandem repeat (STR)

profiling technique will be successful in obtaining a DNA profile from maggot gut content after the maggots fed on decomposing remains (Zehner *et al.*, 2004a).

When considering the possibility of obtaining DNA profiles from the gut content of maggots and finding standard protocols for the preservation and DNA analysis of such samples, it is important to consider the digestive tract of the insect. Little information is available on the morphology of *S. cruentata* due to difficulty in identifying the various species within the family Sarcophagidae. It has further been shown that the species and age of the maggot has an effect on successfully obtaining DNA from the gut content of maggots (Tantawi & Greenberg, 1993). As third instar maggots are preferred for DNA analysis of gut content due to the higher quantity of tissue present in the crop, it is important to understand the movement of food through the gut of the maggot (King *et al.*, 2008). Due to the difficulty in accurately identifying the species of interest (*S. cruentata*), investigators and entomologists have only recently started focusing on the importance of this species at crime scenes. As this species is normally seen as a tertiary fly, it has not received as much attention in a forensic context as other primary and secondary flies found at a crime scene.

1.2. DISSERTATION OUTLINE

This dissertation was written as a collection of individual articles. In **Chapter 1** general information is given pertaining to forensic entomology and how it combines with DNA analysis. It also focuses on the main points of the thesis namely the species of interest, *S. cruentata*; preservation of samples and subsequent DNA analysis and the time period in which DNA profiles can be obtained after the maggots have been removed from its food source. **Chapter 2** focuses on the effect the preservation method had on preserving the integrity of the maggot samples in terms of the morphological condition. It further investigated the possibility to extract DNA from the crops of the maggots using different extraction methods. This chapter also aimed to provide information on the unusual phenomenon of increased DNA analysis success rate with storage of samples over longer periods of time, especially at colder temperatures. **Chapter 3** provides information on the morphology of and digestion in the maggot alimentary canal with special reference to the movement of food through the gut. In **Chapter 4** the research aimed to determine the time

period in which usable DNA profiles could be obtained from the crop after maggots were removed from the food source for increasing periods of time. The result might provide an alternative explanation for the overall low success rate of crop content DNA typing. To bring all the chapters together as a comprehensive unit, **Chapter 5** provides a summary of all the data and findings of the dissertation.

1.3. THE SPECIES SARCOPHAGA CRUENTATA

1.3.1. Morphology

Adult *S. cruentata* (Figure 1-2) have a size range between 8 – 14 mm. The bodies are black but, due to being covered in a white powder, the flies appear to be grey. Although Sarcophagidae and Calliphoridae species are similar in size, Sarcophagidae do not have a metallic coloration. On the thorax there are three black longitudinal lines with the abdomen having a checkerboard appearance. The bodies are abundantly bristled and the eyes are widely separated and bright red in both sexes. In contrast to Calliphorids, the antennae of *S. cruentata* are only plumose at the base and not throughout the whole antenna. *S. cruentata* is also known as the red-tailed flesh fly. This refers to the tip of the abdomen which is bright red. Although it is referred to as the tip of the abdomen this red area is in actual fact external genitalia (Byrd & Castner, 2010). *Sarcophaga cruentata* maggots are usually much larger than that of other fly species present on remains due to the difference in the life cycle.

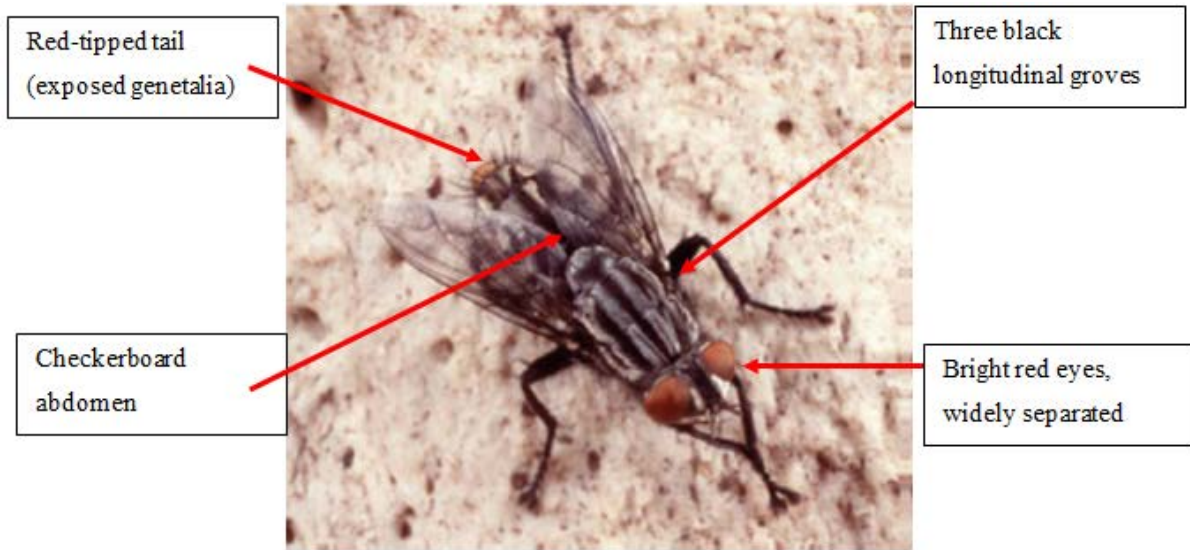


Figure 1-2: Morphology of the adult *Sarcophaga cruentata* (Meigen) (extracted from Byrd & Castner, 2010).

One of the most distinguishing morphological characteristics used to differentiate *S. cruentata* maggots from other fly families is found at the posterior ends of the maggot body. The abdomen of the *S. cruentata* maggots' end in a deep depression in which the posterior spiracles are located. The rim of the depression is lined with fleshy tubercles which is a further distinguishing factor (Figure 1-3). Its larger size also makes the maggots readily detectable in wounds in cases of myiasis (Aspoas 1991; Byrd & Castner, 2010). A study by Aspoas (1991) identified some characteristics of the larvae that can be used to differentiate between the third instars of some of the *Sarcophaga* species. Some of these features include the spinules of the third body segment, the anterior spiracles, the rim of the spiracular atrium and the tubercle.

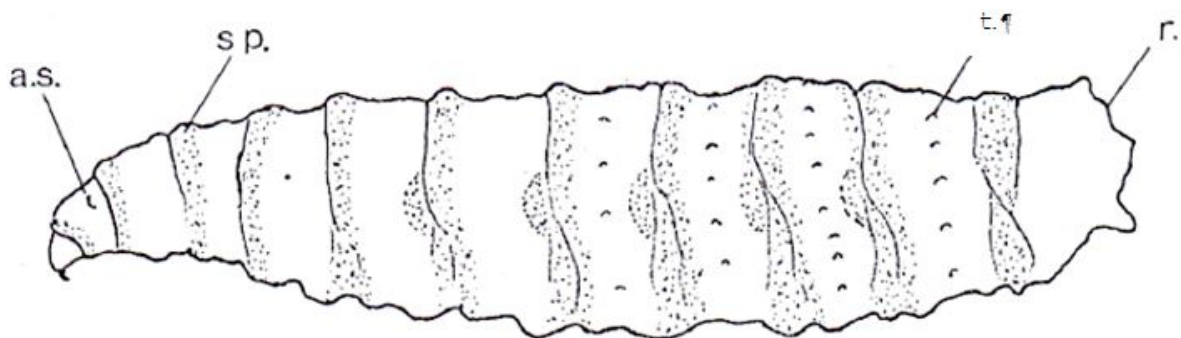


Figure 1-3: Third instar maggot of the family Sarcophagidae, indicating the location of some morphological characteristics used to differentiate between some species; a.s. = anterior spiracles, r = rim of spiracular atrium, t = tubercle, s.p. = spinule (Extracted from Aspoas 1991).

1.3.2. *Sarcophaga cruentata* Larval Alimentary Canal Morphology and Digestion

Very little work has been done on the internal morphology of many species including *S. cruentata* (Buenaventura 2013). As seen in Figure 1-4 (a), it was believed that the digestive system of insects was simply a single tube moving from the mouth to the anus in a straight line.

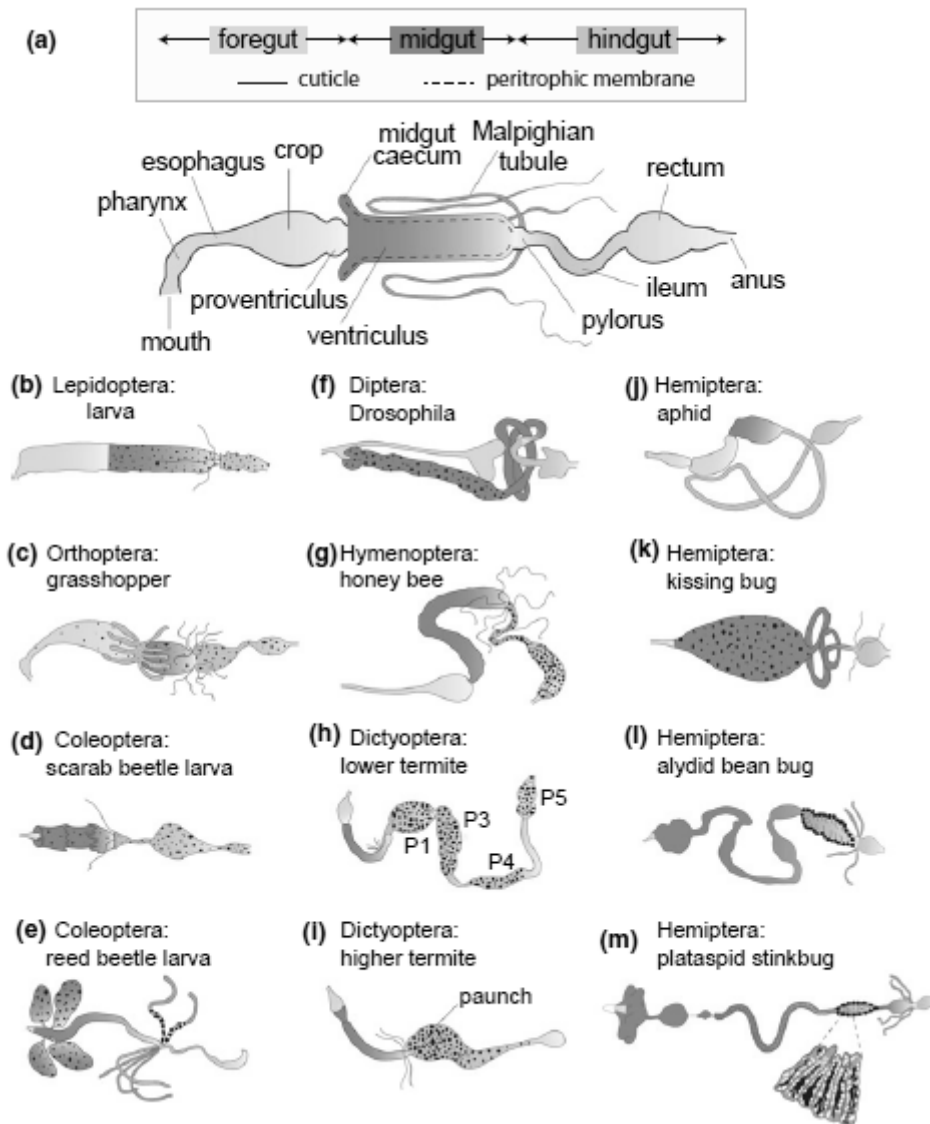


Figure 1-4: Insect alimentary canal structure. (a) Generalized structure of the insect alimentary canal indicating the different parts of the digestive system. (b - m) Structural diversity of some of the insect orders. If not clearly indicated in the picture description as “larval”, the gut portrays that of an adult insect. Stippling on some pictures is indicative of the presence of gut microorganisms (Engel & Moran, 2013).

This theory was changed after studies found that the digestive systems of insects are much more complex in structure and function than initially thought (Boonsriwong *et al.*, 2007). Amongst others, species investigated included the botfly *Dermatobia hominis* (Evangelista & Leite, 2003) and the blackfly *Simulium pertinax* (Cavados *et al.*, 2004). Latest research indicates, depending on the order as well as the species, the digestive system of insects is variable in structure and internal morphology (Figure 1-4 [b] to [m]). Each digestive system is adapted to the type of feeding behavior of the species.

When performing gut content analysis it is important to consider the morphology of a maggot, especially the maggot digestive system (Figure 1-5).

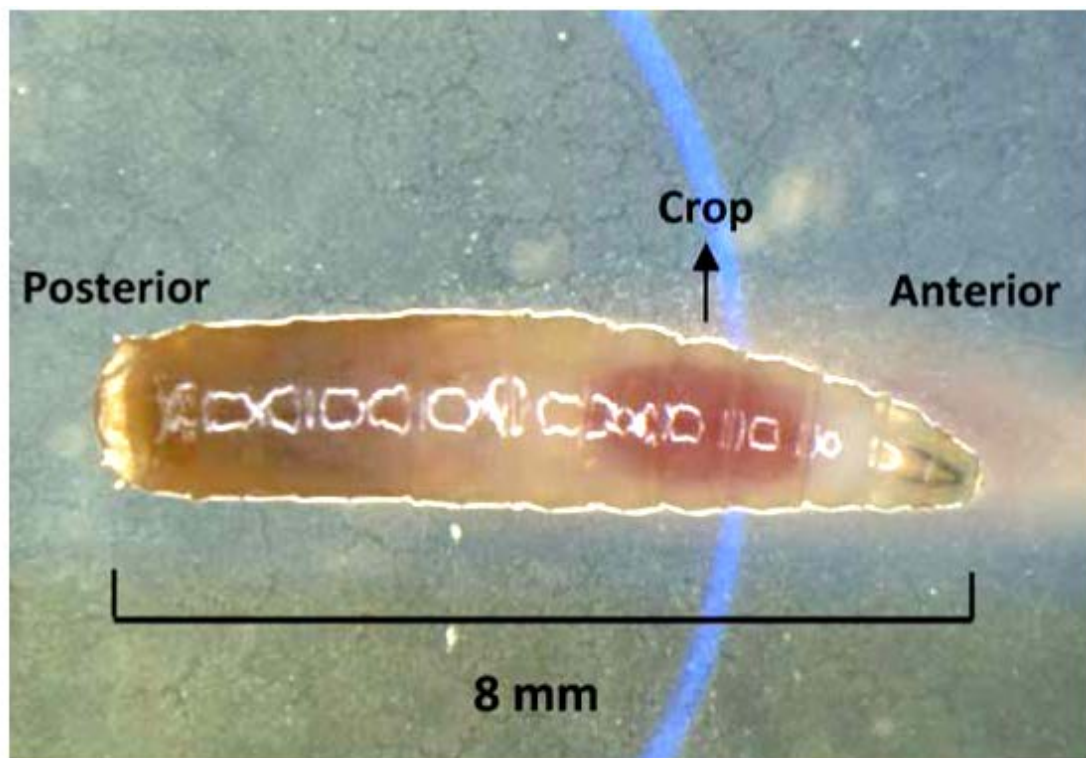


Figure 1-5: Crop content of a maggot (Kondakci *et al.*, 2009).

The insect gut is a long, continuous, convoluted tube that stretches from the mouth to the anus. It is normally about three times the length of the insect body and passes through various regions containing valves and sphincters as it moves from the anterior mouth to the posterior anus (Caetano *et al.*, 2006). As with the digestive system of vertebrates, the insect

gut shows complexity in structure and function, with specialized regions for the digestion and absorption of nutrients, regulation of the insect hemolymph ionic composition and pH, detoxification of the insect body and the production of pheromones (Greenberg & Klowden, 1972; Boonsriwong *et al.*, 2007). The maggot gut is divided into three main areas namely the foregut (stomodaeum), midgut (mesenteron) and hindgut (proctodaeum) (Bursell 1970; Spit *et al.*, 2012). The foregut consists of the mouth (M), crop (C), esophagus (E), cardia (Ca), gastric caeca (GC) and salivary glands (SG). The foregut forms the inlet to the midgut or stomach of the larvae. Some softening of the food and/or digestion may also occur in the foregut. Most of the digestion of the food and some absorption of nutrients occur in the larval midgut (MG). The hindgut (HG) forms the storage area for used food material until this is excreted from the maggot body as well as aids in most of the absorption of nutrients and reabsorption of water and essential ions (Figure 1-6; Figure 1-7) (Snodgrass 1935; Caetano *et al.*, 2006).

As seen in Figure 1-6 the gut is a very long structure and it is clear that most of the gut would not fit into the maggot body if it were to be in a straight line. It is therefore coiled and tangled inside the maggot body in order for the whole system to fit inside the small body (Figure 1-7).



Figure 1-6: Dissected alimentary canal of the third instar maggot of *Chrysomya megacephala* (Diptera: Calliphoridae) showing the different components of the larval digestive system (Boonsriwong *et al.*, 2011). Abbreviations: C, crop; Ca, cardia; E, esophagus; GC, gastric caeca; HG, hindgut; M, mouth; MG, midgut; SG, salivary glands. The alimentary canal is divided into three areas – line 1 indicates the boundary between the foregut and midgut while line two indicates the boundary between the midgut and the hindgut (Boonsriwong *et al.*, 2011).

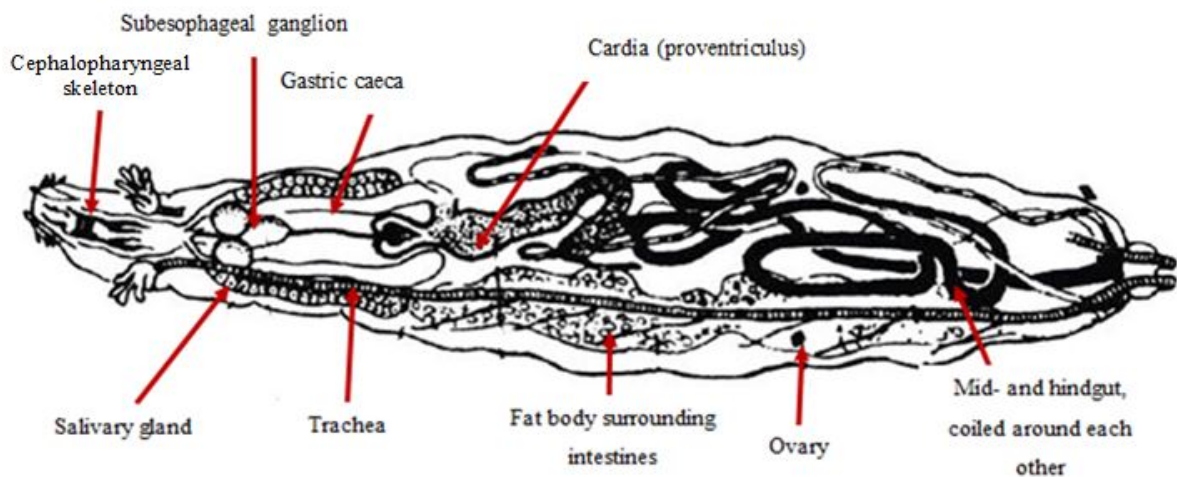


Figure 1-7: Illustration depicting the positioning of the alimentary canal within a *Drosophila* maggot body before dissection (Adapted from Diegelmann *et al.*, 2013).

1.3.2.1. Foregut (Stomodeum)

The foregut is derived from the ectoderm of the body surface and also contains mainly chitin and proteins (Bursell 1970; Spit *et al.*, 2012). When the maggot undergoes molting, the old cuticular lining is sloughed off into the gut where it is digested. Any pieces that are not digested will be excreted in the feces.

The foregut consists of a singular tube divided into different sections as mentioned above. The salivary glands are responsible for the production of the insect saliva and opens into the buccal cavity especially if, as is the case with *S. cruentata*, saliva is excreted onto the food to partially digest it before being consumed by the maggot. Therefore, even though the main function of the foregut is to serve as an access point for the food into the midgut, a great deal of mechanical breakdown as well as some chemical breakdown of food occurs in this part of the maggot gut (Bursell 1970).

In *S. cruentata* as well as other Dipteran species, the foregut develops a diverticulum which forms a much enlarged crop. As food enters the foregut it is either directed into the midgut or to the crop through peristalsis. The crop is in most cases separate from the main food passage (Bursell 1970; Greenberg & Klowden 1972) and mainly plays a role in the storage of food. No digestion occurs in the crop. When fully fed the crop of a maggot will dominate the anterior part of its body. The posterior part of the foregut ends in the cardia (proventriculus). This structure may be muscular and highly sclerotized or may simply form a valve that regulates the entry of food into the midgut (Nation 2008). The posterior part of this structure is also responsible for the production of the peritrophic membrane that lines the midgut (Boonsriwong *et al.*, 2007).

1.3.2.2. Midgut (Mesenteron)

The midgut forms the largest portion of the maggot digestive system and is of epidermal origin (Caetano *et al.*, 2006; Spit *et al.*, 2012). Digestion primarily occurs in this region therefore the midgut has various structural and physiological adaptations including differences in the pH of different parts of the midgut, compartmentalization and variation in the redox potential throughout the midgut. It is composed of a very long, straight tube with numerous blind pouches or caeca. The length is a characteristic of the high protein diet of

Sarcophagidae larvae, allowing longer retention of the protein meal and thus ensuring it is processed more fully (Elmelegi *et al.*, 2006). The midgut emerges from the junction between the gastric caeca and the cardia. The midgut is convoluted and twisted around itself (Hobson 1931) as seen in Figure 1-8. The anterior part of the midgut is responsible for the production of digestive enzymes and in some instances the posterior part of the midgut is a major site for the absorption of some nutrients.

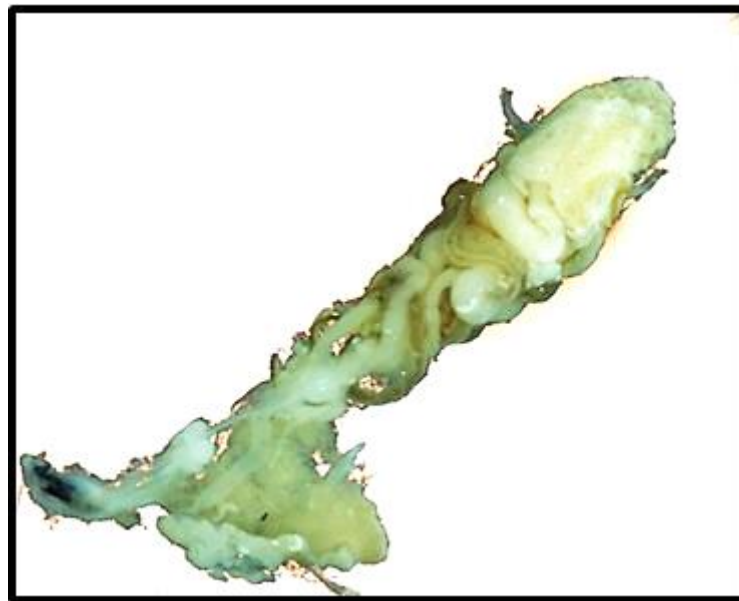


Figure 1-8: Dissected alimentary canal of a third instar *Sarcophaga cruentata* maggot showing the structures coiled around each other before being untangled (4x optical zoom). (Photo by A.M. Barnard).

1.3.2.3. Hindgut (Proctodaeum)

As with the foregut, the hindgut is derived from the ectoderm and is therefore sloughed off during each molt. The main function of the hindgut is to store indigestible remains of food until it is excreted. Some absorption of water and minerals also occurs in the hindgut (Bursell 1970). As with the other parts of the alimentary canal the hindgut consists of different parts namely the pylorus, Malpighian tubules, ileum, colon and rectum and anus (Martoja & Ballan-Dufrançias, 1984; Boonsriwong *et al.*, 2007). The beginning of the hindgut is marked by the Malpighian tubules (Boonsriwong *et al.*, 2007; Nation 2008).

Little information is available on the morphology and histology of *S. cruentata*'s gut. Thus far the gut of *S. cruentata* seems to share morphological and histological similarities with that of *C. megacephala* (Diptera: Calliphoridae) (Sukontason *et al.*, 2014). As Sarcophagidae and other fly larvae do not have any mandibles and can only consume liquefied food, the larvae regurgitate digestive enzymes onto their food medium. This aids in the partial digestion of the tissue prior to consumption. Although no digestion occurs within the crop, the tissue content may get degraded to some extent. Degradation may occur because of the biochemical alterations that occur as a result of digestive enzymes in the saliva of the maggot that is secreted onto the food before consumption (Clery 2001; Zehner *et al.*, 2004a; Kondacki *et al.*, 2009). The extent of the degradation has not been thoroughly investigated, but it is thought to not have such an immense effect on the quality of the ingested material. Thus far most of the degradation of the tissue consumed is thought to be due to the decomposition of the tissue prior to ingestion by the maggot. It remains essential to preserve the maggot effectively to prevent the possibility of any further degradation after the maggot has been killed (Wells & Stevens, 2008; Chávez-Briones *et al.*, 2013). Effective preservation of the maggot also aids in preserving the internal digestive system increasing the possibility of successfully obtaining a DNA profile from the maggot gut content.

Maggot digestive systems are highly differentiated, secreting numerous digestive enzymes during feeding including proteases, lipases and collagenase. The distribution of gut bacteria also differs between orders as indicated by the stippling (Figure 1-4). These microorganisms play an important role in the feeding and digestive behavior of insects. Some serve as actual food for the insects they colonize, others supply essential secondary substances to their hosts while some aid in the digestion of food consumed by the host through their activities (Hobson 1931; Engel & Moran, 2013). Digestion occurs in two stages: *in situ* and *in vivo*. As maggots only have mouth hooks, these insects secrete enzymes onto the decomposing tissue which causes it to liquefy (Hobson 1931; Greenberg & Kunich, 2002). Unlike many other species such as mammals, when maggots feed, the tissue consumed does not go directly to the digestive tract. While a maggot feeds, from emerging until it reaches the third instar, the tissue consumed is digested slower than it is consumed causing the excess to be stored in the crop of the maggot until it reaches the end of the third instar stage. A study by Greenberg & Kunich (2002) indicated that the food will move through the maggot digestive system at a

rate of two millimeters per minute. During the last day of feeding the crop of the maggot will double in size and be visible through the cuticle of the maggot as a dark red oval (Figure 1-5).

1.3.3. Forensic Importance

The Latin name Sarcophagidae translates to “flesh eating”. This more likely refers to the maggots of this family, since the adults are commonly found on flowers, attracted to and feeding on the nectar, sap and honeydew of the plant. The maggots on the other hand feed on carrion, excrement and exposed meat of mammals making it of veterinary, medical, sanitation and forensic importance (Byrd & Castner, 2010). Even though adults of *S. cruentata* mainly feed on nectar the flies are attracted to decomposing remains in order to breed. As other Sarcophagidae species, *S. cruentata* is mostly associated with small carrion but are known to be associated with human corpses as well (Aspoas 1991).

Unlike many Calliphoridae species, *S. cruentata* are attracted to carrion under most environmental conditions including sun, shade, dry, wet and indoor conditions (Aspoas 1991). *Sarcophaga cruentata* are commonly classified as a tertiary fly but this does not mean the flies will always be the last to arrive at carrion. On the contrary, this species is associated with carrion throughout all stages of decomposition. Furthermore since these flies are large and strong *S. cruentata* are in many instances the first to colonize carrion especially if environmental conditions, such as strong winds, prevent other flies from immediately reaching the carrion (Wells *et al.*, 2001; Byrd & Castner, 2010). This fact makes this species of great forensic importance as these flies may provide a more accurate indication of the PMI being the first to arrive at a body in certain conditions. According to Wells *et al.* (2001), maggots from this species may further be the only ones found on a body that is physically isolated e.g. covered by garbage. The large size of the maggots further increases the possibility that investigators will collect *S. cruentata* maggots at a crime scene, especially if collection is done by someone with no entomological background and experience in identifying the different families of flies (Wells *et al.*, 2001).

Since these flies are of such interest in the field of forensic entomology, more research is required to obtain information regarding this family. Lately more emphasis has been placed on Sarcophagidae especially in a forensic sense for the determination of PMI. The use of *S. cruentata* for determination of PMI has been greatly hampered. Reasons for this include difficulty in identification down to species level leading to time-consuming breeding, which can be unsuccessful or impossible if no live maggots were collected (Cherix *et al.*, 2012; Jordaens *et al.*, 2013; Fremdt & Amendt, 2014). Furthermore, control specimens must be collected from a 10 m diameter around the body to determine the natural species diversity in the area. A cause of this wide dispersal of maggots is due to females being unable to deposit maggots directly on the corpse, thus dropping maggots in the vicinity of the corpse. Maggots can further disperse such great distances from the corpse while searching for proper areas to pupate (Jordaens *et al.*, 2013). Lastly, there is a clear lack of information on the feeding and breeding biology of *S. cruentata* as well as other Sarcophagidae species (Cherix *et al.*, 2012; Jordaens *et al.*, 2013).

1.4. GUT CONTENT ANALYSIS

Analysis of gut content has been used in other frameworks of entomology. DNA typing of insect gut content has previously been used to examine predator-prey relationships through identification of host material in an insect predator's gut (Gagnon *et al.*, 2005). Recently the focus has shifted to using gut content analysis in forensic entomology to determine the food source, on which a maggot found at a scene, developed. Maggots will feed on decomposing tissue from the time they hatch until the end of the third instar stage. It is important to remember that one cannot assume that all of the maggots' development occurred on the substrate it was found on, as situations exist where a maggot will leave one substrate and continues feeding on another. In homicide cases determining the insect-corpse association is important (Sharma *et al.*, 2015). As mentioned, *S. cruentata* maggots are in some cases the only species present on a body, especially in instances where the body was isolated (Wells *et al.*, 2001). Gut content analysis could also be useful when working with cases of myiasis. Myiasis-causing Sarcophagidae larvae have been associated with enteric, oral, nasal, urogenital, aural and cutaneous secondary human myiasis (Dutto & Bertero, 2010). This makes the maggots' good indicators of neglect of children and the elderly and useful in determining the period of neglect, especially in a hospital environment by looking at the age

of the maggots (Dutto & Bertero, 2010). Furthermore, if DNA can be extracted from the crops of myiasis-causing Sarcophagidae maggots, it might be possible to determine if the maggots only fed on the affected person or whether the maggots were transferred from another patient also affected by myiasis. This would be a further indication of the sanitary conditions within an institution (Dutto & Bertero, 2010).

Through DNA typing, the gut content's DNA can be compared to known samples of the victim or relatives of the victim to confirm identity (Campbossa *et al.*, 2005). DNA typing can further provide evidence that a maggot used for PMI estimation had in fact fed on the human remains it was collected from (Sharma *et al.*, 2015). In rape cases where pubic lice were transferred between the perpetrator and the victim, the gut content can also be very helpful in confirming victim-suspect interaction (Benecke 1998). DNA typing has previously been used to detect blood meals in hematophagous insects such as mosquitoes (Kreike & Kampfer 1999; Zaidi *et al.*, 1999), ticks, black flies, tsetse flies and lice (Zaidi *et al.*, 1999; Mumcuiglu *et al.*, 2004). Recent gut content analysis has also confirmed that prostate specific antigen (PSA) could successfully be typed from semen ingested by maggots that fed on a murdered rape victim (Benecke 1998; Clery 2001).

The use of DNA typing in gut content analysis is advantageous in that the techniques utilized for this type of analysis is widely known with many already being incorporated into some of the commercially available kits (Cuthbertson *et al.*, 2003). An added advantage is that DNA testing can be performed on any life stage of the maggot (Zehner *et al.*, 2004b). With this new method of investigation in forensic entomology, entomologists are faced with a dual goal when collecting and preserving entomological evidence: they are required to fix and preserve the morphological characters of the maggots while maintaining the molecular composition of the maggot and its gut content for further DNA analysis (Di Luise *et al.*, 2008).

1.4.1. Preservation and Storage of Maggots Collected at the Scene

A major factor that must be taken into consideration when preserving and storing maggots collected at the scene is the fact that DNA is easily degradable. In many laboratory experiments, samples are collected in the laboratory under optimal conditions where it can be processed and analyzed immediately. As biological evidence cannot always be analyzed immediately, it is important to find methods to preserve the maggot sample for as long as possible without affecting the integrity of the host DNA material in the crop or the morphology of the maggot itself (Straube & Juen, 2013). This is a challenge when working with the gut content of maggots as many of the preservation methods traditionally used by forensic entomologists will inhibit DNA analysis (Wells *et al.*, 2001). Furthermore some of the preservation methods suitable for DNA analysis lead to the degradation and desiccation of the sample.

Numerous DNA preservation methods have been verified as can be seen in Table 1-1.

Table 1-1: Summary of previous studies on the effect of preservation method on the morphology of the maggots and/or DNA yield from the maggot gut.

| Reference | Number of samples used | Preservation method(s) | Success/comments |
|--------------------------------|--------------------------------------|----------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Di Luise <i>et al.</i> (2008). | 8 to 10 maggots /batch* ¹ | Waterless 70% EtOH Hot water killed + -20°C | Hot water killed + -20°C provided best preservation. |
| Linville <i>et al.</i> (2004) | 77 (5 control, 3x24 groups) | 70% EtOH 24°C 70% EtOH 4°C 95% EtOH 24°C Kahle's 24°C Formaldehyde 24°C None 24°C None 4°C None -70°C | Most success from storage -70°C no preservation. More success in 70% and 95% EtOH at 24°C and 4°C than formaldehyde. If freezing not possible then EtOH storage preferred above formaldehyde. |
| Adams & Hall, (2003) | 180 | 10% Formalin 80% EtOH | 80% EtOH best overall preservation for morphology. |

| | | 95% EtOH | |
|------------------------------------|----|----------|-----------------------------------------------------------------------------|
| Amendt <i>et al.</i> (2011) | NA | NA | Lit discussion: Best preservation for later DNA analysis in 70 to 95% EtOH. |

*¹Number of batches unknown. EtOH = ethanol

Previous studies (Table 1-1) indicated that the best morphological preservation was achieved when samples were stored at -70°C in no preservation medium. This will lead to the maggot's physical appearance being less degraded providing a better sample for morphological examination and identification of the species, even if this analysis cannot be performed immediately after the sample was collected. If no freezing facilities are available, it is generally recommended that samples be stored in 70 to 95% ethanol both for morphological and DNA analysis. Adams & Hall (2003) found that the morphology of the maggot samples (*Calliphora vomitoria* and *Lucilia sericata*) was best preserved in 80% ethanol. Storage in a higher percentage of ethanol may prove more effective. Storage in ethanol removes water from the sample causing the ethanol to dilute to below sufficient levels required for effective preservation. Storing samples in a higher concentration ethanol prevents dilution to such low levels leading to better preservation of the sample.

Going hand in hand with the preservation method used is the method used to kill the maggot as this may also affect the physical appearance of the maggot and the ability to obtain DNA from the crop. The study of Tantawi & Greenberg (1993) confirmed that the appearance and length of maggots differed when it was placed live into preservatives compared to when it was first hot water killed before being placed in the preservation liquid. Their results indicated that in some instances placement of the maggot live into a preservative caused the maggot to shrink which, as indicated by other studies (Campbossa *et al.*, 2005, study on *Calliphora vicina*), may be due to dehydration. This led to the crop sticking to the maggot body and in some instances breaking during dissection.

In the study of Tantawi & Greenberg (1993), the percentage of shrinkage ranged from 3.2% when preserved in Kerosene to 30.8% when killed and preserved in formalin (depending on

the species¹). Overall the authors found that hot water killing greatly lessens autolysis of the maggots by destroying the digestive enzymes as well as the gut flora of the maggots. Hot water killing further causes the maggots to be fully extended as is the case with a live maggot allowing more accurate age determination. Sharma *et al.* (2015) indicated that an excellent method for preserving maggots was to blanch the maggots in near-boiling water for 60 to 120 seconds, whereafter the maggots must be placed in 80% ethanol. Unfortunately many published studies only mention that samples were preserved without giving more information on the solution(s) used (Klipatrick 2002; Day & Wallman, 2008).

As no standard protocols exist for the preservation of maggot samples to be used in both morphological identification and DNA analysis, this study investigated different preservations methods and its effect on the integrity of maggot morphology and host DNA material in the maggot crop. The objective was to determine which preservation method is most optimal for preserving the external morphology, integrity of the internal maggot structures for dissection as well as the integrity of host material in the maggot crop for DNA typing.

1.4.2. DNA Analysis

As it is already difficult to obtain a full or partial DNA profile from degraded biological samples, it is essential that the sample e.g. maggot crop, be as well preserved as possible. Further factors that must be taken into consideration when working with maggot gut content include the quantity of crop content at the time the maggot is killed. This is why it is preferred to use third instar maggots as this is when maggots are at its peak feeding time. Furthermore it is important to take into consideration the quantity and quality DNA present after the sample was stored for a period of time and DNA extraction and purification of the DNA sample was performed (King *et al.*, 2008).

¹ Tantawi & Greenberg's results of the effect of the killing and preservation method on the length of third instar *P. terraenovae* at peak feeding.

Previous studies have employed the use of various forms of DNA typing including mitochondrial DNA (mtDNA) analysis, single nucleotide polymorphism (SNP) analysis and short tandem repeat (STR) analysis with varying success. The success of STR typing and mtDNA analysis seen in previous studies is summarized in Table 1-2.

Table 1-2: Summary of previous studies on the effect of preservation method on the DNA yield using two types of analysis - STR typing and mtDNA analysis – from DNA extracted from the gut content of maggots.

| Reference | Number of samples used | Preservation method(s) | Extraction method(s) | Type of analysis | Success |
|-------------------------------------------|---------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------|-----------------------|------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Zehner <i>et al.</i>, 2004a | Unknown (13 corpses/cases) | 70% EtOH | Phenol/ Chloroform | STR mtDNA | STR: 7 complete, 2 incomplete, 4 failed mtDNA: successful even if STR fails |
| Linville <i>et al.</i>, 2004 | 77 (5 controls, 3x24 groups) [Thus 9 maggots per method tested.] | 70% EtOH 24°C 70% EtOH 4°C 95% EtOH 24°C Kahle's 24°C Formaldehyde 24°C None 24°C None 4°C None -70°C | QIAGEN DNA easy | STR mtDNA | STR: Best results: -70°C (none) 2 weeks: 83% 8 weeks: 100% 6 months: 100% 2 nd best: 70% EtOH 4°C 2 weeks: 50% 8 weeks: 67% 6 months: 67% mtDNA: 2-8 weeks 100% for all EtOH and none 6 months: 50-100% for all EtOH and none |
| Li <i>et al.</i>, 2011 | Unknown | -70°C (none) | Unknown | STR mtDNA | STR: Complete profiles for all specimens mtDNA: successful for all samples. |
| Chávez-Briones <i>et al.</i>, 2013 | 3 maggots collected from a burned corpse | 70% EtOH at 4°C | Phenol- Chloroform | STR | All 3 provided incomplete profiles but could be used to identify the remains through familial testing. |

Various studies investigated the effect of preservation method on the chance of successfully extracting and obtaining DNA profiles through STR typing and mtDNA analysis of maggot gut content (Table 1-2). The study by Linville *et al.* (2004) provides experimental proof of this effect. During this study maggot samples were stored in different preservation mediums

for two weeks, eight weeks and six months where after either STR typing or mtDNA analysis was performed. When looking at the results of the study, the best results for STR typing was observed if samples were stored at -70°C in no preservation liquid. The second best preservation was seen in samples stored in 70% ethanol at 4°C which is ideal if freezing facilities are not available (Linville *et al.*, 2004). When taking the results of the STR analysis into consideration it can be seen that the success rate increased after the samples were stored for a longer period of time, especially if kept under colder conditions such as -70°C (Linville *et al.*, 2004). This was also observed in a study by Durdle *et al.* (2011).

The effect of the storage medium on the inactivation of bacterial growth and enzymatic actions was suggested as a possible explanation. With colder temperatures such as -70°C , these processes are stopped much faster than at warmer temperatures leading to less breakdown of the DNA in the maggot crops. This is also apparent when looking at the study of Linville *et al.* (2004). Maggots stored in 70% ethanol at 4°C had more degradation of the crop content leading to higher STR typing failure. Since the time required for complete inactivation of the enzymatic processes was longer, the DNA results did not increase as much as the results from the maggots stored at -70°C over the six month storage period. The study also noted that there was no noteworthy improvement in the physical appearance and DNA quality of maggots stored at 4°C when compared to those stored at room temperature. This further supports the reasoning that colder storage will improve the quality of DNA results especially in instances where one works with very low quantities of DNA, such as that found in maggot crops.

As DNA is prone to degradation during transport from the scene to the laboratory, the use of FTA® paper has been suggested due to the fact that samples can be stored at room temperature without degradation of the sample. Harvey (2005) indicated that this was indeed a possibility after obtaining good results using this storage method for analysis of larval, pupal and adult samples (Calliphoridae species). A further consideration includes the effect of starvation on successful DNA typing of maggots, as was further investigated by Njau *et al.* (2016). No DNA profiles could be obtained through DNA typing of maggots

starved for four days or more. Overall, Njau *et al.* (2016) concluded that DNA quantity decreased over time.

Although previous studies indicate that it is possible to successfully perform DNA typing on maggot gut content, results still vary greatly with regards to success rate. Furthermore, the study by Linville *et al.* (2004) did not take into account the effect of photoperiod on the development of maggots as maggots were bred in 24 hour light conditions. Therefore the current study implemented a photoperiod of 12 hours light:12 hours darkness to imitate more natural conditions. Additionally, in the study by Njau *et al.* (2016), the effect of starvation had only been investigated up to days after removal from food source. By assessing the effect of starvation on DNA typing success of crop content by looking at hours instead of days, crop content DNA typing could possibly be used as an alternative to time consuming breeding for PMI estimations.

Standard protocols for the preservation of maggots and subsequent DNA extraction must still be developed as results vary greatly between laboratories as well as species. Furthermore, the time period during which DNA can be successfully typed from the guts of maggots after being removed from its food source has not yet been fully determined (Zehner *et al.*, 2004a; Zehner *et al.*, 2004b; Sharma *et al.*, 2013). This can have serious implications for further studies on DNA typing from gut content, as it may offer an explanation for situations where DNA typing fails. In such cases it is necessary that one be able to determine whether or not a negative result is because the maggot did not feed on a human corpse or because the maggot has been removed from its food source for a long enough period for all of its crop content to have been digested (Zehner *et al.*, 2004a; Zehner *et al.*, 2004b).

1.4.3. Sample Quantity and Quality

When working with gut content very small DNA quantities can be expected. As mentioned, DNA is very sensitive to degradation. In maggots, this is even more apparent as maggots feed on decomposing tissue. As maggots regurgitate digestive enzymes, this degraded tissue

is further exposed to more degradation, decreasing the amount of DNA for analysis. After maggots have been collected from the decomposing body it is thus essential that the maggot be killed immediately to prevent the maggot from digesting the crop content, as the crop will be emptied completely 24 hours after feeding has seized (Wells & Stevens, 2008).

Various challenges are encountered when analyzing and interpreting samples of low quantity and quality. For instance, peak height imbalance occurs due to primers that do not bind equally for each allele at a locus during the first few polymerase chain reaction (PCR) cycles. Sometimes both alleles may be lost completely (Butler & Hill, 2010). The degree to which the stochastic effect will occur is indirectly related to the number of molecules present in the template sample used for the PCR reaction (Budowle & Van Daal, 2009). Stochastic effects include allelic peaks below the detection thresholds, difficulty in profile reproducibility and varying degrees of stutter product in DNA profiles. For this reason it is essential to develop accurate and thoroughly validated extraction and analysis methods when analyzing the gut content of maggots, in order to limit the degradation of DNA during analysis.

As DNA typing of low quantity and quality samples can produce profiles which are difficult to analyze, various methods have been suggested to improve success, including increasing the number of PCR cycles; reducing the reaction volume of the PCR; whole genome amplification prior to the PCR; post-PCR clean-up to remove ions that compete with DNA during electrokinetic injection and concentrate the sample for analysis; and increasing electrophoresis injection time (Budowle *et al.*, 2009; Budowle & Van Daal, 2009).

As with all methods, limitations for DNA typing of low quantity and quality samples exists which impact analysis of maggot samples. Due to the small volume of DNA contained in degraded samples and the high sensitivity of PCR amplification, any background DNA as well as DNA from casual contact (contamination) may be detected. Therefore profiles emerging from this analysis may not necessarily pertain to the case being examined. Interpretation guidelines therefore need to be clearly defined (Conti-Vecchiotti Report; Budowle *et al.*, 2009).

1.5. STUDY RATIONALE

As biological evidence cannot be analyzed immediately, it is important to find methods to preserve the sample for as long as possible without affecting the integrity of the sample. This is a big problem when working with the gut content of maggots as many of the preservation methods traditionally used by forensic entomologists will inhibit DNA analysis (Wells *et al.*, 2001). Other preservation methods suitable for morphological analysis lead to the degradation and desiccation of the sample material, making it unsuitable for DNA analysis. As it is already difficult to obtain a full or partial DNA profile from degraded DNA, it is essential that the sample e.g. maggot crop be as well preserved as possible. Further factors that must be taken into consideration include the quantity of DNA present in the crop at the time the maggot is killed as well as the quantity, and quality of DNA present after the sample was stored for a period of time and DNA extraction and purification of the DNA was performed (King *et al.*, 2008).

Standard protocols must be developed for the preservation of maggots and subsequent DNA extraction as laboratory results vary greatly between laboratories as well as between different species. Furthermore, the time period during which DNA can be obtained after a maggot was removed from its food source has not been determined yet (Zehner *et al.*, 2004a; Sharma *et al.*, 2013). This can have serious implications for further studies on the extraction of DNA profiles from gut content as it may offer an explanation for situations where DNA typing fails.

As DNA analysis can be quite expensive to perform and it is never guaranteed that one will obtain a profile from the gut content, another question that must be answered is whether there is a possibility to follow the movement of food through the digestive tract of the maggot by making use of light microscopy. If a link could be established between the visual movement of food and the quality of DNA at various parts in the digestive tract of the maggot, one could determine whether or not DNA analysis of the gut could be successful or whether DNA analysis should not be attempted as it would be a waste of money.

1.6. AIMS OF STUDY

The aims of the study were to:

1. Determine which of the tested preservation methods was optimal for preserving the morphology of the maggot as well as the integrity of the DNA material in the crop.
2. Investigate the morphology of the maggot digestive system focusing mainly on movement of food in the midgut.
3. Determine the period of time DNA profiles could be obtained after a maggot had been removed from its food source and starved, in order to provide an alternative explanation for the failure of STR typing in some instances.

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CHAPTER 2

Effect of Preservation

2.1. INTRODUCTION

The first paper on the proper collection and preservation of entomological evidence was published in 1983 (Sharma *et al.*, 2015). Since then many advances has been made in the field of forensic science. A basic understanding of insect anatomy and biology is essential for the collection, and subsequent preservation and analysis of insect evidence (Sharma *et al.*, 2015). However, in the past 10 to 15 years researchers have also begun to examine the possibility of using DNA analysis techniques to analyze the gut content of sarcophagus maggots (Chapter 1, Table 1-1). Since sarcophagus maggots develop on decomposing remains by consuming the tissue, some of the host's tissue and DNA will be found in the gut content of the maggots. Saliva containing digestive enzymes is secreted onto the food source to aid in liquefying the food before consumption, leading to some degree of breakdown during consumption of the tissue (Greenberg & Kunich, 2002; Carvalho *et al.*, 2005). No further digestion occurs in the crop. This organ functions only as a storage area for excess food not necessary for the daily development of the maggot until the maggot reaches the third instar, enters the wandering phase and starts to digest the stored food in order to complete its development (Zehner *et al.*, 2004a,b; Campbossa *et al.*, 2005; Li *et al.*, 2011). As food enters the foregut it is either directed into the midgut or to the crop through peristalsis. The crop is in most cases separate from the main food passage (Bursell 1970; Greenberg & Klowden, 1972). Third instar maggots are considered to be the best source of host DNA when performing gut content analysis since the insects are feeding at its peak at this time (Li *et al.*, 2007). Research into this area has been limited mostly due to a lack of standard protocols (King *et al.*, 2008).

A critical factor when performing DNA analysis of the crop content of maggots is to use the most optimal methods for the collection and treatment of the sample prior to DNA analysis (Straube & Juen, 2013). Traditionally, entomological evidence is collected and preserved to

ensure species identification of the insect as well as the developmental stage based on, amongst other factors, the length of the maggot for use in accurate PMI estimation. This has to be done without affecting the morphology of the specimen as the study of morphological characteristics is the main method used for identification (López-Esclapez *et al.*, 2014). With various factors affecting the post mortem integrity of the maggot morphology, one of which is the preservative used (Adams & Hall, 2003), it is essential to ensure that a specimen is preserved as optimally as possible. This is also true if DNA typing of the gut content is also to be performed. As evident from literature, gut content analysis can be utilized in order to associate a maggot with a body (Chávez-Briones *et al.*, 2013). This could aid in identifying the remains if it was too badly decomposed for traditional methods of identification to be used (Chávez-Briones *et al.*, 2013). Entomologists are consequently faced with a dual goal when collecting entomological evidence: the specimens should be preserved in such a way that the morphological characteristics of the maggot remain intact for identification but also use a preservative that would maintain the integrity of the host's tissue and thus DNA in the maggot gut (Di Luise *et al.*, 2008).

As most of the samples in forensic entomological cases are collected in the field, it is essential to avoid or limit the loss of sample quality. Some of the methods for preservation of maggot specimens include freezing, drying out specimens, and preservation in ethanol or buffer solutions such as formalin and Kahle's solution (Linville *et al.*, 2004; Campbossa *et al.*, 2005; Straube & Juen, 2013; Sharma *et al.*, 2015). As it is not practical for investigators to dissect and remove the maggot crop at the scene, proper preservation methods is essential (Linville *et al.*, 2004). This will prevent further degradation of DNA in the crop of maggots, due to continuing action of digestive enzymes as well as prevent the loss of gut content due to crop emptying (Wells & Stevens, 2008). Many of the preservatives traditionally used by entomologists however damage the host DNA in the tissue present in the gut content and hamper downstream analysis of the samples (Wells *et al.*, 2001). When deciding on a suitable preservation method one should keep in mind that the method chosen should be practical. The preservative should be readily available, easy to work with, non-toxic and personnel without extensive training should be able to work with it if following appropriate instructions. This is especially important in instances where the entomologist cannot go the scene of the crime for collection (Day & Wallman, 2008). The preservative

must also not alter the physical characteristics of the maggot e.g. cause shrinkage, dehydration or extension. Dehydration, for instance, will cause the crop to adhere to the maggot body and burst during dissection. The chemical properties of the preservative should also not impede any part of the molecular analysis (Klipatrick 2002; Adams & Hall, 2003; Campbossa *et al.*, 2005).

Linville *et al.* (2004) tested the effect of different preservation methods on successful DNA typing of the maggot gut content. Storage at -70°C in no preservatives proved to be the best method for DNA typing. They found that if freezing facilities were not available, as is the case when collecting specimens in the field, that storage in 70% ethanol was the second best option. In contrast, López-Esclapez *et al.* (2014) observed that specimens stored in 70% ethanol showed slight micro-wrinkles that decreased the quality of ultrastructural details of the maggots. Although the ultrastructure details are affected by ethanol storage, this method has been commonly used for successful storage of entomological specimens. Considering DNA analysis, Campbossa *et al.* (2005) found that the crops of maggots stored in 95% ethanol became very dehydrated and in most cases stuck to the rest of the internal organs. This led to the crops breaking during dissection, leading to the incorporation of the fatty tissue surrounding the alimentary canal into the DNA sample. This fatty tissue contains a high volume of lipids that inhibits downstream DNA analysis, especially in late third instar maggots (Campbossa *et al.*, 2005). Preservatives such as Kahle's solution and formaldehyde-containing solutions inhibit downstream analysis by degrading the high-molecular weight DNA.

An interesting observation that was made in previous studies (Linville *et al.*, 2004; Durdle *et al.*, 2011) is that the success rate of DNA typing increased after longer storage periods. Furthermore, it has been observed that storage in colder temperatures further led to better DNA typing results than storage at room temperature.

Another method that has been suggested is preserving gut content by spreading the gut content on FTA® paper which can be kept at room temperature, without any degradation to

the sample, and will be of practical use for collections made in the field (Harvey 2005). This is an easy and fast method to preserve the gut content of the maggot but will not shorten the processing time of the sample in the laboratory. Furthermore, spreading the gut content on FTA® paper in the field exposes the gut content to possible external contamination from the scene. As it is important to prevent contamination of the sample, this method may not be of practical use in the field.

If analysts are not able to obtain DNA from the crop of the maggot it cannot be assumed that the maggot did not feed on the remains. If no DNA is obtained from the crop it would be useful to know for how long after removal from food source, DNA could still be extracted. Further, as only the crop is currently used in DNA analysis, a further avenue for research is to determine whether or not DNA can be obtained from the mid- and hindgut of the maggot. As DNA analysis is very expensive it is also worthwhile to be able to determine the possibility of successfully obtaining DNA from the maggot gut before any analyses are performed. For instance, is it possible to externally follow food movement through the alimentary canal to determine time since removal from food source, then link this with the possibility of obtaining useful DNA from the crop? Situations exist where only the maggots but no remains of the victim is present at a crime scene. Therefore, to simulate such situations, maggots were removed from the food source in this study. This was done to investigate if it was possible to associate the gut content of a maggot to a specific victim. Quicke *et al.* (1999) suggested that the successful preservation of hymenopteran specimens depended on how fast the preservative could penetrate the specimen. This is especially advantageous when working with larger specimens. As *S. cruentata* maggots are usually quite large, the current study tested the theory that piercing of the maggot cuticle could aid in better preservation of the specimen. The specimens were pierced to test if this would allow more of the preservative to enter the cuticle, possibly leading to better overall preservation of the specimen.

2.2. STUDY RATIONALE

2.2.1 Part One: Preservation of Samples

For ease of analysis, this chapter was divided into two parts (part one and part two). In part one different preservation methods were tested in order to determine which was most optimal for preserving insect morphology and performing alimentary canal dissection. Furthermore it was determined in part one whether piercing the specimens before storage, and thus exposing more of the specimen to the preservative, increased the success of morphological preservation. In this part different preservation methods were tested including room temperature storage in 95% - 100% ethanol and 10% formalin, respectively.

Part one of the study investigated how preservation in ethanol (95% - 100%) and 10% formalin would affect the morphology of the maggot. Storage in 10% formalin has been widely suggested to be one of the best methods of preserving specimens for morphological analysis. This is also routinely used in various mortuaries to preserve maggot specimens (Day & Wallman 2008). As formalin-based preservation cannot be used for downstream DNA analysis of samples as it has been found to significantly decrease DNA recovery, it is necessary to find a preservative that has a similar preservation success with regards to morphology without inhibiting DNA typing of samples. For this reason ethanol was used as comparator to determine how successful ethanol preservation was compared to formalin.

Determining the most effective ethanol concentration was more complicated. For entomological preservation of the morphology of maggots it has been standard practice to preserve specimens in ethanol, with varying success. It has been suggested that successful preservation had been observed in ethanol concentrations ranging from 70% to 95% (López-Esclapez *et al.*, 2014). As part of my honors project which was used as a pilot study, results indicated degradation of the maggot specimens when stored in 70% ethanol (unpub.). As the preservative chosen had to both preserve the morphology of the maggot for entomological and for DNA typing purposes (part two), it was decided against using 70% ethanol and rather considering a higher concentration to be used. Taking this into account, and to determine the ethanol concentration to be used, the study turned to published literature on the matter.

Previous studies have suggested that DNA typing was most successful when samples were preserved in 80% ethanol (Adams & Hall, 2003; Day & Wallman, 2008). Whereas Amendt *et al.* (2011) suggested that storage in 70 – 100% ethanol could provide reasonable amplifiable DNA when samples are kept at low temperatures. As preservation in 70% ethanol had been excluded, the choice of ethanol concentration was narrowed down to 80% to 100%. This choice was made due to the success seen for both morphological analysis and DNA typing of samples stored in this concentration range. Taking into account that specimens were routinely collected and placed in up to 95% ethanol at crime scenes, as well as the fact that successful DNA typing could be achieved in samples stored in up to 100% ethanol, it was decided to investigate the effect of 95% - 100% ethanol on the morphology of the maggot specimens. It was of special interest to the current study to determine to what extent the desiccation effect of 100% ethanol would affect the maggot specimens as previous studies indicated breakage of internal structures at higher ethanol concentrations (Linville *et al.*, 2004). The study was also interested in how the effect would increase the difficulty of dissection as only the crops were used for DNA typing in this study.

2.2.2. Part Two: Effect of Sample Preservation on DNA Analysis

Part two was adjusted based on the results from part one. After part one was performed, the results indicated too high desiccation of maggot specimens when stored in 95% - 100% ethanol, leading to high difficulty with regards to dissection of the maggot crop. In many of the specimens the crop adhered to the maggot cuticle and broke when it was attempted to remove it from the maggot. The presence of ethanol in samples after DNA extraction is known to inhibit PCR-based analyses of samples (Bassetti 2007). Using a higher concentration ethanol for storage would displace more of the water within the maggot body, thus increasing the concentration of ethanol within the maggot *via* the process of osmosis. This would therefore cause inhibition of the PCR process when DNA analysis was attempted due to the presence of ethanol within the sample. Taking this into account as well as the literature studies discussed, it was decided to store maggot specimens used for DNA typing (part two) of the crops in 80% ethanol, and not 95% - 100% ethanol as in part one. This would lessen the degree of crop breakage during removal, while still maintaining a high enough ethanol concentration to prevent degradation of specimens as seen at lower ethanol concentrations.

As discussed, formalin preservation has been indicated to reduce the efficacy of extracting DNA from a sample. For this reason, it was decided to not test DNA typing of the crops of specimens stored in formalin and to purely use this storage method as a comparator of the degree of success achieved using ethanol preservation in part one.

Part two examined the effect of various preservatives and preservation methods on the successful DNA typing of a maggots' gut content. Part two also examined the possibility that the increase in successful DNA typing of gut content is due to storage at lower temperatures, as was observed in a previous study (Linville *et al.*, 2004). This was also observed in an unpublished pilot study. Therefore, it was decided to store maggot specimens at -80°C and 4°C, without any preservation liquid. Linville *et al.*, (2004) further observed that longer storage periods at colder temperatures further affected the chance of successful DNA typing of crop content. Two storage time periods at the two temperatures examined were tested.

2.3. AIMS OF THE STUDY

The primary aim of this part of the study was to determine if a single preservation method can be employed to preserve the morphological characteristics of the maggots as well as for DNA analyses. The study also aimed to test the possibility of following the food movement in the alimentary canal of the maggot visually. This was done to determine if it is possible to determine the position of the food in the digestive system without needing to perform dissection of the alimentary canal which is time consuming and can only be done with someone who has knowledge of entomology as well as the procedure for dissection. Thirdly the study aimed to determine whether piercing the insect specimens would have any effect on the successful preservation of the specimens or not.

The results can be used to create standardized protocols for the preservation of *S. cruentata* maggots for morphological analysis as well as DNA typing of the gut content. Results may also serve as baseline information for creating protocols for other forensically important species.

2.4. MATERIALS AND METHODS

Chicken liver bait traps were set up in an open field on the west campus of the University of the Free State (29°8'S; 26°10'E, ± 1560 m above sea level) from which adult Sarcophagidae spp. flies were sourced. The traps also contained egg and early instar larvae, which was moved to the insectarium of the Department of Zoology and Entomology. Once larvae developed to the third instar stage where it could positively be identified as *Sarcophaga* larvae using the keys from Brink (2009), larvae were collected from the content of the bait traps and moved to containers with suitable rearing medium. Both the adult flies and those reared from the larvae collected from the field made up the stock colony for the study.

Maggot development occurred at insectarium conditions of 28°C ± 2°C with a day/night cycle of 12 hours/12 hours. Adults were fed sugar and water until a protein meal was provided approximately within 1 week of emergence to ensure maturation of the ovaries. Bovine meat was used as breeding medium as molecular analysis was performed using bovine STR markers. Meat was added daily to ensure that sufficient breeding medium was available to the flies and maggots.

When *S. cruentata* maggots reached the third instar stage, the maggots were allowed to feed for approximately 24 hours after which the maggots were removed from the food source. The maggots were placed into containers containing a thin layer of sawdust for protection, with no food. As mentioned, this part of the study was divided into two subdivisions. A summary of each subdivision's study outline is given in Table 2-1 below.

Table 2-1: Study outline and outline of maggot sampling.

| Objective(s) | Number of maggots sampled | Sampling period | Storage method(s) | DNA extraction method(s) |
|------------------------------------------------------------------------------------------------------------------------------------------|---------------------------|-------------------------------------|--------------------------------------------------------------|--------------------------|
| <u>Part 1:</u> Investigate the effect of different preservation methods on the morphology of the maggot body. Preservation methods | 180 (60 x 3 repeats) | 0 to 30 hours (6 hour intervals) | 100% ethanol at room temperature 10% formalin at room | Not applicable |

| Objective(s) | Number of maggots sampled | Sampling period | Storage method(s) | DNA extraction method(s) |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------|-----------------|-----------------------------------------------|--------------------------------------------------------------------------------------------------|
| <p>included:</p> <ul style="list-style-type: none"> • 100% ethanol, pierced • 100% ethanol, unpierced • 10% formalin, pierced • 10% formalin, unpierced | | | temperature | |
| <p><u>Part 2</u></p> <p>Extract DNA from the crops of maggots stored in 80% ethanol and at -80°C. Investigate if storage time and condition had an effect on successfully obtaining DNA from the crop. Specimens in each storage condition were stored for two weeks and for two months, respectively. Storage conditions included:</p> <ul style="list-style-type: none"> • 80% ethanol at 4°C • -80°C frozen | <p>24 (12 x 2 storage methods)</p> | 0 hours | <p>80% ethanol at 4°C</p> <p>-80°C frozen</p> | <p>QIAmp DNA Mini kit (QIAGEN)²</p> <p>Phenol/Chloroform/Isoamyl (PCI) extraction</p> |

² QIAmp DNA mini kit by QIAGEN®, a registered trademark of the QIAGEN Group (QIAGEN N.V.), Venlo, The Netherlands.

2.4.1. Part One: Effect of Preservatives on the Morphology of the Maggot

2.4.1.1. Preservation for Analysis of Maggot Morphology (100% Ethanol vs 10% Formalin)

Twelve maggots were sampled at 6-hour intervals, for up to 30 hours post removal from the food source (Table 2-2). Half of the specimens at each time interval were pierced twice through the maggot cuticle, while the other half remained unpierced. Preservation methods are indicated in Table 2-2.

Table 2-2: Different preservation methods tested over a 30 hour period of sampling.

| Preservation method | 6 hrs | 12 hrs | 18 hrs | 24 hrs | 30 hrs |
|--------------------------------------|-------|--------|--------|-----------|--------|
| 95% - 100% ethanol unpierced | 3 | 3 | 3 | 3 | 3 |
| 95% - 100% ethanol pierced | 3 | 3 | 3 | 3 | 3 |
| 10% formalin unpierced | 3 | 3 | 3 | 3 | 3 |
| 10% formalin pierced | 3 | 3 | 3 | 3 | 3 |
| TOTAL MAGGOTS USED PER REPEAT | | | | 60 | |

2.4.1.2. Preservation for Optimal Dissection and the External Visual Movement of Food in the Gut

Maggots were dissected using the method as described by Boonsriwong *et al.* (2011) for examination of the alimentary canal. For all morphological and preservation analysis, the preserved specimens were stored at room temperature for ten days whereafter dissection of the alimentary canal was attempted. Various assessments (Table 2-3) were made before, during and after dissection of the alimentary canal. The cumulative analyses score for all maggots collected over the 30 hour period for all three repeats, i.e. the total score out of 216, was calculated for each of the preservatives and used in further analysis. The possible total score of 216 points for each time-point was calculated by multiplying the amount of possible points a maggot could score (24 points) by the number of maggots examined at each time-point (9 [three maggots per repeat]). The experiment was performed in triplicate to ensure reliability of the results.

Table 2-3: Criteria for measuring the success of the preservation method tested.

| Criteria for success of preservative | None/easy | Some problems | Difficult |
|----------------------------------------------------|------------------|-------------------------------|--------------------------------|
| Outer appearance of maggot | 3 White | 2 Brown | 1 Black |
| Difficulty cutting through cuticle | 3 | 2 | 1 |
| Inner appearance of maggot | 3 White | 2 Brown/spots | 1 Black |
| Difficulty extracting alimentary canal | 3 | 2 | 1 |
| Difficulty distinguishing different parts of canal | 3 | 2 | 1 |
| Difficulty untangling midgut | 3 | 2 | 1 |
| Sensitivity of midgut to breakage | 3 | 2 | 1 |
| Appearance of food bolus in alimentary canal | 3 Good/intact | 2 Some breakage/leakage | 1 High degree of leakage |

Statistical analysis of the data was done using Microsoft Excel (2010) and GraphPad InStat® Version 3.0. Statistical analysis included performing a Student's T-test, per time interval, as well as one-way analysis of variance (ANOVA), over the 30-hour period, to determine if there were any significant differences within the data obtained. A 95% confidence interval was used.

2.4.2. Part Two: Analysis of the Crop Content after Preservation Using Storage in 80% Ethanol and at -80°C

2.4.2.1. Sampling and Preservation for Part Two

For part two, 24 maggots were hot water killed after which half (12) were stored at -80°C (no preservative) while the other half (12) was stored at 4°C (in 80% ethanol). For each of these preservation methods tested, three maggots were sampled per extraction method per period of storage (Table 2-4).

Table 2-4: Summary of the different preservation methods and subsequent DNA extraction methods tested during part two.

| Preservation method | DNA extraction methods | | | | Total maggots per preservation method |
|-------------------------------------------------|------------------------|---------|-------------------|---------|---------------------------------------|
| | QIAGEN | | Phenol/Chloroform | | |
| | 2 weeks | 8 weeks | 2 weeks | 8 weeks | |
| 80% ethanol at 4°C | 3 | 3 | 3 | 3 | 12 |
| -80°C frozen | 3 | 3 | 3 | 3 | 12 |
| Total maggots per DNA extraction methods | 12 | | 12 | | 24 |

2.4.2.2. DNA Analysis of Samples

2.4.2.2.1. Dissection of Maggot Crops

For all DNA typing, dissection of maggot crops occurred as per Figure 2-1 below. An incision was made at the third anterior body segment of the maggot to remove the maggot head just after the cephalopharyngeal skeleton. Light pressure was applied on the posterior sections of the maggot body to lightly guide the crop out of the maggot body into a microcentrifuge tube for further analysis. The rest of the maggot body was discarded following the laboratory's standard operating procedures, by placing the maggot bodies back into the microcentrifuge tubes and placing these in the biohazard containers for proper disposal of the biological material.

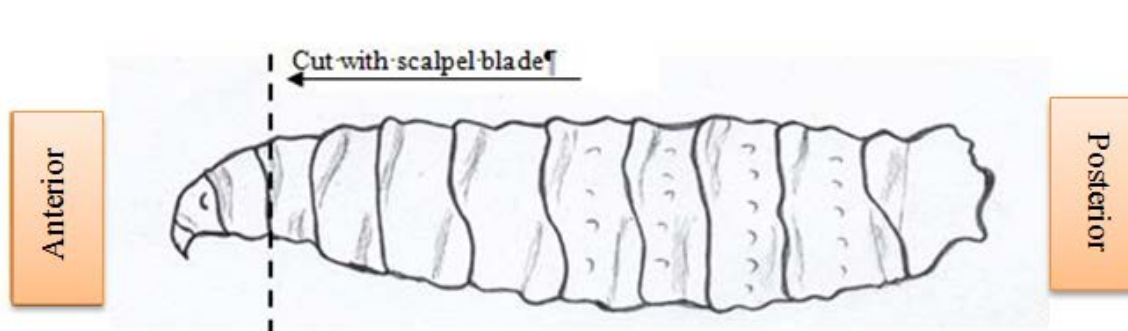


Figure 2-1: Dissection of crop from maggot body. (Sketch by A.M. Barnard [2014]).

2.4.2.2.2. DNA Extraction

The maggot crops were removed prior to DNA extraction. Each group underwent DNA extraction using the following methods: QIAmp DNA Mini kit (QIAGEN) following the manufacturer's protocol, and standard phenol/chloroform/isoamyl extraction. A brief description of each is given below.

i. QIAmp DNA Mini Kit

After the crop was dissected from the maggot body, the crop was placed into a 1.5 ml microcentrifuge tube and 180 μ l of the tissue lysis buffer was added to the tube. Proteinase K (20 μ l) was added to the tube and the mixture briefly vortexed to mix the contents. The sample was incubated at 56°C overnight. After incubation, the sample was briefly centrifuged to remove any drops from the inside of the lid whereafter 200 μ l of an additional buffer was added to the sample. The content was mixed by briefly vortexing the sample for 15 seconds. The samples were left to incubate at 70°C for 10 minutes. After incubation, 200 μ l ethanol (96 – 100%) was added to the sample and mixed by briefly vortexing for 15 seconds. The mixture was carefully applied to a QIAmp DNA Mini spin column without wetting the rim of the tube. The samples were centrifuged at 8000 rpm for 1 minute. After centrifugation the QIAmp DNA Mini spin column was placed into a clean 2 ml collection tube, the filtrate was discarded. After placing the spin column in a clean collection tube, 500 μ l of the first wash buffer was added to the tube. The sample was centrifuged at 8000 revolutions per minute (rpm) for 1 minute. The QIAmp DNA Mini spin column was placed into a clean 2 ml collection tube, the filtrate discarded and 500 μ l of the second wash buffer was added to the tube. The sample was centrifuged at 14 000 rpm for 3 minutes. The spin column was placed into a clean collection tube and the step repeated. The spin column was placed into a clean microcentrifuge tube, the filtrate discarded and 200 μ l of the elution buffer was added. The sample was incubated at room temperature for 1 minute whereafter the sample was centrifuged at 8000 rpm for 1 minute. The spin column was discarded and the filtrate stored at -20°C until further analysis was performed.

ii. Phenol/Chloroform/Isoamyl Extraction

After the maggot crop was dissected from the maggot body, the crop was placed in a 1.5 ml microcentrifuge tube. Proteinase K (20 µl) was added to the tube together with 70 µl 1X TE buffer (pH 7.5). The samples were incubated overnight at 55°C until completely lysed. Following incubation, 100 µl PCI (25:24:1) was added to the samples. Samples were vortexed for 20 seconds and centrifuged for 5 minutes at 14 000 rpms. The supernatant was transferred to a clean tube where after 400 µl ice-cold ethanol (100%) and 50 µl 5M sodium chloride (NaCl) was added. The samples were vortexed for 20 seconds and centrifuged for 5 minutes at 14 000 rpm. The supernatant was discarded and 400 µl ice-cold 70% ethanol were added followed by a 20 second vortex and centrifugation for 5 minutes at 14 000 rpms. The supernatant was discarded and the tube left to air-dry for 30 minutes. In order to re-suspend the pellet, 30 µl 1X TE buffer was added to the pellet and the samples were incubated at 55°C for 30 minutes (samples were vortexed for 10 seconds every 10 minutes).

2.4.2.2.3. DNA Clean-up

A clean-up procedure was performed to purify extracted DNA. This was done to remove buffer salt, enzymes and other substances that could affect downstream applications. In short, 10 µl of the extracted DNA sample was diluted to a total volume of 20 µl by adding 10 µl nuclease free, PCR-grade water. Before the samples were incubated at -20°C for 1 hour, 2 µl 3M sodium acetate, 1 µl glycogen and 50 µl 100% ethanol was added. After incubation the samples were centrifuged at 10 000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet rinsed with 70% ethanol. Samples were again centrifuged and rinsed with 70% ethanol. The supernatant was discarded and the pellet left to air-dry for approximately 15 minutes. The pellet was dissolved in 20 µl 1X TE buffer.

2.4.2.2.4. DNA Quantification

After DNA extraction and clean-up, the quantity and quality of the extracted DNA was measured using the NanoDrop™ Lite spectrophotometer³. The NanoDrop™ Lite

³ Nanodrop is a registered trademark of Thermo Fisher Scientific., Wilmington, Delaware, USA

spectrophotometer calculates the concentration of nucleic acids by measuring the absorbance value of light at a wavelength of 260 nanometer (nm). The quantity of the DNA present in a sample is measured in nanograms per microliter (ng/μl), based on the absorption at 260 nm, while the purity is presented by the 260/280 absorption ratio. An absorption ratio between 1.8 and 2.2 is indicative of pure DNA.

2.4.2.2.5. Polymerase Chain Reaction

DNA amplification was performed using PCR. The 12 μl PCR reaction consisted of 6 μl KAPA⁴ HiFi PCR mix, 0.83 μM of each primer, 1 μl PCR-grade water and 3 μl DNA. Primers used included BM2113, BM1329, MAF46 and ETH225 (Table 2-5). During fragment analysis it was decided to run DNA samples using two PCR plexes – plex 1 with primers BM2113 and BM1329, and plex 2 with primers MAF46 and ETH225. The PCR program used consisted of a denaturation step of 95°C for 10 minutes; 35 cycles consisting of 95°C for 45 seconds, 58°C for 45 seconds and 72°C for 1 minute; final elongation step of 72°C for 10 minutes and an infinite hold at 4°C.

Table 2-5: Primers used during PCR analysis including the primer sequence and fluorescent dye of each.

| Primer # | Scale | Probe/primer name | Primer sequence (5' – 3') | 5' Dye |
|----------|--------|-------------------|----------------------------|--------|
| 1 | 80nmol | BM2113 F | GCTGCCTTCTACCAAATACCC | 6'FAM |
| | | BM2113 R | CTTCCTGAGAGAAGCAACACC | None |
| 2 | 80nmol | ETH225 F | GATCACCTTGCCACTATTTCT | NED |
| | | ETH225 R | ACATGACAGCCAGCTGCTACT | None |
| 3 | 80nmol | MAF46 F | AAATACCCTATAAGGCACAGTACCAC | PET |
| | | MAF46 R | CACCATGGCCACCTGGAATCAGG | None |
| 4 | 80nmol | BM1329 F | TTGTTTAGGCAAGTCCAAAGTC | VIC |
| | | BM1329 R | AACACCGCAGCTTCATCC | none |

⁴ KAPA is a trademark of Kapa Biosystems, Wilmington, Massachusetts, USA.

Fragment analysis was performed using the ABI Genetic Analyzer 3130 (Applied Biosystems) and GeneMapper software Version 3.2.

2.5. RESULTS

2.5.1. Preservation Part One: Preservation for Analysis of Maggot Morphology (100% Ethanol vs 10% Formalin)

The best overall preservation, according to the assessments performed, was achieved for unpierced maggots stored in 10% formalin (Table 2-6). Unpierced maggots stored in 95% - 100% ethanol were least effective method of preservation for morphological analysis (Table 2-6).

Table 2-6: Summary of the assessment results obtained from preserving third instar *Sarcophaga cruentata* maggots in 95% - 100% EtOH and 10% Formalin for 10 days. Each hour's specimens are summarized as a mark out of 216. Values in brackets represent the percentage value of success for each of the hours within each treatment. The total mark is a value out of a possible 1080 points for each of the treatments.

| Time since removal from food source | Criteria examined | Unpierced specimens | | Pierced specimens | |
|-------------------------------------|-------------------------------------------|--------------------------|---------------------|----------------------|--------------------------------------|
| | | Ethanol | Formalin | Ethanol | Formalin |
| 6 hrs | Outer appearance | Brown | Cream/white | Brown | Cream/white, some specimens browning |
| | Inner appearance | White | White | White | White |
| | Dissection difficulty | Minimal difficulty | Easy | Difficult | Easy |
| | Sensitivity | Very sensitive | Sensitive | Sensitive | Sensitive |
| | Leakage | High degree of leakage | Some leakage | Some leakage | Some leakage |
| | Assessment score (mark out of 216) | 120 (55.56%) | 151 (69.91%) | 139 (64.35%) | 135 (62.50%) |
| 12 hrs | Outer appearance | Higher level of browning | Cream/white | Brown, turning black | Cream/white |
| | Inner appearance | White | White | White | White |
| | Dissection difficulty | Difficult | Easy | Difficult | Difficult |
| | Sensitivity | Sensitive | Sensitive | Sensitive | Sensitive |
| | Leakage | High degree of leakage | Some leakage | Some leakage | Some leakage |
| | Assessment score (mark out of 216) | 103 (47.69%) | 166 (76.85%) | 111 (51.39%) | 147 (68.06%) |

Table continued on the next page

| Time since removal from food source | Criteria examined | Unpierced specimens | | Pierced specimens | |
|--------------------------------------------------|-------------------------------------------|------------------------|---------------------|------------------------|---------------------|
| | | Ethanol | Formalin | Ethanol | Formalin |
| 18 hrs | Outer appearance | Brown | Cream/white | Brown, turning black | Cream/white |
| | Inner appearance | White | White | White | White |
| | Dissection difficulty | Difficult | Some difficulty | Difficult | Difficult |
| | Sensitivity | Sensitive | Sensitive | Sensitive | Sensitive |
| | Leakage | High degree of leakage | Some leakage | Some leakage | Some leakage |
| | Assessment score (mark out of 216) | 80 (37.04%) | 156 (72.22%) | 113 (52.31%) | 130 (60.19%) |
| 24 hrs | Outer appearance | Brown | Cream/white | Brown, turning black | Cream/white |
| | Inner appearance | White | White | White | White |
| | Dissection difficulty | Difficult | Easy | Some difficulty | Easy |
| | Sensitivity | Sensitive | Sensitive | Sensitive | Sensitive |
| | Leakage | High degree of leakage | Some leakage | High degree of leakage | Some leakage |
| | Assessment score (mark out of 216) | 107 (49.54%) | 139 (64.35%) | 122 (56.48%) | 147 (68.06%) |
| 30 hrs | Outer appearance | Brown/black | Cream/white | Brown/black | Cream/white |
| | Inner appearance | White | White | White | White |
| | Dissection difficulty | Difficult | Some difficulty | Difficult | Some difficulty |
| | Sensitivity | Sensitive | Sensitive | Sensitive | Sensitive |
| | Leakage | High degree of leakage | Some leakage | High degree of leakage | Some leakage |
| | Assessment score (mark out of 216) | 87 (40.28%) | 81 (37.50%) | 57 (26.39%) | 92 (42.59%) |
| Total assessment score out of 1080 points | | 497 (46.02) | 693 (64.17%) | 542 (50.19%) | 651 (60.28%) |

Except for the maggots retrieved after 30 hours post removal from the food source, unpierced maggots stored in 95% - 100% ethanol were better preserved with regards to external features if compared to the pierced specimens (Table 2-6). Upon dissection the majority of specimens stored in 95% - 100% ethanol were found to be very dehydrated. This increased the difficulty of removing the alimentary canal intact. Distinguishing different parts within the maggot gut were also extremely difficult to perform on dehydrated specimens. Most of the specimens proved to be very fragile leading to many of the alimentary canals breaking during the dissection. Some of the specimens had such a high degree of dehydration that dissection was not pursued. Therefore the concentration ethanol to be used for part two of the study was decreased to 80% (see Rationale for Preservation methods used), as was suggested by previous studies (Adams & Hall 2003; Day & Wallman 2008). A Student's T-test found that there was no significant difference in the success of preservation between the unpierced and pierced specimens stored in 100% ethanol ($p = 0.5779$).

Unpierced maggots stored in 10% formalin were better preserved if compared to the pierced specimens (Table 2-6). Upon dissection it was found that the cuticle of the maggot specimens was very soft and similar to that of a freshly sampled maggot. In the majority of the specimens the alimentary canal was intact and could be dissected out of the maggot body without any breakage. Some of the specimens' digestive tracts were very wet and broken when dissected. A Student's T-test found that there was no significant difference in the success of preservation between the unpierced and pierced specimens stored in 10% formalin ($p = 0.6554$).

A Student's T-test was performed to compare the unpierced and pierced specimens (ethanol vs formalin) from each of the preservation methods, respectively. Although no significant difference was observed between the preservation success for the pierced specimens ($p = 0.2378$) between the preservatives, the test did find a significant difference between the preservation success of the unpierced specimens stored in 95% - 100% ethanol vs 10% formalin ($p = 0.0465$). A One-way ANOVA test was also performed to test the overall success between the different preservatives used. This test found no significant difference between the different preservation methods and time intervals ($p = 0.1104$).

2.5.2. Preservation for Optimal Dissection and the External Visual Movement of Food in the Gut

The type of preservative used did not have an effect on the position of the food bolus within the digestive tract when compared to the specimen prior to preservation (Table 2-2 and Appendix A).

During the external analysis of maggots to determine if it was possible to track food movement without dissecting the maggot, the maggots stored in 95% - 100% ethanol was compared to those stored in 10% formalin (Figure 2-2 and Figure 2-3 respectively). As seen in Figure 2-2 and Figure 2-3, tracing the movement of the food was challenging as the exact location of the food bolus could not always be determined. It was concluded that at the early stages after removal from food source, the bolus in the crop was more visible than later stages. In later stages, the bolus in the crop was lighter, smaller and less visible while darker areas were seen in other parts of the maggot body. In some instances (Figure 2-2 [e] and Figure 2-3 [d]), the presence of food within the mid- to hindgut region of the digestive tract was seen clearly. These images also show the coiled structure of the digestive system within the maggot body. When comparing the two storage methods (Appendix A, Figure 1 through Figure 30) it was clear that storage in 10% formalin proved to be better suited for tracking food movement through external analysis.



Figure 2-2: Visual analysis of food movement from outside the maggot body, specimens stored in 100% EtOH. Photos (4x optical zoom) indicate the movement of food through the digestive system of the maggot at various intervals post removal from food source. (a) 6 hours, (b) 12 hours, (c) 18 hours, (d) 24 hours and (e) 30 hours. Only specimens which indicate the movement of food are presented above. Overall, specimens had high levels of discoloration.

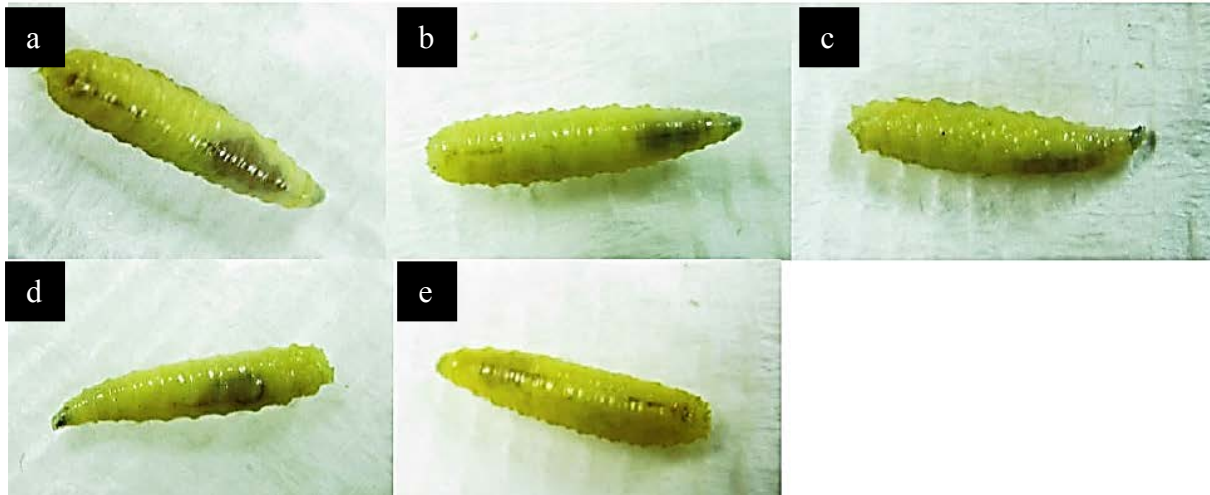


Figure 2-3: Visual analysis of food movement from outside the maggot body, specimens stored in 10% Formalin. Photos (4x optical zoom) indicate the movement of food through the digestive system of the maggot at various intervals post removal from food source. (a) 6 hours, (b) 12 hours, (c) 18 hours, (d) 24 hours and (e) 30 hours.

The internal structures of some of the maggots stored in 95% - 100% ethanol were liquefied which made it difficult to impossible to distinguish the different structures as well as track the movement of the food bolus. Furthermore, 90% of specimens turned brown and in some cases black when stored in the ethanol.

In two maggot specimens stored in 10% formalin liquefaction of the inner organs was noted. In some cases the posterior part of the hindgut would contain some digested material present from the digestion of food during the development up to the third instar stage. With regards to the visual movement of food from outside the maggot body, specimens stored in 10% formalin indicated that it was possible to see food from outside the maggot body. It could be determined without any difficulty whether or not the crop of a maggot contained any material that could prove useful for further analysis. It was not possible to determine the exact location of the food within the alimentary canal if the presence of food was seen further on down the digestive tract as the mid- and hindgut are coiled around one another.

2.5.3. Part Two: Analysis of the Crop Content after Preservation Using Ethanol and Freezing Specimens at -80°C

As discussed, 95% - 100% ethanol storage was replaced with 80% ethanol storage, while formalin storage was not used to attempt DNA extraction for reasons already discussed. Storage and extraction method combinations used are denoted in Table 2-7.

Table 2-7: Storage and extraction combinations

| Abbreviation | Definition |
|--------------|------------------------------------------------------------------------------------|
| WEP | Two week storage in 80% ethanol at 4°C, extracted using phenol/chloroform/isoamyl |
| WEQ | Two week storage in 80% ethanol at 4°C, extracted using QIAmp DNA Mini kit |
| WFP | Two week storage at -80°C, extracted using phenol/chloroform/isoamyl |
| WFQ | Two week storage at -80°C, extracted using QIAmp DNA Mini kit |
| MEP | Two month storage in 80% ethanol at 4°C, extracted using phenol/chloroform/isoamyl |
| MEQ | Two month storage in 80% EtOH at 4°C, extracted using QIAmp DNA Mini kit |
| MFP | Two month storage at -80°C, extracted using phenol/chloroform/isoamyl |
| MFQ | Two month storage at -80°C, extracted using QIAmp DNA Mini kit |

2.5.3.1. DNA Quantification

Quantification results of the DNA extracted from the crops of the maggots are indicated in Table 2-8 and Figure 2-4 and Figure 2-5. DNA quantification was not species specific therefore, although utmost care was taken to prevent addition of any maggot DNA, tissue surrounding the crop could have been incorporated into the sample upon dissection of the crop.

Table 2-8: Summary of DNA concentration and purity (A260/280) data of specimens collected and analyzed (part two).

| Storage time | Storage method | DNA extraction method | Specimen | Quantity | Purity |
|-----------------|----------------|-----------------------|----------|----------|----------|
| | | | | ng/μl | A260/280 |
| 2 weeks | 80% ethanol | PCI (WEP) | 1 | 109.0 | 1.94 |
| | | | 2 | 51.1 | 1.73 |
| | | | 3 | 184.2 | 1.84 |
| | -80°C frozen | PCI (WFP) | 1 | 90.1 | 1.92 |
| | | | 2 | 140.4 | 1.95 |
| | | | 3 | 188.2 | 1.97 |
| | 80% ethanol | QIAmp kit (WEQ) | 1 | 9.8 | 1.48 |
| | | | 2 | 36.2 | 1.84 |
| | | | 3 | 28.6 | 1.80 |
| | -80°C frozen | QIAmp kit (WFQ) | 1 | 4.3 | 1.80 |
| | | | 2 | 18.5 | 1.33 |
| | | | 3 | 28.6 | 1.80 |
| 2 months | 80% ethanol | PCI (MEP) | 1 | 123.4 | 1.70 |
| | | | 2 | 94.2 | 2.01 |
| | | | 3 | 113.0 | 1.63 |
| | -80°C frozen | PCI (MFP) | 1 | 243.5 | 1.86 |
| | | | 2 | 121.9 | 1.99 |
| | | | 3 | 93.3 | 1.96 |
| | 80% ethanol | QIAmp kit (MEQ) | 1 | 11.6 | 1.51 |
| | | | 2 | 46.7 | 2.64 |
| | | | 3 | 10.5 | 1.45 |
| | -80°C frozen | QIAmp kit (MFQ) | 1 | 18.7 | 1.81 |
| | | | 2 | 12.5 | 0.72 |
| | | | 3 | 18.5 | 1.62 |

Abbreviations: PCI = phenol/chloroform/isoamyl

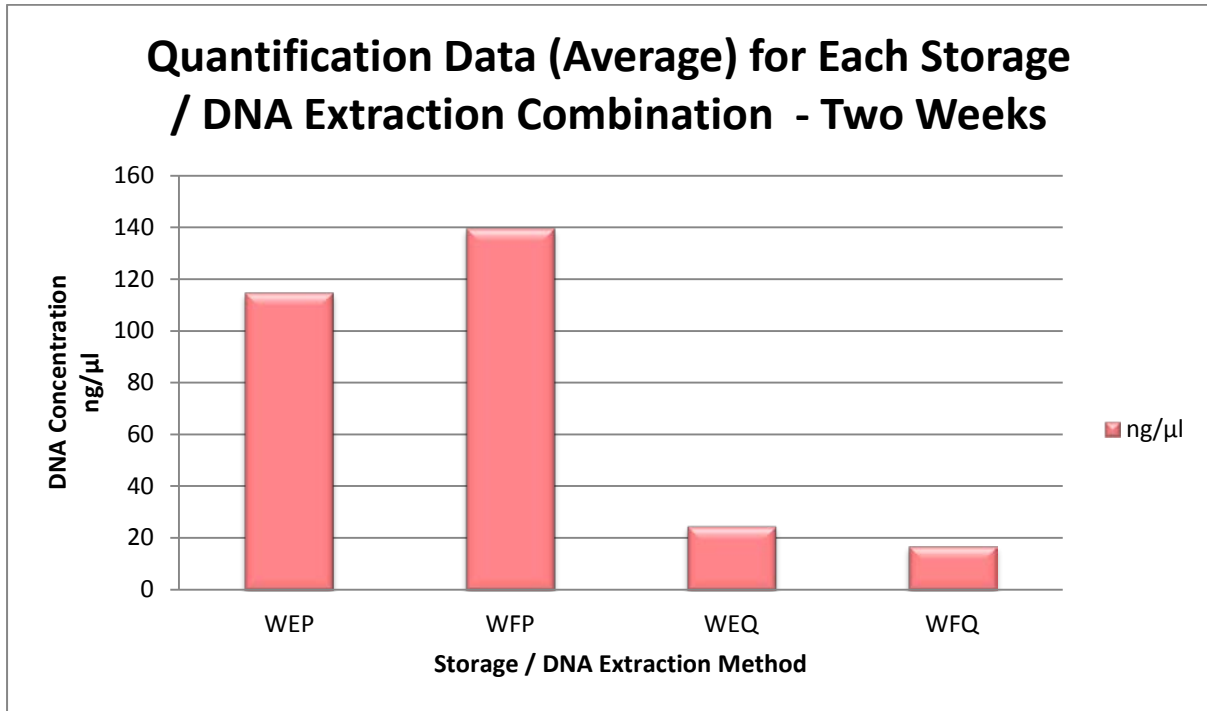


Figure 2-4: Quantification data (average concentration of the three maggots collected per storage / DNA extraction method) for each storage / DNA extraction method tested – two weeks. For definitions of abbreviations please see Table 2-7.

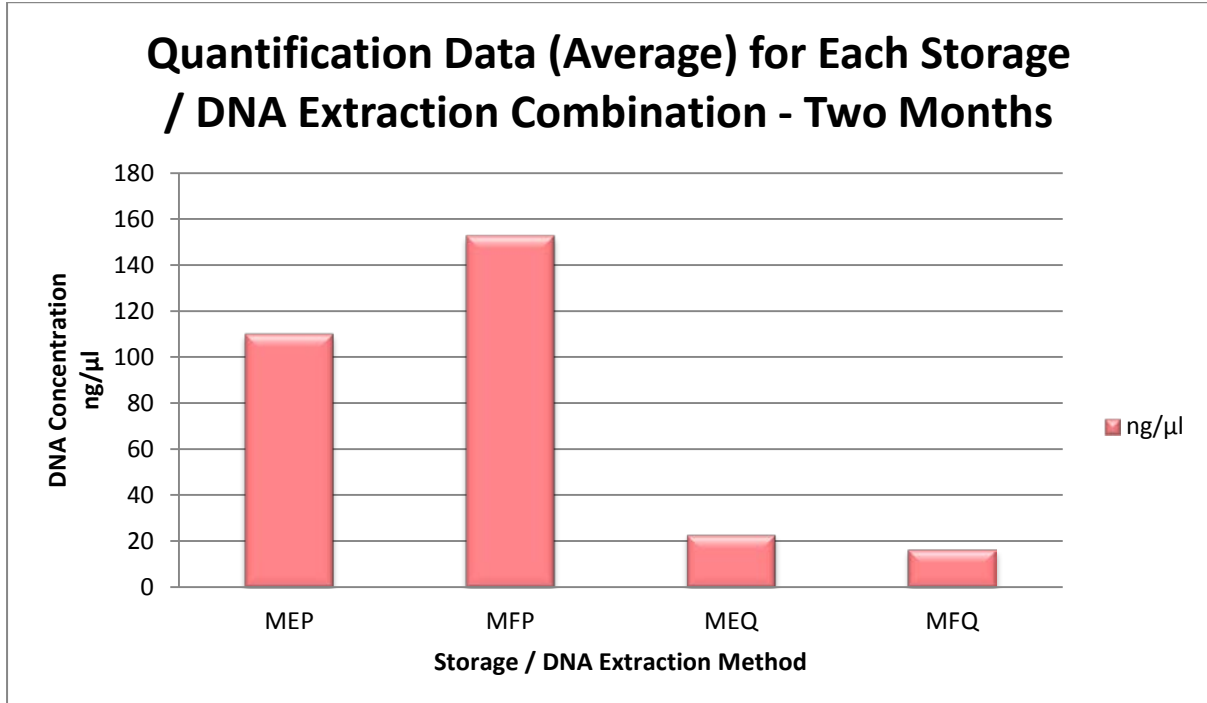


Figure 2-5: Quantification data (average concentration of the three maggots collected per storage / DNA extraction method) for each storage / DNA extraction method tested – two months. For definitions of abbreviations please see Table 2-7.

As seen, the highest average DNA concentration was observed when specimens were frozen at -80°C and DNA extraction was performed using the phenol/chloroform/isoamyl method, for both two weeks and two months storage (with purity [A260/280] of 1.95 and 1.94, respectively). The least effective storage/DNA extraction method combination with regards to the quantity DNA extracted was when specimens were stored frozen and DNA extraction was performed using the QIAmp DNA Mini kit, for both the two weeks and two months storage period (with purity [A260/280] of 1.64 and 1.38, respectively).

2.5.3.2. DNA Analysis

A tissue sample was used as reference (Table 2-9).

Table 2-9: Control sample DNA profile used as reference

| Primer | Sample profile |
|---------------|----------------|
| BM2113 | 139 / 139 |
| BM1329 | 144 / 151 |
| MAF46 | 102 / 102 |
| ETH225 | 143 / 155 |

DNA analysis was performed on specimens stored for two weeks or two months, respectively, in 80% EtOH or at -80°C using the QIAmp DNA mini kit or phenol/chloroform/isoamyl extraction. As indicated in Table 2-10, MAF46 and ETH225 did not amplify in the samples. Primer BM2113 proved to be the most successful. Most samples did not yield DNA profiles that complied with analysis standards set for the study, and was subsequently not analyzed.

Table 2-10: DNA analysis of specimens stored for two weeks and two months in 80% EtOH or frozen at -80°C followed by DNA extraction using the QIAmp DNA mini kit or phenol/chloroform extraction. A full colored block indicates that both alleles were present while a half-colored block indicates that only one allele was present.

| Sampling conditions | BM2113 | BM1329 | MAF46 | ETH225 |
|----------------------------------------------------------------------------------------------|--------|--------|-------|--------|
| Two week storage in 80% ethanol at 4°C – phenol/chloroform/isoamyl extraction (WEP): | | | | |
| Replicate 1 | Full | Full | Full | Full |
| Replicate 2 | Full | Full | Full | Full |
| Replicate 3 | Full | Full | Full | Full |
| Two week storage at -80°C – phenol/chloroform/isoamyl extraction (WFP): | | | | |
| Replicate 1 | Full | Full | Full | Full |
| Replicate 2 | Half | Full | Full | Full |
| Replicate 3 | Full | Full | Full | Full |
| Two week storage in 80% ethanol at 4°C – QIAmp extraction (WEQ): | | | | |
| Replicate 1 | Full | Full | Full | Full |
| Replicate 2 | Full | Full | Full | Full |
| Replicate 3 | Full | Full | Full | Full |
| Two week storage at -80°C – QIAmp extraction (WFQ): | | | | |
| Replicate 1 | Full | Full | Full | Full |
| Replicate 2 | Full | Full | Full | Full |
| Replicate 3 | Full | Full | Full | Full |
| Two month storage in 80% ethanol at 4°C – phenol/chloroform/isoamyl extraction (MEP): | | | | |
| Replicate 1 | Full | Full | Full | Full |
| Replicate 2 | Half | Full | Full | Full |
| Replicate 3 | Full | Full | Full | Full |
| Two month storage at -80°C – phenol/chloroform/isoamyl extraction (MFP): | | | | |
| Replicate 1 | Full | Full | Full | Full |
| Replicate 2 | Full | Full | Full | Full |
| Replicate 3 | Full | Full | Full | Full |
| Two month storage in 80% ethanol at 4°C – QIAmp extraction (MEQ): | | | | |
| Replicate 1 | Full | Full | Full | Full |
| Replicate 2 | Half | Full | Full | Full |
| Replicate 3 | Full | Full | Half | Full |

| Sampling conditions | BM2113 | BM1329 | MAF46 | ETH225 |
|-------------------------------------------------------------|--------|--------|-------|--------|
| Two month storage at -80°C – QIAmp extraction (MFQ): | | | | |
| Replicate 1 | | | | |
| Replicate 2 | | | | |
| Replicate 3 | | | | |

Key:

| | |
|-------------------|--|
| Allele(s) present | |
| Allele(s) absent | |
| Partial profile | |

2.5.3.3. Success Rate of Genotyping

The above results were further analyzed to determine how successful the process of DNA typing of maggot gut content were using the different conditions tested in terms of the successful genotyping. Figure 2-6 summarizes the results obtained.

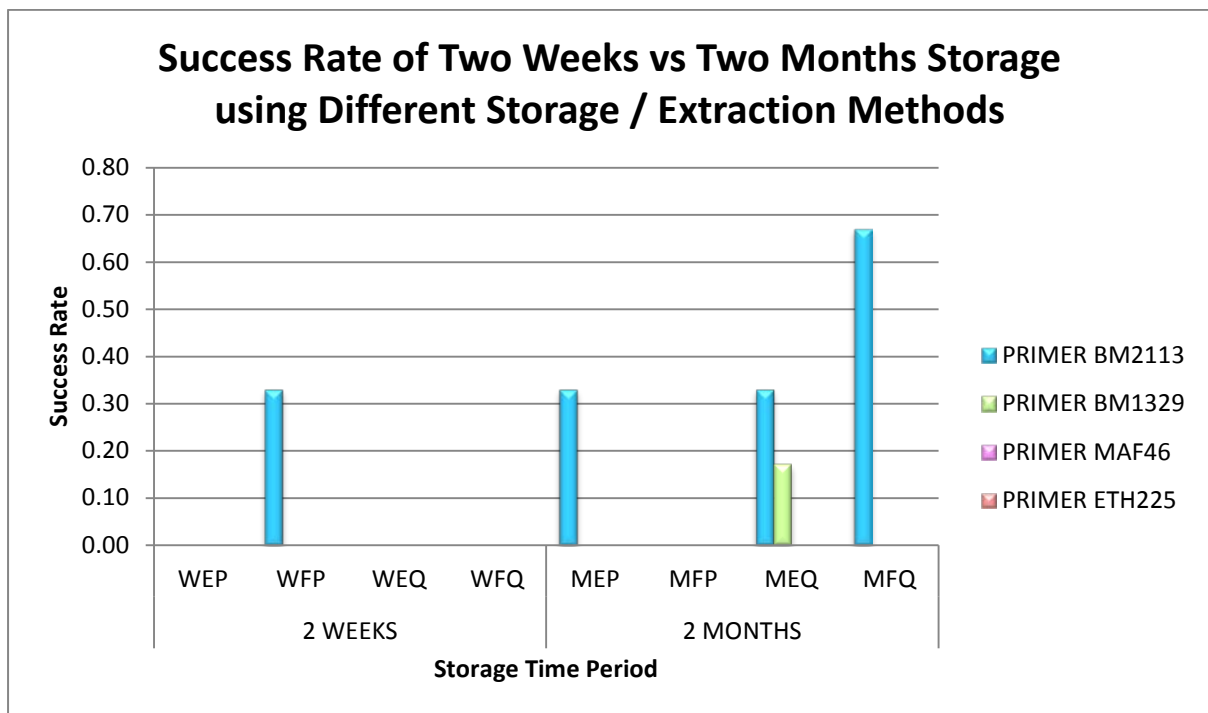


Figure 2-6: Summary of success rate for specimens stored for two weeks and two months when analyzed using different storage and DNA extraction methods. Abbreviations: WEP - 2 week storage in 80% EtOH at 4°C,

using PCI extraction; WFP - 2 week storage at -80°C, using PCI extraction; WEQ - 2 week storage in 80% EtOH at 4°C, using QIAGEN extraction; WFQ - 2 week storage at -80°C, using QIAGEN extraction; MEP - 2 month storage in 80% EtOH at 4°C, using PCI extraction; MFP - 2 month storage at -80°C, using PCI extraction; MEQ - 2 month storage in 80% EtOH at 4°C, using QIAGEN extraction; MFQ - 2 month storage at -80°C, using QIAGEN extraction.

As seen in Figure 2-6, clear differences were noted between the two week and two month storage periods. For the two week storage period, only one out of the 12 samples tested amplified successfully. The best results were seen when specimens were stored at -80°C and extracted using the QIAGEN extraction. Samples did not amplify successfully for the other preservation / extraction methods tested. Linear regression analysis for the two week storage indicated no significant difference between the different storage and DNA extraction method combinations tested ($p = 0.7418$).

The highest success rate with regards to the number of primers that successfully amplified was observed for specimens stored in 80% ethanol, extracted using the QIAmp DNA Mini kit. Linear regression analysis for the two month storage nonetheless indicated no significant difference between the different storage and DNA extraction method combinations tested ($p = 0.3194$).

Only primer BM2113 (bovine) amplified successfully for both the two week and two months storage specimens (Figure 2-6). No amplification was observed for the primers MAF46 (ovine) or ETH225 (bovine). A two-tailed t-test was performed to compare the results obtained during the two week storage and two month storage periods. Results indicated that there was no statistically significant difference between the two week and two month storage of the specimens ($p = 0.1274$). Looking at Figure 2-7, it can be seen that the two month storage had higher overall means between the samples tested. The standard deviation for the two month storage was also much higher than for the two week storage.

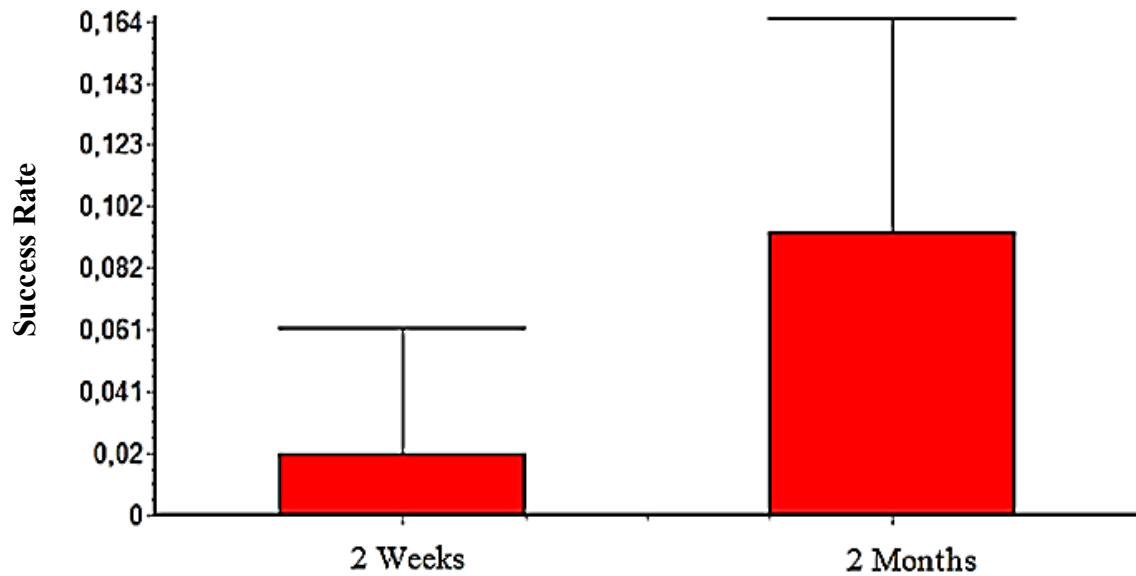


Figure 2-7: Success rate for two weeks vs two months storage when analyzed using different storage and DNA extraction methods indicating the mean and standard deviations for each.

2.6. DISCUSSION AND CONCLUSION

The aim of this section was to find the most optimal preservation method to both preserve the morphology of the maggot and aid in successful DNA analysis of the crop content of the maggot.

With regards to the most optimal preservation of maggots for alimentary canal dissection or visual assessment of the position of the food bolus, and specifically the fullness of the crop, it was observed that better results were obtained if specimens were stored in formalin. As seen in the results, for preservation of the maggots' external characteristics as well as internal structures, formalin provided the best preservation. The finding is consistent with previous published articles which also recommend storage in formalin-based solutions (Linville *et al.*, 2004; Campbossa *et al.*, 2005). This is because formalin-based solutions prevent the degradation of the internal organs within the maggot body. However, due to its inhibiting effect on DNA analysis, this method is not the most optimal for preservation of specimens for DNA typing. Storage in ethanol is often recommended as the best alternative, especially for storage in the field. Results from this study indicated that the specimens became very dehydrated when stored in a high percentage of ethanol, with dissection of many

of the specimens impossible. The longer the specimens were stored, the more dehydrated the specimens became increasing the difficulty of dissecting the alimentary canal successfully. Alimentary canals were fragile and broke easily during dissection. This is due to the fact that ethanol replaces the water molecules in samples leading to morphological disturbances such as shrinking, hardening and distortion (Carter 2003). All these characteristics were also observed in specimens stored in ethanol. Tracking the food movement, without performing dissection of the alimentary canal, proved challenging as the exact location of the food bolus could not always be determined through external visual inspection. For specimens stored in formalin, no difficulty was experienced in determining whether or not food was present in the crops of the maggots. Due to the coiled structure of the maggot digestive track (Chapter 3), more difficulty was experienced in determining the exact location of the food bolus when the food was present in the mid- or hindgut.

The current study hypothesized that piercing the maggot cuticle, hence allowing more of the preservative to penetrate the maggot, would increase the successful preservation of the specimen. When comparing the treatments (pierced vs unpierced) between each of the storage methods tested, no statistical significant difference was noted when specimens were pierced with a p-value of 0.2378. In contrast, a statistically significant difference was found when specimens were stored unpierced in formalin and 95% - 100% ethanol, with $p = 0.0465$. In unpierced maggot specimens stored in ethanol, not enough ethanol was able to pierce the cuticle in order to preserve the internal morphology of the maggots. This led to many of the specimens being highly decomposed on the inside and turning black on the outside. Discoloration increased the difficulty of determining if there was any food present in the crop without having to perform dissection. Formalin was very successful in penetrating the cuticle, even if no incisions were made to aid this, further proving its high success in preservation of entomological specimens.

Several extraction methods including PCI and QIAGEN extraction were tested in this study which yielded varying results. The highest average DNA concentration was observed when specimens were stored at -80°C and DNA extraction performed using the phenol/chloroform/isoamyl extraction method. Colder temperatures proved most effective in

the current study for yielding the highest DNA concentrations, which was similar to previous studies (Linville *et al.*, 2004; Campbossa *et al.*, 2005; Di Luise *et al.*, 2008; Chávez-Briones *et al.*, 2013). This could be due to inactivation of enzymatic activity and bacterial growth that leads to degradation of specimens. In contrast, when DNA extraction was performed using the QIAmp DNA Mini kit, the best results was observed in specimens stored in 80% ethanol at 4°C. Although phenol/chloroform/isoamyl extraction yielded the highest concentration DNA in samples, not all of the DNA material present in the extracted sample is host DNA material. Although great care was taken to limit the addition of maggot tissue in the current study, it cannot be discounted completely. The maggot digestive system is surrounded by fats and tissues that may also be removed during dissection and added to the sample. These tissues contain lipids and proteins that inhibit PCR reactions. With the QIAmp DNA Mini kit, samples underwent various purification steps to remove such contaminants from the sample. Further, it contains two wash steps to remove the ethanol from the sample, another factor that normally contributes to PCR inhibition. The phenol/chloroform/isoamyl extraction does not have these extra steps to ensure the purity of the DNA extracted from a sample. This could possibly explain the greater success observed in DNA typing when samples were extracted using the QIAmp DNA Mini kit. To further aid in limiting the presence of any proteins and lipids in the samples, all samples were put through clean-up.

High DNA concentrations with low amplification success were observed in samples extracted using the phenol/chloroform/isoamyl method, and *vice versa* when using the QIAmp DNA Mini kit. DNA concentration however is not a reliable measure of the possibility of successfully obtaining a DNA profile from maggot specimens. This type of contradiction has also been previously observed (Carter 2003) where it was found that the spectrometry readings for DNA concentration are not a reliable measure of the true host DNA content of the sample. This might be due to pigments or other cellular components that co-purify with the extracted DNA, leading to inaccurate readings during quantification, as discussed previously. Clean-up of samples is therefore highly recommended by the current study when working with maggot gut content analysis and further development of protocols for clean-up is still required.

This study further investigated the greater success rate observed when storing specimens at colder temperatures by storing specimens for two weeks and two months at 4°C and -80°C before DNA analysis was attempted. Higher reproducibility was observed in specimens stored for two months. Overall, the two month storage had the highest mean DNA concentrations. The standard deviation was also much higher for the two months storage than the two week storage period, indicating greater variation between specimens with regards to the DNA concentration of the extracted samples following storage. Although colder temperatures is believed to inactivate enzymatic activity and bacterial growth faster, limiting decomposition of the specimens during storage, it is still unknown why longer storage periods lead to higher reproducibility of DNA typing results. No statistical significant difference was observed between the two weeks vs. two months storage periods ($p = 0.1274$). Considering this result, the study results indicated that longer storage of specimens will not necessarily guarantee better results when performing DNA analysis of the samples.

Various recommendations can be made based on the results of the current study. It is recommended that maggot specimens to be used for gut content analyses, should be stored at -80°C. Freezing facilities are not readily available in the field, thus it is suggested that specimens be hot water killed then placed on dry ice to achieve the same temperatures. In situations where dry ice cannot be obtained, as well as due to its dangerous nature, the study would recommend storage in ethanol at temperatures no higher than 4°C after collection in the field. Specimens should only be stored in ethanol for a short period of time, where after the specimens should be removed and stored using more appropriate methods such as freezing at -80°C. Similar to Linville *et al.* (2004), the current study found that storage at colder temperatures increased the possibility of successful DNA typing from the crop content of maggots. The period of time that specimens were stored did not have a significant effect on the possibility of successful typing of the maggot gut content. It was also concluded that piercing of specimens did not have a statistically significant effect on the preservation of specimens.

Overall, results concluded that it is possible to extract DNA from the crop of the maggot although full profiles will in all likelihood not be obtained due to the degraded nature of the samples used. Traditional methods such as phenol/chloroform/isoamyl extraction yields higher levels of DNA but is not always the best method for DNA profiling of maggot specimens due to the inconsistent results observed. Traditional methods such as phenol/chloroform/isoamyl extraction are furthermore dangerous and time consuming. With the higher sensitivity and clean-up procedures of modern forensic DNA kits, it is suggested that kits such as the QIAmp DNA Mini kit be used for extraction.

More work is nonetheless still needed to optimize the preservation methods and DNA typing methods used when analyzing maggot crop content. Various factors that may influence the success of DNA typing from maggot crop content must still be investigated. This includes whether or not the type of species will have an influence on the success of DNA typing from the crop as well as the amount of material ingested at varying temperatures. Furthermore whether or not the digestive enzymes secreted onto the food has an effect on the ingested material after ingestion, and to what degree the material is affected, to name just a few.

The study indicated that this is a promising area for future research with many avenues still unexplored.

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CHAPTER 3

Gut Morphology

3.1 INTRODUCTION

Flies within the family Sarcophagidae are notoriously difficult to identify to the species level, leading to very little work being done on this family in the past (Aspoas 1991; Cherix *et al.*, 2012; Buenaventura 2013; Jordaens *et al.*, 2013, Freemdt & Amendt, 2014; Pai *et al.*, 2014). These flies are synantrophic, found in close association to human settlements and animal surroundings (Byrd & Castner, 2010). Although adult for breeding purposes, the larval stages complete their development on decaying flesh (Lima de Azerede-Espin & Lessinger, 2006; Shiravi *et al.*, 2011). This makes the maggots of great forensic importance (Buenaventura 2013). In the past 10 to 15 years more emphasis has been placed on this family of flies. Since these flies are robust, strong flyers as well as able to survive in a variety of environmental conditions, it is very common to find maggots from this family on decomposing remains (Wells *et al.*, 2001). It has also been observed that in cases where the remains were found indoors, members of this family were the dominant species present (Aspoas 1991).

When performing gut content analysis it is important to consider the morphology of a maggot, especially the maggot digestive system. It was originally believed that the maggot gut was a single tube moving from the mouth to the anus. This theory was changed after studies were done on various species including the botfly *Dermatobia hominis* (Evangelista & Leite, 2003) and the blackfly *Simulium pertinax* (Cavados *et al.*, 2004). These studies indicated that the digestive systems of maggots are much more complex in structure and function than initially thought (Boonsriwong *et al.*, 2007). Each digestive system is adapted to the type of feeding of the species. The maggot gut is a long, continuous, convoluted tube that stretches from the mouth to the anus. It is normally about three times the length of the insect body and passes through various regions containing valves and sphincters as it moves from the anterior mouth to the posterior anus (Fishman *et al.*, 1984; Caetano *et al.*, 2006). As with the digestive system of vertebrates, the insect gut shows

complexity in structure and function, with specialized regions for the digestion and absorption of nutrients, regulation of the insect hemolymph ionic composition and pH, detoxification of the insect body and the production of pheromones (Greenberg & Klowden, 1972; Boonsriwong *et al.*, 2007). The maggot gut is divided into three main areas namely the foregut (stomodaeum), midgut (mesenteron) and hindgut (proctodaeum) (Bursell 1970; Spit *et al.*, 2012). The foregut consist of the mouth (M), crop (C), esophagus (E), cardia (Ca), gastric caeca (GC) and salivary glands (SG). The foregut forms the inlet to the midgut or stomach of the maggot. Some softening of the food and/or digestion may also occur in the foregut (Caetano *et al.*, 2006). Most of the digestion of the food and some absorption of nutrients occur in the larval midgut (MG). The hindgut (HG) aids in most of the absorption of nutrients and reabsorption of water and essential ions as well as forms the storage area for used food material until this is excreted from the maggot body (Snodgrass 1935; Caetano *et al.*, 2006). The alimentary canal is coiled and tangled inside the larval body in order for the whole system to fit inside the small body (Figure 3-1).

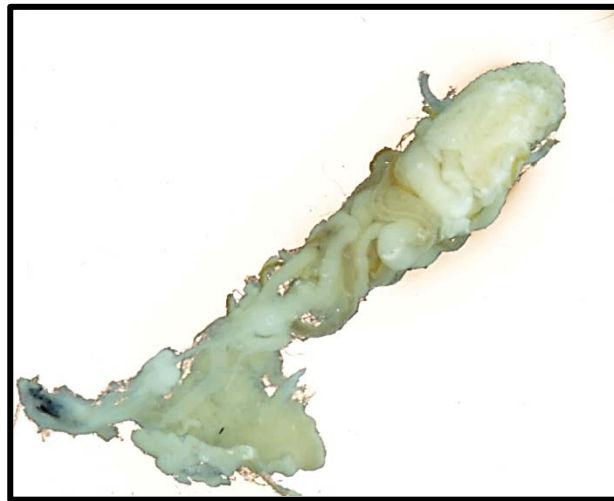


Figure 3-1: Dissected alimentary canal of a third instar *Sarcophaga cruentata* maggot showing the structures coiled around each other before being untangled (4x optical zoom). (Photo by A.M. Barnard).

As a maggot feeds, from emerging from the egg until it reaches the third instar stage, the tissue consumed is digested slower than it is consumed causing the excess to be stored in the crop of the maggot. A study by Zdarek (1985) indicated that the food will move through the maggot digestive system at a rate of two millimeters per minute. During the last day of feeding the crop of the maggot will double in size and be visible through the cuticle of the maggot as a dark red oval (Greenberg & Klowden, 2001).

Very little work has been done on the alimentary canal of maggots including *S. cruentata* (Buenaventura 2013). In order to better understand the maggot gut, the internal structures of the *S. cruentata* maggot gut was investigated. Literature indicates that the alimentary canals *S. cruentata* and *C. megacephala* maggots share many similarities (Boonsriwong *et al.*, 2007). As with the other parts of the alimentary canal the hindgut consists of different parts namely the pylorus, Malpighian tubules, ileum, colon and rectum and anus (Martoja & Ballan-Dufrancias, 1984; Boonsriwong *et al.*, 2007).

This study aimed to examine the alimentary canal of laboratory bred *S. cruentata* flies to get acquainted with the overall layout of the digestive system. The study further aimed to track the movement of the food bolus within the maggot alimentary canal over a 30 hour period. This examined the period for which food will be present in the crop to be used in further DNA typing procedures and to examine the tempo of gastric emptying in third instar *S. cruentata* maggots.

3.2 MATERIALS AND METHODS

Third instar maggots were removed from the food source (bovine muscle) after being allowed to feed to capacity for 24 hours and placed into a separate container with no food.

Five samples were analyzed to determine the different structures present in the maggot alimentary canal of *S. cruentata* maggots. The alimentary canal was dissected following the procedure as described by Boonsriwong *et al.* (2011) to determine the exact location of the food bolus within the alimentary tract. The full alimentary canal was dissected out to further confirm similarity with that of *Chrysomya megacephala*. Photographs were taken using an AZ microscope as well as a Leica EZ4 HD microscope.

3.3 RESULTS

3.3.1. Gross Morphology of the Alimentary Canal

The midgut and hindgut of the larvae are coiled around each other and surrounded by fat bodies (Figure 3-2). The crop was located dorsal in relation to the cardia and salivary glands.

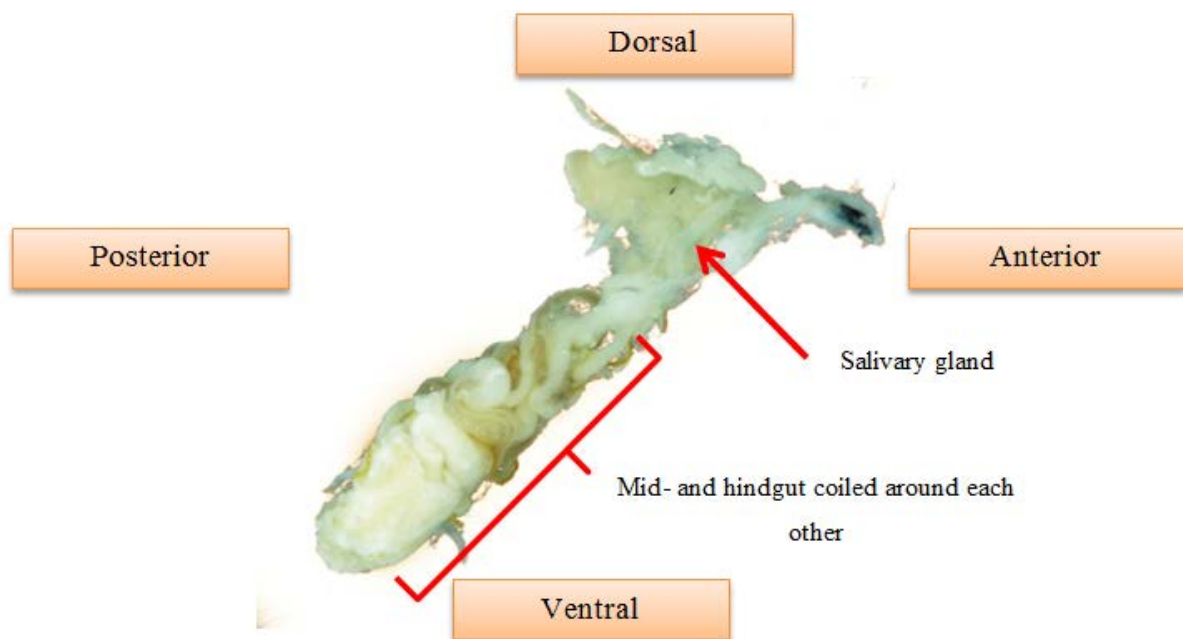


Figure 3-2: Dissected third instar *Sarcophaga cruentata* larval alimentary canal indicating the structural composition before untangling of the digestive tract. Notice the coiled formation of the midgut and hindgut (4x optical zoom)

Paired salivary glands are in a ventral position. These glands opened into narrow efferent ducts and finally converged to form one deferent duct which leads to the oral cavity. An enlarged structure, the crop, was visible on the dorsal side of the maggot body. The crop formed the largest organ within the uncoiled maggot body when full and fully extended (Figure 3-2, Figure 3-3, Figure 3-4 and Figure 3-6). Following the connections of the crop and salivary gland is the cardia. The cardia with the gastric caeca marked the junction between the foregut and midgut (Figure 3-3). The midgut (Figure 3-3 a) forms the biggest part of the alimentary canal followed by the hindgut (Figure 3-3 b). The Malpighian tubes forms the junction between the midgut and hindgut (Figure 3-3).

Along the length of the maggot, thin tubes (tracheal tubes) were connected to various parts of the maggot body as well as two large tracheal tubes. These two tracheal tubes originate from the posterior spiracles and extend throughout the rest of its body; most of the tracheal tubes were however removed or broke during the dissection.



Figure 3-3: Complete alimentary canal of a third instar *Sarcophaga cruentata* larva indicating the different structures present. Photo taken with the AZ microscope. The stippling indicates the different sections of the digestive system: (from left to right) foregut, midgut and hindgut. (a) Start of midgut; (b) Start of hindgut.

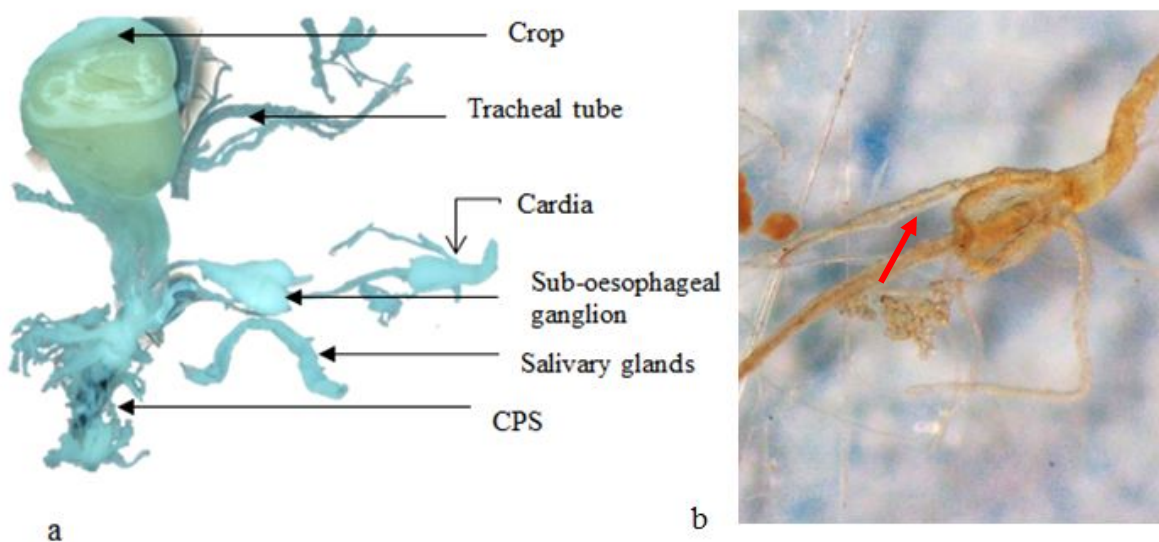


Figure 3-4: a) Close-up view of the anterior part of the maggot digestive system indicating the position of the cephalopharyngeal skeleton (CPS), crop, salivary glands, sub-oesophageal ganglion and cardia. b) Cardia of a third instar *Sarcophaga cruentata* maggot.

The cardia forms a ball like casing around the foregut-midgut junction. From this emanates the gastric caeca (see Figure 3-6). The midgut consisted mainly of one long tube. The end of the midgut is signified by the presence of Malpighian tubules. This forms the beginning of the hindgut which, similarly to the midgut, is also a long, thin, fragile structure until it reaches the anus.

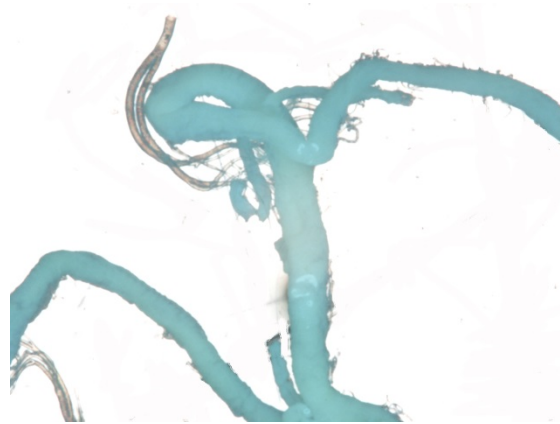


Figure 3-5: Part of the midgut of the alimentary canal indicating the connection and presence of two tracheal tubes to the digestive system.

In Figure 3-5 part of a tracheal tube is seen clinging to the midgut of the larvae, indicating its connection to the alimentary canal. These tubes were more firm to the touch and hollow. These tubes originated at the posterior end of the maggot (near the spiracles) and radiated to the anterior part of the maggot.

3.3.2. Timeline of food movement through the digestive tract

Figure 3-6 indicates the movement of food through the maggot gut. The 6 hour interval was chosen to indicate the movement of the food bolus as the crop was most visual through the cuticle prior to dissection.

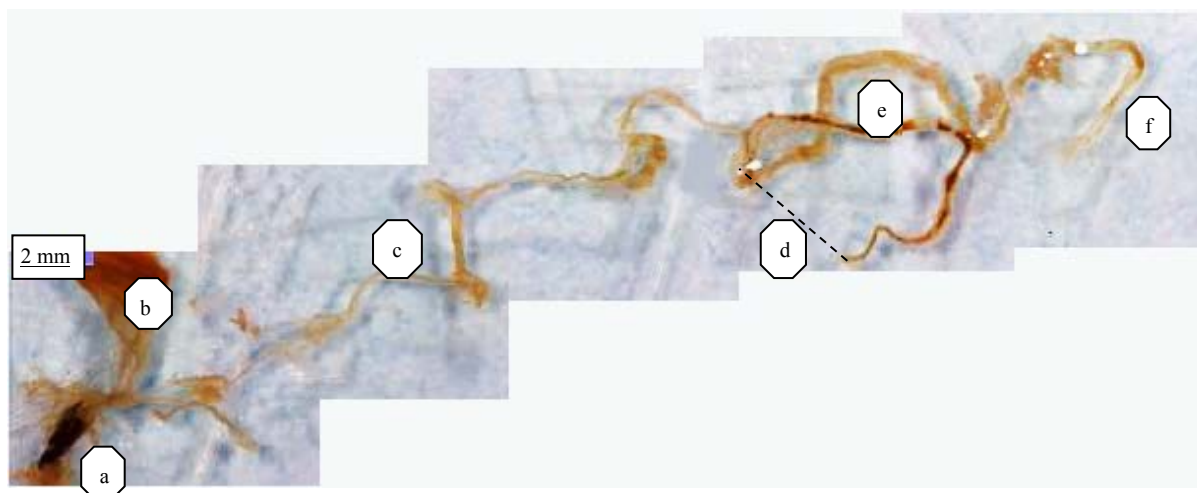


Figure 3-6: Dissected maggot gut indicating the movement of food within the alimentary canal. The maggot was collected and killed 6 hours post removal from food source and stored in 10% Formalin. Photos were taken using the Leica EZ4 HD microscope. (a) Cephalopharyngeal skeleton; (b) Crop; (c) Midgut; (d) Tear at junction between midgut and hindgut, location of Malpighian tubes; (e) Anterior hindgut; (f) Posterior hindgut.

The timeline for movement of food within the maggot alimentary canal is illustrated in Figure 3-7 (formalin) and Figure 3-8 (ethanol). The degree of fullness was denoted as full (100%), partially full to full (75%), partially full (50%), partially full to empty (25%) and empty (0%).

3.3.2.1. Tracking the Movement of Food through the Alimentary Canal of Samples Preserved in Formalin

Six hours post removal from the food source the crop (Figure 3-6 a) of the maggot was full. The crops of samples were completely empty after 30 hours post removal from food source. In the midgut, some food was noted during the first 6 hours post removal from food source. No food was present at 12 hours post removal. From 18 hours through 30 hours post removal, there was a steady increase in the content of the midgut. Similarly, food was observed in the anterior hindgut (Figure 3-6 e) while the posterior hindgut was partially full to empty (Figure 3-6 f) at 6 hours post removal. No food was observed in the hindgut at 12 hours post removal from food source. The content of the hindgut increased at 18 hours and 30 hours post removal with a slight decrease observed at 24 hours post removal from food source (Figure 3-7).

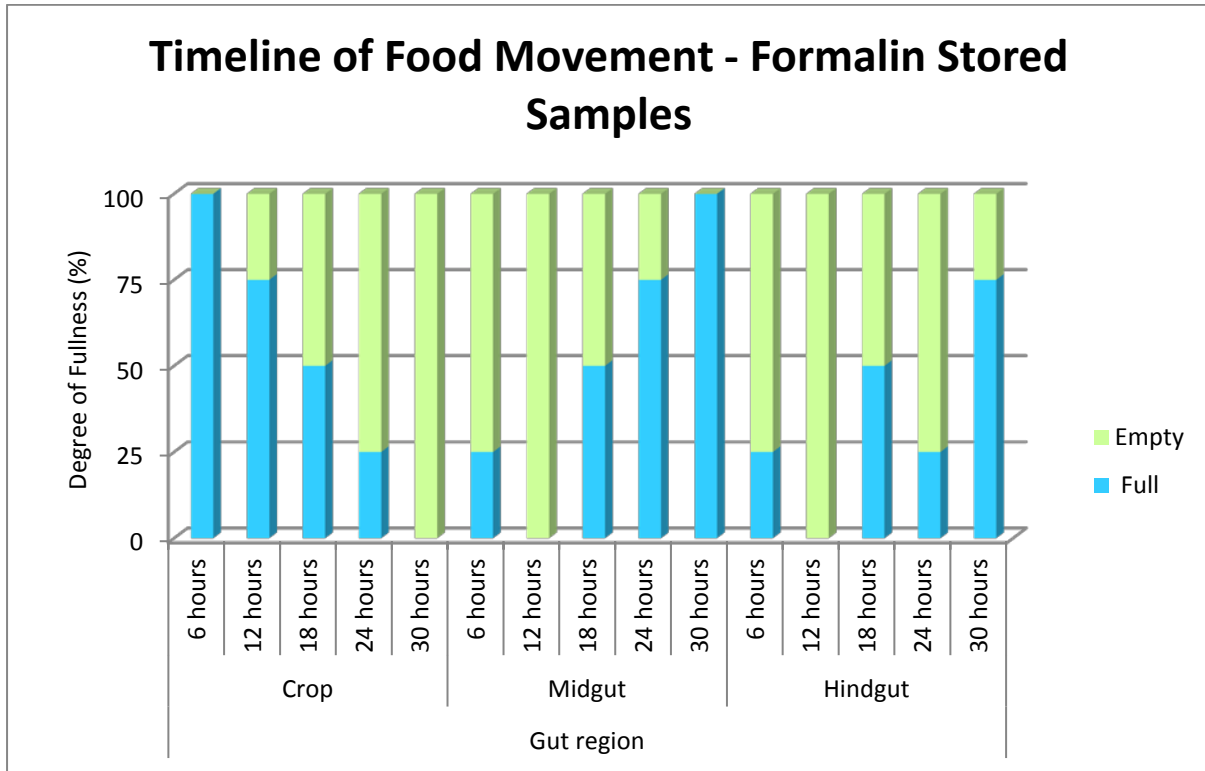


Figure 3-7: Time course of food movement within the alimentary canal of third instar maggots stored in 10% formalin.

3.3.2.2. Tracking the Movement of Food through the Alimentary Canal of Samples Preserved in Alcohol

The sequential emptying over time was not evident in samples stored in ethanol as it was in formalin. Unlike formalin stored samples, the crops of samples stored in ethanol was full up to 12 hours post removal from food source. At 18 hours post removal a rapid decline in crop content was observed with the crops fully emptied by 24 hours post removal. In the midguts no food was observed up to 12 hours post removal. Thereafter there was a rapid increase in midgut content which increased gradually up to 24 hours and remained steady for the remaining period of evaluation (Figure 3-8).

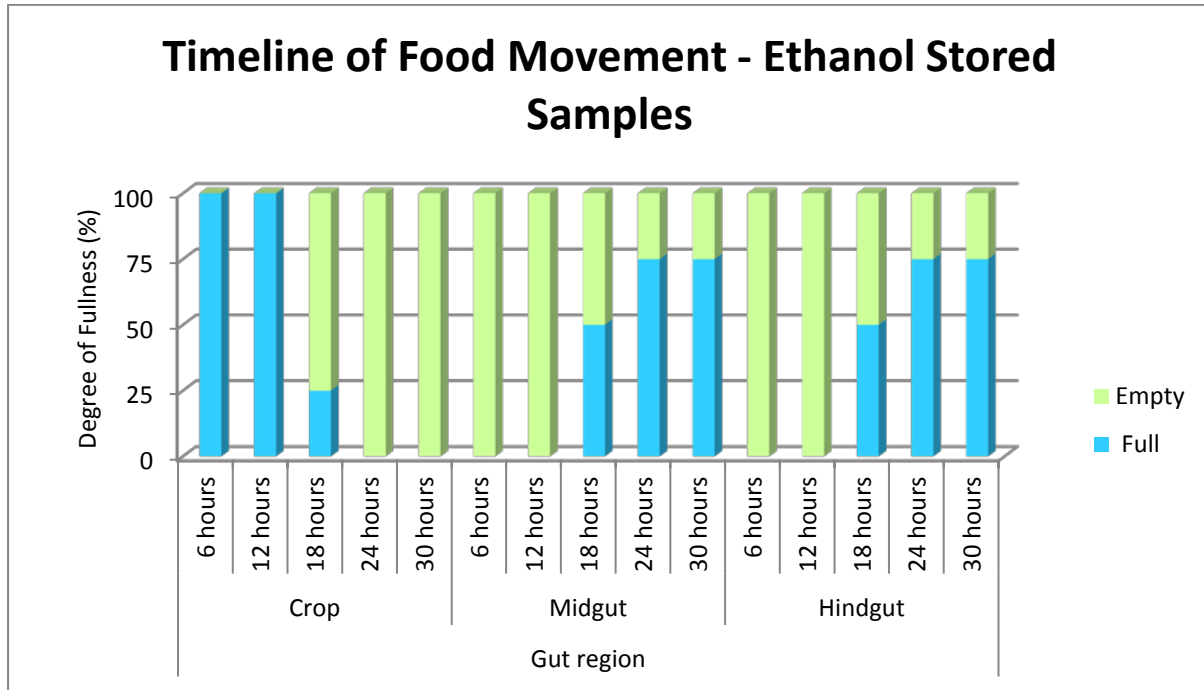


Figure 3-8: Time course of food movement within the alimentary canal of third instar maggots stored in 100% EtOH.

More difficulty was experienced in tracking the movement of food in the alimentary canals of samples stored in ethanol compared to those stored in formalin. Sharp increases and decreases were observed in the content of the different sections of the gut of samples stored in ethanol which was followed by plateaus over multiple evaluation intervals. In formalin stored samples, the content of the different regions of the gut increased and decreased gradually over the evaluation time periods. When the maggot began to digest the food stored in the crop only a continuous dark line was visible in the midgut and in later cases in the hindgut, when assessing the dissected digestive tract. No clear distinction could be made with regards to the specific position of the food bolus within the maggot gut, as indicated by the continuous black line observed after the food in the crop was digested.

Unfortunately, when assessing the alimentary canal before uncoiling the gut, it was not possible to pinpoint the exact location of the food bolus in the alimentary canal. Without performing dissection, it was possible to see a visual shift in the position of the food bolus between the different sections of the maggot body.

3.4 DISCUSSION AND CONCLUSION

In preparation for the pupal stage, that require high energy stores for this drastic development from the maggot stage to the adult stage, necrophages maggots will feed ferociously to gain fat stores. It is expected that different species of necrophages Diptera maggots will have morphologically similar digestive systems. This was confirmed for the sarcophagid maggots examined in this study, which was on gross anatomical level similar to *C. megacephala* when viewed microscopically (Figure 3-3; Boonsriwong, *et al.*, 2007).

Digestion begins with the chemical breakdown of food by the enzymes in saliva that is secreted onto the food source. This saliva begins the process of digestion, making it easier for the maggot to consume the tissue as maggots do not have mandibles to tear off the tissue. As food enters the foregut it either passes into the crop or through to the midgut. The enlarged crop functions as a storage organ in which the maggots store the excess food they consume which is not required for the daily functioning of the maggot. No absorption occurs in the crop and esophagus. The foregut is connected to the midgut through the esophagus which main function is to pass food forwards to the midgut (Lee *et al.*, 1998).

Analysis of the alimentary canal showed that the mid- and hindgut of the larvae are coiled around itself in the abdomen of the maggot body (Figure 3-2). This coiling is most likely due to the length of the maggot gut, which would not fit into the maggot body otherwise. These structures are very fragile with thin walls, possibly to increase the process of absorption between the gut and the hemolymph and are surrounded by a layer of fat bodies which provide protection to these structures. Tracheales are present along the length of the maggot body with an elevated presence surrounding the midgut of the maggot. The elevated presence of trachea reflects the high level of oxygen required by the gut to supply sufficient energy for the process of digestion, absorption and to perform the peristaltic movements to transfer the food along the digestive tract (Mohamad *et al.*, 2015). The tracheal tubules originated at the posterior end of the maggot and radiated to the anterior part of the maggot. This direction mainly focusses the attention on the feeding behavior of maggots as these larvae can usually be found buried in decomposing tissue with their heads thus only having their posterior parts exposed to clean air. After untangling the gut it was observed that the midgut forms the largest part of the alimentary canal. The end of the foregut and the start of

the midgut are marked by the presence of the cardia and gastric caeca (Mohamad *et al.*, 2015). The midgut forms the main organ of the maggot's alimentary canal functioning primarily in the digestion of food and absorption of nutrients which is apparent by its length as well as the presence of fat in the anterior and posterior regions of the midgut (Hobson 1931). The beginning of the hindgut is marked by Malpighian tubules.

Although formalin storage did not affect the ability to observe the position of the food bolus within the digestive tract without dissection of the alimentary canal, it was difficult to follow the movement of the food. This is because the mid- and hindgut is coiled around each other making an accurate distinction of the exact location of the food impossible without dissecting the alimentary canal for further inspection. The preservative did affect the quality of the digestive tract, which was discovered after dissection of the alimentary canal. Furthermore, as the integrity of the alimentary canal was affected in some instances, an increase in the level of difficulty experienced when dissecting the alimentary canal was experienced which led to more breakage of the canal and thus subsequent leakage of the gut content. Results indicated a clear decrease in the crop content of the maggots over the 30 hour evaluation period indicating that digestion of the stored food stuffs occurred.

It was established during this study that formalin preserved specimens were better preserved in terms of integrity of the alimentary canal as well as tracking of the movement of the food bolus. It was postulated for this study that once maggots were removed from the food source, the ingested food would be passed through the alimentary canal in a logical manner. It was further postulated that the food stored in the crop would become less as time progressed. The same pattern, lagging behind the emptying of the crop was expected to occur in the mid- and hindgut.

The movement of food was observed in a logical manner with the midgut filling up as the crop began to empty. In the hindgut, the increase in the content of the gut was not consistent as was observed in the midgut. A possible reason for this might be due to the absorption of different nutrients at various areas within the midgut and hindgut. Hobson (1931) indicated the midgut could be divided into different sections: the anterior midgut, the middle midgut and the posterior midgut. Each section has varying numbers of microvilli, increasing and decreasing the absorption within each part (Khedre 2002). The middle midgut can further be

divided into three parts. Hobson (1931) reported that the food consumed is concentrated in the second segment of the middle midgut. Hobson (1931) suggested that this section of the midgut was the main part where water absorption occurred as food passed rapidly to this section after a starved maggot was fed. It was also suggested by Hobson (1931) that the main site for protein digestion was the posterior midgut. Typically, the presence of microvilli is indicative of areas where large levels of absorption or secretion occur (Boonsriwong *et al.*, 2007). With the variation observed in the different sections of the midgut, each section was characterized by absorption of specific nutrients as well as different absorption rates depending on, for instance, the volume of microvilli present in the section. This varying absorption pattern could influence the volume of waste material that is moved to the hindgut. The primary function of the midgut is absorption of nutrients.

To summarize, these results indicated that the anterior and posterior midgut as well as the third portion of the middle midgut were the most important sites for the absorption of nutrients. The presence of the large number of microvilli resulted in an enormous increase in surface area for absorption. The well-developed basal infoldings further provide cells with the capacity to transport water and ions in the openings formed. These restricted openings which are formed also concentrate solutes (Khedre 2002). This creates an osmotic pressure gradient between the opening and the lumen which might assist in the absorption of water (Terra *et al.*, 2006). Therefore, as various parts of the midgut aids in the absorption of different nutrients and the fact that the rate of absorption differs between the various sections, a possible explanation for the inconsistent increase in hindgut content is provided. As food moves through the digestive system, nutrients are absorbed at different areas within the gut. Most insects have a counter-current movement of fluid in the midgut caused by the secretion of fluid in the posterior midgut and its absorption in the anterior midgut (Mohamed *et al.*, 2015). Any unabsorbed particles moves through to the hindgut where it is stored until excretion. Due to the counter-current flow of food particles in the midgut, the amount of unabsorbed particles which enter the hindgut at each evaluation time-point could possibly vary depending of the degree of absorption that occurred. This theory could offer an explanation for the decrease in hindgut content observed at 24 hours. Any water excreted by the Malpighian tubules goes through reabsorption in the hindgut. As more food goes through the process of digestion in the midgut, more unabsorbed particles move through to the hindgut. A further factor that influences the rate of digestion, in unregulated environments,

includes the temperature which can increase or decrease the rate of digestion in the maggot gut. The maggot mass present will also influence the temperature surrounding the maggot. This inconsistent result could additionally be due to external factors which do not involve the rate of digestion within the maggot or the structures present in the gut. As mentioned, a high degree of gut breakage was observed in some instances which led to much of the gut content leaking out. The inconsistent results observed in this study could therefore also be due to the influence of this factor.

Food movement in samples stored in ethanol after removal from the food source did not follow the same, expected patterns as samples stored in formalin. A rapid decrease in the content of the crop was observed at 18 hours and it was completely empty 24 hours post removal from the food source. It is possible that the dehydrating effect of ethanol could distort the position of food in the gut. Ethanol caused the maggot body to shrink leading to the sides sticking to one another, possibly due to rapid displacement of the water from the maggot body. This shrinkage could cause the food content to be moved within the gut of the maggot possibly explaining the difference seen compared to formalin stored samples. As the formalin was a buffered solution, displacement of water occurred less rapidly.

Although it is possible to observe the presence of food within the maggot from outside through visual inspection, it is not possible to exactly pinpoint the location of the food within the alimentary canal. Using maggots stored in alcohol for determining how long a maggot has been removed from the food source is not advised. Although formalin storage proved more accurate, various aspects has to be researched before this method can be used to make an assessment on how long ago the maggot was removed from the food source. These include the type of food consumed; the influence of toxins, hormones and foreign substances in the food consumed; if results are consistent between different species and the effect of fluctuating temperatures on the rate of digestion, to name a few. Determining the time period for which the maggot has been removed from the food source using this method of analysis is not yet reliable as it is subjective to the analysts' opinion. This increases the possibility of inconsistent results being reported. With further research into this area of forensic investigation, limiting the variable that affect the interpretation of results, this method may prove effective in the future.

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CHAPTER 4

Analysis of *Sarcophaga cruentata* Crop Content

4.1. INTRODUCTION

Modern forensic investigations first started using insects in the early 19th century. Today insects are routinely used in homicide and other medicolegal investigations. Even though morphological analysis has been widely used in the past for the study of insects, modern forensic entomology has begun to move more towards the use of molecular approaches. The molecular approach most used in forensic entomology is the identification of species with the use of DNA barcoding (Kondacki *et al.*, 2009; Kester *et al.*, 2010). Recently investigators have begun to focus more on molecular analysis of the gut content of larvae from forensically important species. This technique appears to be a promising development with many advantages. These include providing information on entomotoxicology, relocation of the remains following death, determining the number of food sources the maggot has fed on and identifying a victim if the remains are too badly decomposed for traditional methods of identification to be performed (Li *et al.*, 2007; Kondacki *et al.*, 2009; Kester *et al.*, 2010; Stuyt *et al.*, 2010). The first forensic case, where host DNA from tissue evidence in a maggot alimentary canal was used, was documented by Chávez-Briones *et al.* (2013). In this case the remains of a badly burned body were identified by comparing the DNA of the host tissue in the gastrointestinal content of three maggots to that of the alleged father of the victim.

Dipteran maggots feed on decomposing tissue from the time the maggots hatch until the maggots reach the third instar. At this stage the maggots' alimentary canal is fully extended causing receptors in its digestive tract to signal it to seize feeding (Carvahlo *et al.*, 2005; Di Luise *et al.*, 2008). Situations do exist nevertheless where a maggot may leave its food source before being fully fed. Examples include when it is disturbed or when the food source is depleted before the maggot was fully developed. In these situations it is important to confirm the maggot's food source especially if the maggot was removed from a crime scene (Campbossa *et al.*, 2005; Li *et al.*, 2007; Stuyt *et al.*, 2010; Amendt *et al.*, 2011). In the past

molecular typing of the insect digestive tract has been successful in flies (Wells *et al.*, 2001), beetles (DiZinno *et al.*, 2002), bedbugs (Szalanski *et al.*, 2006) and human lice (Mumcuoglu *et al.*, 2004).

It has been shown that third instar maggots are the best source of host DNA because the maggot is at its peak feeding period, reaching its maximum length and crop size (Sharma *et al.*, 2015). This theory was supported by, to name one, the study by Stuyt *et al.* (2010). These authors attempted to test from which developmental stage of *Protophormia terraenovae* it was possible to extract usable host DNA to obtain good DNA profiles. Their findings suggested that it was possible to extract usable DNA from day two of the second instar to the fourth day of the third instar when larvae were reared at 26°C. No amplifiable DNA could be extracted from first instar, early second instar, late third instar, pupae or adult flies (Stuyt *et al.*, 2010). First instar and early second instar larvae crops may still be too small to contain sufficient tissue for DNA analysis (Campbossa *et al.*, 2005; Carvahlo *et al.*, 2005), while the failure to amplify DNA in the late third instar larvae may be due to maggot migration. When the maggot is fully fed at the end of the third instar, it starts to move away from the food source. During this stage it searches for an appropriate area to pupate and complete its immature development using the food stored in the crop for nutrients (Singh & Bala 2009). Amendt *et al.* (2011) nevertheless found that it was possible to obtain DNA specific to the food source from two-day old pupae of *Ceriodaphnia dubia*. This brings to mind the question whether or not the success of alimentary canal analysis might be species specific or not? This avenue has not yet been fully investigated and more research is needed before a conclusion can be drawn.

Although tissue is stored in the crop while the maggot is feeding, when it starts moving away from the food source it begins to digest this tissue and the crop will empty rapidly. It has been shown that in some species the crop can empty completely within 24 hours after removal from the food source (Campbossa *et al.*, 2005; Wells & Stevens, 2008). When the crop starts to empty the tissue contained in the crop is rapidly degraded by digestive enzymes in the midgut of the maggot, which could lead to it not being usable for molecular analysis.

This is important to keep in mind when working with gut content samples as it may provide an explanation for obtaining negative results. A study by Li *et al.* (2007) indicated that the rate of food digestion is further affected by the ambient temperature to which the maggot is exposed. These authors found that the rate of digestion was slower with decreasing temperature except when the maggot was exposed to 28°C. At this temperature the digestive enzyme activity of *Aldrichina grahami* maggots was at its highest. Therefore it is essential that the temperature should be measured when collecting maggot specimens.

While this seems to be a promising field in forensic entomology, attention has only been given to this subject in the past 10 years. This deficiency can mainly be contributed to the lack of standard protocols for the analysis of gastrointestinal content samples (Clery *et al.* 2001; Li *et al.*, 2007). Problems are also experienced with regard to the storage of samples for this type of analysis (Linville *et al.*, 2004; Di Luise *et al.*, 2008). Traditional entomological methods used to preserve samples for morphological analysis have been proven to cause degradation of the samples making them unusable for molecular analysis (Linville *et al.*, 2004; Campbossa *et al.*, 2005). Additionally, some commonly used preservatives such as Kahle's solution inhibit the PCR reactions preventing further DNA analysis (Linville *et al.*, 2004). The storage method and storage time also has an impact on the success of obtaining DNA profiles as seen in the study by Linville *et al.* (2004). Linville and his co-authors had a success rate of between 83% after storage for two weeks and 100% after storage for six months when samples were stored at -70°C, while only being successful in 67% of the cases when storage was in 70% ethanol at 4°C for two weeks and six months respectively.

Even though these authors have indicated the possibility of extracting usable DNA from the gut content of maggots many questions still exist pertaining to this technique. Standard protocols must be developed for the storage and subsequent molecular analysis of samples. Furthermore it is unclear for how long usable DNA can still be obtained from the crop after the maggot was removed from its food source before reaching the post-feeding period (Li *et al.*, 2007; Stuyt *et al.*, 2010). The latter prompted this research paper. The aims of this

study was therefore to determine for what period of time, after removal from its food source, usable DNA could be obtained from the crop content of third instar maggots. The result may provide an alternative explanation for situations where DNA profiling of the alimentary canal content fails in that it may show that the maggot was in fact only removed from the food source for a sufficient time for the gut content to be digested.

4.2. MATERIALS AND METHODS

4.2.1. Breeding, Sample Collection and Storage

Details on breeding and sampling of maggots are outlined in Chapter 2. Third instar *S. cruentata* maggots were allowed to feed on bovine meat for approximately 12 to 18 hours. Thereafter the maggots were removed from the breeding cages and put into separate containers containing a thin layer of sawdust to draw up any excess moisture that may be present and to provide some shelter for the maggots. Three maggots were removed immediately, killed by placing it into $60^{\circ}\text{C}\pm 2^{\circ}\text{C}$ warm water for 30 seconds, dried on a paper towel, placed into microcentrifuge tubes and frozen at -80°C with no preservative. After this initial sampling, three maggots were collected at three hour intervals (for up to 24 hours or until the maggots reached pre-pupae stage) and killed and preserved as above (see also Table 4-1). All samples were frozen at -80°C until DNA analysis was performed. Sampling was done in triplicate for each of the three trials. Samples were taken of the bovine meat to serve as a reference to which the maggot gastrointestinal genotypes could be compared.

Table 4-1: Proposed sampling of *Sarcophaga cruentata* maggots for gastrointestinal analysis.

| Time since removal from food source (hrs) | Number of samples for crop content analysis |
|--------------------------------------------------|----------------------------------------------------|
| 0 | 3 |
| 3 | 3 |
| 6 | 3 |
| 9 | 3 |
| 12 | 3 |
| 15 | 3 |
| 18 | 3 |
| 21 | 3 |
| 24 | 3 |
| TOTAL | 27 |

4.2.2. Maggot Dissection and DNA Analysis

Dissection was performed according to the procedure outlined in Chapter 2, Part 2. DNA analysis was performed using standard PCI extraction (Chapter 2, Part 2). Chapter 2 also provides the details on the clean-up, quantification and amplification of maggot samples.

4.3. RESULTS

The sampling strategy aimed to collect maggots from 0 to 24 hours post removal from the food source or until the maggots began to enter pre-pupae stage (Table 4-1). However after 12 hours post removal from the food source the maggots entered pre-pupae stage and were therefore only sampled up until this time.

4.3.1. Quantification of Results

DNA of sufficient quantity and quality was obtained from the control samples (0 hours) in all three trials (Table 4-2). As can be seen, DNA concentrations varied greatly across the

different time intervals. In two out of the three trials (Trial 2 and Trial 3), the 0 hours samples had the highest DNA concentration (289.87 and 411.53 ng/μl, respectively), with purity (A260/280) of 1.92 and 1.96, respectively. In all three trials the DNA concentration decreased with increasing time since removal from food source.

Table 4-2: Summary of DNA concentration and purity (A260/280) data of samples collected and analyzed.

| Time since removal from food | Sample | Trial 1 | | Trial 2 | | Trial 3 | |
|------------------------------|--------|---------|----------|---------|----------|---------|----------|
| | | ng/μl | A260/280 | ng/μl | A260/280 | ng/μl | A260/280 |
| 0 | 1 | 137.3 | 1.76 | 413.0 | 1.98 | 341.4 | 2.04 |
| | 2 | 97.3 | 1.92 | 270.6 | 1.95 | 277.5 | 1.98 |
| | 3 | 468.1 | 1.94 | 186.0 | 1.84 | 615.7 | 1.86 |
| 3 | 1 | 502.2 | 1.82 | 68.8 | 1.87 | 162.4 | 1.88 |
| | 2 | 187.0 | 1.95 | 151.2 | 1.98 | 70.4 | 1.84 |
| | 3 | 265.7 | 1.92 | 217.8 | 1.89 | 174.9 | 1.88 |
| 6 | 1 | 127.1 | 1.87 | 251.5 | 1.79 | 214.8 | 2.18 |
| | 2 | 102.8 | 1.83 | 107.7 | 1.96 | 79.1 | 1.77 |
| | 3 | 227.4 | 1.98 | 157.6 | 1.78 | 162.9 | 1.80 |
| 9 | 1 | 206.8 | 1.93 | 62.7 | 2.04 | 185.0 | 1.78 |
| | 2 | 173.0 | 1.89 | 183.6 | 1.92 | 93.0 | 1.90 |
| | 3 | 637.2 | 2.03 | 77.4 | 1.92 | 73.1 | 1.89 |
| 12 | 1 | 91.1 | 1.87 | 66.4 | 1.86 | 438.8 | 2.22 |
| | 2 | 182.0 | 1.90 | 41.9 | 1.43 | 61.1 | 1.85 |
| | 3 | 149.4 | 1.92 | 72.1 | 1.79 | 70.6 | 2.04 |

For the assessment of the DNA concentrations over time, the average concentration of the DNA (Table 4-2) obtained for the three maggots per each collection time was calculated, as indicated in the figure below (Figure 4-1). As seen, results varied between the three trials. For sampling performed at 9 hours post removal, there appears to have been an increase in the DNA concentrations obtained from the crop analysis in Trial 1 and Trial 2. This was also

observed at 12 hours post removal for Trial 3. DNA quantification used in this study was not species specific. Therefore, the increases in DNA quantification results at the mentioned time points could have been caused by the incorporation of maggot DNA. Although utmost care was taken to remove only the crop of the maggot during dissection, some of the maggot's surrounding tissue could have been added with the crop before DNA extraction was performed on the sample.

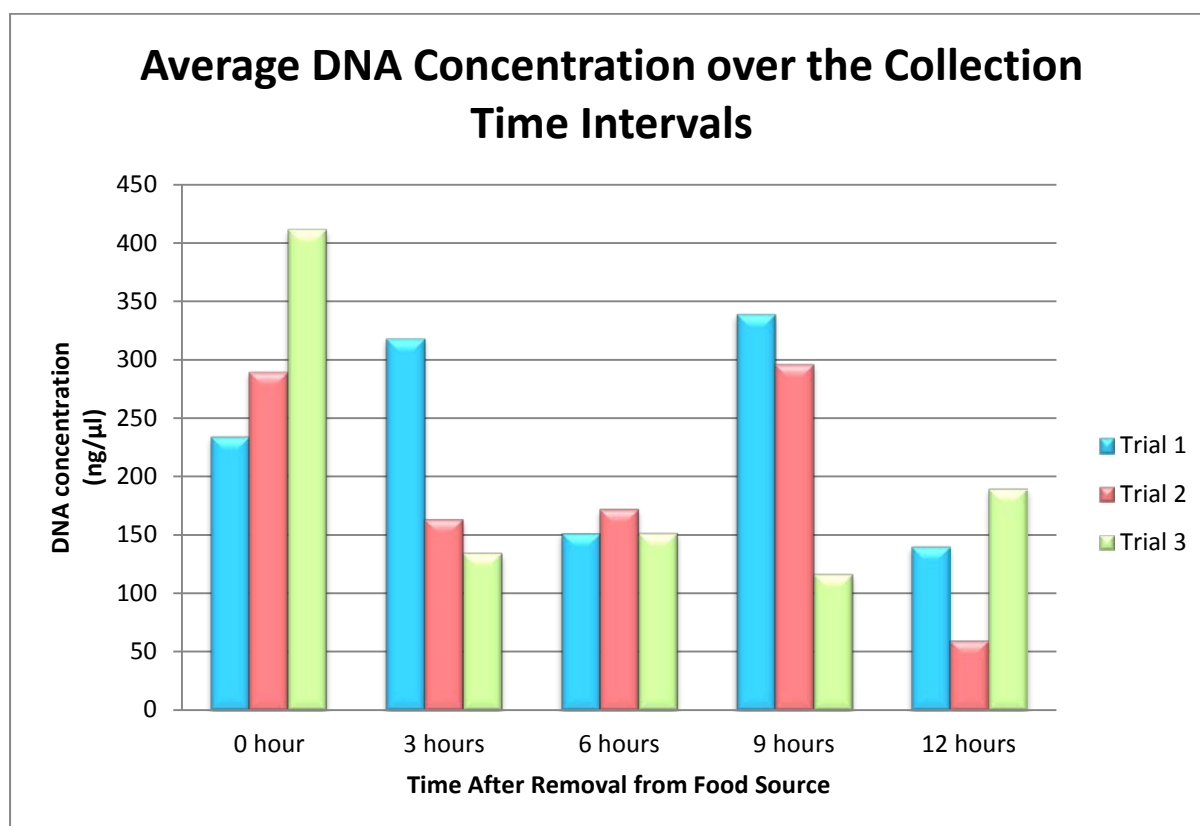


Figure 4-1: Average DNA concentration (average values of the three samples collected per collection time interval) for the collection intervals since removal of maggots from the food source – Trial 1, Trial 2 and Trial 3.

4.3.2. DNA Analysis

A sample was taken from the meat on which the maggots were reared serving as the reference sample for the maggot crop analysis. As seen in Figure 4-2, all primers amplified successfully in the reference sample with little background noise and artefacts.

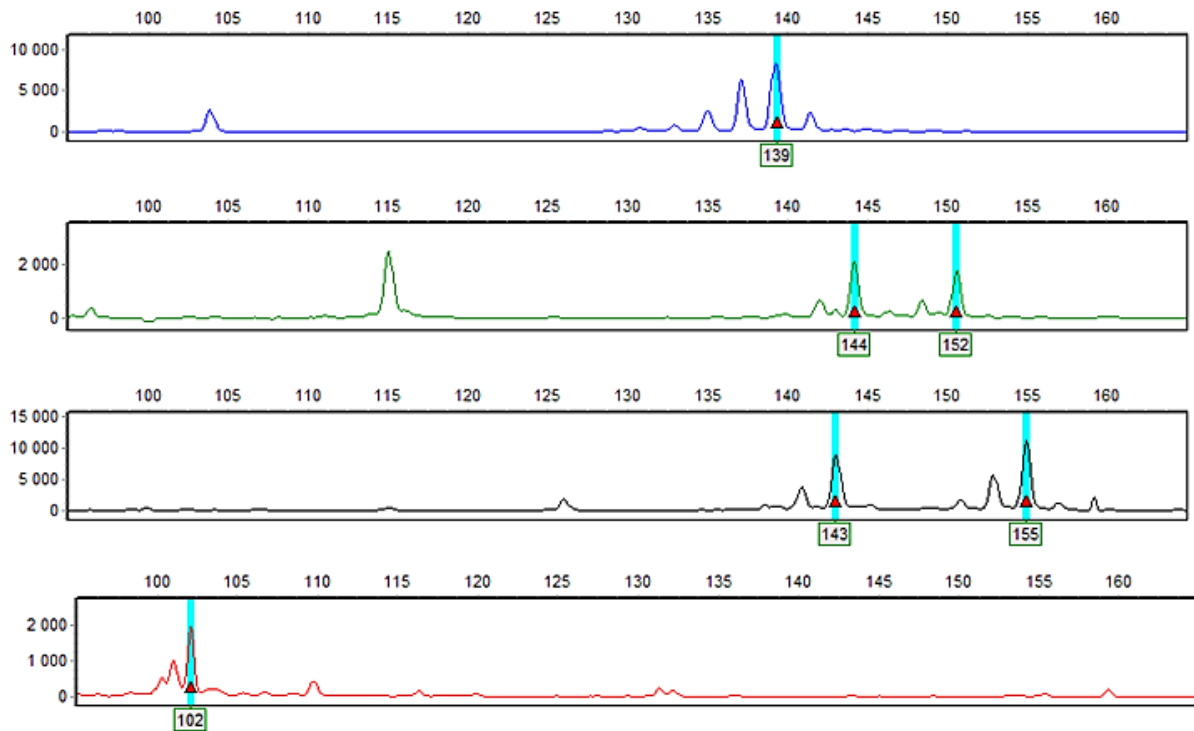


Figure 4-2: Electropherogram representing the DNA profile of the reference sample (bovine meat). Primers used included BM2113 (blue), BM1329 (green), ETH225 (yellow, indicated above as black) and MAF46 (red).

Amplification of the maggot samples proved to be more challenging. DNA amplification only provided partial profiles (Table 2-10, Chapter 2). However, the alleles that did amplify successfully in the partial profiles correlated with the reference sample. Maggot sample DNA profiles had high levels of background noise and artefacts, making interpretation of the profiles difficult. High variation in profiles obtained was observed between the maggots. In many maggot samples, no target DNA could be detected after amplification.

4.3.3. Amplification Success Rate

The amplification success rate throughout the three trials is indicated in Figure 4-3. The individual amplification success rate per primer, per sample, was measured on a scale of 0 to 1, where 0 indicated no amplification of either alleles, 0.5 indicated one allele amplified and 1 indicated both alleles amplified successfully. Therefore, for each primer three

measurements were obtained, one for each sample or repeat in that specific time interval. The average success rate of each primer for the three samples collected per time interval was calculated by adding the three individual measurements (0 to 1) and dividing the total by three. To calculate the success rate at each collection interval, the average per primer calculated was divided by four and the answers were converted to an average percentage success rate for each collection time interval. Results are presented as combined percentage successful amplification of primers per collection time interval. Results varied greatly between the three trials. See Table 2-10 (Chapter 2) for further information on the genotypes obtained during the study.

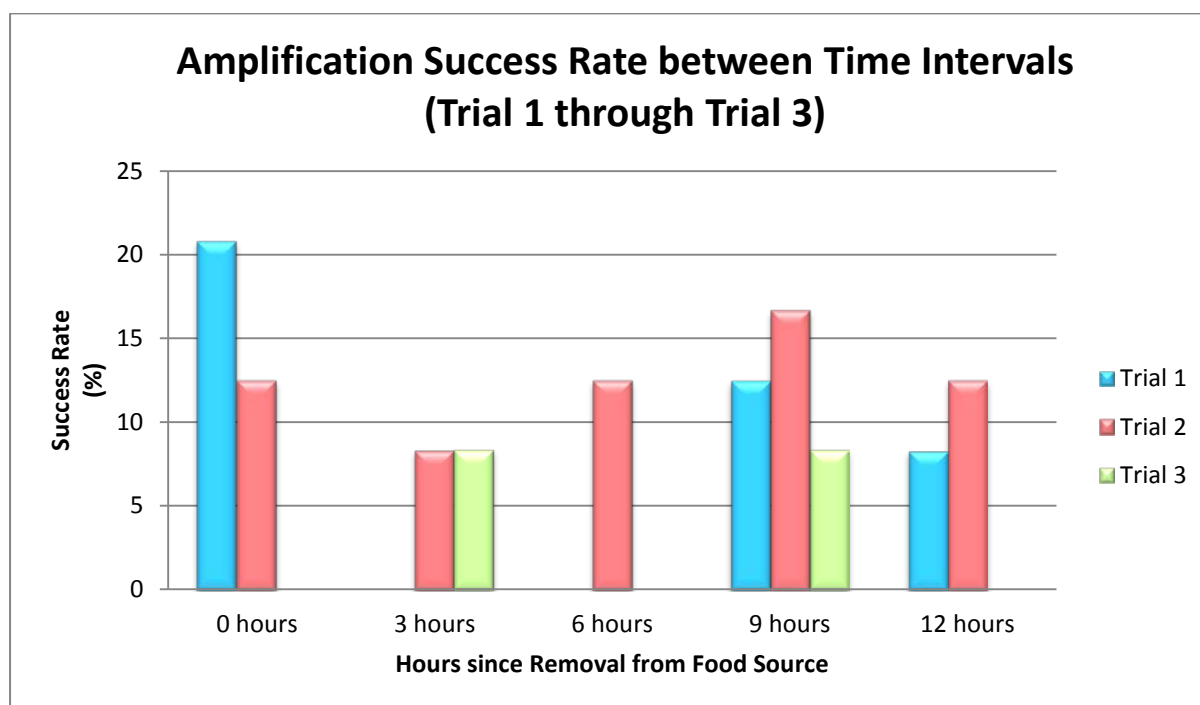


Figure 4-3: Amplification success rate between time intervals 0 through 12 hours post removal from food source for the three trials tested. In each time interval, the combined success rate of the nine maggots analyzed per time interval is depicted.

4.3.3.1. Trial 1

Allelic dropout was observed for primer BM1329 at 9 hours post removal from food source, as well as primer ETH225 at 0 hours post removal from food source. None of the primers

amplified successfully during the 3 to 6 hours post removal time intervals (Figure 4-3). During the 0 through 6 hours and 12 hour post removal time intervals, only one primer amplified successfully. During the 9 hour post removal time interval two primers amplified successfully in one maggot. As seen in Figure 4-3, amplification success rate generally declined with longer periods post removal from food source. Linear regression analysis indicated no significant difference between reproducibility of results with increasing time increments since removal from food source ($p = 0.7176$).

4.3.3.2. Trial 2

The highest overall amplification success was observed in Trial 2 (Figure 4-3). At most, two primers per time interval successfully amplified. For each individual maggot analyzed, only one out of the four primers tested amplified successfully. Allelic dropout at primers BM1329 and ETH225 was observed in time intervals 0 hours, 6 hours and 12 hours post removal from food source. No clear trend was observed when looking at the percentage of successful amplification of primers over the 12-hour period. Linear regression analysis results indicated no significant difference between the successful amplification of samples over the collection time intervals ($p = 0.4472$).

4.3.3.3. Trial 3

In terms of amplification success the third trial was the most unsuccessful. Only partial amplification was observed in two of the time frames, 3 hours and 9 hours post removal. Allelic dropout could be observed for the 9 hour post removal samples for both the loci that showed amplification. No amplification was observed for the time intervals 0 hours, 6 hours and 12 hours post removal from food source (Figure 4-3). Linear regression analysis indicated no significant difference between the collection time intervals, with a p-value of 0.9999.

4.3.3.4. Overall Amplification Success Rate of Results

In order to determine if there was any significant difference in the results obtained between the different trials, an ANOVA analysis was performed using the GraphPad InStat® software program, Version 3.00.

When comparing the ANOVA results between the various trials, Trial 2 had the highest mean result between the three trials analyzed (Figure 4-4). This indicates Trial 2 had the highest success in amplification of DNA obtained in the maggot crops analyzed in this trial. Trial 2 also had the lowest standard deviation indicating the lowest variation between results of samples analyzed in this trial. Trial 1 had the second highest mean result while Trial 3 had the lowest mean result. The greatest variation in successful amplification of DNA in samples analyzed was observed in Trial 1, as seen by the standard deviation value of 0.08838. Overall, ANOVA results indicated no statistical significant difference in amplification success rate between the trials ($p = 0.0916$).

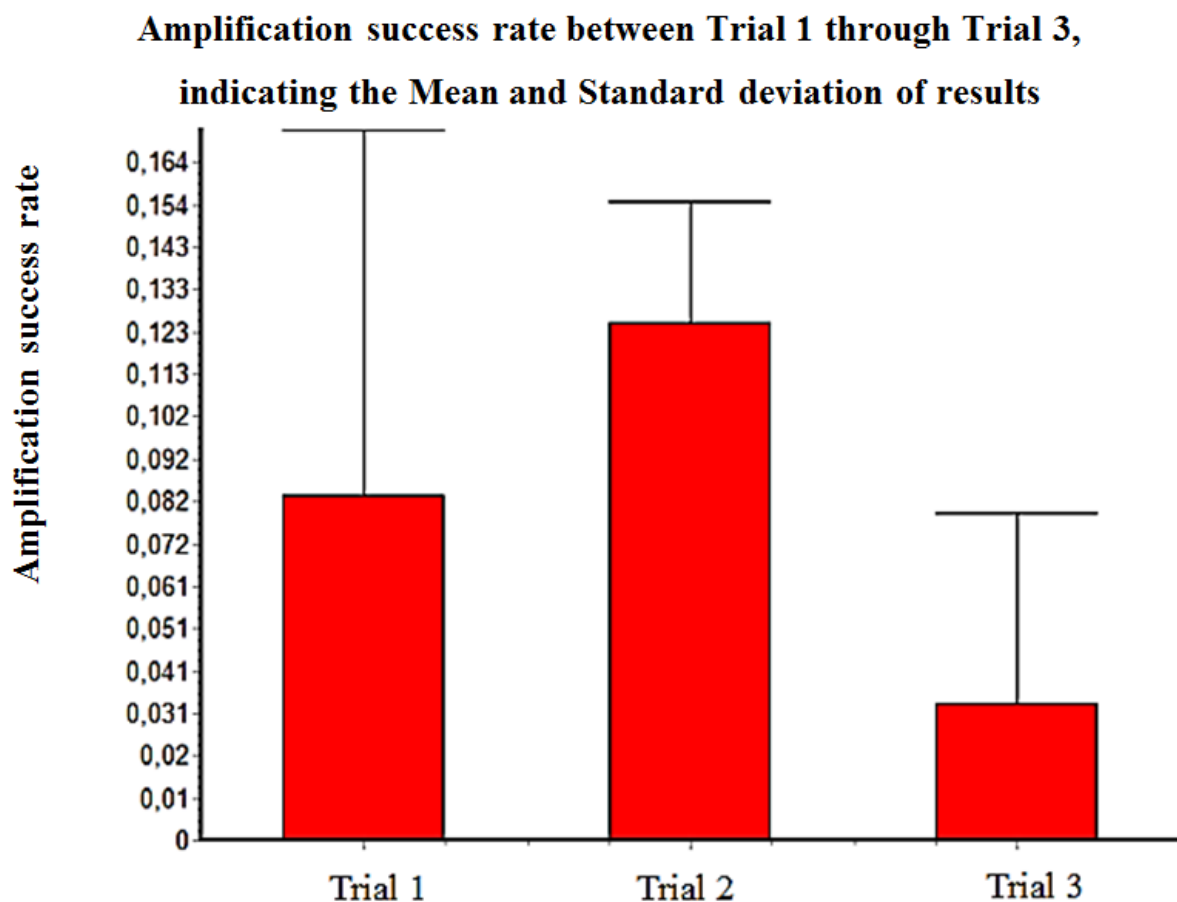


Figure 4-4: ANOVA results indicating the mean and standard deviation results between Trial 1, Trial 2 and Trial 3.

4.4. DISCUSSION AND CONCLUSION

With the advances in DNA technology, more focus has been put on using molecular methods in forensic entomology, not only for species identification but also in attempting to identify the maggot's food source (Kondacki *et al.*, 2009; Kester *et al.*, 2010). In instances where bodies discovered at crime scenes are too badly decomposed to perform traditional methods for identification of the deceased, it would be extremely useful to be able to identify the body through genetic testing of the gut content of the maggots found on the deceased. In extreme cases, instances could arise where only maggots are left at the scene of a crime, but no body is present. In such instances, it would be advantageous to be able to compare the DNA found in the gut of the maggots with a known reference sample of missing person suspected to be the deceased (Li *et al.*, 2007; Kondacki *et al.*, 2009; Kester *et al.*, 2010; Stuyt *et al.*, 2010).

An unexpected result drawn from this study was shortened developmental time leading to sampling only being possible up to 12 hours post removal. After this time interval, maggots began to enter the pre-pupae stage, which was faster than expected when compared to available literature. In a study by Byrd and Butler (1998) on the development of *Sarcophaga cruentata* maggots, results indicated that the maggot development up to third instar took approximately 86 hours (3.5 days) at a cyclic temperature of 26.7°C, and up to 142 hours (6 days) until pupa. In this study larvae were left to feed until they reached the fully fed stage and entered the wandering phase. Sarcophagidae maggot development is highly dependent on the temperatures to which the maggots are exposed. In the current study, maggots were left to develop at 28 ± 2°C. This higher temperature excelled maggot growth and development between the instars. Similar results of shortened developmental times was also observed in a study by Singh and Bala (2009) when *Chrysomya megacephala* maggots were starved. In this study, third instar maggots reached the post-feeding (wandering) larval stage earlier than would be expected. Therefore, the results suggest that starvation has an apparent acceleration effect on the development of the maggots. Results indicate the importance of considering the presence or absence of food when collecting *S. cruentata* maggots from a crime scene. This is an unexpected result drawn from the results of the current study, and should be considered in future research.

Although previous studies have indicated the possibility of obtaining useful DNA profiles from maggot gut content analysis, it still remained unclear for what period of time DNA can be obtained from the gut after the maggot has been removed from the food source. With regards to the molecular results, DNA concentrations varied greatly between the different time intervals analyzed. Overall, linear trend lines drawn from the data for each of the trials indicated that the DNA concentration over the time intervals tested decreased with increasing time since removal from food source, even though DNA quality was in most instances within the preferred range of 1.8 to 2.2 for pure DNA (Table 4-2).

Amplification results varied greatly between maggot samples, with no target DNA observed in many instances and amplification yielding only partial profiles in all samples. The

incomplete profiles from the maggots could be expected due to the high degree of degradation of the maggots' food source. Similar inconsistent results using phenol/chloroform extraction was observed by Stuyt *et al.* (2010) when performing a similar study using *Calliphora vicina* maggots. As in the study by Stuyt *et al.* (2010), the current study also obtained good results with regards to DNA quantity after DNA extraction using phenol/chloroform. However, DNA profiles could not consistently be obtained from the extracted DNA in order to identify the source. Another reason for the inconsistent results might be due to the high protein and lipid content of larval bodies, which could interfere with DNA purification. Another interesting aspect of this study was that the authors were unable to consistently detect the beef liver that was the food source for some of the maggot samples. The authors suggested that this result may reflect the differential effect of the salivary proteinase that is present in the maggot saliva on the beef tissue. Although this result was observed when analyzing a different species, the current study suggests that this might offer an explanation for the similar results observed when analyzing *S. cruentata* maggots. Degradation of the crop content may occur because of the biochemical alterations that occur as a result of the digestive enzymes in the saliva of the maggot that is secreted onto the food before consumption. These enzymes are mixed in with the tissue they consume, breaking the tissue down to monomers which cause some degradation of the DNA within (Carvahlo *et al.*, 2005). Further research on this matter is needed in the future before a conclusion can be drawn. Interpretation of results were further complicated due to the presence of high levels of background noise and artefacts such as split peaks, pull-up and dye blobs.

The current study also confirmed the lack of consistency between samples, as found by Stuyt *et al.*, (2010). Results were not only inconsistent between trials, but also inconsistent between maggots collected within the same time interval. In Trial 1, amplification success rate decreased with increasing time since removal from food source. Trial 2 results indicated an increase in amplification success rate over time while Trial 3 results were inconclusive in this regard due to very low overall amplification success. It is expected that the DNA concentration contained within the maggot crop is to decrease with increasing time since removal from food source due to digestion of the crop content. With increasing time since removal from food source, more of the content within the crop of the maggot will be

digested, leading to a decrease in the material available for DNA extraction and amplification from the crops of maggots (Stuyt *et al.*, 2010). This was not consistently observed in the current study. In Trial 1, the expected results were observed. When looking at the results of Trial 2, higher success was observed at 9 hours post removal from food source than at 3 hours post removal from food source. In Trial 3 in contrast, amplification success was consistent between 3 and 9 hours post removal from food source. Although no statistical difference was observed, as can be seen by ANOVA results, the average mean values for successful amplification of DNA samples differed substantially. The standard deviation between the maggot samples in each trial was also considerable indicating high variation between samples. This further indicated that results could not be obtained consistently using maggot gut content analysis. Due to the lesser degree of success with STR analysis, it has been suggested that greater success will be obtained by using SNP analysis due to the degraded nature of the samples (Kondacki *et al.*, 2009).

Although consistency in results was problematic in the current study, partial DNA profiles could still be obtained from *S. cruentata* maggot crops for up to 12 hours post removal from food source. Various factors contribute to successfully obtaining DNA from crop contents. This includes the quality and quantity of the food ingested by the maggot, the quality and quantity of the DNA present after extraction and amplification of the samples and the methods used to preserve the samples and perform DNA extraction (Carvalho *et al.*, 2005). It has further been suggested that the species of maggot used for crop content analysis might impact the success of obtaining DNA profiles. This is due to the varying degree and speed of digestion between different carrion feeding species after cessation of feeding (Gachuri 2013).

Therefore future research is needed to determine the rate of digestion for other species of forensic interest and to determine for what period DNA can still be obtained from the crop content of maggots. Overall, this study forms a baseline for further research into this topic.

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CHAPTER 5

SUMMARY OF RESULTS

Morphological analysis of insect evidence plays a significant role in crime scene investigation. With the influence of DNA analysis in forensic cases, which now also plays a key role in forensic entomology, more emphasis has been placed on a dual preservation goal when collecting insect evidence. Previous studies indicated that it might be possible to identify the last meal of sarcophagid maggots using gut content combined with DNA profiling. For gut content analysis it is imperative to be familiar with the internal morphology as well as maggot gastric emptying. However, insufficient information is available on the morphology of *Sarcophaga cruentata* maggot alimentary canals as well as the rate of maggot gastric emptying. Also, considering the use of insects for both PMI and DNA analysis, the current preservation methods are not necessarily suitable for maggot preservation for DNA analysis of the crop content.

Various preservation methods were examined for optimal preservation of both morphology and gut content. In order to understand gastric emptying, the internal gut structures and movement of food through the gut was examined. Fully fed maggots were removed from the food source and hot water killed at 3 hour intervals for up to 30 hours to investigate gastric emptying. Crop content DNA analyses were performed to attempt identification and DNA profiling of the maggots' last meal.

Due to the inhibiting effect of formalin on DNA analysis and the extensive dehydration of prolonged ethanol storage, -80°C was investigated for sample preservation which generally provided good results. Inconsistent results were obtained using the various preservation and DNA analysis combinations tested.

Sarcophagidae gut morphology analysis indicated gross anatomical similarity to *Chrysomya megacephala*. External tracking of food movement proved difficult. After digestive tract dissection it was found that the mid- and hindgut coiled around each other. Due to the coiled structure of the gut, the exact location of the food bolus could therefore not be determined without dissection.

The expected gastric emptying was not consistently observed with pronounced variability in results. Nevertheless, it was observed that the maggot crops were completely empty by 30 hours post removal from food source.

DNA profiling of the crop content supported previous findings, although only partial STR profiles were obtained. It is unlikely that full profiles will be obtained when analyzing gut content due to the degraded nature of the food source, as well as the effect of digestive enzymes present in the maggot saliva regurgitated onto the food source.

Various recommendations can be made based on the results. At crime scenes maggots should be killed in warm water ($\pm 60^{\circ}\text{C}$ for 30 seconds), dried on paper towel and stored in 80% ethanol while transported to the laboratory. Samples should be removed from the ethanol within 24 hours after collection, dried and stored individually in microcentrifuge tubes at -80°C (or similar low temperatures) until analysis is performed. During analysis samples should be handled on ice, ensuring the integrity of the sample, as it was found that samples defrosted rapidly after removal from the -80°C storage. It is further recommended to use commercially available kits when analyzing maggot gut samples due to the additional clean-up steps present in the kits. These clean-up steps aid in limiting the addition of fats and lipids from the maggot internal structures that could inhibit downstream DNA analysis of samples.

Overall this study reinforced the possibility of using maggot crop content for providing STR profiles of the victim and/or perpetrator. Although only partial STR profiles were obtained, it

indicated that, with further investigation and optimization, this is an interesting avenue for future research with many unexplored avenues for aiding in crime scene investigation.

Keywords: alimentary canal, dissection, DNA, ethanol, formalin, gut content, maggot, preservation, Sarcophagidae, STR

CHAPTER 6

OPSOMMING VAN RESULTATE

Morfologiese analise van insek bewyse speel 'n noemenswaardige rol in misdaadtoneel ondersoek. Met die invloed van deoksiribonukleïensuur (DNS) analise in forensiese sake, wat nou ook 'n sleutelrol speel in forensiese entomologie, word meer klem geplaas op 'n dubbele bergingsdoel met versameling van insek bewyse. Vorige studies dui aan dat dit moontlik kan wees om die laaste maaltyd van sarcophagid larwes te identifiseer deur gebruik te maak van dermkanaalinhoud gekombineer met DNS profielbepaling. Vir dermkanaalinhoudsbepaling is dit noodsaaklik om vertrouwd te wees met die larwale interne morfologie as ook larwale gatriese lediging. Onvoldoende inligting is egter beskikbaar oor die morfologie van *Sarcophaga cruentata* spysverteringskanale asook die tempo van larwale gatriese lediging. Verder, met die gebruik van insekte vir beide PMI en DNS analise, is die huidige bergingsmetodes nie noodwendig geskik vir larwale berging vir DNS analyses van die dermkanaal inhoud nie.

Verskeie bergingsmetodes vir optimale storting vir beide morfologie en dermkanaalinhoud analise is ondersoek. Om die gatriese-lediging te verstaan is die interne spysverteringskanaal struktuur asook die beweging van voedsel deur die dermkanaal ondersoek. Ten volle gevoede larwes is vanaf die voedselbron verwyder en met warm water doodgemaak teen 3-uur intervalle vir 30 ure na verwydering vanaf die voedselbron. Kropinhoud analise was uitgevoer in 'n poging tot identifikasie en DNS profielbepaling van die larwe se laaste maaltyd.

Weens die inhiberende effek van formalien op DNS analise asook die ekstensiewe dehidrasie effek van langdurige etanol berging, is -80°C berging ondersoek wat oor die algemeen goeie resultate verskaf het. Strydige resultate is verkry met gebruik van die verskillende bergings en DNS analise kombinasies getoets.

Morfologiese analise van Sarcophagidae dermkanale het aangedui dat dit ooreenstem met die van *Chrysomya megacephala*. Beweging van voedsel kon nie ekstern maklik gevolg word nie. Disseksie van die spysverteringskanaal het aangetoon dat die middel en agterderm om mekaar gedraai is. Die presiese posisie van die voedselbolus kon weens hierdie gedraaide struktuur van die dermkanaal, nie bepaal word sonder disseksie nie.

Die verwagte gastriese-lediging is nie konsekwent waargeneem nie, met merkbare variasie in resultate. Nietemin is dit waargeneem dat larwale kroppe teen 30 uur na verwydering van die voedselbron, heeltemal leeg was.

DNS profielbepaling het vorige bevindings ondersteun al is slegs gedeeltelike profiele verkry. Dit is egter onwaarskynlik dat vol profiele verkry sou word weens die gedegradeerde natuur van die voedselbron, asook die effek van verteringsensieme teenwoordig in die larwale spoeg, wat help met gedeeltelike vertering van die voedselbron.

Verskeie voorstelle kan gemaak word vanaf die resultate. By misdaad tonele moet larwes doodgemaak word in warm water ($\pm 60^{\circ}\text{C}$ vir 30 sekondes), drooggemaak word op papierdoek en gestoor word in 80% etanol tydens vervoer na die laboratorium. Monsters moet binne 24 uur verwyder word uit die etanol, drooggemaak en individueel gestoor word in buise teen -80°C (of soortgelyke temperature) tot analise uitgevoer word. Tydens analise moet eksemplare op ys hanteer word aangesien daar waargeneem is dat larwes baie vinnig ontdooi na verwydering uit -80°C berging. Daar word verder voorgestel dat kommersieel beskikbare stelle gebruik word om larwale dermkanaal inhoud te analiseer aangesien dit addisionele skoonmaakstappe vereis. Hierdie skoonmaakstappe beperk die invloed van vette en lipiede vanaf die larwale interne strukture wat DNS analise van monsters kan inhibeer.

Oor die algemeen versterk hierdie studie die moontlikheid dat larwale kropinhoud gebruik kan word om STR profiele van die slagoffer en/of oortreder op te lewer. Al was slegs gedeeltelike STR profiele verkry dui dit steeds daarop dat, met verdere navorsing en

optimisering, hierdie 'n interessante veld vir toekomstige navorsing met verskeie onverkende moontlikhede is wat hulp kan verskaf in die ondersoek van misdaadtonele.

Sleutelwoorde: dermkanaalinhoud, disseksie, DNA, etanol, formalien, larwe, preservering, Sarcophagidae, spysverteringskanaal, STR

APPENDIX A

ETHANOL PRESERVATION - TRIAL 1



Figure 1: Sarcophagidae third instar maggots 6 hours post removal from food source Trial 1. Comparison of pierced and unpierced maggot samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's A.M. Barnard 29/08/14.)



Figure 2: Sarcophagidae third instar maggot 12 hours post removal from food source, Trial 1. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 3: Sarcophagidae third instar maggot 18 hours post removal from food source, Trial 1. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 4: Sarcophagidae third instar maggot 24 hours post removal from food source, Trial 1. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 5: Sarcophagidae third instar maggot 30 hours post removal from food source, Trial 1. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).

ETHANOL PRESERVATION - TRIAL 2



Figure 6: Sarcophagidae third instar maggot 6 hours post removal from food source, Trial 2. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 7: Sarcophagidae third instar maggot 12 hours post removal from food source, Trial 2. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 8: Sarcophagidae third instar maggot 18 hours post removal from food source, Trial 2. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 9: Sarcophagidae third instar maggot 24 hours post removal from food source, Trial 2. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 10: Sarcophagidae third instar maggot 30 hours post removal from food source, Trial 2. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).

ETHANOL PRESERVATION - TRIAL 3

Figure 11: Sarcophagidae third instar maggot 6 hours post removal from food source, Trial 3. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 12: Sarcophagidae third instar maggot 12 hours post removal from food source, Trial 3. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 13: Sarcophagidae third instar maggot 18 hours post removal from food source, Trial 3. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 14: Sarcophagidae third instar maggot 24 hours post removal from food source, Trial 3. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 15: Sarcophagidae third instar maggot 30 hours post removal from food source, Trial 3. Comparison of pierced and unpierced samples stored in 100% EtOH for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).

10% FORMALIN PRESERVATION - TRIAL 1



Figure 16: Sarcophagidae third instar maggot 6 hours post removal from food source, Trial 1. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row has not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 17: Sarcophagidae third instar maggot 12 hours post removal from food source, Trial 1. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 18: Sarcophagidae third instar maggot 18 hours post removal from food source, Trial 1. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 19: Sarcophagidae third instar maggot 24 hours post removal from food source, Trial 1. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 20: Sarcophagidae third instar maggot 30 hours post removal from food source, Trial 1. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).

10% FORMALIN PRESERVATION - TRIAL 2



Figure 21: Sarcophagidae third instar maggot 6 hours post removal from food source, Trial 2. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 22: Sarcophagidae third instar maggot 12 hours post removal from food source, Trial 2. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 23: Sarcophagidae third instar maggot 18 hours post removal from food source, Trial 2. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29).

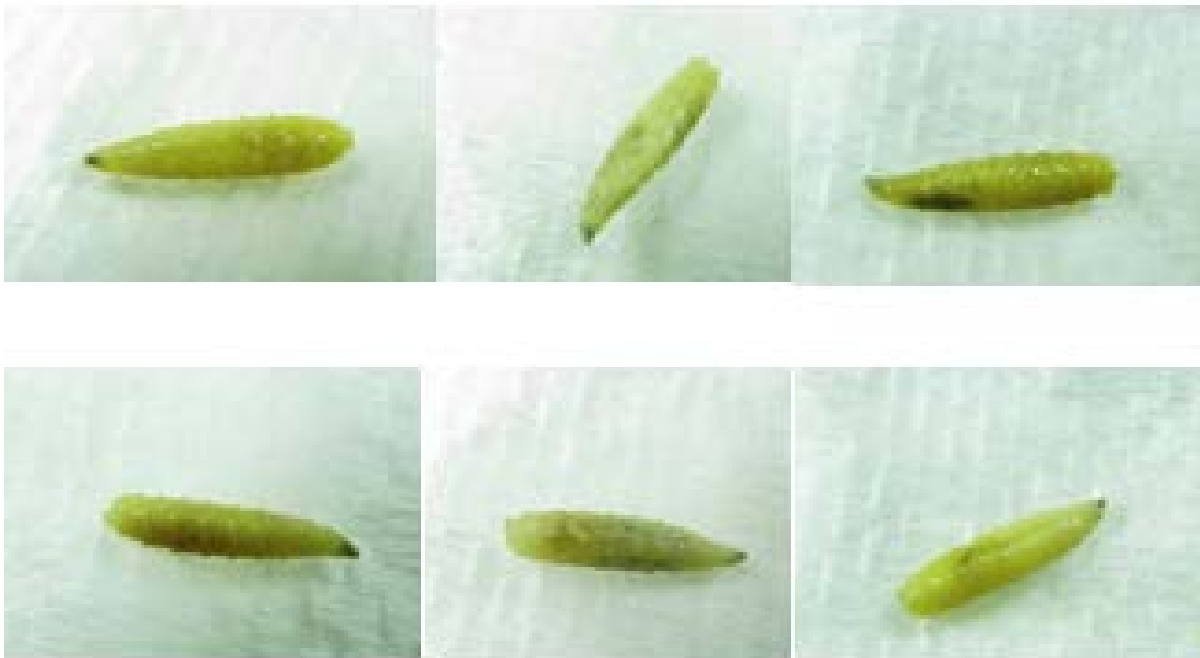


Figure 24: Sarcophagidae third instar maggot 24 hours post removal from food source, Trial 2. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 25: Sarcophagidae third instar maggot 30 hours post removal from food source, Trial 2. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).

10% FORMALIN PRESERVATION - TRIAL 3



Figure 26: Sarcophagidae third instar maggot 6 hours post removal from food source, Trial 3. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 27: Sarcophagidae third instar maggot 12 hours post removal from food source, Trial 3. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).



Figure 28: Sarcophagidae third instar maggot 18 hours post removal from food source, Trial 3. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).

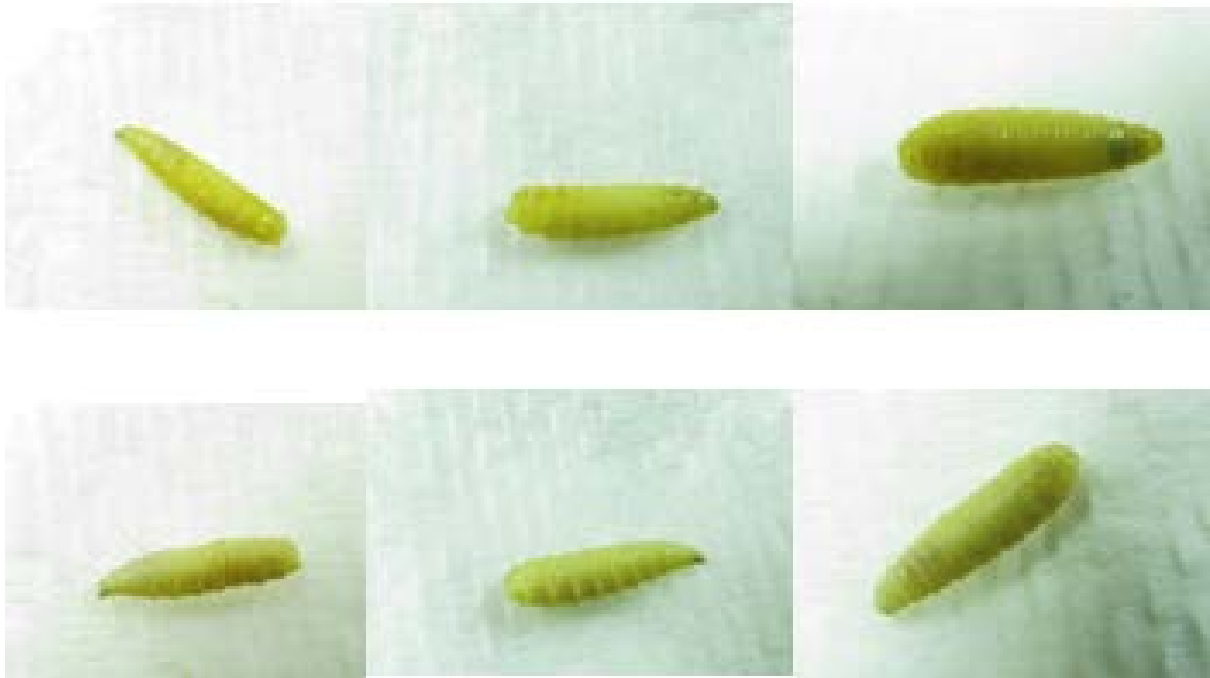


Figure 29: Sarcophagidae third instar maggot 24 hours post removal from food source, Trial 3. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).

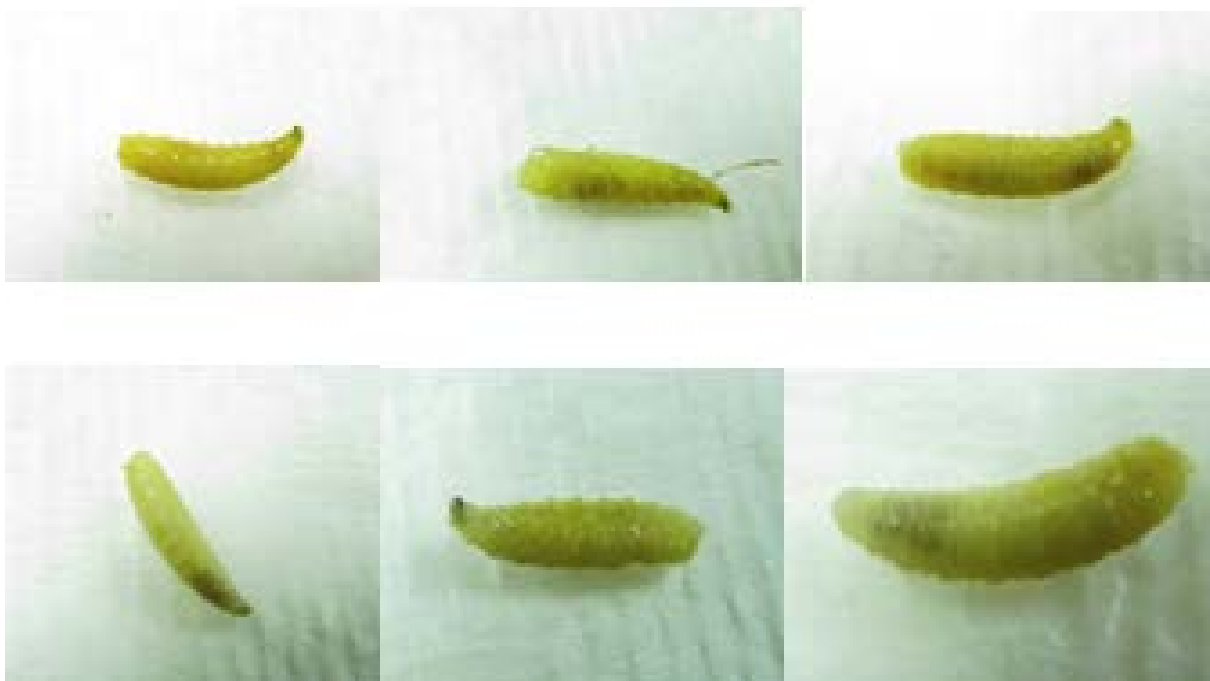


Figure 30: Sarcophagidae third instar maggot 30 hours post removal from food source, Trial 3. Comparison of pierced and unpierced samples stored in 10% Formalin for 10 days. Top row was not pierced while bottom row was pierced twice (Photo's by A.M. Barnard 29/08/2014).