The cascading trophic accumulation of aldicarb in a carrion ecosystem: the forensic implications.

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DECLARATION

I, Tshepiso Christinah Motolo, declare that the Master's Degree research dissertation or interrelated, publishable manuscripts/published articles, or coursework Master's Degree mini-dissertation that I herewith submit for the Master's Degree qualification in Entomology at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

Tshepiso Christinah Motolo

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ABSTRACT

Entomotoxicology is a relatively new discipline in forensic entomology which deals with the study of drugs in insects of forensic importance. The toxicant under investigation is the active ingredient found in an insecticide commonly known as “Two steps”. This was the first study to investigate the forensic implications of the toxicant, aldicarb, in a carrion ecosystem.

Insects that feed on a deceased person who had toxicants in his or her system will also ingest the toxicants. In cases where the decomposed tissues of the deceased have degraded and can no longer be used for traditional toxicological analysis, insect specimens can be utilised as an alternative toxicology matrix. A high performance liquid chromatograph-Ultra Violet detector (HPLC-UV) was used to detect the toxicant in entomological specimens. Varying concentrations of aldicarb were mixed with chicken livers and presented to *Chrysomya chloropyga* fly larvae and *Thanatophilus micans* adult beetles. There was a correlation between the aldicarb concentrations found in entomological specimens and the concentration present in the chicken livers. An experiment was also set up to test for the accumulation of aldicarb in a secondary trophic level of a carrion food chain. To this end, *C. chloropyga* larvae that were exposed to the toxicant at varying concentrations were presented as a prey item to predatory *Chrysomya albiceps* fly larvae and adults of the predaceous *Saprinus splendens* beetle. Although the toxicant was recovered from these predators, there was no correlation between the toxicant concentrations in them and that that the *C. chloropyga* larvae were exposed to.

Another iteration of this experiment was to test which of the post-feeding life stages of flies can also be used as an alternative toxicological matrix. It was postulated that the toxicant would be eliminated during the post-feeding stages of the fly and that some of the toxicant might be deposited in the cuticle of larvae and consequently also in the pupal casings. It was found that since the emergent adult flies of *C. albiceps* and *S. cruentata* still contained trace amounts of the toxicant, the pupae of these two species

would still be suitable alternative toxicology sources. The toxicant was not be picked up in the emergent adult flies of *C. chloropyga* and it is unclear up to what point the toxicant remains present in its pupae. The toxicant was not picked up in the pupal casings of any of the flies. However since the extraction method used might have been inadequate to release the toxicant from the chitinised matrix of the pupal casing, a verdict cannot be made regarding excluding the pupal casings as alternative toxicology source.

When calculating a post mortem interval (PMI) based on the Developmental Model, it is imperative to know whether or not the deceased was exposed to toxicants since toxicants can potentially influence the growth rate of forensic indicator species. To test the effect of aldicarb on the development of forensic flies, *C. chloropyga*, *C. albiceps* and *Sarcophaga cruentata* larvae were exposed to a lethal dose of aldicarb. Larvae were measured and weighed at 24 hour intervals, pupal development was tracked by noting morphological landmarks every 24 hours and adult fitness was assessed based on the ability to reproduce. Aldicarb slowed down the total development rate of *C. chloropyga* and accelerated that of *C. albiceps* but had no effect on *S. cruentata*. The necessary PMI adjustments should be made for the calliphorid life stages that were exposed to the toxicant as larvae. It was furthermore noted that the toxicant did not affect the reproductive fitness of all species examined.

Keywords: Entomotoxicology, Aldicarb, *Chrysomya chloropyga*, *Chrysomya albiceps*, *Sarcophaga cruentata, Thanatophilus micans, Saprinus splendens*

LIST OF ABBREVIATIONS

AAS	- anabolic androgen steroids
AChE	- acetylcholinesterase
ADH	- accumulative degree hours
ANOVA	- Analysis of Variance
BBB	- blood brain barrier
CNS	- central nervous system
g	- gram
h	- hours
HPLC (UV)	- high liquid chromatography ultra violet
LD□	- lethal dose
mg/kg	- milligram per kilogram
mL	- millilitre
MS	- mass spectrometry
nm	- nanometre
PAI	- pre-appearance interval
PIA	- period of insect activity
PMI	- post mortem interval
PMR	- post mortem redistribution
ppm	- parts per million
rpm	- revolutions per minute
WHO	- world health organisation

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

★Picture taken by: Z. Mbo

1. INTRODUCTION AND LITERATURE REVIEW

1.1 MEDICO-LEGAL ENTOMOLOGY

Forensic pathologists use a range of interval changes a body undergoes upon death to determine the post mortem interval (PMI), however, the precision of this estimate declines within 72 hours (Tempelman-Kluit 1993). Catts & Goff (1992) defined forensic entomology as the application of the study of insects and other arthropods to legal issues especially in a court of law. It consists of three areas: urban, stored products and medico-legal entomology (focus of the study). When a body covered in necrophagous insects is found, medicolegal entomology comes into play. Medico-legal entomology is a branch of forensic entomology whereby insects are used as evidence to shed light in legal matters when death was not witnessed or is suspicious (Gennard 2007; Amendt *et al.* 2011; Sharma *et al.* 2015). It provides data that cannot be obtained by using standard pathology particularly in cases whereby death arose beyond three days (Amendt *et al.* 2011; Sharma *et al*. 2015) or in cases where the body is compromised as in the case of charred remains (Bugelli *et al.* 2017).

Entomologists analyse the species composition and collect insects from and around the body to determine the lifecycle stage and species of the oldest insects thus giving rise to the time of colonisation otherwise known as the minimum PMI (Catts 1992; Mullany *et al.* 2014; Sharma *et al.* 2015). Except for the medico-legal entomological application of PMI estimation, insects can also be used to determine whether a body had been relocated (Benecke 1998; Campobasso *et al.* 2001; Thyssen & Grella 2011). Flies are known to show preferences for carcasses in different environments, therefore, a body would have been tampered with if found out in the field covered in eggs or larvae of flies that usually reside indoors. Insect evidence can further be used to determine whether or not the deceased was neglected and for how long the person was neglected prior to death (Benecke & Lessig 2001). Furthermore, arthropods can also help in identifying a suspect in sexual abuse cases by examining insects sampled from victims (Campobasso & Introna 2001). Lastly, insect evidence can be used to determine the presence of drugs (Amendt *et al.* 2011). The complete absence of

insects means that the corpse had probably been frozen or sealed in a securely tight container.

The main application of medico-legal entomology, is the correct estimation of a PMI (Definis-Gojanovic *et al.* 2007; Velez & Wolff 2008; Shiravi *et al.* 2011; Sharma *et al.* 2015). PMI is defined as the period between death and the discovery of the body (Amendt *et al.* 2011). The interval resembles the point at which the first insects to arrive deposited their eggs on the body which is actually the period of insect activity (PIA). According to Brown (2012), the minimum PMI is longer than the PIA considering the additional time it takes for the insects to locate the body and factors that delay colonisation. These include: wind speed and direction, rainfall, location and the covering of the body. Furthermore, flies are not known to be active at night. Forensic entomologists therefore, need a background knowledge of the distribution, biology, ecology and behaviour of insects associated with decomposing corpses to make an informed PMI estimation (Velez & Wolff 2008; Sharma *et al.* 2015).

Brown (2012) stated that, the determination of the PMI incorporates numerous fields within entomology. These include: the use of morphological and molecular approaches to identify insects to species level, entomotoxicological analysis, decomposition and succession patterns, and developmental data. PMI estimates can be narrowed down to days or even hours depending on: the condition the corpse was found in, the technique used for entomological sampling, species composition as well as their stages of development.

There are currently two methods used to determine the PMI estimate and the use of each, depends on the state of decomposition the body is in (Sankhla *et al.* 2017). The first method focuses on the timely arrival of insects (insect succession) and the second method focuses on the development of immature flies (development model). Sankhla *et al.* (2017) further stated that, factors such as season, climate, location and treatment of the corpse also determine which method should be used. Succession patterns are usually used in the later stages of decomposition after the first colonisers have

completed a lifecycle. For insects that colonise the body at later stages, a pre-appearance interval (PAI) is used instead (Bajerleina *et al.* 2018). Richards *et al.* (2009) gives a good overview of a PMI based on the development method of PMI determination. In the article, they explained that the determination of PMI involves the use of four models namely: curvilinear regression, isomegalen diagrams, isomorphen diagrams and thermal summation models. Isomegalen and isomorphen diagrams however, are only applicable to bodies found indoors where the temperature is roughly constant (Sharma *et al.* 2015). These diagrams illustrate the developmental pattern at different temperatures from which age estimation can be done.

1.2 A CARRION ECOSYSTEM

1.2.1 TROPHIC LEVELS

Soon following death, chemicals are released from the body and are detected by insects leading to the arrival of these insects. The first insects to arrive are necrophages; arthropods that feed directly on the carcass. Shortly thereafter, predators, parasites and omnivores make their way to the carcass. Predators are arthropods that feed on necrophages as well as on the eggs of other insects (Boucher 1997). Parasites inject their eggs or larvae into necrophages and their larvae feed on necrophages in order to develop. Omnivores are arthropods that feed on both the carcass as well as the insects feeding on the carcass (Boucher 1997). Opportunists' are arthropods that use the body as part of their habitat or are just there by chance and serve no forensic importance. A carrion ecosystem is therefore, the interaction of arthropods and their environment on a carcass. A schematic diagram is shown in Figure 1.1.

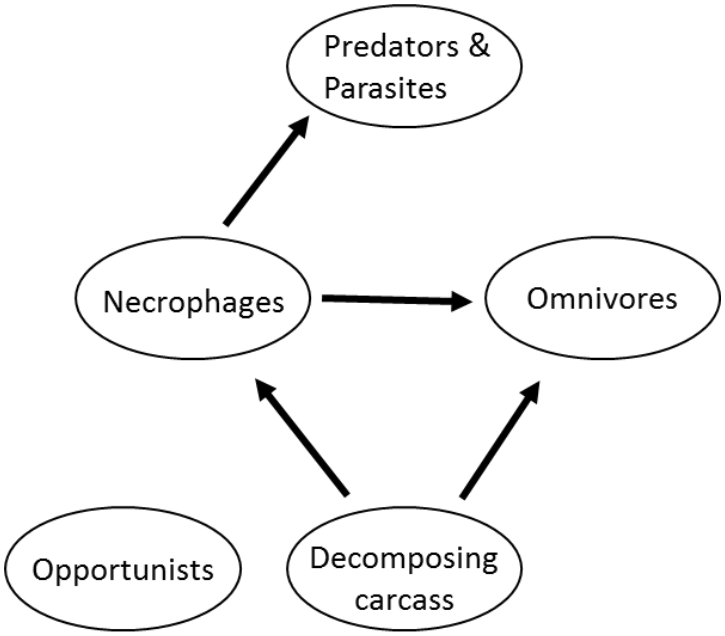

Figure 1.1: Diagrammatic representation of the trophic levels in a carrion ecosystem.

1.2.2 DECOMPOSITION

Kocarek (2003) defined decomposition as the process whereby dead materials are broken down to simpler matter. Under normal circumstances, decomposition consists of five stages namely: fresh, bloat, active decay, advanced decay and skeletal decay. In the absence of scavengers, the breakdown of tissues is largely dependent on insects (Carter *et al.* 2007). Maggots are usually found in abundance and are thus the driving force of decomposition. The more necrophagous insects are present, the faster the rate of decomposition. The rate of decomposition is to some degree, slowed down by the presence of predators, parasites and omnivores (feeding on necrophages).

1.2.3 INSECT SUCCESSION

The forensic indicators of utmost importance are Diptera and Coleoptera (Greenberg 1991; Kelly 2006; Huchet 2010). This is because they are the first to arrive, are found in abundance, they complete their life cycle on the body and their biology and ecology are well established (Kintz *et al.* 1990; Gosselin *et al.* 2011a). According to Byrd & Castner (2010), the different stages of decay will attract and repel certain species

leading to the predictable appearance of insects known as succession. Succession is defined as the sequential colonization of arthropods in an orderly manner.

Succession begins with an influx of primary flies during the first wave. According to Louw & van der Linde (1993) and Kovler (2009), the South African species of primary flies are: *Lucilia* sp. Robineau-Desvoidy, 1830, *Chrysomya marginalis* (Wiedemann, 1830), *Chrysomya chloropyga* (Wiedemann, 1818) and *Calliphora vicina* Robineau-Desvoidy, 1830. Of the primary flies, this study only focused on *C. chloropyga*. *Chrysomya chloropyga* are commonly known as the “green bottle blowflies”. As an adult, it is easily distinguished from other blow flies by the omega symbol on its prothorax (Fig. 1.2). It is medium sized with a wingspan of 18 mm (Picker *et al.* 2002). It is widely distributed around South Africa and is usually found in winter and spring months (Picker *et al.* 2002; Kovler 2003). Adults oviposit eggs on carrion which hatch into feeding larvae. Its larvae are necrophagous.

Figure 1.2: Photograph of *Chrysomya chloropyga* with its distinctive feature clearly visible on its prothorax (Picture taken by Gernus Terblanche).

The second wave of colonisers follows thereafter, with the arrival of secondary flies and the only known species in South Africa is *Chrysomya albiceps* (Wiedemann, 1819). It is commonly known as the “banded blowfly” and is a cosmopolitan species (Grassberger *et al*. 2003). The adults are metallic green with dark abdominal bandings (Fig. 1.3). They are medium in size with a wingspan of 20 mm (Picker *et al.* 2002). The adults oviposit eggs on the carrion which hatch into feeding larvae. The larvae are easily identifiable from other species by being “hairy” i.e. fleshy protrusions covering their body (Huchet 2010). The first instar larvae are said to be necrophageous while the second and third instars are predaceous in addition to being necrophageous (Grassberger *et al*. 2003; Salimi *et al.* 2018). According to Faria *et al.* (1999), predation involves: encounter, attack, capture and ingestion of prey. *Chrysomya albiceps* larvae are generalist predators and predation is strongly influenced by age and size structure (Reigada & Godoy 2005). Cannibalism (organisms that feed on their own kind) has been reported in the larvae of this species (Reigada & Godoy 2005; Brink 2009; Salimi 2018; personal observation). This behaviour is a means of reducing competition and having enough food for oneself (Faria *et al.* 2004).

Figure 1.3: Photograph of *Chrysomya albiceps* commonly known as the “banded blowfly” due to the presence of distinct bands found on its abdomen (Picture taken by Gernus Terblanche).

Tertiary flies are represented by a single Family: Sarcophagidae. The species mostly found in Bloemfontein is *Sarcophaga cruentata* Meigen, 1826. Tertiary flies are said to exhibit adaptations that allow them to be found during the later stages of wet decay. Sarcophagids are commonly known as 'flesh flies". The adults are large in size with a wingspan of 28 mm (Picker *et al.* 2002). They have a black thorax with grey stripes (Fig. 1.4) and are not metallic (Triplehorn & Johnson 2005). Unlike *C. chloropyga* and *C. albiceps*, *S. cruentata* are larviparous (lay live larvae). The larvae are easily distinguished from other species by their size and deep posterior spiracular cavity. Sarcophagid larvae are necraphagous.

Figure 1.4: Photograph of *Sarcophaga cruentata* commonly known as "flesh flies" with their non-metallic grey appearance and striped thorax.

Coleoptera are also a major component of the arthropods found on a decomposing body. Forensic beetles use the carrion as a breeding medium, as a food source for the adult and immature stages and as a hunting ground for other necrophagous insects present on the body. According to Boucher (1997), the families that are prevalent on carrion in the central Free State are: Siphidae, Dermestidae, Histeridae, Staphynilidae, and Cleridae. This study only focused on the necrophagous Silphidae (*Thanatophilus micans*) and the predatory Histeridae (*Saprinus splendens*).

Histeridae beetles are commonly known as “clown beetles” (Triplehorn & Johnson 2005). They are small to medium in size with a body length ranging between 1-20 mm (Picker *et al.* 2001). Histeridae elytra are shiny and do not cover the last 2 segments of the abdomen. *Saprinus splendens* are oval shaped and black or green in colour (Fig. 1.5). They are predaceous in both adult and larval form.

Figure 1.5: Light micrograph of the histerid beetle, *Saprinus splendens*, used during this study (Picture taken by Thabo Moeti).

Silphidae beetles are flattened and metallic green or blue. They are known as “Carrion beetles” (Triplehorn & Johnson 2005). They have vertical ridges on their elytra and their elytra do not cover the last four abdominal segments (Fig. 1.6). According to literature adults and larvae are necrophagous. Larvae can however be cannibalistic (personal observation).

Figure 1.6: Light micrograph of a Silphidae beetle, *Thanatophilus micans*, with its short elytra leaving the last four abdominal segments exposed (Picture taken by Thabo Moeti).

1.3 ENTOMOTOXICOLOGY

There has been an increase in the number of drug related deaths and by the time most of the bodies are recovered, the decomposed tissues of the deceased have long degraded (Sankhla *et al.* 2017). In such cases, toxicological analysis will largely depend on the examination of entomological evidence (larvae, pupae, puparial cases, exuviae and beetle frass) gathered on and near the corpse (Kintz *et al.* 1990; Gagliano-Candela & Aventaggiato 2003; Thyssen & Grella 2011; Sankhla *et al.* 2017). According to Definis-Gojanovic (2007), toxicological analysis using entomological specimens is similar to when using human tissue or fluids.

Entomotoxicology is the field of study where insect evidence is utilised in forensic toxicology investigations. Insects can be used where traditional toxicology screening cannot be performed due to the extent of decomposition of a body. The insect samples collected from the deceased are analysed to determine the presence of toxicants that accumulated in the insects in the process of feeding on the body (Oliveira *et al.* 2014). Larvae are usually used because they remain on the body for a long period, are easy to sample and have fewer matrix effects which makes them easier to examine (Hutchet

2010; Franca *et al.* 2015). According to Franca *et al.* (2015), drug concentrations are more stable in entomological specimens than in putrefied tissue. The qualitative application of entomotoxicology is and has been well established in the police industry (Sankhla *et al.* 2017). Insects are very resourceful in drug related cases and drugs extracted from them can be presented as evidence in court (Bushby *et al.* 2012; Verma & Paul 2013). Entomotoxicology also includes studying the effects of drugs on insect physiology and their growth rates (Gosselin *et al.* 2011b; Sankhla *et al.* 2017). Drugs may potentially alter the development rate of forensic flies leading to erroneous PMI estimates.

Although entomological specimens make excellent alternative toxicological specimens, there is still a gap of knowledge and research in entomotoxicology (Sankhla *et al.* 2017). According to Liu *et al.* (2009), entomotoxicology is a new field in entomology which has not yet proven to be reliable. One of its limitation is that it only proves the presence of toxicants in the deceased but does not trace the cause of death back to the toxicants. Sankhla *et al.* (2017) explained that this is because its quantitative application is still inadequate and there are no concrete equations to calculate the initial concentration that was ingested by the deceased from that found in the entomological specimens. In addition to that, toxicants can only be detected in larvae that are still in the feeding stage because then, the rate of absorption exceeds the rate of elimination (Verma & Paul 2013; Sankhla *et al.* 2017).

Numerous studies have proposed that there could be a correlation to some degree (between the drug concentrations in the entomological samples in comparison to the drug concentration in the food source) even though, it has not yet proven to be a relevant correlation. One the other hand, there have been studies that claim to have achieved a fixed regression equation for entomotoxicological analysis (Sankhla *et al.* 2017). It is difficult to interpret quantitative analysis because there is still a broad spectrum of factors that are currently unexplored and unpredictable (Gosselin *et al.* 2011a; Franca *et al.* 2015). For now, drug concentrations detected in entomological samples and cadaver tissue still cannot be interpreted (Dayananda & Kiran 2013).

1.4 ALDICARB

Entomotoxicological studies have mostly focused on prescription medicine and examined the effects of frequently abused drugs on the growth and mortality rates of insects (Verma & Paul 2013). Prescription drugs and pesticides have led to many fatalities (Franca *et al.* 2015). Even though poisoning patterns vary throughout the world, pesticides have contributed to many drug related deaths (Franca *et al.* 2015). Death statistics are easily obtainable for most drugs but not for aldicarb. A poison expert by the name of Gerhard Verdoorn, told the Sowetan newspaper that aldicarb kills at least one hundred people per year in Johannesburg alone. This study focused on aldicarb which is an active ingredient found in a carbamate used in agriculture as an insecticide, acaricide and nematocide (Covaci *et al.* 1999; Proenca *et al.* 2004; Damasceno *et al.* 2008).

Aldicarb is the common name for 2-methyl-2(methylthio) propionaldehyde-O-(methylcarbamoyl) oxime (Proenca *et al.* 2004). Its molecular formula is $C_7H_{14}N_2O_2S$. It is produced by Bayer Cropscience and is sold under the tradename “Temik”. Aldicarb is sold in granular form. It is dark grey in colour and is said to have no taste or distinctive smell, making it difficult to detected (Durao & Machado 2016). Aldicarb is quickly oxidized to aldicarb sulfoxide by hepatic microsomal enzymes and then gradually metabolized to aldicarb sulfone by oxidation and hydrolysis before undergoing hydrolytic breakdown to noncholinergic agents (Baron 1994; Damasceno *et al.* 2008; Blondet *et al.* 2015). Aldicarb sulfoxide is considered to be more toxic than aldicarb itself, while aldicarb sulfone is less potent than aldicarb, the original sulphide (Baron 1994). The two metabolites are then detoxified by hydrolysis oximes and nitrites. According to Trehy *et al.* (1984), aldicarb oxime and aldicarb nitrile are the major by-products of aldicarb formed in spiked anaerobic water samples.

According to the World Health Organisation (WHO), aldicarb is a persistent pesticide with a degradation half-life of up to two months in soil and several years in acidic groundwater. In agriculture it is used as an insecticide, acaricide and nematocide and due to its persistence, farmers do not need to apply it multiple times. It is highly toxic

and classified as a restricted use pesticide but is illegally sold as a rodenticide in most parts of the world. Baron (1994) stated that, aldicarb has a mammalian toxicity of LD_{50}=0.3-1.5 mg/kg when given orally to laboratory animals. The oral LD_{50} for humans is 0.80 mg/kg (Arnot *et al.* 2011).

In South Africa aldicarb is commonly known as “Two steps” because after ingestion a person only takes two steps before they die. There is not much literature available on aldicarb intoxication autopsies regardless of its many fatalities (Durao & Machado 2016). Thieves usually mix it with meat baits to kill dogs thus gaining easy access to people’s properties (Arnot *et al.* 2011). Due to its easy obtainability, it has been reported in homicidal, accidental as well as suicidal deaths. In homicidal attempts, aldicarb is usually mixed with dark coloured foods (Durao & Machado 2016). Aldicarb is rapidly absorbed by the digestive tract, skin, and can be detected in the bloodstream after ingestion. It is highly toxic by all routes of entry whether orally, dermally or subcutaneously (Covaci *et al.* 1999; Proenca *et al.* 2004). Its metabolites are also toxic therefore, protective gear always has to be worn when handling this toxicant (Durao & Machado 2016).

Aldicarb’s mode of action involves the inhibition of acetylcholinesterase (AChE) (Arnot *et al.* 2011; Durao & Machado 2016). This is a vital enzyme in charge of the chemical reaction that transforms acetylcholine (a neurotransmitter) into choline in the nervous systems of insects and mammals. The inhibition of this enzyme leads to the build-up of acetylcholine which interferes with the transmission of nerve impulses between nerve junctions (Arnot *et al.* 2011; Durao & Machado 2016). This results in the loss of muscular coordination, convulsions and death. It takes several hours for aldicarb to separate from AChE meaning the inhibition is actually reversible. This is said to occur even after death. This reversibility of the AChE inhibition is what differentiates carbamate from organophosphate insecticides (Durao & Machado 2016).

Durao & Machado (2016) mentioned the following neurological and cholinergic symptoms due to aldicarb poisoning: sweating, salivation, miosis, bronchial hyper

secretion, respiratory failure, bronchospasm, cough, vomiting, mental confusion, and seizures. Aldicarb also triggers a depression of foetal blood and brain acetylcholinesterase activity. Studies done on animals show that aldicarb has no effect on the reproduction, fertility, gestation, viability and lactation (Baron 1994). In addition to this, no congenital malformations were observed. Just like in animals, studies done on human volunteers also showed rapid acetylcholinesterase inhibition followed by a rapid recovery. Baron (1994) concluded that toxicological responses of humans to aldicarb and its metabolites are the same as those of experimental vertebrate animals (Baron 1994).

1.5 AIMS AND OBJECTIVES

The **aim** of the study was to determine the forensic significance of aldicarb in a carrion ecosystem. This was achieved by the following **objectives**:

1. Testing the bio-accumulation of aldicarb in a carrion ecosystem (Chapter 2)
2. Testing the elimination of aldicarb in the post feeding stages of some flies of forensic importance (Chapter 3).
3. Testing the effect of aldicarb on the larval development rate of some flies of forensic importance (Chapter 4)
4. Testing the effect of aldicarb on pupal development and the adults of some flies of forensic importance (Chapter 5)

1.6 RATIONALE

Not all the species of the central Free State carrion system were examined. Species were selected based on their trophic level and successional “wave”. *Chrysomya chloropyga* was chosen as a calliphorid representative of a primary fly whose larvae feed exclusively from the carrion itself. The calliphorid *Chrysomya albiceps* represented a secondary fly whose larvae feed from the carrion, but also maximise its feeding gains in a carrion food chain by cannibalism and by predating on the larvae of other fly species larvae. Necrophagous *Sarcophaga cruentata* larvae is the only

tertiary fly and consistent sarcophagid species in this particular carrion food chain. *Thanatophilus micans* beetles were chosen because they are necrophagous and *Saprinus splendens* because of their predaceous nature. Aldicarb was investigated because it has been reported in homicidal, accidental as well as suicidal deaths.

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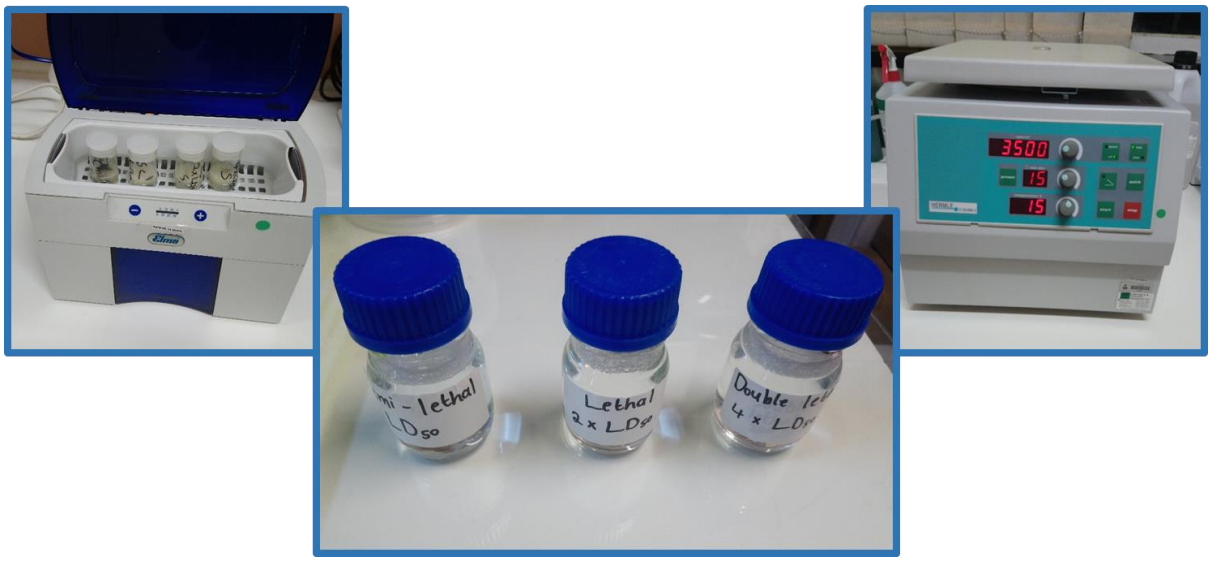
CHAPTER 2

Bio-accumulation of aldicarb in a carrion ecosystem



2. BIO-ACCUMULATION OF ALDICARB IN A CARRION ECOSYSTEM

2.1 INTRODUCTION

Entomotoxicology is a relatively new branch of forensic entomology whereby insects are used as an alternative source for toxicological screening (Gosselin *et al.* 2011a). Traditional toxicology is not always efficient or possible for highly decomposed or skeletonised remains. In such cases, the insect stages that have ingested drugs (and/or metabolites) present in a deceased drug overdosed victim can be used as a source for toxicological analysis (Mullany *et al.* 2014). Drug concentrations are more stable in entomological samples than in decomposed tissue (Franca *et al.* 2015).

When larvae are exposed to toxicants whilst feeding on a contaminated substrate, two processes are known to occur: bioaccumulation and elimination of the toxicant and its metabolites (Carvalho *et al.* 2001). Gobas *et al.* (2016) defined bio-accumulation as "a process by which chemicals are taken up by an organism either directly from exposure to a contaminated medium or by consumption of food containing the chemical". When the concentration of a chemical in a trophic level exceeds that of the food source, it is referred to as bio-magnification (Gobas *et al.* 1999). Usually when bioaccumulation occurs in larvae, the toxicant has a significant effect on their development and mortality (Carvalho *et al.* 2001). Previous studies have also shown that toxicant bioaccumulation may also affect the morphology of forensic flies (Fathy *et al.* 2008). To curb the harmful effects of a toxicant, insects have the ability to eliminate toxicants via various mechanisms.

Not much is known about the bioaccumulation of drugs in a carrion based ecosystem but toxicants are known to not bio-accumulate throughout the life-cycle of an insect (Sadler *et al.* 1995). Toxicants can only be detected when the rate of absorption exceeds the rate of elimination (Sadler *et al.* 1995; Verma & Paul 2013). Toxicants are any man-made substances that can cause harm to the body such as: prescription medicine, illegal drugs and poisons. This means that, insects need to be in constant

supply of a drug for it to be detected. Furthermore, entomologists also have to sample insects where the drugs are known to accumulate because different drugs accumulate in different parts of the body (Dayananda & Kiran 2013; Franca *et al*. 2015). According to Sankhla *et al.* (2017), toxicants accumulate at different sites of the body according to their physicochemical properties. This is why insects sampled at different sites on the body may have different toxicant concentrations even though they had been feeding on the same corpse.

The use of insects as an alternative matrix for toxicological screening is widely accepted, however, the interpretation of the results is still problematic considering the lack of knowledge surrounding drug metabolism in insects and also the lack of equipment sensitive enough to detect the drugs. The concentration of the drug in the larvae therefore has to be well above the detection limit of the selected analytical method (Sadler *et al.* 1995). Some drugs go unnoticed because not much is known about their accumulation, metabolism and elimination (Fathy *et al.* 2008). Chemical bioaccumulation models for aquatic insects are well established but are almost non-existent for terrestrial insect despite the crucial role insects play in maintaining ecosystem function and transfer of contaminants in food webs (Gobas *et al.* 2016).

Toxicological analysis in post-mortem cases is quite challenging compared with clinically derived specimens (Drummer 2004). According to Pelissier-Alicot *et al.* (2003), post-mortem drug concentrations do not represent the exact concentrations the deceased was exposed to at the time of death. Most toxicants are sequestered ante-mortem in organs such as the gastrointestinal tract, viscera, liver, lungs and myocardium. These organs serve as “reservoirs” and the toxicants are redistributed post-mortem (Pelissier-Alicot 2003; Mullany *et al.* 2014). Variations in drug concentrations also occur in the corpse post-mortem and this is known as post-mortem drug redistribution (PMR) (Pelissier-Alicot *et al.* 2003; Gosselin *et al.* 2011b; da Silva *et al.* 2017). PMR occurs due to the rupturing on cell membranes which allow toxicants to diffuse through different tissues thus leading to discrepancies in the toxicant concentrations (da Silva *et al.* 2017). These variations further depend on the tissue type, sampling site, time between death and collection, autolysis of cells and

putrefaction processes (Pelissier-Alicot *et al.* 2003; Mullany *et al.* 2014). It is therefore, crucial to sample at different sites because these variations are site and time dependent. According to da Silva *et al.* (2017), the pathways and rates of metabolism are influenced by intrinsic factors such as: species, heredities, gender, age, hormone activity, pregnancy and disease. Extrinsic factors such as the diet and the environment also play a role.

Drug concentrations in biological matrices are important when determining the abuse of drugs prior to death and therefore, it is crucial to know how stable substances are in tested tissues (Drummer 2004). This is applicable in forensic toxicology whereby some tissues remain exposed to toxicants over extended periods of time. During this period, the PMI may be affected as a result of chemical change or metabolism. Da Silva *et al.* (2017) stated the possibility of estimating the quantity of a toxicant in a body by empirically accounting for metabolic processes. This however, requires the period of exposure to be known. Some drugs have an unstable nature and concentration changes may arise even if the PMI is relatively short. The following qualities are of importance: sampling technique, the state and quality of the specimen, stability of drug in the case generally and in the specimen particularly, redistribution and the effects of any drug diffusion away from or to other tissues (Drummer 2004). This could be why toxicological analysis is only interpreted qualitatively.

Many entomotoxicological studies have been published on the presence of drugs in feeding larvae. Analytical techniques include: gas chromatography coupled to a nitrogen-phosphorus detector or mass spectrometry (MS), and liquid chromatography coupled to a UV detector, MS or MS–MS (Franca *et al.* 2015). The study by Franca *et al.* (2015), was the first to validate a method for the simultaneous determination of substances from different chemical classes in larvae. In the current study, an HPLC was used to determine the presence or absence of the aldicarb in the test groups. The HPLC has been validated to test for aldicarb (Damasceno *et al.* 2008).

Drug concentration variability has been reported in larvae. This could be as a result of the stage of development the larvae was in and also the feeding site that the larvae was collected. Larvae tend to move around on the body to regulate optimum temperature and end up feeding from different sites. This results in toxicant tropism which requires multiple specimens for an accurate toxicological analysis (Da Silva *et al.* 2017). In addition to that, there is still no definite correlation between drug concentrations detected in larvae compared to those in their food source. The detection of a drug in the feeding larvae depends on the ingestion and excretion rates. Furthermore, the drug is stored in different compartments within the body of the developing insect. These include the haemolymph, fat body and cuticle. Not all drugs that are ingested, cross the digestive system into the rest of the insect's body. Some are contained in the digestive tract within the Peritrophic membrane and are excreted in the faeces. The concentration of a toxicant within a forensic indicator species largely depends on the rate of toxicant intake across the midgut and secretion by the Malpighian tubules. The rate of ingestion boils down to the concentration gradient across the midgut (Gosselin *et al.* 2011b; Parry *et al.* 2011). Larvae reared on a high concentration of a toxicant will experience a higher concentration gradient across their midgut resulting in them having a higher concentration of the toxicant within them. The larvae of *C. stygia* that were reared on meat spiked with a high concentration of morphine contained a higher concentration compared to those that fed on lower concentrations of the same drug (Parry *et al.* 2011).

This experiment was conducted to test the bioaccumulation of aldicarb in carrion insects. This was to establish to what extent insects can and may be used as an alternative when traditional toxicological analysis cannot be performed due to the degradation of body tissues. It was hypothesised that aldicarb would be detected in the test groups that were exposed to the toxicant.

2.2 MATERIALS AND METHODS

The bio-accumulation of aldicarb in a central Free State carrion ecosystem was explored in terms of two fly species (*Chrysomya chloropyga* and *Chrysomya albiceps*)

and two beetle species (*Thanatophilus micans* and *Saprinus splendens*). These species were selected based on their trophic levels. *Chrysomya chloropyga* is a primary fly and its larvae are necrophagous. *Chrysomya albiceps* is a secondary fly and its larvae are necrophagous and known predators of primary fly larvae. *Thanatophilus micans* is a necrophagous beetle whereas, *Saprinus splendens* beetle is a predator of fly larvae in a carrion ecosystem.

2.2.1 SAMPLING AND BREEDING OF INSECTS

2.2.1.1 Sampling

Specimens were collected from a caged pig carcass placed on an open field (Fig 2.1) on the grounds of the University of the Free State (29°8'S; 26°10'E). This summer rainfall grassland area experiences summer temperatures between 21°C and 43°C, winter temperatures from 12°C to 27°C and an average annual rainfall of 300-400 mm (van der Merwe 2016). After collection, the specimens were transported to the insectarium at the Department of Zoology and Entomology at the University of the Free State.

Figure 2.1: Aerial view of the sampling site (yellow block) on the University of the Free State grounds (Image altered from Google Maps, accessed 08 June 2018).

2.2.1.2 Breeding of flies

Specimens for the study were sampled together with a bit of the decaying meat from the west campus using baited fly traps. Third instar larvae were sorted and identified to species level (using Brink 2009 and personal experience) at the laboratory. Larvae were reared on an adequate food source (chicken livers) in the insectarium at ±23ºC until pupation. Pupae from each bucket were removed and placed in fly cages made of mesh (Fig. 2.2). Adults of all fly species were sustained on sugar and tap water. A few days ahead of the experiment, chicken livers were placed at the bottom of the cage for 24 hours for the females to gain a protein meal for ovary development. The livers were then removed for 48 hours and only water and sugar were left in the cages. This was done to “imitate” the time it takes the female to find a suitable substrate in nature. Meat starvation gives the females enough time to mature their eggs, it mimics the time required for females to locate a carcass (which could take days or weeks) and the re-introduction of meat allows oviposition prediction (Brown 2012). The liver breeding media were monitored every 30 minutes from the time they were placed to ensure that eggs used were of the same age. As soon as oviposition took place, the eggs were carefully removed from the cages to begin the experiment.

Figure 2.2: Mesh cages that were used for the breeding of flies.

2.2.1.3 Breeding of beetles

Pig carcasses used for medical experiments were repurposed for forensic entomological studies. Adult beetles were sampled together with a bit of soil from the area surrounding the carcass. Adult *S. splendens* and *T. micans* were maintained in the insectarium at ±23 ºC. The soil from the collection site was used as a dry substrate. A small wet cotton ball was placed in the container to maintain the humidity of the soil. Predaceous *S. splendens* were reared on an adequate supply of fly larvae and the necrophagous *T. micans* were reared on chicken livers. At the time of the experiment, fifty *S. splendens* beetles were removed from the colony and placed in a separate container. The beetles were starved for five days in preparation for the experiment. The same was done with *T. micans* beetles.

2.2.2 STUDY DESIGN

2.2.2.1 Aldicarb preparation

Arnot *et al.* (2011) determined that, the oral LD_{50} of aldicarb in humans is 0.8 mg/kg. Initially, aldicarb (an active ingredient of a pesticide commonly known as “Two steps”) was added to the liver as granules. This approach did not work because this allowed larvae to seek out uncontaminated parts of the liver.

Because aldicarb is only semi-soluble in water, an ethanol TritanX diluent was used to make the dilutions. The diluent consisted of 98 mL absolute ethanol (99,9% ethanol) mixed with 2 mL of Triton X-100 to give a 97,9% ethanol, 2% Triton X solution. Of this solution, 4 mL was mixed with 396 mL of distilled water to give a 0,979% ethanol and 0, 02% Triton X solution. Two liters of this solution was prepared for the experiment.

Aldicarb was crushed to a fine powder using a micro-pestel in a 1.5 mL Eppendorf tube in a fume cabinet. A stock solution was made with 0.032 g of aldicarb and 1000 mL diluent. It was mixed by manual shaking for 5 minutes and a magnetic stirrer for an hour. The stock solution was poured into a 100 mL bottle and taken as the double lethal dose ($4xLD_{50}$). In another 100 mL bottle, 50 mL of the stock solution was added together with 50 mL of the diluent to make the lethal dose ($2xLD_{50}$). To make a semi-lethal dose (LD_{50}), 25 mL of the stock solution was mixed with 75 mL of the diluent in

a 100 mL bottle. All bottles were labelled accordingly and “toxic” warning signs were pasted on them.

It was calculated that 1 mL of the dilutions had to be added per 10 g of livers for the correct exposure of the larvae to each concentration. A syringe and needle was used to inject the toxicant in the livers at different places. To avoid contamination, a different syringe was used for each concentration. The livers were patted dry with paper towel before being spiked with the toxicant.

2.2.2.2 Experimental set-up

To test **bio-accumulation**, the experiment involved four food chains commonly found in a carrion ecosystem. The food chains were as follows:

1. Food source→ primary fly.
2. Food source→ necrophagous beetle.
3. Food source→ primary fly→ secondary fly.
4. Food source→ primary fly→ predaceous beetle.

The following procedure was followed for the first two food chains:

Four plastic containers (Fig 2.3) were used and each container was lined with five layers of paper towel and 50 g (five pieces of 10 g) of fresh chicken livers were placed on top of the paper towel. Nothing was added to the livers in the first container and it was taken as the control. The livers in the second container were injected with a semi-lethal dose of aldicarb, those in the third container were injected with a lethal dose of aldicarb and those in the third container were injected with a double lethal dose of aldicarb. A new clean syringe was used for each toxicant solution to avoid contamination. All containers were labelled accordingly and container number 2 to 4 had “toxic” warning signs attached to them.

Figure 2.3: The plastic containers used to carry out the experiments.

Food chain number one:

Eggs of *C. chloropyga* were weighed to 0.02 g (±100 eggs) and placed in the tissue folds of each container. As soon as the larvae reached the third instar stage, 10 from each container were collected, labelled accordingly and tested for toxicant bio-accumulation. A schematic diagram of the experimental set-up is shown in Figure 2.4.

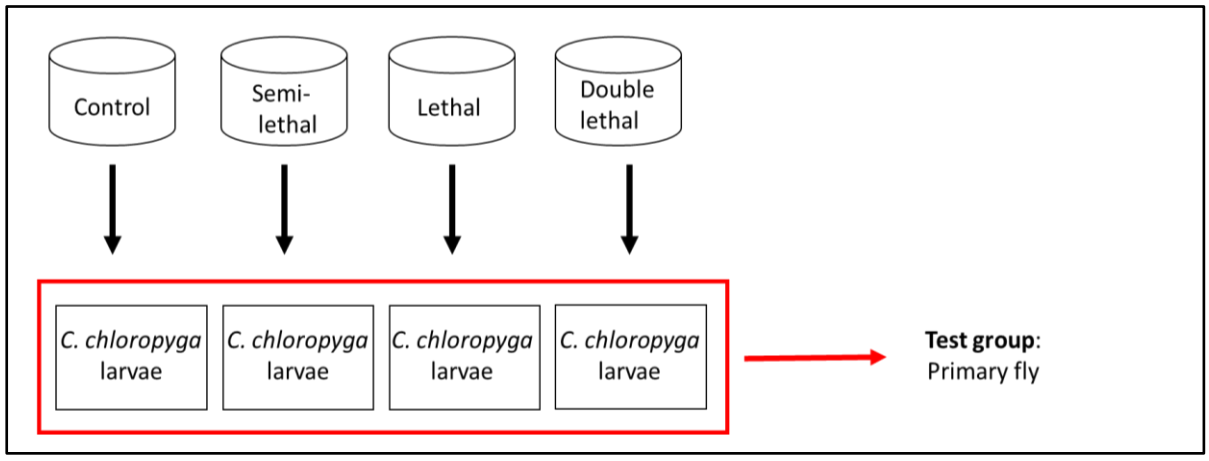


Figure 2.4: Diagrammatic representation of the experimental set-up of the first food chain with the primary fly as the test group.

Food chain number two:

Ten *Thanatophilus micans* beetles that had been starved for five days were placed in each container. Forty eight hours later, five beetles were randomly picked from each container, labelled accordingly and tested for toxicant bio-accumulation. The remaining five were kept as a reserve during the testing procedures. A schematic diagram of the experimental set-up is shown in Figure 2.5.

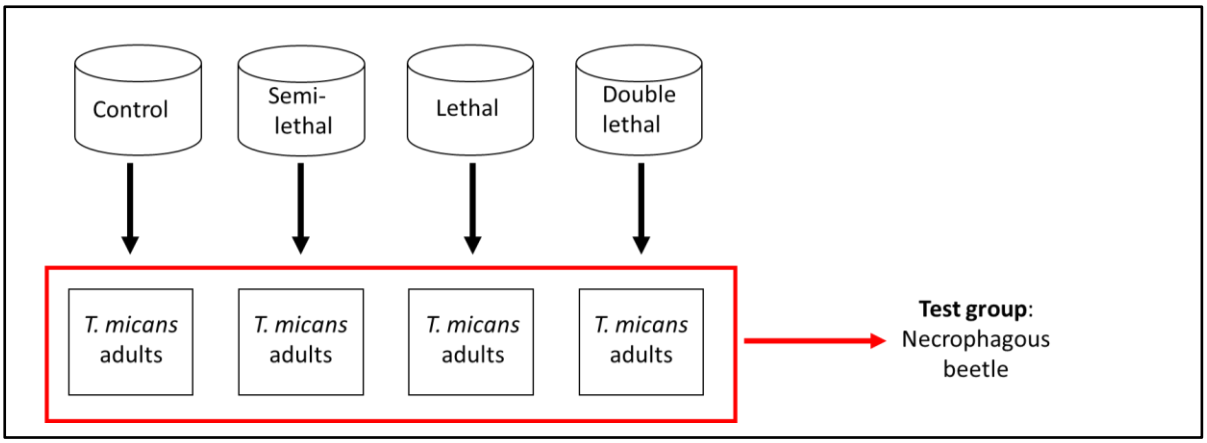


Figure 2.5: Diagrammatic representation of the experimental set-up of the second food chain with the necrophagous beetle as the test group.

<u>The following procedure was followed for the last two food chains:</u>

These food chains were conducted with the use of eight plastic containers aligned in two rows (Fig. 2.6). The first row of containers (Container 1-4) each had five layers of paper towel and 50 g of fresh chicken livers. Nothing was added to the livers of the first container and it was taken as the control. The livers in the second container were injected with a semi-lethal dose of aldicarb, those in the third container were injected with a lethal dose and those in the fourth container were injected with a double lethal dose. A new clean syringe was used for each container to avoid contamination. All containers were labelled accordingly and Container 2-4 and 6-8 had “toxic” warning signs attached to them.

Figure 2.6: The alignment of the plastic containers to carry out the experiments.

Food chain number three:

Eggs of *C. chloropyga* were weighed to 0.02 g and placed in the tissue folds. When the larvae reached late second instar or early third instar stage, they were transferred to the second row of containers which each contained 40 early third instar *C. albiceps* larvae. Forty eight hours later, 10 *C. albiceps* larvae from each container were collected, labelled accordingly and tested for toxicant bio-accumulation. A schematic diagram of the experimental set-up is shown in Figure 2.7.

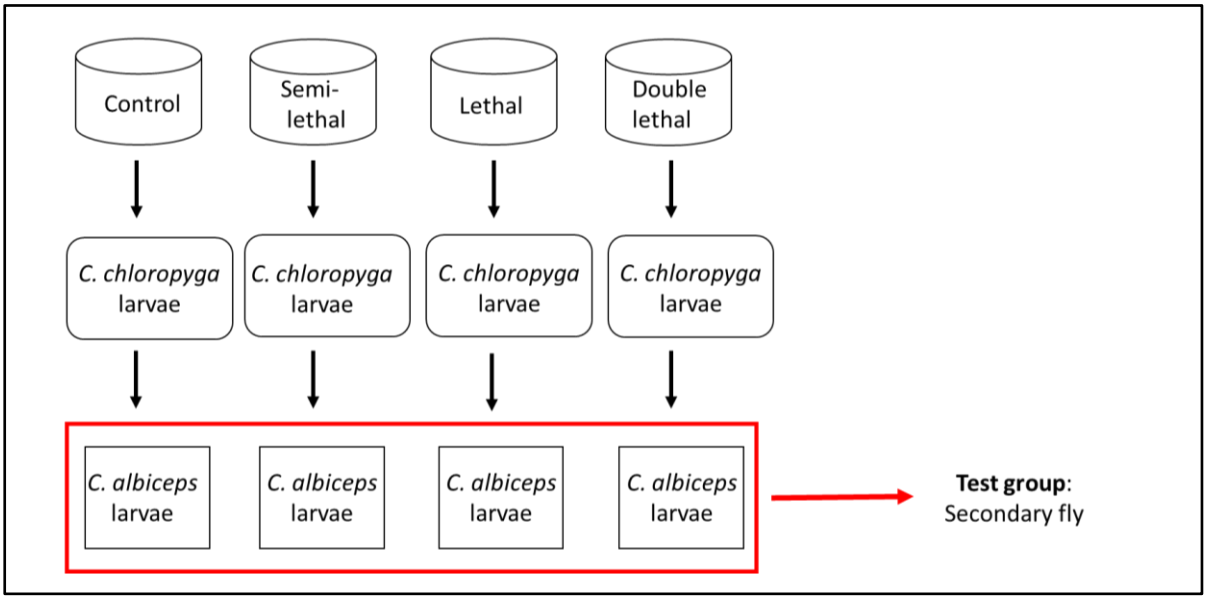


Figure 2.7: Diagrammatic representation of the experimental set-up of the third food chain with the secondary fly as the test group.

Food chain number four:

Eggs of *C. chloropyga* were weighed to 0.02 g and placed in the tissue folds. When the larvae reached late second instar or early third instar stage, they were transferred to the second row of containers which each contained 10 adult *Saprinus splendens* beetles that had been starved for five days. Five beetles from each container were collected 48 hours later, labelled accordingly and tested for toxicant bio-accumulation. A schematic diagram of the experimental set-up is shown in Figure 2.8.

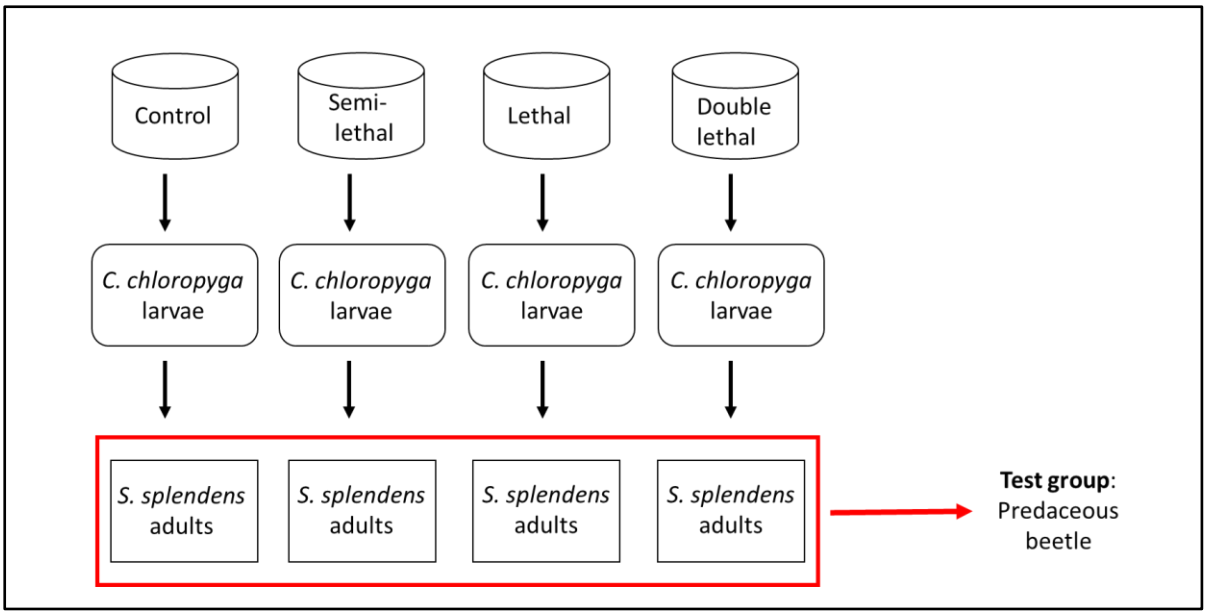


Figure 2.8: Diagrammatic representation of the experimental set-up of the fourth food chain with the predaceous beetle as the test group.

Food chain 1-4: All containers used in the experiments were closed with gauze centred lids to allow air circulation and prevent the larvae from escaping. Throughout the experiment, food was monitored and added so that it was not a limiting factor (the bigger they got, the more food they received [still spiked for the treated groups]). The experiment was carried out in the insectarium at ±23 ºC and it was done in triplicate.

2.2.2.3 Entomotoxicological analysis

All specimens were killed with boiling water and rinsed with cold water. They were then dried with paper towel. For the extraction, all samples were homogenised separately in Eppendorf tubes then transferred to polytopes and 7 mL of acetic acid (1%) was added to each homogenate. It was sonificated at 25 vibrations/sec for 20 minutes to separate the analyte and centrifuged with a Hermle Z 233 MK-2 at 3500 rpm for 15 min at 15˚C. The bottles were marked in correlation to the containers they were taken from. A standard solution (half the aldicarb stock solution and half acetonitrile) was prepared and run on a Waters HPLC (Fig 2.9) to determine the retention time of the chemical of interest and concentration calculations of samples. The mobile phase consisted of ACN 320 mL, dH2O 20 mL, TBac 0.4 g, NaH2PO4 0.1248 g, with a column of Venusil XPB, C18 2, 5Um, 100A, 4.6x150mm and a pressure : 26-28Psi.The area underneath the peak of the standard was calculated to

represent 16 ppm aldicarb. The samples were run for ten minutes each and peaks were detected at a wavelength of 290 nm The test sample area underneath the peak detected at 290 nm at around 1.66 minute elution, was then compared to the standard peak area to calculate the concentration of the test sample.

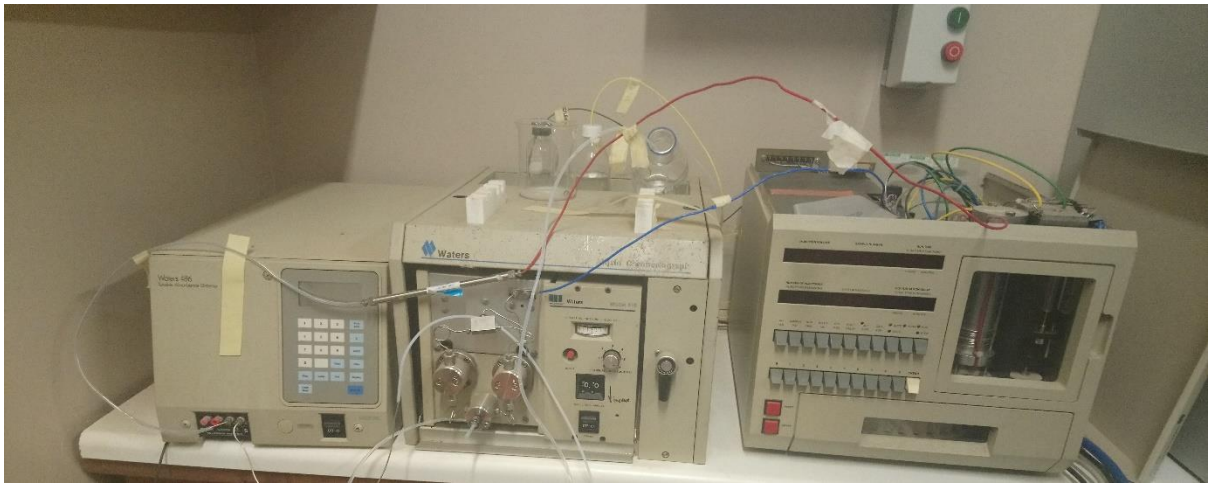


Figure 2.9: The High Performance Liquid Chromatography (HPLC) used to test the samples for the presence or absence of the toxicant.

2.2.3 ETHICAL AND BIO-SAFETY CONSIDERATIONS

The 3Rs (Replacement, Reduction, and Refinement) as specified by the Animal Welfare Act were applied as far as possible. Research using animals in South Africa is guided by the Animals Protection Act (Act No. 71 of 1962).

- **Reduction**: by re-using pig carcasses (previously used for medical experiments) as a source of carrion to attract insects for forensic entomological studies, the number of animal killings was reduced.
- **Refinement**: pig baits were suitable for the study and specimens were euthanized which is considered a humane way to kill an animal (minimum stress and pain).
- **Replacement**: to avoid any suffering such as poisoning a live vertebrate animal (for example: mice and rats), the toxicological part of the study was not conducted on live vertebrate animals. Store bought chicken livers were used instead. The study thus replaced the use of a live animal with store bought chicken livers.

The study impacted more regarding bio-safety issues. Safety precautions that were taken included wearing a lab coat, gloves, goggles, a mask and working in a fume cabinet. The drug was locked in a cool dry place out of reach of people. The cabinet was marked with a “danger” label pasted on the door. All waste products were discarded in clearly marked disposal bags indicating that the plastic contained hazardous waste and it was disposed of by a registered waste company.

2.3 RESULTS AND DISCUSSION

A standard solution of 1:1 aldicarb stock solution and acetonitrile was prepared and run on an HPLC to determine the concentration of the chemical of interest and for comparison with the other samples. From the stock solution, it was determined that the standard solution contained 16ppm of aldicarb. The area of the standard solution peak was 1569.57 mV.s. To determine the concentration of aldicarb in the samples, the following formula was used:

$$\text{Concentration (ppm)} = \frac{\text{Designated ppm} * \text{ area of test sample}}{\text{area of the standard solution}}$$

Food chain 1

The first food chain represented necrophagous *C. chloropyga* fly larvae feeding on carrion. The control sample showed no peak at 1.66 minutes elution. Peaks were noted in fly larvae that fed on livers spiked with a semi lethal (LD_{50}), lethal ($2xLD_{50}$) and double lethal ($4xLD_{50}$) dose of the aldicarb. The detection of peaks at around 1.66 minutes indicated the presence of aldicarb and its metabolites in the test samples. Table 2.1 shows the aldicarb concentration found in each sample. A pattern could be seen whereby, the higher the concentration the larvae were exposed to, the higher the concentration was in the larval samples. Bio-magnification of aldicarb was observed.

Table 2.1: The detection of aldicarb in the test samples of *Chrysomya chloropyga* larvae.

Test sample	Area of HPLC peak (mV.s)	Concentration (ppm)
Control	-	-
Semi-lethal	6875.79	70.09
Lethal	7226.54	73.67
Double lethal	7864.49	80.17

Food chain 2

The test subjects for the second food chain were the adult beetles of *Thanatophilus micans*. These beetles feed directly on the carcass in a carrion ecosystem. Table 2.2 shows the HPLC results of the second food chain samples. A similar pattern to the previous food chain was observed with regards to the concentration. Bio-magnification of aldicarb was also observed in this food chain but not to the same extent as seen in the first food chain.

Table 2.2: The detection of aldicarb in the samples of the adult *Thanatophilus micans* beetles.

Test sample	Area of HPLC peak (mV.s)	Concentration (ppm)
Control	-	-
Semi-lethal	2696.82	27.45
Lethal	3185.08	32.47
Double lethal	4068.24	41.47

Food chain 3

The third food chain represented the larvae of the secondary fly, *Chrysomya albiceps*, predating on primary fly larvae on a carrion. *Chrysomya chloropyga* was presented as prey. Table 2.3 shows that peaks were only observed in the samples that were

exposed to the toxicant. This proved that aldicarb was present in these samples and that secondary bioaccumulation of aldicarb occurred. There was, however, no pattern between the toxicant concentration in the samples and what they were exposed to.

Table 2.3: The detection of aldicarb in the samples of *Chrysomya albiceps* larvae.

Test sample	Area of HPLC peak (mV.s)	Concentration (ppm)
Control	-	-
Semi-lethal	1439.81	14.68
Lethal	1552.33	15.82
Double lethal	907.10	9.25

Food chain 4

The fourth food chain represented *Saprinus splendens* beetles predating on fly larvae on carrion. Larvae of a primary fly, *C. chloropyga* were presented as prey. Table 2.4 shows the HPLC results of the fourth food chain. Peaks showed that secondary bioaccumulation of aldicarb occurred in the samples that were exposed to the toxicant. The pattern in this case seemed to be indirectly proportional, the lowest concentration was found in the sample that was exposed to the highest concentration.

Table 2.4: The detection of aldicarb in the samples of adult *Saprinus splendens* beetles.

Test sample	Area of HPLC peak (mV.s)	Concentration (ppm)
Control	-	-
Semi-lethal	1021.36	10.41
Lethal	902.17	9.20
Double lethal	858.88	8.76

The results showed the absence of the toxicant in all the control samples and aldicarb was only detected in the samples that were exposed to the toxicants. Bio-magnification of aldicarb was observed in the first two food chains. The concentration of aldicarb in the test samples exceeded that of the standard solution. Secondary bio-accumulation was observed in the last two food chains. Aldicarb was detected in the samples but at lower concentrations. This could have been because, their prey were already undergoing elimination of the toxicant as a result of being removed from their food source. Insects need to be in constant supply of the toxicant because toxicants can only be detected when the absorption exceeds the rate of elimination (Sadler *et al.* 1995; Verma & Paul 2013). Parry *et al.* (2011) stated that, these elimination mechanisms are so effective that they facilitate the insect's ability to maintain a lower toxicant concentration compared to the food source.

Previous studies have mostly used laboratory animals to simulate the effect of drugs in humans but it has recently been reported that drug metabolism is species-specific (Mullany *et al.* 2014). Just like in the current study, Goerge *et al.* (2009) substituted drugging a live animal with lethal doses by spiking a food source. The chosen food source for their study was pet mince whereas in this study, chicken livers were used. Goerge *et al.* (2009) stated that, animal models do not guarantee a perfect simulation of human overdoses and should be used with caution. This is because, not much is known about the bio-accumulation, metabolism and elimination of toxicants in some animals. Also, the rates at which these take place may differ considerably between humans and laboratory animals since they are species specific (Mullany *et al.* 2014). There have also been reports on differential drug metabolism in humans compared to laboratory animals. According to Goerge *et al.* (2009), about five morphine metabolites have been documented in laboratory animals but have not been seen in the metabolism of morphine by humans. Furthermore, secondary metabolites may impose different effects from the toxicant itself and the effects may also differ between species.

Mullany *et al.* (2014) also used HPLC chromatograms to determine the presence or absence of methamphetamine. Sadler *et al.* (1995) used an HPLC as well as gas chromatography/MS to determine the accumulation and elimination of amitriptyline

and nortriptyline. It was found that the compounds were detected in the groups that were exposed to the toxicants but absent in the control groups. The same was observed in the current study. In a study by Kharbouche *et al.* (2008), codeine was also present in all the treated groups but not in the control groups. In a study by Goff *et al.* (1992), the entomotoxicological analysis could not detect the presence of the methamphetamine with the use of radioimmunoassay techniques. Gunn *et al.* (2006) emphasised the strong need for research on extraction procedures in entomotoxicological analysis. Furthermore, analytical techniques should be rapid, safe, simple and cost effective.

The fact that toxicants can be picked up in feeding larvae makes entomological specimens a good alternative for toxicological analysis when the body has long degraded. Nonetheless, quantitative analysis still cannot be explained by entomological analysis due to the wide inter-site and intra-site variations of drug concentration. Toxicants have been observed to show strange transfer patterns to entomological specimens which are species specific. According to da Silva *et al.* (2017), tropism patterns largely depend on the age and sex of the body. They further mentioned that, tropism in insects is not an issue unless the toxicological analysis is based on the frass or exoskeletons. Regardless of this, toxicants are known to accumulate in fly larvae and may later be found in pupa intestines and the meconium of adults. This explains why toxicants may not be detected in adults. Also, drug concentrations in entomological specimens are affected by: toxicant tropism in the body, pre- and post-mortem changes and the extraction and detection efficiencies of the analytical techniques (da Silva *et al.* 2017).

Many studies have reported on the existence of a correlation. Parry *et al.* (2011), observed a correlation between the concentrations in fly larvae relative to the concentrations in the diet. The concentration of morphine found in the larvae was relative to the concentration in the food source resulting in larvae that were reared on a high morphine concentration having a higher drug concentration than those that were reared on a lower concentration. In a study by Kharbouche *et al.* (2008), there was also a correlation between the drug concentration in the diet and the drug

concentration in the feeding larvae. The concentration in the larvae was directly proportional to the concentration in the food source. That is, the higher the concentration was in the food source, the higher the concentration was found in the larvae. The results obtained by Kharbouche *et al*. (2008) showed a correlation between codeine and necrophagous larvae reared on it but this, however, was only witnessed in the larval stage. This was supported by two studies done on morphine. Introna *et al.* (2001) observed a correlation between morphine concentrations in third instar larvae of *Calliphora vicina* and human liver. Similarly, Hedouin *et al.* (1999) reported a significant correlation between morphine concentrations in rabbit tissues and concentrations found in the third instar larvae of *Lucilia sericata*. In the current study, this was only witnessed in necrophagous insects (insects that fed directly on the food source). This correlation was thus only seen in the first two food chains. A higher concentration of aldicarb was observed in larvae and necrophagous beetles that were reared on a food source spiked with a double lethal dose ($4xLD_{50}$) than those that were reared on a lethal ($2xLD_{50}$) or semi-lethal dose (LD_{50}).

Larvae of *C. albiceps* are known to be predaceous and cannibalistic. This behaviour is triggered by food competition, palatability, and action of pheromones (Faria *et al.* 2004; Souza *et al.* 2011). The cannibalism and predation mode of *C. albiceps* was orchestrated during this experiment by starving them, then introducing *C. chloropyga* larvae to predate on them. *Saprinus splendens* is naturally predaceous. No pattern or correlation was observed in these last two food chains. In a quest to determine secondary bio-accumulation in a carrion ecosystem, the results proved positive for accumulation but negative for a correlation.

To conclude, when a body is only recovered 72 hours later following death, its tissues are no longer suitable for toxicological analysis due to the extent of decomposition. In such cases, entomological specimens may be used as alternative toxicological indicators. Bioaccumulation and bio-magnification (in primary trophic levels) of aldicarb were observed in the carrion ecosystem. Forensic Diptera and Coleoptera can thus be utilised as toxicological indicators where traditional methods fail. The hypothesis was therefore proven.

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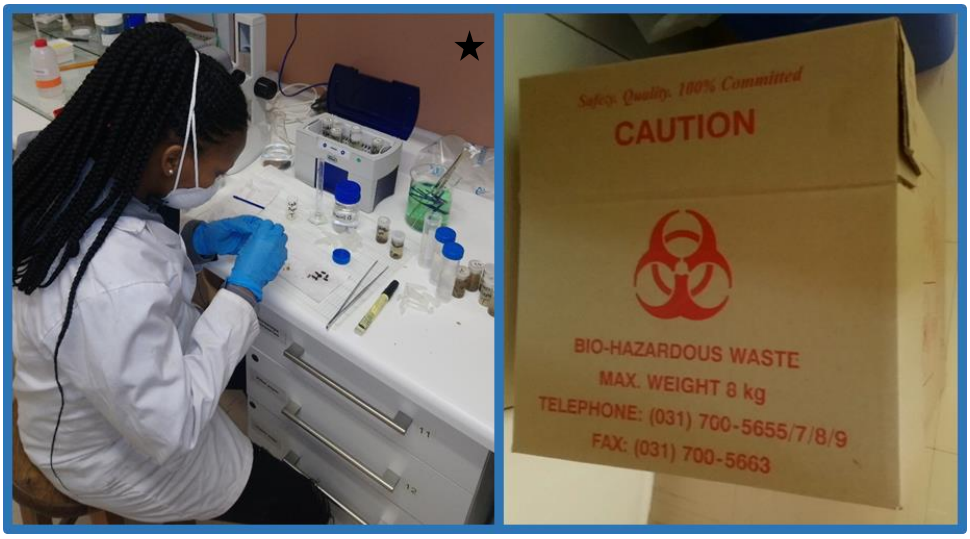
CHAPTER 3

Elimination of aldicarb in the post-feeding stages of some flies of forensic importance.



★Picture taken by: N. Ndlovu

3. ELIMINATION OF ALDICARB IN THE POST-FEEDING STAGES OF SOME FLIES OF FORENSIC IMPORTANCE

3.1 INTRODUCTION

In feeding larvae, toxicants can only be detected when the absorption exceeds the rate of elimination (Sadler *et al.* 1995; Verma & Paul 2013). In drug accumulation studies, it has been shown that the highest drug concentrations are found in the larval form, more so in the third instar larvae (Sadler *et al.* 1995; Kharbouche *et al.* 2008; Monthei 2009).

In a study to test the bio-accumulation of codeine, it was observed that the concentration measured in the pre-pupae of *L. sericata* was far less than the concentration found in the larvae (Kharbouche *et al.* 2008). Sadler *et al.* 1995 also confirmed that, amitriptyline and temazepam concentrations were higher in the larvae of *C. vicina* than in the pupae. These studies proved that the drug concentration in maggots decreased when they entered the post feeding stage. Post feeding larvae have also been reported to actively eliminate toxicants during pupariation (Sadler *et al.* 1995; Monthei 2009). This is due to the continuous elimination and lack of ingestion thus resulting in the rate of elimination exceeding the rate of absorption. In the study by Kharbouche *et al.* (2008), it was noted that the rate of elimination of codeine in *L. sericata* exceeded the rate of absorption after 60-70 hours. It is at the post feeding stage that the concentration levels of opiates in the larvae are greatly reduced.

Entomotoxicology is seen as a “scientific imposter” or a “laboratory curiosity” (Campobasso *et al*. 2004; Gosselin *et al*. 2011b). This is mainly due to previous studies not being able to show a definite correlation between toxicant concentrations in the immatures and their feeding substrate (Campobasso *et al.* 2004; Liu *et al*. 2009; Gosselin *et al*. 2011b Franca *et al.* 2015; Sankhla *et al.* 2017). Furthermore the processes of elimination mechanisms for different classes of drugs and toxicants in different species, across the different life stages and in different trophic levels of the food chain is not fully explored yet.

In a study to determine the accumulation, metabolism and excretion of morphine, Parry *et al.* (2011) exposed *C. stygia* to different concentrations of morphine and measured the concentration of the drug in all the life stages of the insect. This was done to determine the ability of the immatures to maintain the drug at lower concentrations and also examine the presence of morphine in the pupal cases as well as in the meconium. The larvae indeed maintained lower drug concentrations than their food source. The remaining drug was mostly excreted with the meconium and only a small percentage was found in the body of the adult fly. The drug concentration found in the adult fly was lower than the drug concentration found in the larvae. The presence of elimination mechanisms is witnessed in the fact that drugs do not bio-accumulate throughout the lifecycle of forensic insects (Monthei 2009). Previous studies have reported on the elimination of drugs prior to metamorphosis.

When determining the PMI estimate, it is important to know whether the completion of a lifecycle has occurred on that carcass. This is made known by the presence of puparia. Puparia are usually used in relation to old cases or burials. The puparium is a protective cover made of chitin which is resistant to chemical attack, microorganisms, weather conditions and remains for long periods of time (Huchet 2010; Gosselin *et al.* 2011b). These puparia are used to determine month and location of death or burial since certain species are seasonal and some are only found in specific geographic regions and environments. Morphological characteristics of the pupal casings are usually used to identify the species, however, if the puparia are damaged (too weathered of fragmented) then the alternative methods include: DNA extraction and subsequent COI sequence analysis (Brown 2012). Monthei (2009) found high morphine concentrations in larvae (second and third instars) but only trace amounts were found in the cuticle of the pupae. This then raised an important question…can empty pupal casings found at a crime scene be used for toxicological analysis?

This experiment aimed to test for elimination of aldicarb in the post feeding stages of some forensic fly species and also determine if aldicarb can be detected in empty puparial casings. It was hypothesised that aldicarb would be eliminated in i) pre-pupae,

ii) pupae and iii) adults of the test groups. It was furthermore hypothesised that aldicarb would be detected in empty pupal cases of the test groups.

3.2 MATERIALS AND METHODS

To test for **elimination**, three fly species: *Chrysomya chloropyga* (primary fly), *Chrysomya albiceps* (secondary fly) and *Sarcophaga cruentata* (tertiary fly) were exposed to a lethal dose of aldicarb.

Six plastic containers were used, each containing sawdust, five layers of paper towel and 50 grams of fresh chicken livers. The first container for each species was taken as the control (nothing was added to the livers) and labelled accordingly (control group, date and the species name). The last container for each species was taken as the treated group and the livers in those containers were each injected with 5 mL of the lethal dose (2 x LD_{50}) of aldicarb and labelled accordingly (treated group, date and the species name). Containers of the treated groups had a “toxic” warning sign attached to them. The eggs of *C. chloropyga* and *C. albiceps* were carefully weighed (0.02 g per container) and placed in the tissue folds of each container. The eggs of *C. chloropyga* were placed in the first two containers (control and treated), eggs of *C. albiceps* were placed in the next two containers (control and treated) and *S. cruentata* 1^{st} instar larvae were placed in the last two containers (control and treated). A schematic diagram of the set-up is shown in Figure 3.1. Eggs and larvae were reared in the insectarium at ±23°C. As soon as the immature stages reached the post feeding stage, 10 pre-pupa from each group for each species were sampled and taken for toxicological analysis (as described in chapter 2). Toxicological analysis was also performed 24 hours into pupariation (10 pupae) and within 10 hours of adult emergence (10 adults and 10 empty pupal casings) for each group and species. Throughout the experiment, food was monitored and added so that it was not a limiting factor (the bigger they got, the more food they received). The experiment was done in triplicate.

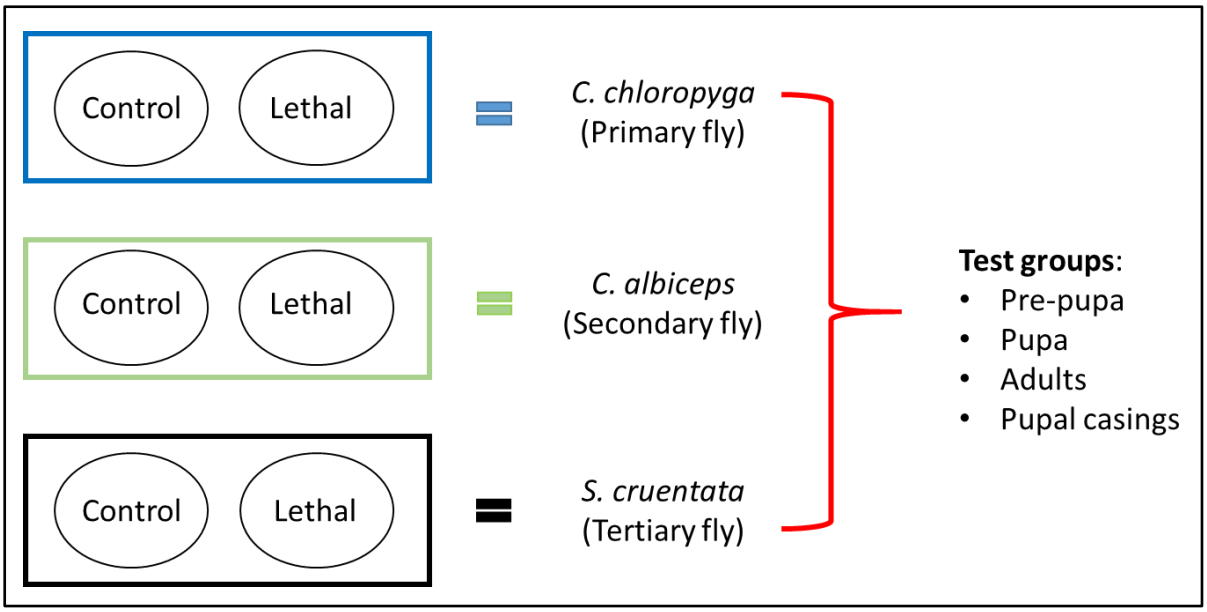


Figure 3.1: Diagrammatic representation of the experimental set up to test the elimination of aldicarb in the post feeding stages of some flies of forensic importance.

3.3 RESULTS AND DISCUSSION

Peaks were only detected in treated samples of *C. chloropyga* pre-pupae (308.11 mV.s) and pupae (8.88 mV.s) but no peaks were detected in newly emerged *C. chloropyga* adults and empty pupal casings. The concentration of aldicarb was lower in the pupae compared to the pre-pupae and it was undetectable in the newly emerged adults and empty pupal casings (Table 3.1). This is an indication of the extent at which the toxicant was eliminated for this particular species.

Table 3.1: The detection of aldicarb in the test samples of *Chrysomya chloropyga*.

Test sample	Control	Treated
Pre-pupae	-	3.14 ppm
Pupae	-	0.09 ppm
Adults	-	-
Empty pupal casings	-	-

The HPLC results showed peaks in the pre-pupae (525.81 mV.s), pupae (22.92 mV.s) and adult (7.23 mV.s) samples of *C. albiceps* that were exposed to a lethal dose of aldicarb as maggots. The concentration of the toxicant progressively decreased with the development of the fly (Table 3.2).

Table 3.2: The detection of aldicarb in the test samples of *Chrysomya albiceps*.

Test sample	Control	Treated
Pre-pupae	-	5.36 ppm
Pupae	-	0.23 ppm
Adults	-	0.07 ppm
Empty pupal casings	-	-

Peaks were detected in the treated pre-pupae (347.55 mV.s), pupae (17.67 mV.s) and adults (3.95 mV.s) of *S. cruentata*. The concentration of aldicarb decreased as developed progressed. This showed that the toxicant was eliminated in the post feeding stages.

Table 3.3: The detection of aldicarb in the test samples of *Sarcophaga cruentata*.

Test sample	Control	Treated
Pre-pupae	-	3.54 ppm
Pupae	-	0.18 ppm
Adults	-	0.04 ppm
Empty pupal casings	-	-

In this study, a comparison was made between post feeding larvae, pupae, adults and pupal casings. This was done to determine whether drug elimination occurred during the post feeding stages. The results for all the control samples tested negative for

aldicarb. It was observed that aldicarb concentrations progressively decreased as the pupae developed, i.e. the concentration found in adults (if any) was less than what was found in the pupae or pre-pupae. This proves that toxicants were eliminated throughout the course of the immature non-feeding stages. In all species, the results showed a correlation that was proportionally indirect to the stage of development. The more the species developed the less toxicant was detected. The results of all the pupal casings for all species tested negative for aldicarb. Pien *et al*. (2004) were able to detect nordiazepan and its metabolite oxazepam in puparia of *C. vicina*.

According to Sadler *et al.* (1995), many studies have reported on drugs being present in pupae, however, their study failed to detect any drugs in *C. vicina* pupae. They reasoned that, it could have been that the analytical method selected may have not been sensitive enough. Furthermore, the rinsing of samples to remove surface contamination prior to the toxicological analysis may have contributed to the negative results. It was expected regardless that, concentrations measured in the pupa would have been less than those reported in larvae. The current study also failed to detect aldicarb in all the empty pupal casings meaning that aldicarb does not accumulate within the pupal casing itself. Nonetheless the rinsing of the casings to eliminate surface contamination may have, to some degree (however minimal), influenced the results.

Kharbouche *et al.* (2008) found that, the concentration of morphine, codeine and norcodeine in *L. sericata* decreased the more the insect developed. Further elimination of the toxicant was noted for post feeding stages of *L sericata*. The same was witnessed in the current study for all species tested on aldicarb. The concentration of aldicarb reduced as development progressed. Little to none concentrations of aldicarb were found in the later stages of development. This proves the existence of these elimination mechanisms which render the effective excretion of toxicants.

According to Williams & Villet (2014), maggots have the ability to eliminate, metabolise or sequester a wide variety of drugs. Not much is known about the physiological

mechanisms that aid in toxicant secretion. Nonetheless, the Malpighian tubules or nephrocytes are believed to be responsible for this since they are accountable for the active secretion of a variety of organic compounds (Gosselin *et al.* 2011a; Parry *et al.* 2011). The gut is aligned with p-glycoproteins (organic cation transporters) which facilitate excretion (Parry *et al.* 2011). Furthermore, the secretion of a toxicant by the Malpighian tubules occurs rapidly when a forensic indicator species continues to feed on tissue containing the toxicant. The concentration of morphine in *C. stygia* was observed to be lower than the concentration in the food source due to the rapid secretion of the drug by Malpighian tubules (Parry *et al.* 2011). The secretion of toxicants by the Malpighian tubules was thus significant considering toxicants were found at lower concentrations in entomological sample than in the food source (Parry *et al.* 2011). The rate at which Malphigian tubules secrete toxicants differs amongst species (Rheault *et al.* 2006). In Chapter 2 it was observed that the concentrations of the toxicant in the predaceous *C. albiceps* fly species larvae and *S. splendens* beetles were lower than the concentration in the prey *C. chloropyga* fly larvae.

When insects get to the wandering phase, they stop feeding. As a result, the insect is left with an empty gut and when this happens, the concentration gradient reverses and the toxicant diffuses from the haemolymph back to the gut (Bourel *et al.* 2001). The Malpighian tubules continue secreting toxicants during the post-feeding stage resulting in the excretion of toxicants. Those that remain thereafter, are stored in the haemolymph and in-between the endo and exocuticle in some species. During pupariation, some of the drug may be incorporated into the cuticle of the puparium (Gosselin *et al.* 2011a). At this stage, a layer of fat is deposited just beneath the cuticle and this functions as a storage organ. Most toxicants probably remain in that layer. When the larvae enter the pupal stage, the Malpighian tubules stop functioning due to their degradation and toxicants remain within the pupae. Toxicant secretion resumes as soon as the adult Malpighian tubules are reformed in the pupa. The toxicant can further be excreted together with the meconium (first stools) when the adult emerges. Some toxicants fuse to the cuticle and remain within the pupal cases (Parry *et al.* 2011). Gosselin *et al.* (2011b), emphasised the need for immune-histochemical studies to determine the localisation of toxicants.

Aldicarb is classified as extremely hazardous by the world health organisation (WHO 2001). It is said to have a log Kow of 0.053 which means it is unlikely to concentrate in fat deposits. From studies done on vertebrate animals to test the metabolism of this toxicant, it was observed that aldicarb is rapidly excreted and does not concentrate in the tissues, milk or eggs of animals. No articles are available regarding specifics of the metabolism of aldicarb in the immature stages of flies of forensic importance, however from this study it is excreted totally by the adult stage and not caught up in fat bodies or other structures of the emerging flies. It is also clear that none of the toxicant was accumulated in the larval skin, i.e. the pupal casing.

Morphine is a hydrophilic compound which dissolves within the haemolymph as witnessed in *C. vomitoria* (Bourel *et al.* 2001). According to Gosselin *et al.* (2011a), hydrophilic molecules are usually excreted from the haemolymph via the Malpighian tubules of fly larvae. Insoluble toxicants on the other hand, convert to hydrophilic molecules within the insect via biotransformation. These hydrophobic substances are deposited near the pore canals in the cuticular matrix. EDDP is a hydrophilic metabolite and therefore does not accumulate in the tissues of the insect's body. The effective elimination of a toxicant largely depends on its chemical complexities and lipid/water solubility. Furthermore, it also boils down to the larvae's competence to metabolise and defecate the toxicant which is why only a few studies have reported on a correlation between drug concentrations in larvae and their feeding substrate. Gosselin *et al.* (2011a) observed that methadone could be detected in the cuticle of the puparia as well as the meconium of adults. When it can no longer accumulate in the cuticle, it is stored in the fat bodies. The drug thus accumulates in the insect's remains. The accumulation of drugs in adipocytes has been documented before with a possible interference of fat bodies in such reserves. Many entomotoxicological studies have shown a lot of variability in entomological specimens thus, the absence of drugs in immatures does not necessarily mean they were never present in the food source (Gosselin *et al.* 2011a).

Sadler *et al.* (1995), reared *C. vicina* on muscle that was spiked with a high concentration of paracetamol, but however, could not detect the drug in the larvae. It

was reasoned to possibly be as a result of the rapid elimination that occurs of this particular drug in this particular species. This ability to eliminate drugs was also observed at a slower rate during larval diapause. This is because, during this phase the metabolic activity is greatly reduced. Kharbouche *et al.* (2008) reported codeine elimination during the larval stages and it not being limited to the inactive phase in *L. sericata*. There is still a gap in the knowledge surrounding the accumulation, metabolism and elimination of toxicants found in carrion insects. As a result this leads to difficulties in analysing entomotoxicological results. Kharbouche *et al.* (2008) emphasised that, a better understanding of drug metabolism in Diptera is a necessity and noted that drug elimination in Diptera may be linked to their chemical structure and pharmacological properties.

Although more research is still required on entomotoxicological sampling techniques, one should always keep in mind that, the concentration of the drug in the sample must be well above the detection limit of the selected analytical method (Sadler *et al.* 1995). The pupal stage is a non-feeding stage thus drug concentrations are minimal, even more so in puparial cases therefore, drug analysis methods need to be quite sensitive (Gosselin *et al.* 2011b). Further research needs to be done with regards to insect physiology in respond to drugs and elimination mechanisms.

To conclude, little to no concentrations of aldicarb were found in the adults of the species tested that were exposed to the toxicant as maggots. Aldicarb therefore, does not bio-accumulate throughout the lifecycle of *C. chloropyga*, *C. albiceps* and *S. cruentata* and there are elimination mechanisms present in the post feeding stages of these species (hypothesis proven). The forensic implication is that pre-pupae and 24 h old pupae of *C. chloropyga*, *C. albiceps* and *S. cruentata* can be utilised as alternative toxicology sources. Because the pupal stage lasts for five, four and eleven days respectively (chapter 5), it would be beneficial to test older pupa to see for what period of time pupae can still be useful in toxicological analysis. In addition to this, aldicarb was not detected in empty pupal cases of the test species therefore, pupal casings of these species cannot be used for toxicological analysis of aldicarb (hypothesis not proven). These results however, are only applicable when larvae are

exposed to a lethal dose (2 x LD_{50}) of aldicarb reared under ±23°C and using the HPLC-UV detector. With additional analyses and more sensitive methods could yield different results.

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CHAPTER 4

The effect of aldicarb on the larval development of some flies of forensic importance

4. THE EFFECT OF ALDICARB ON THE LARVAL DEVELOPMENT OF SOME FLIES OF FORENSIC IMPORTANCE

4.1 INTRODUCTION

One of the applications of forensic entomology is to determine a PMI based on the developmental stage of the first generation of the first colonisers (I.e. the calliphorid and sarcophagid flies) from a crime scene (Kelly 2006; Sharma *et al.* 2015). Forensically important insects not only feed on the corpse but also oviposit their eggs which then hatch into feeding larvae (Bushby *et al.* 2012). Chemosensory stimuli attract flies to the corpse and a vast number of eggs (or larvae) are laid in natural orifices of the corpse because they provide a humid shelter which prevents desiccation (Brown 2012). The eggs then hatch into feeding larvae that moult into three different intars (Shiravi *et al.* 2011). These intars can be distinguished by the number of respiratory slits present on their posterior ends (Reibe *et al.* 2010). While feeding together as a maggot mass, larvae generate a temperature that differs from the ambient temperature reading (Kovler 2003). They also move around the body to maintain optimal temperatures. According to Arnott and Turner (2008), the third instar stage lasts longer than the first two instars and is divided into a feeding and a post-feeding phase.

Forensic entomologists are called in to analyse the species composition and collect the insects from and around the body. These insects are used to determine the lifecycle stage and species of the oldest insects (Amendt 2011; Sharma *et al.* 2015). Insects collected from the crime scene are taken as reference and are compared to data that is locally available (if any). The reference collection is also compared to specimens reared in the laboratory under conditions that are similar to those of the crime scene (Ahmad *et al.* 2018). Knowing the age of the insects allows us to determine their time of colonisation thus making it possible to determine the PMI. The age is determined by the length and mass of larvae and the largest are considered the oldest specimens (Mullany *et al.* 2014).

A number of factors are known to alter the development of necrophagous insects leading to errors in PMI estimations. These factors include: temperature, time of day, humidity, larval density, the environment that the body was found in, artificial diet and presence of drugs (Campobasso & Introna 2001; Velez & Wolff 2008; Goerge *et al.* 2009; Reibe *et al.* 2009; Richards *et al.* 2009; Rueda *et al.* 2010; Gosselin *et al.* 2011b; Shiravi *et al.* 2011; Verma & Paul 2013; Salimi *et al.* 2018).

When calculating a post mortem interval (PMI) based on the Developmental Model it is imperative to know whether or not the deceased was exposed to toxicants since toxicants can potentially affect the growth rate of forensic indicator species. (Monthei 2009; Rueda *et al.* 2010; Verma & Paul 2013). According to Cutler (2013), the effect of a toxicant is dependent on a number of factors, however, the dose is the main key that triggers a response. Verma & Paul (2013) stated that, not all drugs require to be in high concentrations for their effects to be observed, with some drugs their mere presence will cause an effect. According to Gosselin *et al.* (2011a), some physiological parameters such as sex-ratio and mortality (although not studied enough) could to a certain extent, have an influence on the development of forensic Diptera. The effect of a toxicant on the development of larvae depends on its concentration and metabolism within the insect. According to Parry *et al.* (2011), the entry of morphine into the central nervous system of *C. stygia* is prohibited due to the presence of the perineurium. This is a simple epithelium that covers and protects the central nervous system thus preventing the entry of any toxic organic compounds. This protective cover is facilitated by a p-glycoprotein transporter that actively pumps out toxicants to prevent them from reaching the central nervous system (CNS). Arnot *et al.* (2011) stated that, carbamates are less likely to cause CNS toxicity because do not readily cross the blood-brain barrier (BBB).

The effect of toxicants on the growth rate of insects needs to be understood and taken into consideration for the correct PMI estimation. There have been studies reported on the effects of a few toxicants on the development of forensic flies. Codeine accelerated the development of *C. albiceps* (Fathy *et al.* 2008) and *L. sericata* (Kharbouche *et al.* 2008). Morphine decelerated the growth rate of *L. sericata* (Bourel *et al.* 1999) but had no effect on the development rate of *C. stygia* (George *et al.* 2009).

De Carvalho *et al.* (2012) found that cocaine accelerated the development time of *C. albiceps*. Goff *et al.* (1991) observed an acceleration in the larval growth rate of *Boettcherisca peregrina* exposed to heroin. Buscopan decelerated the development rate of *Chrysomya megacephala* (Oliveira *et al*. 2009). Goff *et al.* (1994) emphasised the importance of entomotoxicological testing for accurate PMI estimations.

There is a lot of literature highlighting the effect of toxicants on the growth rate of forensic flies, however, there are no publications on aldicarb. This is the first study to determine the forensic implications of aldicarb in a carrion ecosystem. The aim of this part of the study was to assess the effect aldicarb had on the development of the larvae of *Chrysomya chloropyga*, *Chrysomya albiceps* and *Sarcophaga cruentata*. This was done to determine to what extent an altered growth rate would influence the PMI.

Hypothesis:
H_a= aldicarb would have an effect on the larval development of the test groups.
H_o= aldicarb would not have an effect on the larval development of the test groups.

4.2 MATERIALS AND METHODS

4.2.1 Experimental set-up

The eggs of larvae used in this experiment were sourced from stock colonies maintained in an insectarium at the Development of Zoology and Entomology, University of the Free State. The flies in these colonies were collected from carrion baits placed in a natural veld area that is relatively isolated from the build-up, inhabited areas of the University of the Free State.

Thirty plastic containers (ten for each species) were used for the experiment. The bottom of each container was covered with sawdust, followed by five layers of paper towel. Five containers for each species were taken as the control (nothing was added to the 50 g of fresh chicken livers placed on top of the paper towel in each container) and labelled accordingly (control group, date and the species). The 50 g of fresh chicken livers per container in the remaining five containers designated for each

species were each injected with 5 mL of the lethal dose (2 x LD_{50}) and the containers were labelled accordingly (treated group, date and the species name). Containers of the treated groups had a “toxic” warning sign attached to them. The eggs of *C. chloropyga* and *C. albiceps* were carefully weighed to 0.02 grams and placed in close proximity of the chicken livers between the tissue folds in the designated containers. *Sarcophaga cruentata* first instar larvae were placed in the ten containers designated for the species (five control and five treated). A schematic diagram of the experimental set-up is shown in Figure 4.1. All containers were closed with gauze centred lids to allow ventilation and prevent larvae from escaping. The containers were kept in the insectarium and reared at ±23ºC. Larvae were reared on a cyclic light (light switched on at 8am and switched off at 6pm). The day the eggs were placed was designated as Day 0. Throughout the experiment, the feeding substrate was monitored and replenished. This was done to ensure that larval development was not influenced due to possible starvation.

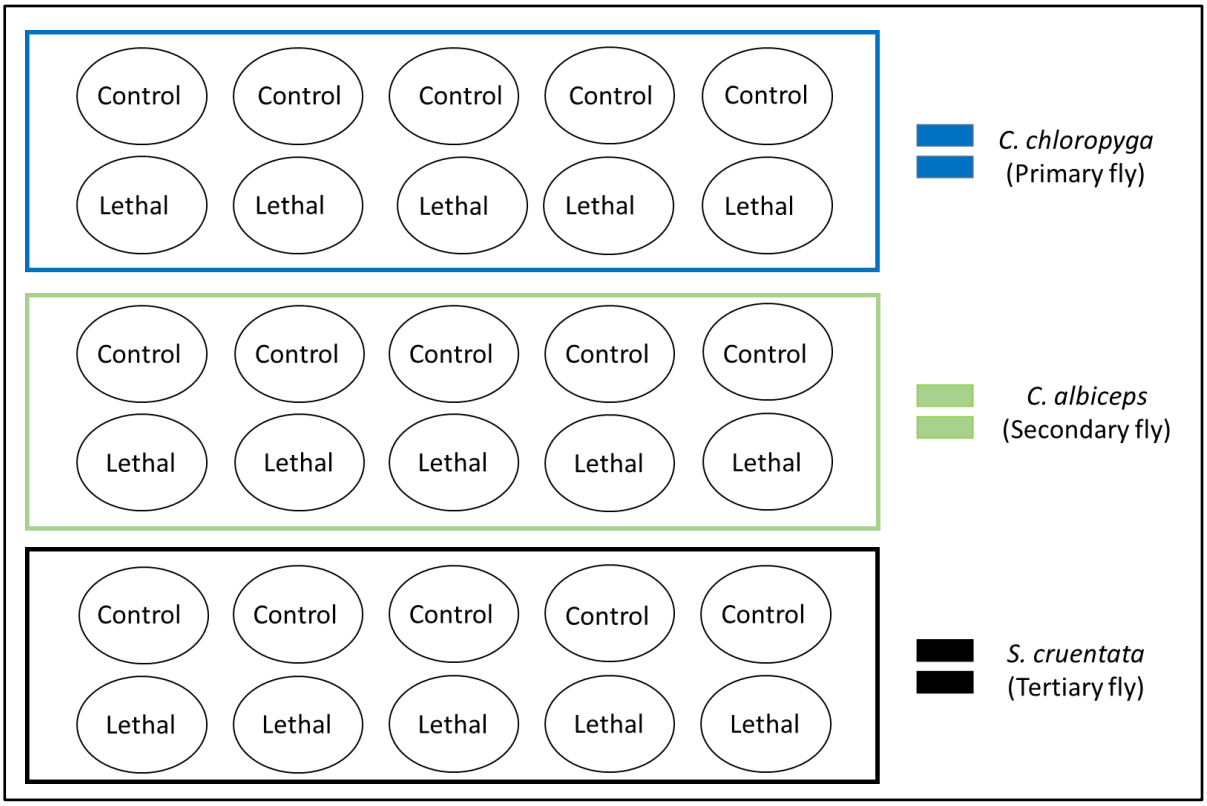

Figure 4.1: Diagrammatic representation of the experimental set-up to test the effect of aldicarb on the development rate of forensic flies.

Forty five larvae were collected on a daily basis from each group (control and treated) for *C. chloropyga* and *C. albiceps*. Only 15 larvae for *S. cruentata* were collected because they are larviparous and do not larviposit as much progeny. All specimens were killed with near boiling water, transferred to 70% ethanol and refrigerated (4°C). For accurate measurements, larvae need to be killed with hot water to prevent curvature and shrinkage. The killing and preservative methods are crucial in maintaining size and morphology (Brown 2012). They were then measured and weighed. This process continued until pupation. For accuracy, the experiment was done in triplicate.

4.2.2 Statistical analysis

Statistical analysis was conducted with Statistica (Version 13.0; Dell, 2015). Anova was used as statistical analysis of the differences among group means for larval development. Significance was accepted at the level of $p<0.05$.

4.3 RESULTS

4.3.1 *Chrysomya chloropyga*: primary fly.

The development of *C. chloropyga* larvae over a period of 168 hours is shown in Figures 4.2 and 4.3. The results showed the larvae from the treated group to be significantly larger and heavier than the larvae from the control group. The length and weight of the larvae progressively increased and larvae from both groups reached their maximum growth at 120 hours (Figs. 4.2 and 4.3). Thereafter, the length and weight decreased until the next stage of development. The larvae in the control group went into pupation at 144 h whereas those in the treatment group pupated at 168 h. The lengths and weights of the larvae of the control and treated groups differed from 72 h onwards.

The results of the current study showed that the *C. chloropyga* larvae of the treated group were larger and heavier than the larvae of the control group. As a result, *C.*

chloropyga larvae that fed on aldicarb would appear to be older than what they are. The presence of aldicarb could result in the PMI being overestimated. It cannot, however, be said that aldicarb accelerated the development time of this species because the treated larvae took longer to pupate than the larvae that were not subjected to the toxicant. The presence of aldicarb had an effect on when the final stage of larval development was reached. The total larval stage of the treated groups lasted 24 hours longer than that of the control group. The duration of the larval stage was thus decelerated by the presence of aldicarb.

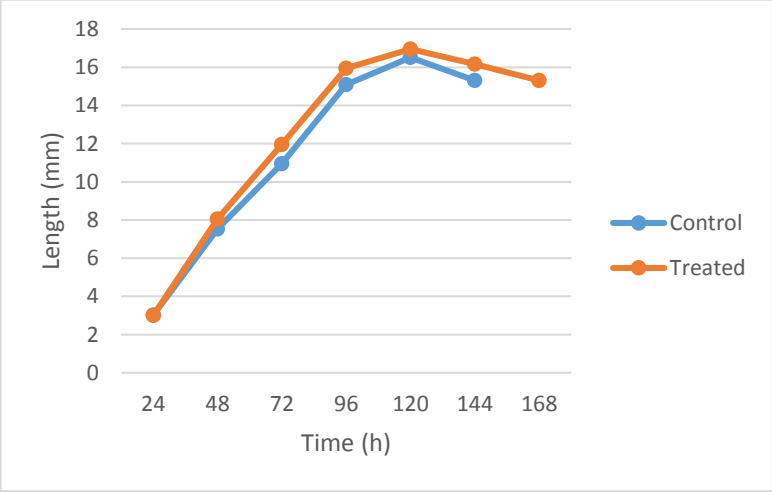

Figure 4.2: The average length of *Chrysomya chloropyga* larvae over a period of 168 hours showed that, overall, the treated groups were larger than the control groups.

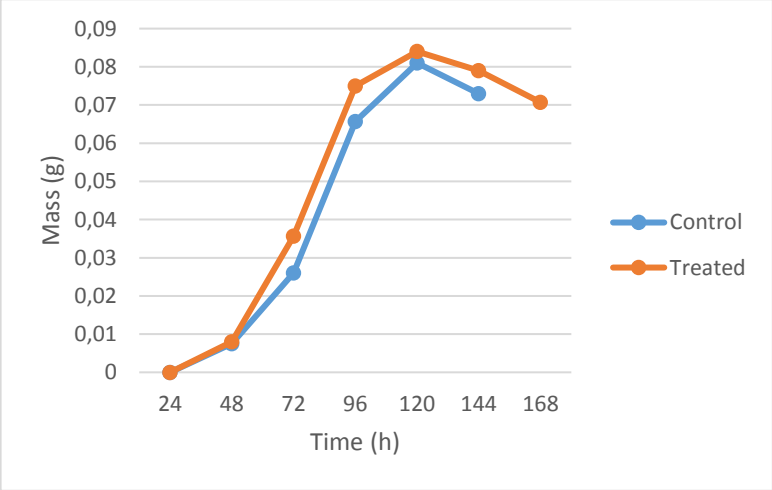

Figure 4.3: The average mass of *Chrysomya chloropyga* larvae over a period of 168 hours showed that, overall, the treated groups were heavier than the control groups.

Statistical analysis of the data showed that there were no significant differences in the development of the larvae on Day 1 ($F= 2.047$, $p= 0.156$) for *C. chloropyga*. Significant differences were observed from Day 2 onwards and the interval between the initial and the final larval development was significant between the groups. There was a significant difference in the mean lengths ($F=27.396$, $p<0.0001$) and weights ($F= 19.674$, $p<00001$) of *C. chloropyga*. The larval phase was significantly longer for those that were exposed to aldicarb than for the control group. Aldicarb thus, decelerated the development rate of *C. chloropyga* larvae.

4.3.2 *Chrysomya albiceps:* secondary fly.

The development of *C. albiceps* larvae over a period of 192 hours showed that the treated larvae were larger and heavier at 72 hours but from 96 hours, the control group larvae appeared to be larger and heavier than the treated group larvae (Fig. 4.4 and 4.5). The larvae gradually developed until the maximum size was reached at 144 hours for both groups. After this point in time the post-feeding larvae became smaller (i.e. decreased in length and weight).

Chrysomya albiceps larvae differed from *C. chloropyga* larvae and also from the trend observed by other researchers (Carvalho *et al.* 2001; Mullany *et al*. 2014; Kharbouche *et al*. 2008) in that a consistent accelerated growth for treated larvae was not observed. *Chrysomya albiceps* larvae exposed to aldicarb were larger and heavier than the larvae from the control group on at 72 hours, but thereafter the results shifted to the control groups being larger and heavier than the treated ones. The presence of aldicarb in a corpse may result in PMI underestimation. The presence of aldicarb in the livers shortened the larval stage by 24 hours. (Figs 4.4 and 4.5). The treated group went into pupariation before the control group. The duration of the larval stage was thus accelerated by the presence of aldicarb.

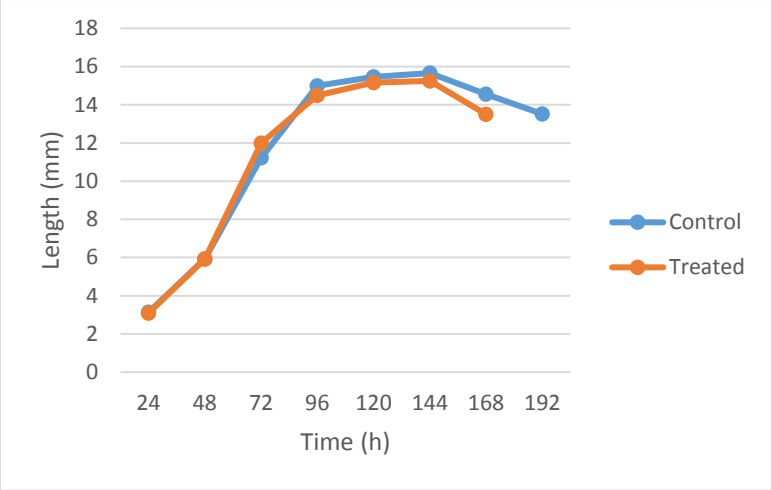

Figure 4.4: The average length of *Chrysomya albiceps* larvae in the experiment over a period of 192 hours.

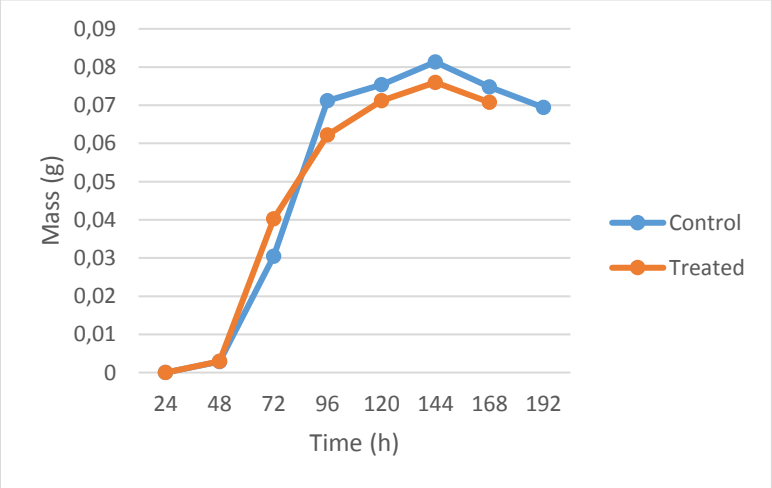

Figure 4.5: The average mass of *Chrysomya albiceps* larvae in the experiment over a period of 192 hours.

Aldicarb seemed to have an opposite effect on *C. albiceps* in that, it accelerated the growth rate of this species. There were significant differences in the mean lengths ($F= 67.931$, $p<0.0001$) and weights ($F=9.5744$, $p<0.00001$) of the control and treated groups. The duration of the larval phase was significantly shortened by 24 hours.

4.3.3 *Sarcophaga cruentata*: tertiary fly.

The development of *S. cruentata* larvae over a period of 120 hours showed no significant difference in the length (Fig. 4.6) and mass (Fig. 4.7) between the control

and the treated group. The size of the larvae increased and both groups reached their maximum growth at 96 hours. Thereafter, the size decreased as the larvae progressed to the post feeding stage. The development of *S. cruentata* was not affected by the presence of aldicarb. Both the control and treated group developed at a similar rate.

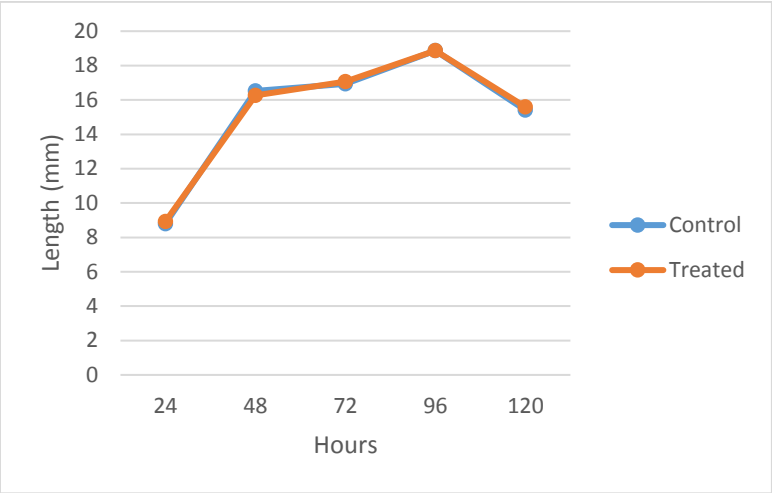

Figure 4.6: The average length of *Sarcophaga cruentata* over a period of 120 hours showed that the control groups were almost the same length as the treated groups.

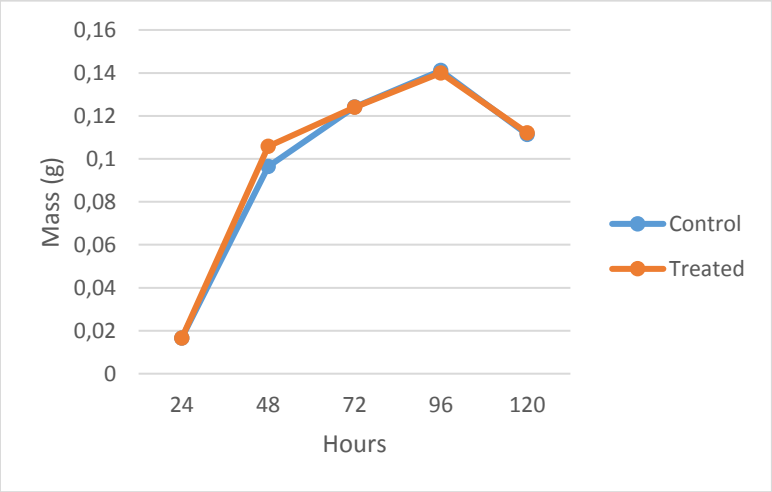

Figure 4.7: The average mass of *Sarcophaga cruentata* over a period of 120 hours showed that both groups had an approximately similar mass.

Aldicarb had no significant effect on the growth rate of *S. cruentata*. There were no significant differences in the mean lengths ($F=0.38115$, $p>0.05$) and weights

(F=0.74581, p>0.05) of the control and treated group. The duration of the larval stage was the same for both groups.

4.4 DISCUSSION

Rates of larval development were determined by increases in length and mass. The larval stage of development was based on the number of slits on the posterior spiracles and the size of the cephalopharyngeal skeleton. The posterior spiracle is not only used for species identification but to also determine the age of larvae (Zou *et al.* 2013). The number of body segments and the number of branches found on the anterior spiracles may also be used (Visser 2016).

The effect of aldicarb on the development of *C. chloropyga* and *C. albiceps* larvae was not evident at the onset of the experiment. The control larvae did not exhibit a differential growth rate compared to the treated larvae during the initial few days. Differences in growth (length and mass) became evident after 48 hours. Just like in the present study, in a study done by Carvalho *et al.* (2001), the effect of diazepam was also not witnessed from the get go and differences were only seen after 18 hours.

The presence of toxicants in the food source may affect the development rate of insects and lead to erroneous PMI estimates. Morphine was observed to decelerate the growth rate of *L. sericata* (Bourel *et al.* 1999). Oliveira *et al*. 2009 also observed a decelerated growth rate when *C. megacephala* were exposed to buscopan. The presence of cocaine in the food source resulted in an accelerated growth rate in both *C. albiceps* and *C. putoria* larvae (de Carvalho *et al.* 2012). Mullany *et al.* (2014) also witnessed a shortened larval stage when larvae of *Calliphora stygia* were treated with methamphetamine Souza *et al.* (2011) also observed that, *C. albiceps* larvae that were exposed to anabolic-androgenic steroids (AAS) weighed less than the larvae that were not exposed to the steroid. Similar results were also reported for larvae of *C. putoria* treated with the same steroid. The presence of AAS (Souza *et al.* 2011),

methylphenidate hydrochloride or phenobarbital (Rezende *et al.* 2014) and aldicarb could lead to the PMI being underestimated. George *et al.* (2009) observed all concentrations of pure morphine to have no effect on the growth rate of *C. stygia* larvae whether it was exposed to the drug or not. Depo-Provera and Nur-Isterate had no effect on the growth rate of *C. chloropyga* (da Silva & Villet 2006).

Mullany *et al.* (2014) emphasised that ignoring the presence of drugs in the corpse may lead to incorrect PMI estimates. Carvalho *et al.* (2001) noticed that, a PMI error of between 18 and 54 hours may arise when the presence of diazepam in the corpse is ignored and the estimation is based on total weight of the larvae. Based on the results of the current study, it was observed that an error of up to 24 hours may result in the PMI estimate if the presence of aldicarb in the tissues is not considered in the total larval development time of *C. chloropyga* and *C. albiceps*.

The effect of aldicarb on the development of *C. chloropyga* and *C. albiceps* showed different results even though both species are from the same genus. The same was also witnessed by Souza *et al.* (2011) when they tested the effect of nandrolone decanoate on three *Chrysomya* species. When cocaine was fed to species from the same genus: *C. albiceps* and *C. putoria* larvae, de Carvalho *et al.* (2012) observed that, the drug accelerated the development of *C. putoria* but decelerated the development of *C. albiceps* when compared to the controls (at 6th- and 18th-hour observations).

Rezende *et al.* (2014), compared two studies that were conducted to determine the effect of a lethal concentration of scopolamine on forensic species. In the first study, done by Thyssen & Grella (2011), *C. putoria* larvae were reared on an artificial diet spiked with the medication while in the second study, conducted by Oliveira *et al.* (2009), *C. megacephala* were reared on rats that were exposed to the medicine. The results showed scopolamine caused a 48 h delay in the development time of *C. putoria* whereas a 54 h delay was observed in the development time of *C. megacephala*.

Furthermore, the final size of *C. megacephala* was reduced. One should therefore, not generalize the effect of a toxicant for all flies of a specific genera (Rezende *et al.* 2014).

In conclusion, the main application of medico-legal entomology is the correct estimation of the PMI. It is important for errors to be avoided by all means. In this part of the study it was found that aldicarb delayed the total larval duration of *C. chloropyga* (null hypothesis rejected), accelerated that of *C. albiceps* (null hypothesis rejected) but had no effect on the duration of the larval development of *S. cruentata* (failed to reject the null hypothesis). This shows that different fly species of forensic importance may respond differently to the same drug. This thus shows that the effect of a drug on one species should not be extrapolated for all species of the same genus. More research still needs to be done to establish baseline data on the forensic implications of aldicarb and other toxicants in a carrion ecosystem. This will provide information about the necessary corrections factor to apply which will lead to more accurate post-mortem interval estimates.

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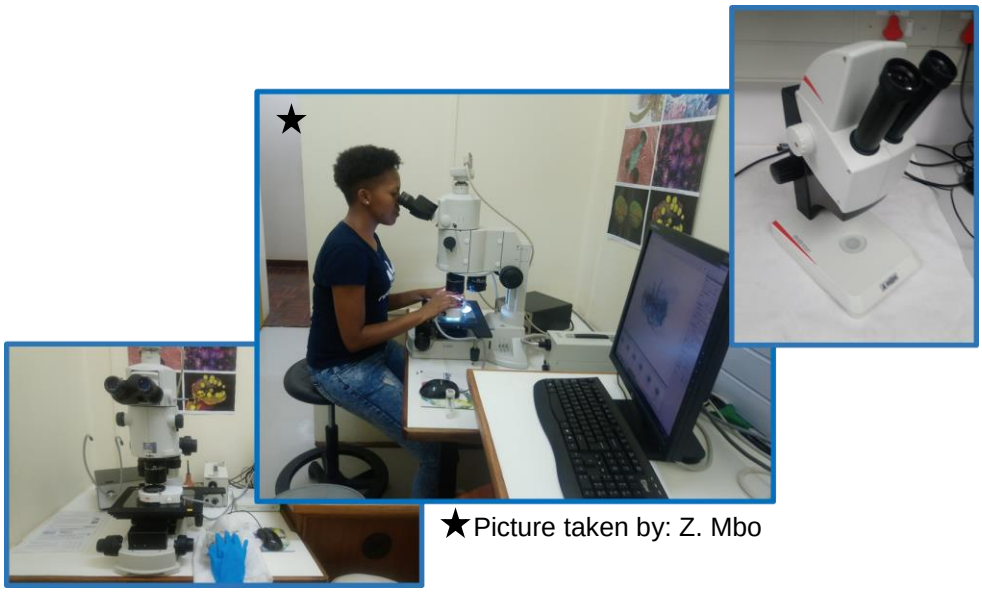
CHAPTER 5

The effect of aldicarb on pupal development and the adults of some flies of forensic importance



★Picture taken by: Z. Mbo

5. THE EFFECT OF ALDICARB ON PUPAL DEVELOPMENT AND THE ADULTS OF SOME FLIES OF FORENSIC IMPORTANCE

5.1 INTRODUCTION

The determination of a PMI is based on the oldest specimens collected (Sharma *et al.* 2015). If pupae are recovered from or around the body then they should be used for this purpose. The post-feeding stage is entered soon after the larvae reach their maximum size. Once in the post feeding phase, the larvae wander off and empty their gut to find a suitable place for pupariation (Gosselin *et al.* 2011b). The pre-pupal or wandering phase is marked by the decrease in size (Goff *et al.* 1991). Greenberg (1991) stated that, the post feeding stage covers approximately one third of the pre-adult development time.

The pupal stage is a non-feeding dormant stage that lasts approximately half of the total developmental time (Turner 2009; Reibe *et al.* 2010). According to Finell & Jarvilehto (1983), the pupal stage consists of three phases: histolytic changes, histogenetic changes and differentiation. The histolytic phase is the dissolving of larval organs. The histogenetic phase is the formation of adult fly organs. Differentiation is the changing of proteins from insoluble to soluble types. At this stage of development, the larvae undergoes developmental changes into an adult within the puparium (Gosselin *et al.* 2011b). These developmental changes leading to the emergence of an imago are known as metamorphosis (Gaudry *et al.* 2006). The puparium is a protective cover made of chitin which is resistant to chemical attack, microorganisms, weather conditions and remains for long periods of time (Huchet 2010; Gosselin *et al.* 2011b). During eclosion, adults emerge and ovaries mature within six days (Brown 2012). From there on, adults will mate, lay eggs and revisit the corpse depending on the amount of food.

Unlike with larvae, age estimation of pupae does not involve measuring their size. Instead, they can be reared under controlled conditions (the same as those from the

death/crime scene) until the adult stage and the PMI is back-calculated from eclosion. Pupae are reared under the assumption that they are alive and that conditions are favourable. This includes no parasitism, diapause or any disturbance to the lifecycle duration (Brown 2012). The use of pupae to determine the PMI is usually a slow process because some species take long to complete their lifecycle. According to Brown (2012), *C. vicina* takes up to 11 days to emerge when reared under 22°C. Although there are some published studies on the external pupal morphology of forensic indicator species, none of them can really be used for PMI estimations. A PMI estimate based on the puparial cases of *Phormia regina* was rendered unreliable (Bajerlein *et al.* 2018).

There are currently two methods used to determine the PMI estimate (narrowing it down to days or weeks) using pupae. The first method uses the colouration of the puparium which, depending on the species and environmental conditions, takes 12-24 hours to change from white to brown. In the first 24 hours of pupal development, age estimation is largely based on the colouration of the pupae in conjunction with the respiratory horn eversion (Brown 2012). When pupae are recovered with a pale colour, it means that the duration since pupation is earlier than 550 ADH (Brown 2012). This method, however, may be unreliable for age estimation considering that, the complete change of colour (to a dark brown) does not always occur. Furthermore, the colouration rates of different species as well as the final colour intensities are unknown. Due to the lack of research and knowledge surrounding pupal development, pupae are usually categorised as relatively “young” when light in colour or “old” when the pigmentation has darkened. This then leads to a PMI estimate with a window of approximately one week. As mentioned before, the age of the pupa cannot be determined based on size, it is therefore, based on morphological changes that occur as a result of metamorphosis. These to mention a few include: wings, legs and head development; eye colour change; mouth parts and antennal development; and sclerotisation.

As with larvae, an age estimation of pupae based on morphological markers can be influenced by various other factors, including the effect of toxicants ingested by the

larvae. In this chapter, the possible effect of aldicarb on pupal development, survival and a fitness parameter (ability to reproduce viable eggs or larvae) of newly emerged adults of some species of forensic importance was investigated. The aim of this part of the study was to evaluate the effect aldicarb had on the development of pupae and on the adults of *Chrysomya chloropyga*, *Chrysomya albiceps* and *Sarcophaga cruentata* exposed to the pesticide as larvae. It was hypothesised that aldicarb would have an effect on the pupal development and the adults of the test groups.

5.2 MATERIALS AND METHODS

This experiment continued from the experiment as set-up in the previous chapter. Five pupa from each group for each species were collected at 24 hour intervals. They were killed with boiling water, transferred to 70% ethanol and stored in a fridge. The pupal cases were removed and developmental landmarks of the pupae (adapted from Brown 2012) were assessed with either a Nikon multipurpose zoom microscope multizoom AZ100 or EZ4 StereoZoom® HD dissection microscope. The experiment was repeated to confirm results.

5.3 RESULTS

5.3.1 THE EFFECT OF ALDICARB ON PUPAL DEVELOPMENT OF SOME FLIES FORENSIC OF IMPORTANCE

5.3.1.1 *Chrysomya chloropyga*: primary fly

Day 7 of total development:

Control group (first day of intra-puparial development): three body regions were not yet distinctly defined (Fig. 5.1A). The entire body was cream in colour. When viewed laterally (Fig. 5.1B) and ventrally (Fig. 5.1C), the legs and wings appear attached to the body with the wings ending just behind the anterior part of the abdomen whereas the legs stretch to the posterior end of the abdomen (ventrally).

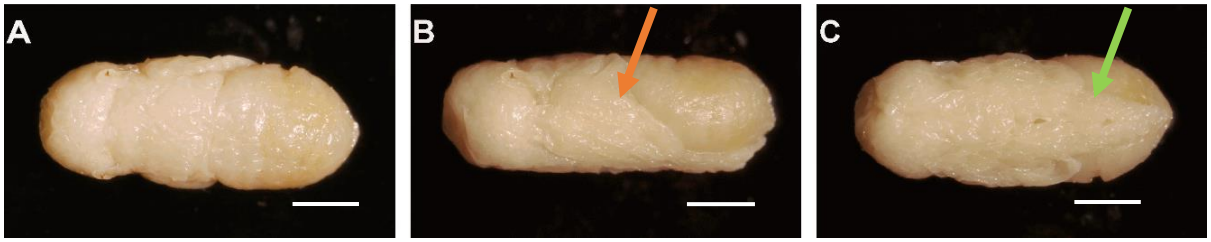


Figure 5.1: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group of a developing *Chrysomya chloropyga* pupa on Day 7 of total development. The orange arrow indicates the wing and the green arrow indicates the legs (Scale = 2 mm).

Treated group: Post feeding larvae had not yet undergone pupariation.

Day 8 of total development:

Control group (second day of intra-puparial development): The body of the control group remained cream but had divided into three distinct body regions: head, thorax and abdomen (Fig 5.2A-C). Although still cream in colour, the wings and legs had become distinctly visible. There seem to be projections near the wings resembling halters (Fig. 5.2A). The oral lobes (mouthparts) were slowly starting to develop taking position on the ventral side above and between the legs (Fig 5.2C). The antennae had also developed antero-laterally to the head.

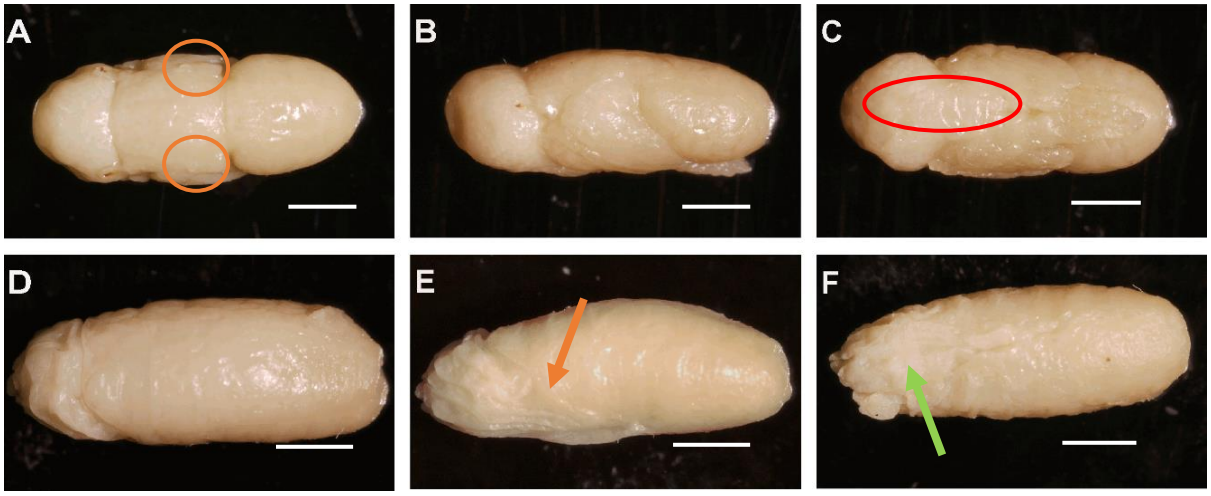


Figure 5.2: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and the dorsal (D), lateral (E) and ventral (F) view of the treated group of *Chrysomya chloropyga* pupae on Day 8 of total development. The orange circles indicate the "halters", the red oval the developing mouthpart, the orange arrow the wing and the green arrow the developing legs (Scale = 2 mm).

Treated group (first day of intra-puparial development): The body was cream and not defined into body regions (Fig. 5.2D). Although the head had not yet developed, undefined, weakly formed wings and short legs were noted (Fig. 5.2E and F).

Day 9 of total development:

Control group (third day of intra-puparial development): Whereas the compound eyes were not visible on Day 8 due to being the same colour as the body, they were now clearly visible due to their red colour (Fig. 5.3A-C). The oceli was also visible and well developed (Fig. 5.3A). The antennae and arista were well developed and distinctly visible even though they had no colour (sclerotisation) (Fig. 5.3C). The wings were folded with a bit of venation visible. The abdomen was a shade darker than the rest of the body (it appeared light brown and slightly banded).

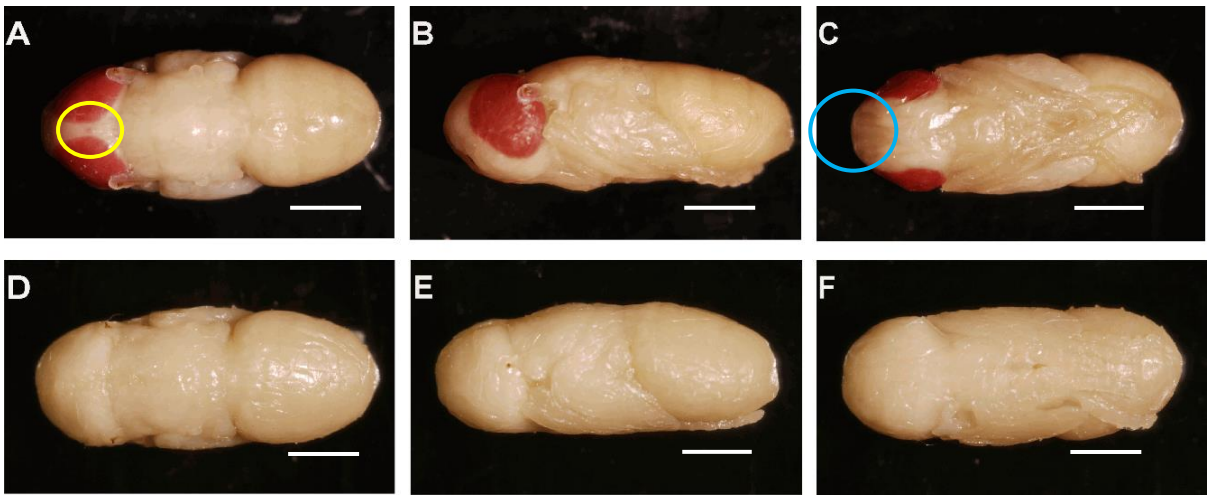


Figure 5.3: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and the dorsal (D), lateral (E) and ventral (F) view of the treated group of *Chrysomya chloropyga* pupae on Day 9 of total development. The yellow circle indicates the oceli and the blue circle the antennae (Scale = 2 mm).

Treated group (second day of intra-puparial development): The developing pupa was pale coloured (Fig. 5.3D-F). The three regions were clearly defined i.e. the development was the same as that of the control group on the 8th day of the total development.

Day 10 of total development:

Control group (fourth day of pupal development): The head (antennae, compound eyes mouthparts), thorax, appendages and abdomen were well developed and the pharate was now an adult fly waiting to emerge. The colour of the compound eye had darkened. Facial, thoracic and abdominal bristles were visible (i.e. were black). The abdomen was clearly banded. Complete sclerotisation had taken place (Fig. 5.4A-C).

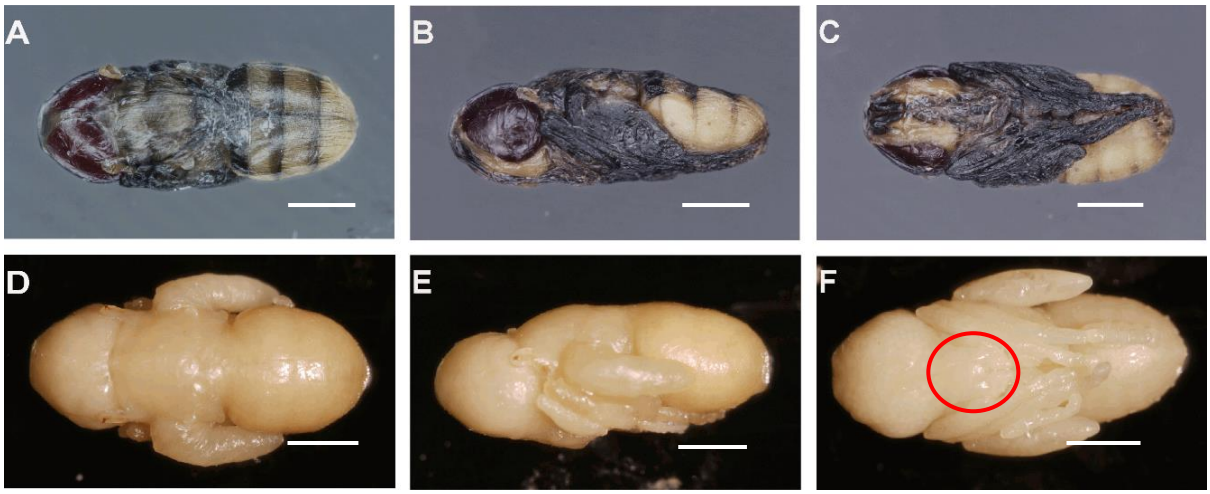


Figure 5.4: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and the dorsal (D), lateral (E) and ventral (F) view of the treated group of *Chrysomya chloropyga* pupae on Day 10 of total development. The red circle indicates the mouthparts (Scale = 2 mm).

Treated group (third day of inta-puparial development): The whole body was still pale (Fig. 5.4D-F). The mouthparts had started to develop taking position on the ventral side between the anterior of the legs (Fig. 5.4F). The wings were inflated and unfolded (Fig. 5.4D-F). The legs were also inflated.

Day 11 of total development:

Control group: Emerged as adults (Fig. 5.18A-C).

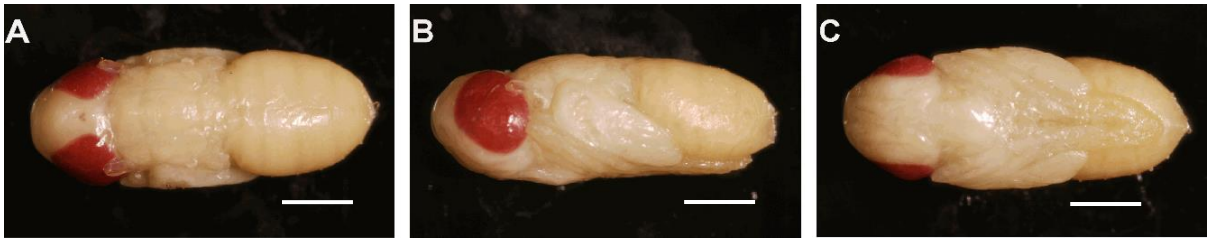


Figure 5.5: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the treated group of *Chrysomya chloropyga* pupa on Day 11 of total development (Scale = 2 mm).

Treated group (fourth day of intra-puparial development): The compound eyes had turned red (Fig. 5.5A-C) and the development was the same as that of the control group on Day 9 of the total development.

Day 12 of total development:

Control group: Already emerged as adult flies.

Figure 5.6: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the treated group of *Chrysomya chloropyga* pupa on Day 12 of total development (Scale = 2 mm).

Treated group (fifth day of intra-puparial): Complete sclerotisation had occurred (Fig. 5.6A-C) and the development was the same as that of the control group on Day 10 of the total development. Adults emerged on Day 13 (Fig. 5.18D-F)

5.3.1.2 *Chrysomya albiceps*: secondary fly.

Day 8 of total development:

Control group: Had not yet undergone pupariation.

Figure 5.7: Dissection micrographs of the dorsal (A), lateral (B) and ventral (C) view of the treated group of *Chrysomya albiceps* pupa on Day 8 of total development. The orange arrow indicates the wing and the green arrow the legs (Scale = 2 mm).

Treated group (first day of intra-puparial development): The whole body was pale and not defined into body regions (Fig. 5.7A-C). Regardless of there not being a head formed, the legs and wings had already started forming (Fig. 5.7B and C).

Day 9 of total development:

Control group (first day of intra-puparial development): The body was pale and not yet divided into body regions (Fig. 5.8A-C). The cephalopharyngeal skeleton was still attached (Fig. 5.8C).The legs and wings had started to form (Fig. 5.8B and C).

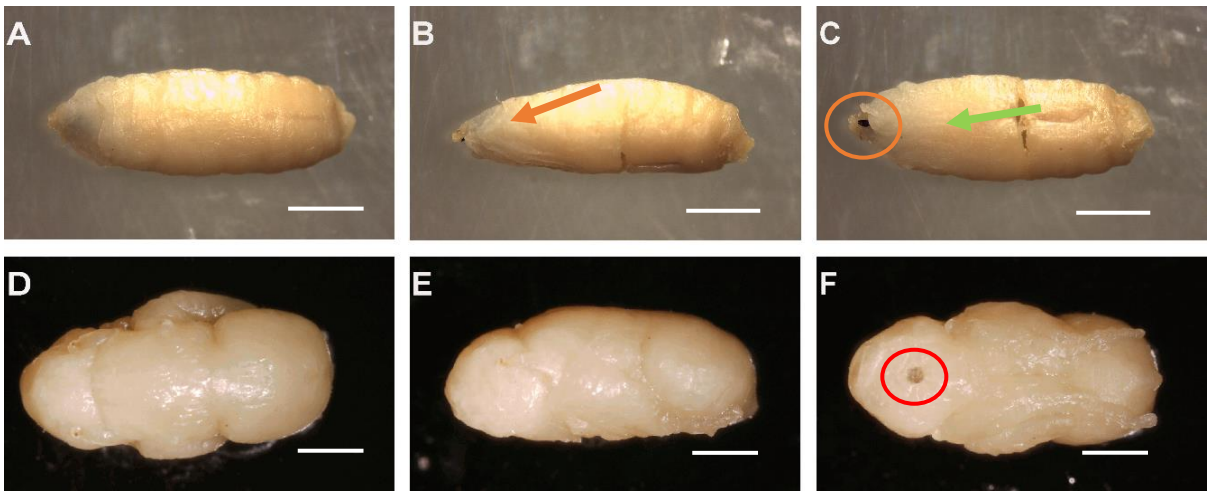


Figure 5.8: Dissection micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and stereo light micrographs of the dorsal (D), lateral (E) and ventral (F) view of the treated group of *Chrysomya albiceps* pupae on Day 9 of total development. The orange arrow indicates the wing, the green arrow the legs, the orange circle the cephalopharyngeal skeleton and the red circle the greyish spot (Scale = 2 mm).

Treated group (second day of intra-puparial development): The body was pale and distinctly divided into three body regions (Fig. 5.8D-F). There was a small greyish circle that remained beneath where the cephalopharyngeal skeleton detached (Fig. 5.8F). The appendages (especially the wings) were inflated.

Day 10 of total development:

Control group (second day of intra-puparial development): The body was cream with the abdomen appearing to have a darker shade than the rest of the body (Fig. 5.9A and B). When the pupal case was removed, the cephalopharyngeal skeleton remained and was still attached with a small greyish circle beneath it (Fig. 5.9C). The legs and wings were clearly visible but were not inflated.

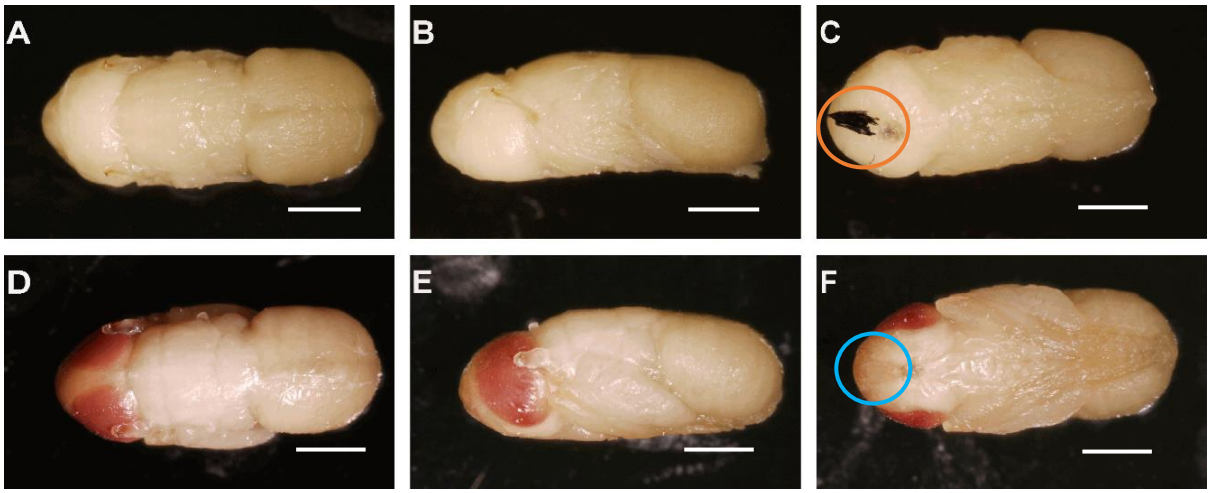


Figure 5.9: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and the dorsal (D), lateral (E) and ventral (F) view of the treated group of *Chrysomya albiceps* pupae on Day 10 of total development. The orange circle indicates the cephalopharyngeal skeleton and the blue circle the antennae (Scale = 2 mm).

Treated group (third day of intra-puparial development): The colour of the compound eyes had changed to red (Fig. 5.9D-F). Antennal development was evident at the anterior of the head (Fig. 5.9 F). The legs and wings were close to the body and no longer inflated.

Day 11 of total development:

Control group (third day of intra-puparial development): The body was still cream and the compound eyes were clearly visible. The compound eyes were still undergoing a colour change from pale to red (Fig. 5.10A-C). Antennae were visible on the anterior of the head (Fig. 5.10C). Abdominal banding was slight (Fig. 5.10A).

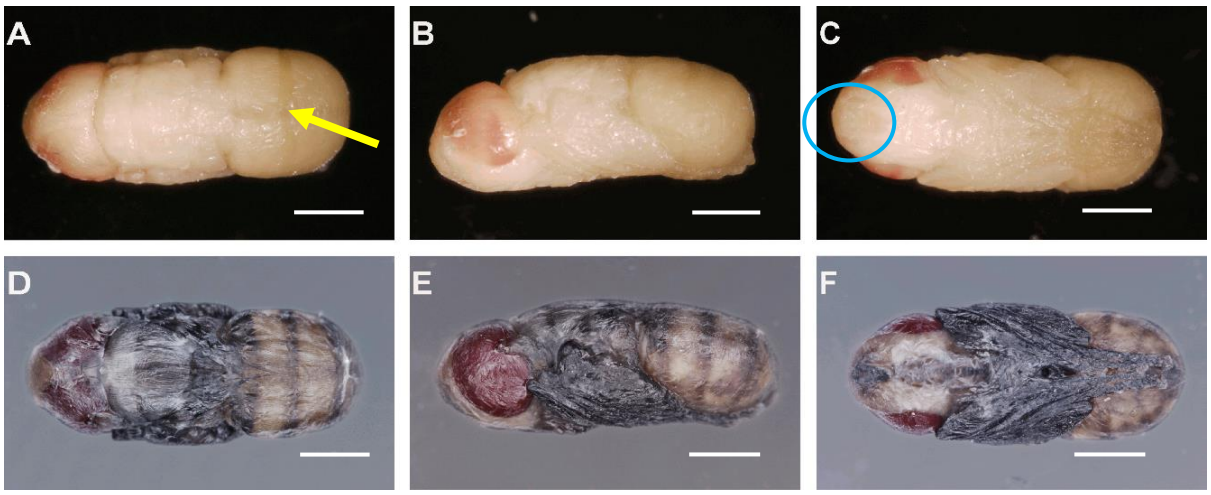


Figure 5.10: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and the dorsal (D), lateral (E) and ventral (F) view of the treated group of *Chrysomya albiceps* pupae on Day 11 of total development. The yellow arrow indicates the abdominal banding and the blue circle the antennae (Scale = 2 mm).

Treated group (fourth day of intra-puparial development): The compound eyes had darkened to a reddish brown colour. Complete sclerotization had taken place and all the bristles and setae had darkened to a black colour (Fig. 5.10D-F). Abdominal banding was evident and the pharate was well developed and ready to emerge.

Day 12 of total development

Control group (fourth day of intra-puparial development): The pharate was completely sclerotised (Fig. 5.11A-C) and the development was the same as that of the treated group on Day 11 of the total development. Adults emerged on Day 13 (Fig. 5.19A-C).

Figure 5.11: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group of *Chrysomya albiceps* pupa on Day 12 of total development (Scale = 2 mm).

Treated group: Emerged as adults (Fig. 5.19D-F).

5.3.1.3 *Sarcophaga cruentata*: tertiary fly.

Day 6 of total development

Control and treated group (first day of intra-puparial development): It was very difficult to remove the pupal case as it was still connected to the larvae (Fig. 5.12A and C). Upon the successful removal of the pupal casing, it was noted the larval form remained. The inside looked like a blob of larva (Fig. 5.12B and D).

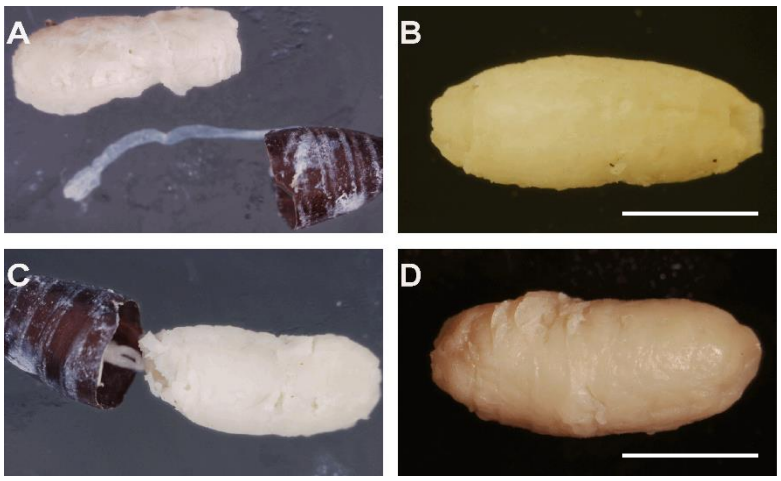


Figure 5.12: Stereo light micrographs of the development of the control (A and B) and treated (C and D) group of *Sarcophaga cruentata* pupae on Day 6 of total development (Scale = 5 mm).

Day 7-9 of total development:

Control and treated group (second to fourth day of intra-puparial development): The whole body was pale. The head had not yet developed and the cephalopharyngeal skeleton was still intact and could be seen at the anterior end (Fig. 5.13A and C). The legs and wings had already developed regardless of there not being a head (Fig. 5.13B and D).

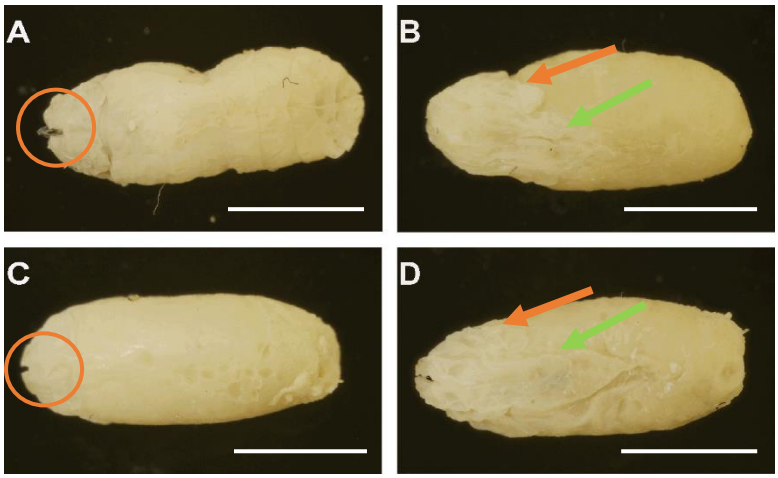


Figure 5.13: Stereo light micrographs of the dorsal (A) and ventral (B) view of the control group and the dorsal (C) and ventral (D) view of the treated group of *Sarcophaga cruentata* pupae on Day 7-9 of total development. The orange circles indicate the cephalopharyngeal skeletons, the orange arrows the wings and the green arrows the legs (Scale = 5 mm).

Day 10-13 of total development:

Control and treated group (fifth to eighth day of pupal development): The body was divided into three distinct body regions. The legs and wings were clearly distinct and inflated (Figs. 5.14A-F). The legs had ridges (were not smooth) and were already shaped like those of an adult fly.

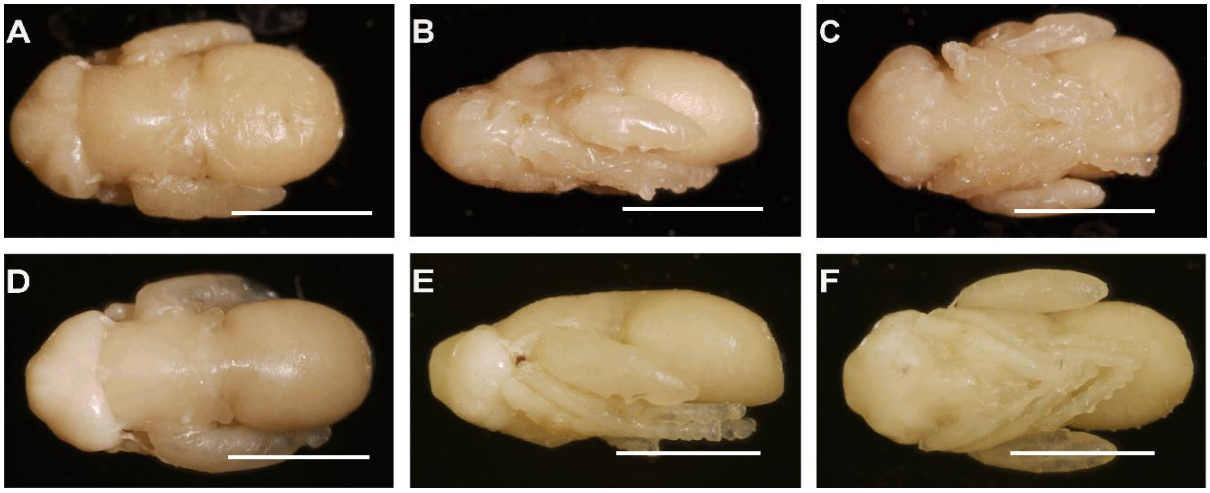


Figure 5.14: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and the dorsal (D), lateral (E) and ventral (F) view of *Sarcophaga cruentata* pupae on Day 10-13 of total development (Scale = 5 mm).

Day 14 of total development:

Control and treated group (ninth day of intra-puparial development): The compound eyes were pale but visible (Fig. 5.15B and E). Antennae were starting to develop as well as the mouthparts (Fig 5.15C and F). The legs and wings were less inflated and were close to the body (Fig. 5.15A-F).

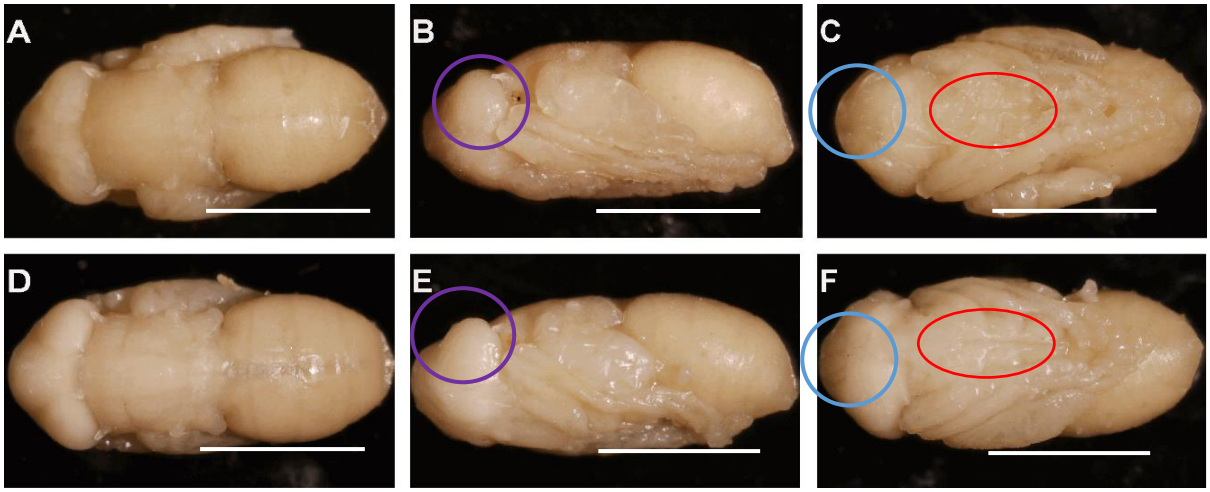


Figure 5.15: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and the dorsal (D), lateral (E) and ventral (F) view of the treated group of *Sarcophaga cruentata* on Day 14 of total development. The purple circles indicate the compound eyes, the blue circles the antennae and the red circles the mouthparts (Scale = 5 mm).

Day 15 of total development

Control and treated group (tenth day of intra-puparial development): The compound eyes had turned red. The antennae and mouthparts were well developed. The body had undergone moderate sclerotisation (Fig. 5.16A-F). Abdominal banding was partially visible (Fig. 5.16 A and D).

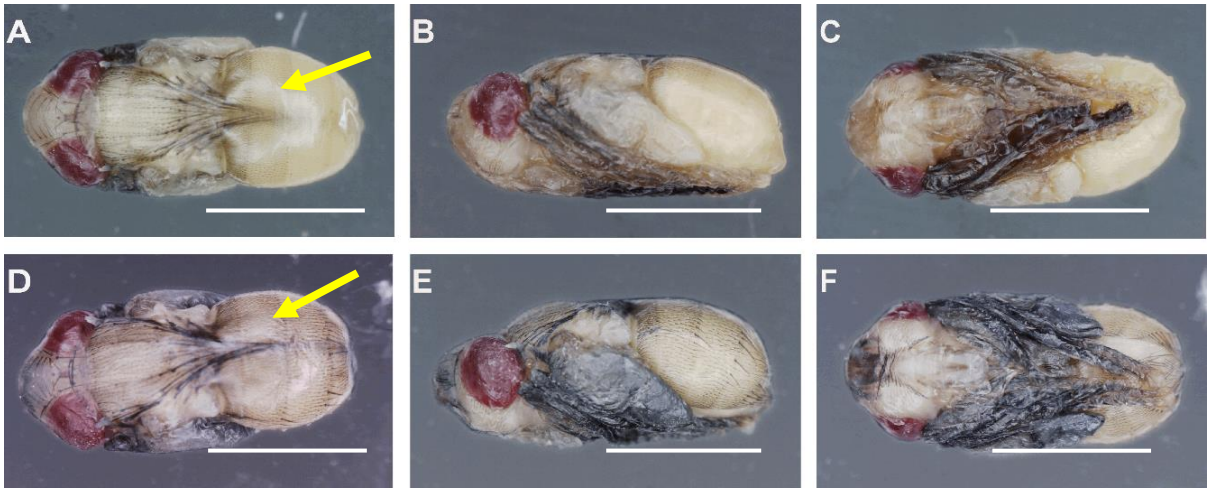


Figure 5.16: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and the dorsal (D), lateral (E) and ventral (F) view of the treated group of *Sarcophaga cruentata* pupae on Day 15 of total development. The yellow arrows indicate the abdominal banding (Scale = 5 mm).

Day 16 of total development:

Control and treated group (eleventh day of intra-puparial development): The body was completely sclerotised (Fig. 5.17A-F). The pharates were ready to eclode. Adults emerged on Day 17 (Fig 5.20A-F).

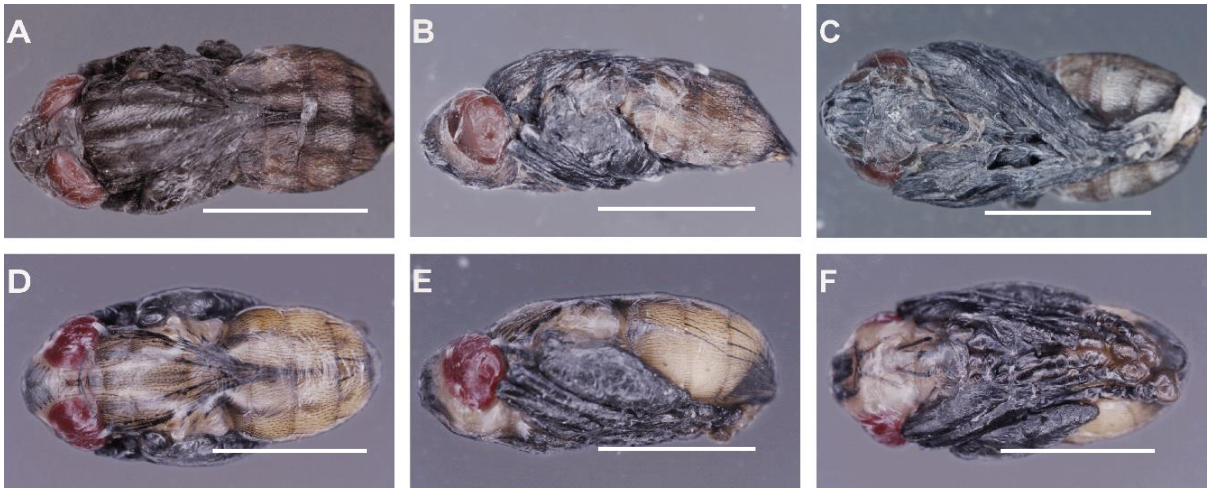


Figure 5.17: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and the dorsal (D), lateral (E) and ventral (F) view of the treated group of *Sarcophaga cruentata* pupae on Day 16 of total development (Scale = 5 mm).

5.3.2 THE EFFECT OF ALDICARB ON SOME FLIES OF FORENSIC IMPORTANCE THAT WERE EXPOSED TO THE TOXICANT AS MAGGOTS.

Entomotoxicological studies have shown that the bioaccumulation of toxicants in insects may significantly affect the morphology, the growth rate and survival of forensic flies. In this study, however, there were no mortalities throughout the study. There were no morphological defects noted in the adults that were exposed to aldicarb as maggots (Figs. 5.18-5.20).

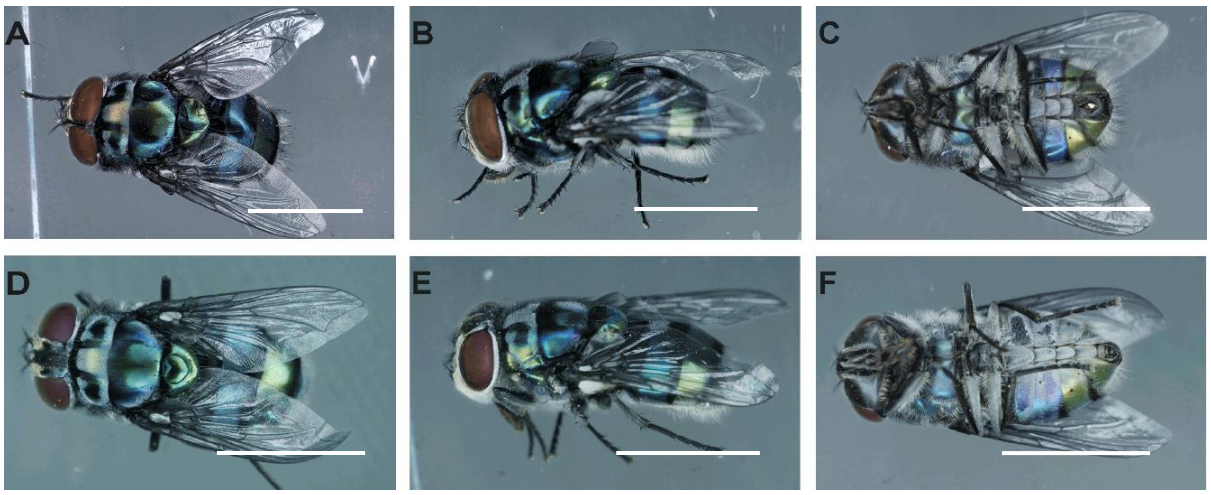


Figure 5.18: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and the dorsal (D), lateral (E) and ventral (F) view of the treated group of *Chrysomya chloropyga* adults on the day of eclosion (Scale = 5 mm).

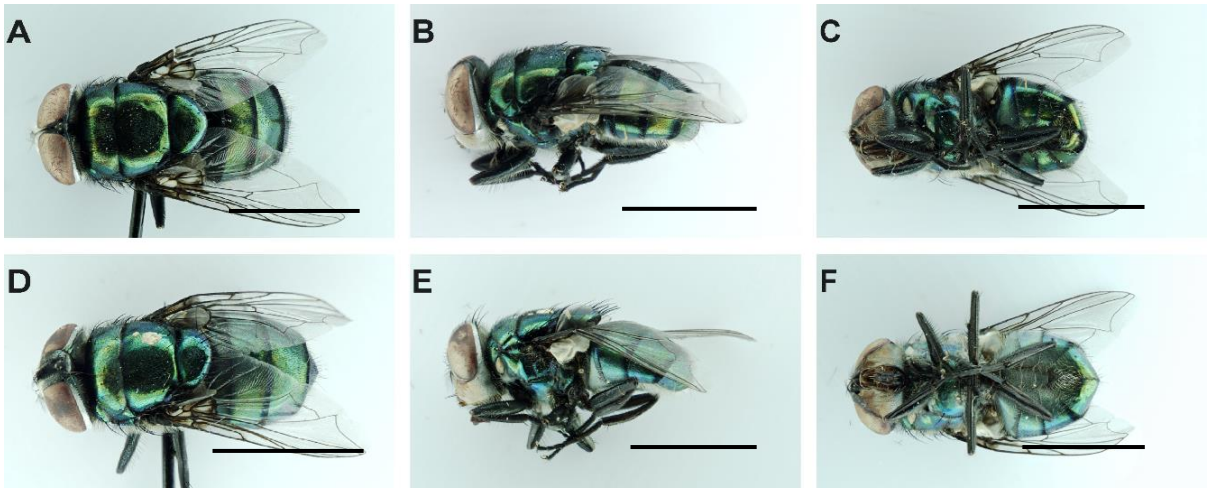


Figure 5.19: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and the dorsal (D), lateral (E) and ventral (F) view of the treated group of *Chrysomya albiceps* adults on the day of eclosion (Scale = 5 mm).

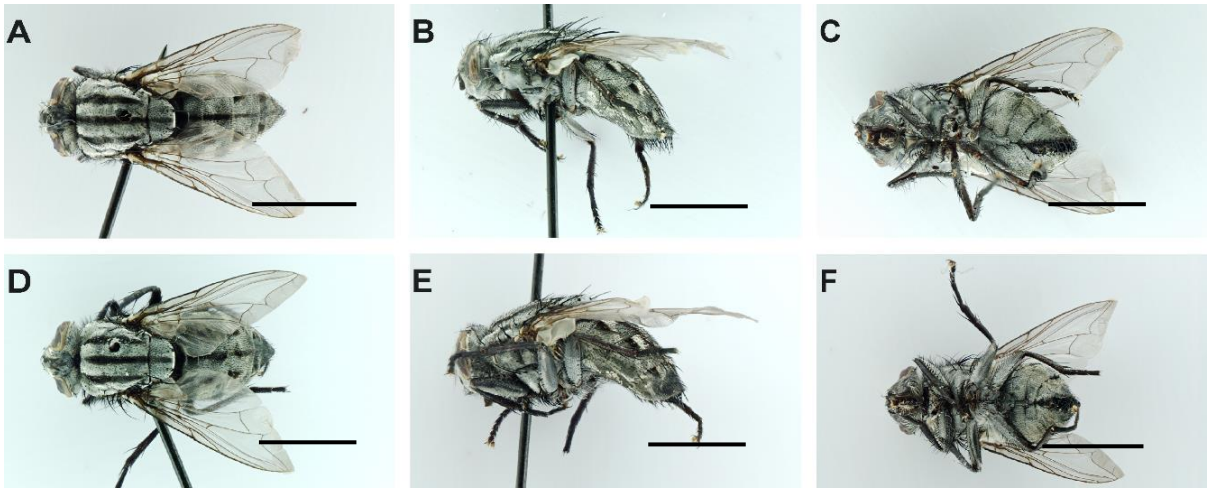


Figure 5.20: Stereo light micrographs of the dorsal (A), lateral (B) and ventral (C) view of the control group and the dorsal (D), lateral (E) and ventral (F) view of the treated group of *Sarcophaga cruentata* adults on the day of eclosion (Scale = 5 mm).

5.4 DISCUSSION

In this study pupariation was first observed in the control group of *C. chloropyga*. Pupal development was significantly longer in the treated group and adult emergence occurred two days earlier in the control group. Aldicarb thus, decelerated the pupal development rate of *C. chloropyga*. In a study by Goff *et al.* (1992), pupariation was significantly longer in the treated group compared to the control group. The presence

of methamphetamine may lead to PMI estimates that are erroneous by 48 hours based on pupal development. Goff *et al.* (1991) also noted that the pupal stage lasted significantly longer for those that were exposed to heroin compared to the control group with eclosion first occurring in the control group.

Aldicarb resulted in pupariation and adult emergence first occurring in the treated group due to the accelerated development experienced during larval development. There was however, no significant difference in the duration of pupal development. The pupal stage for both the control and treated group lasted four days each. Carvalho *et al.* (2001) noted a significant difference in the time required for pupariation due to the presence of diazepam. There was a significant difference in adult emergence with eclosion first occurring in *C. putoria* then in *C. albiceps*. There was a significant difference between the control and the treated group with the colour change in pre-pupa occurring 44 hours earlier in the treated group (Mullany *et al.* 2014). Pupariation also ended significantly earlier in those that were exposed to methamphetamine and its metabolite.

Aldicarb had no significant effect on the pupal development rate of *S. cruentata*. The duration of the pupal stage was the same for both groups. Furthermore adult emergence occurred at the same time for both groups. Souza *et al.* (2011) also found no significant difference between the control groups of *Chrysomya* species and those that were exposed to AASs.

The effect of aldicarb on the morphological markers showed no common trend regarding intra-puparial development for the three species investigated that were exposed to the toxicant as larvae. At any given time along the duration of the pupal stage, the *C. chloropyga* pupae exposed to aldicarb as larvae appeared younger compared to the pupae that were not exposed to aldicarb as larvae. The *C. albiceps* pupae exposed to aldicarb as larvae appeared older compared to the pupae that were not exposed to aldicarb as larvae The *S. cruentata* pupae exposed to aldicarb as larvae appeared to be the same age compared to pupae that were not exposed to

aldicarb as larvae. It was not possible to compare the results against any literature since there are no previous studies that have investigated the effect of a toxicant on inta-puparial developmental markers. Furthermore, there are no studies on the effect of this specific chemical on any of the other forensic species.

More research is still needed on pupal development since methods currently used to determine the PMI with the use of pupae are inaccurate and unreliable. This inaccuracy may be due to the fact that, with pupal development there are two possible starting points: from oviposition or start of pupariation (Brown 2012). Different starting points may give different results thus leading to incorrect PMI estimates. Oviposition is considered a good reference point since it is precise and easily controlled.

Regardless of it being the longest development stage and undergoing drastic changes, the pupal stage still remains understudied. According to Brown (2012), it is an unexploited resourceful PMI tool. The scarcity of studies done on pupal development of forensic flies constitutes to the underutilisation of pupae when determining the PMI. This stage of development is still missing basic standard protocols on sample preservation and baseline information on morphological and molecular developmental data. The documentation and publication of these would render this developmental stage as reliable and increase its usage in PMI estimations.

Few studies done in entomotixicology reported on the effect of the drug on the emerging adults. An increase in metabolic rate may be seen as an accelerated growth rate resulting in larger immature and adult specimens (Mullany *et al.* 2014). According to Gosselin *et al.* (2011b) drugs are excreted within the first two days of adult emergence. In studies done on the effect of drugs on forensic insects, Goff *et al.* (1991) and Williams & Villet (2014) found that the adults in their studies were fecund and fertile. They mentioned that adult flies oviposited eggs that hatched. The same was observed during the current study and adults of all species exposed to aldicarb as larvae produced viable eggs or larvae when supplied with chicken livers. According to Baron (1994), experiments conducted on laboratory vertebrate animals resulted in

aldicarb having no effect on the reproduction, fertility, gestation, viability and lactation. There were no congenital malformations reported in the adult flies of the larvae that were exposed to aldicarb.

An in depth study on pupal development under different temperature regimes outlining the timeline of developmental changes would ease age estimations and maximise the utilisation of this developmental stage in PMI estimations. According to Brown (2012), the rearing of pupae under controlled conditions with high temporal resolution would lead to accurate age estimations. PMI estimations from pupal development is still not yet established or well documented. The use of pupa greatly depends on morphological features which require excellent preservation techniques. For now, these techniques have not yet been described. Brown (2012) used optimal and universal preservatives, but mentioned that those preservatives, however, were not tested after months of storage. Ideally, when protocols are adhered to, specimens should be good to use for at least a year. Until such protocols are published, it is vital that one observe and analyse entomological specimens soon after collection.

In conclusion, intra-puparial development was initiated earlier in the control group of *C. chloropyga* (null hypothesis rejected), later in the control group of *C. albiceps* (null hypothesis rejected) but at the same time for both groups of *S. cruentata* (failed to reject null hypothesis). The development of fly larvae is well established but there is still a huge gap of knowledge on pupal development. Although larvae provide the best results when it comes to accuracy, pupae should be used when recovered on or near a body because PMI estimates are based on the oldest specimens. Developmental markers should be used to narrow down the PMI.

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CHAPTER 6

Discussion and Conclusion

Picture taken by: Z. Mbo

6. DISCUSSION AND CONCLUSION

Both aspects of entomotoxicology were investigated during this study: (i) the usability of insect specimens as an alternative source for toxicologal analysis and (ii) the effect of a toxicant on the life stages of flies of forensic importance and its potential effect on PMI_{min} estimations. This was the first study to test the entomotoxicological implications of aldicarb, a carbamate nematocide, insecticide and acaricide, in a carrion ecosystem. The following insect species were used for various parts of the study: *Chysomya chloropyga*, a first wave calliphorid fly species whose larvae are necrophagous; *Chrysomya albiceps*, a second wave calliphorid fly species whose larvae are necrophagous, but who also maximise its nutritional gains through cannibalism and predation of other necrophagous fly larvae; *Sarcophaga cruentata*, a second wave sarcophagid fly species whose larvae are necrophagous; *Thanatophilus micans* a necrophagous beetle species and *Saprinus splendens*, a beetle that predates on fly larvae.

6.1 USABILITY OF INSECT SPECIMENS FOR TOXICOLOGY

When a body is only recovered once the tissues of the corpse have degraded, toxicological analysis largely depends on the necrophagous and predacious insects that were feeding on the body. These insects ingest toxicants that the deceased was exposed to prior to death. The larvae of the three fly species and the adults of the beetle species that were exposed to aldicarb showed primary and secondary bio-accumulation of the toxicant. They can therefore be utilised as alternative toxicological sources where traditional corpse tissue samples are too degraded for toxicological screening. The correlation between the aldicarb concentrations found in entomological specimens was only evident in specimens that fed directly on the treated livers (primary bio-accumulation). A higher concentration of aldicarb was detected in specimens that were reared on livers spiked with a higher concentration of aldicard. No correlation was observed in the test subjects that ingested the toxicant through secondary mechanisms (i.e. predating on larvae that was exposed to the toxicant).

The elimination of a toxicant occurred in the post-feeding stage when the elimination rate exceeded the absorption rate. This part of the experiment was only performed for the immature stages of the flies. The pre-pupal stage contained a higher toxicant concentration of the toxicant compared to the subsequent life stages. Minute traces of aldicarb were detected in the adults of *C. albiceps* and *S. cruentata* but not in the adults of *C. chloropyga*. This could be because, their treated group entered pupariation after the control group giving them enough time to eliminate the toxicant. It can thus be concluded that the pupae of *C. albiceps* and *S. cruentata* are suitable alternative toxicology sources. Further studies still need to be performed on *C. chloropyga* pupae to test until what age the pupae can still be utilised as an alternative toxicological source. The toxicant was not picked up in the puparial casings of any of the test subjects. This does not imply that the toxicant was not deposited in this structure during the toxicant elimination process. The pupal casings were only macerated before toxicology testing; this might have been inadequate to release the toxicant from the chitinized matrix of the pupal casing. Further experiments should be embarked upon before a verdict can be made regarding the usability of the pupal casings of these test subjects as alternative toxicology source.

6.2 EFFECT OF ALDICARB ON THE LIFE STAGES OF THE TEST SUBJECTS

The second part of the study was to test the effect of aldicarb on the development of the various fly species. Toxicants are one of the factors that can greatly influence the precision of a PMI_{min}. It is therefore, crucial to know whether or not the deceased was exposed to any toxicants before using insect evidence to determine a PMI_{min}.

The rate of larval development is reflected by the increases in length and mass. Toxicants have the potential to alter the size of feeding fly larvae. Therefore, the size of larvae feeding on tissue containing aldicarb may lead to incorrect age estimations. The effect of aldicarb on the growth rate of the test subjects differed. It delayed the total development time of *C. chloropyga* by 48 hours, it accelerated the development time of *C. albiceps* by 24 hours but had no effect on the development rate of *S.*

cruentata. This proves that no generalizations can be made about the effect of a toxicant. This also underpins the notion that understanding the effects of drugs on the development of larvae is essential and that not taking it into consideration can lead to errors in determining a PMI

The complete lifecycle of *C. chloropyga* took 11 days for the control and 13 days for the treated group (Fig. 6.1). The larval stages (first, second and third instars plus post feeding larvae) lasted six days for the control and seven days for the treated group. Aldicarb decelerated the duration of larval development; a fact that should be considered when calculating a PMI estimate. The pupal stage lasted five days for the control and six days for the treated group. At any given time along the total development timeline, the pupae that was exposed to aldicarb as larvae appeared to be younger compared to the pupae that was not exposed to the toxicant as larvae. There seems to be a 24 hour lag in the development of *C. chloropyga* pupae between those exposed to toxicant as larvae compared to those not exposed to the toxicant as larvae. The adults of the control group emerged on Day 11 but those of the treated group only emerged on Day 13.

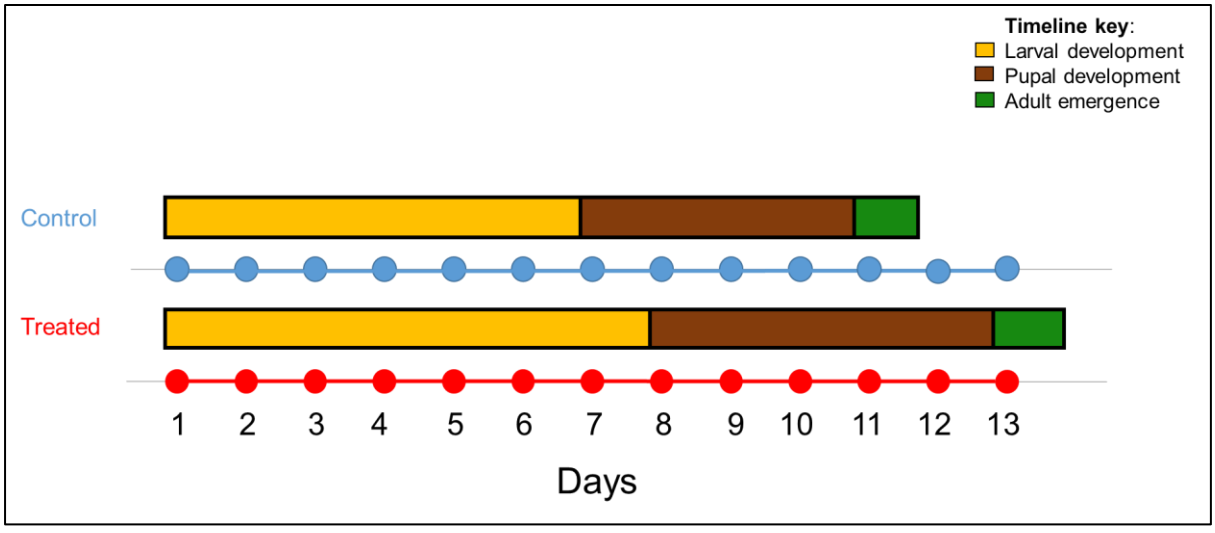

Figure 6.1: The development time of *Chrysomya chloropyga* showed that the adults of the control group emerged before those of the treated group.

The total development for *C. albiceps* lasted 13 days for the control group but only 12 days for the treated group (Fig.6.2). The larval development lasted eight days for the control but only seven days in the treated group. Larval development was accelerated during the last days of the active feeding stage; an appropriate PMI adjustment should be made for this specific time period. The pupal development lasted four days for both groups. However, at any given time along the total development timeline, *Chrysomya albiceps* pupae that were exposed to aldicarb as larvae appeared to be older compared to the pupae that were not exposed to aldicarb as larvae. This lag in development is more than a day, but less than two days. Eclosion occurred on Day 12 for the treated group whereas it only occurred on Day 13 for the control group.

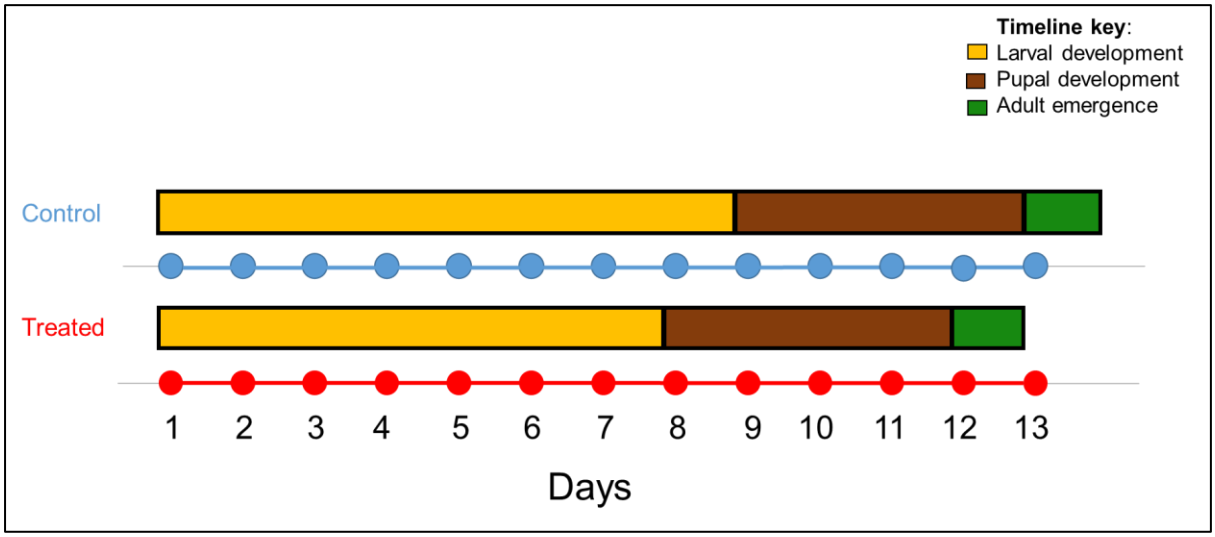

Figure 6.2: The developmental rate of *Chrysomya albiceps* showed that eclosion first occurred in the group that was exposed to aldicarb.

The growth rate of *S. cruentata* was unaffected by aldicarb (Fig. 6.3). Consequently, no adjustment in a PMI_{min} estimate needs to be done for either the larval stages or the pupal stages. The larval stage lasted for five days. Pupariation commenced on Day 6 for both groups and lasted for 11 days. Adults emerged on Day 17.

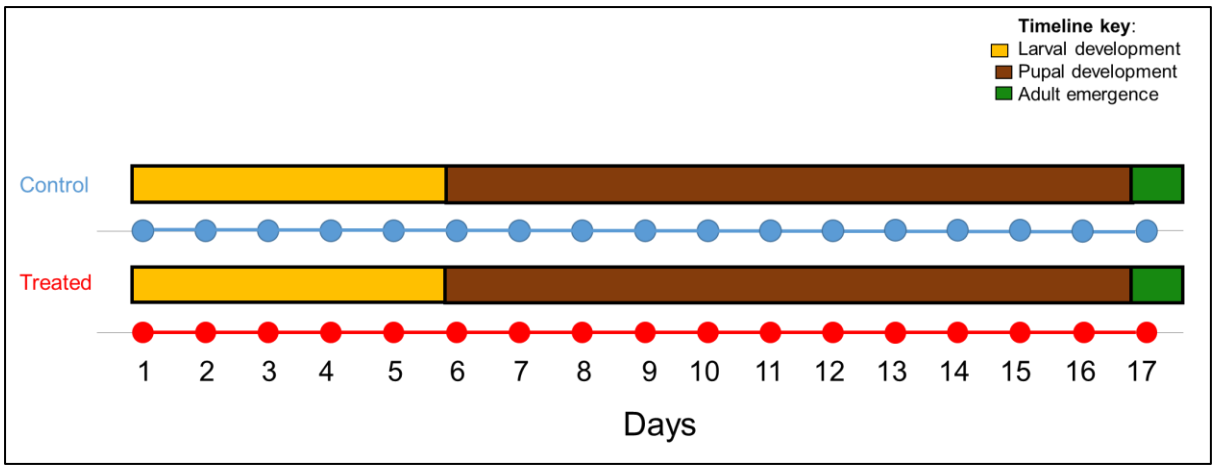

Figure 6.3: The developmental rate of *Sarcophaga cruentata* was the same for both groups.

Aldicarb did not affect the fitness of adult flies that were exposed to the toxicant as maggots. Entomotoxicological studies have reported on toxicants affecting the morphology, growth rate and survival of blowflies. In this study, however, there were no mortalities and no obvious alterations to the adult morphology.

6.3 CONCLUDING REMARKS

More research still needs to be done on the effect of aldicarb at different temperatures and concentrations as well as on other blowfly species. Entomological results have to be strong enough to withstand cross examinations in court, thus, the presence of drugs in a corpse should by all means be considered. There is still a gap in the knowledge on larval drug bio-accumulation and elimination and also the effect of drugs on intra-puparial development of forensic flies.

6.4 LIMITATIONS AND SUGGESTIONS

Initially, Silphidae larvae were used however, due to their cannibalistic behaviour, starving them was rather impossible therefore, adults were used instead. The larvae require to be kept in isolation (bred individually in petri dishes).

The cannibalistic behaviour of *C. albiceps* larvae also made starving the population difficult and breeding them individually led to early pupariation (diapause mechanism). What helped was starving a large population ensuring that the required number of the test group was obtained on the test day.

When doing the extractions, have enough equipment for each concentration. Contamination must be avoided by all means therefore, have a forceps (etc.) for each concentration and label the test groups accordingly to avoid any mix ups.

Although the pupal cases do not need to be killed, hot water should still be added before rinsing with the cold water. This makes it easier to remove contaminants (livers, paper towel etc.) that may have attached to the pupal casing. I do not suppose this is done in real life cases as that would reduce the concentration or chances of detecting the toxicant. However, in this study the aim was to determine whether pupal cases may be used thus the rinsing of the cases was to minimise surface contamination which may occur due to the limited space to ‘wander off’.

The stage of larval development should also be based on the number of posterior spiracles and not solely on the size to ensure that specimens that appear larger are indeed older and not just big or “fat”.

APPENDIX

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

22 August 2018

Dear Dr. S Brink

ENQUIRY WITH REGARD TO ETHICAL APPROVAL FOR TWO COMPLETED RESEARCH PROJECTS

Background:
Both the research projects were registered in 2016 as part of MSc qualifications. These projects were approved by a Research evaluation committee.Ethical approval for these projects were not requested. Both projects was entomological (using insects). Carcasses (sheep and pig) were used. The carcasses were obtained from the animal research unit. The carcasses were from other approved research projects in the Faculty of Health Sciences. Furthermore, chicken liver and offal obtained from a butcher as bait to catch flies were used. Maggots used for the one experiment were reared on chicken livers.

Regarding ethical approval:
The Interfaculty Animal Ethics Committee (IAEC) of the University of the Free State (UFS) do not issue retrospective ethical approval for research studies. Since these studies commenced in 2016 and is almost completed in 2018, **the IAEC cannot give Ethical Approval for these studies**.

Insects are not defined as animals according to the South African National Standards (SANS10386:2008). Research projects on insects do not require ethical approval from an Animal Ethics Committee (AEC) according to National (NHREC), SANS10386:2008, and UFS regulations. **These studies therefore did not, and still don't, need ethical approval from and AEC**.

When a research study utilize carcasses and organs from dead animals, it is the responsibility of the IAEC to ensure these animal tissue were obtained according to ethical research practices. The carcasses and animal organs used in this study was from ethical approved research projects (UFS), and from well known, ethically approved sources (registered butcher). The IAEC are satisfied that the **carcasses and animal organs used were sourced from ethically acceptable suppliers.**

In 2016 the UFS did not require ethical approval for studies that did not involve humans or animals as defined by the SANS10386:2008. **These studies therefore did not require ethical approval according to the UFS regulations** of 2016.

Implications:
The IAEC cannot issue Ethical Approval for these studies.
Both these studies were performed according to the National and UFS guidelines and regulations with regard to research and ethical approval.
The IAEC were aware of these studies and as far as the IAEC could determine, these studies were performed according to ethical acceptable standards.

Recommendation:
The IAEC recommend that the research projects, results from these research projects, and publications from these research (including MSc-thesis) be accepted as ethically performed research.

Gerhard van Zyl
CHAIR: Interfaculty Animal ethics Committee

Yours Sincerely

Mr. Gerhard Johannes van Zyl
Chair: Animal Research Ethics Committee