The influence of Methylphenidate Hydrochloride on the development of the forensically significant blow fly *Chrysomya chloropyga* (Diptera: Calliphoridae) in the Western Cape, South Africa

University of Cape Town • iYunivesithi yaseKapa • Universiteit van Kaapstad

by

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Study Summary

The ability to establish the post mortem interval (PMI), or time since death, forms an integral part of a forensic investigation. The PMI is used as a timeline to include or exclude suspects, and assists in the identification of the decedent. A forensic entomologist is able to estimate the PMI using necrophagous arthropods, and this is particularly useful in cases where the time since death exceeds 72 hours, after which medical manifestations of death becomes less reliable. A forensic entomologist will typically rely on the pattern of succession and/or development of necrophagous insects as a means to estimate the PMI.

As one of the earliest colonisers of a corpse, Calliphoridae (blow flies) provides the most accurate entomological means of estimating the PMI. The underlying assumption for the use of insect development to estimate the PMI, is that developmental data of immature insects can be used to determine insect age, which can be related to the time of colonisation, from which the PMI can be inferred. However, several studies have indicated that factors such as: climatic conditions, particularly temperature, geographic location, maggot masses and xenobiotics have an effect on the development of blow flies. Consequently, it has become important to consider these and other factors when using developmental data to estimate the PMI.

Forensic entomological/entomotoxicological research on the local blow fly populations of the Western Cape, South Africa is in its infancy. This study offers background information on some of the limitations faced during the undertaking of such research. Furthermore, this study provides a brief visual documentation of the life stages of *Chrysomya chloropyga* and certain identifying characteristics of blow fly species that were present in the study

population. This study investigated the influence of Ritalin (methylphenidate hydrochloride) (MPH) on the development of *C. chloropyga*. *C. chloropyga* larvae were reared on MPH spiked porcine liver and sequentially sampled at specific time intervals during which length and weight measures were collected. However, the possible influence of confounding factors complicated inferences from these measurements. Assessment of the larval and pupal development duration however, suggested a shortened larval development by up to 17 hours and a prolonged pupal development by up to 16 hours. These findings may have important implications as it suggests that PMI estimates based on these stages may be in error if the effects of MPH are not considered.

The entomotoxicological feasibility of *C. chloropyga* larvae were assessed in terms of detecting MPH. Interestingly, despite its labile nature, MPH was detected from larvae incubated at ~30°C, and sampled up to 3.5 days after replenishing the treated food source. Furthermore, during a comparison between ~ same aged larval specimens killed with near boiling water (> 90°C) and preserved in 70% ethanol at 4°C and those killed and preserved by freezing at - 20°C, MPH could still be detected in ethanol preserved specimens, albeit at a lower level than frozen specimens. This may suggest that, although frozen specimens are apparently more suitable for toxicological analysis, those preserved in ethanol may, under certain conditions, be of equal qualitative value. These findings suggest that *C. chloropyga* may be particularly suitable for the detection of MPH and possibly other xenobiotics and are therefore important to forensic investigations of drug related deaths. This is the first entomotoxicological data on MPH in the Western Cape, South Africa and may establish the way forward for future studies and the eventual construction of reference data that may assist forensic investigations.

Chapter 1: Research Proposal

The influence of Methylphenidate on the development of the forensically significant blow fly *Lucilia sericata* (Diptera: Calliphoridae) in the Western Cape Province

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1.1. Purpose of the study

The proposed study will investigate the hypothesis that methylphenidate hydrochloride (MPH) can affect the development rate of Calliphoridae subspecies (spp.). Therefore, the objective of this study is to determine the influence of MPH on the development rate of *Lucilia sericata* larvae prevalent in the Western Cape Province, with the aim of generating reference data specific to the Western Cape.

1.2. Background

Forensic investigators are frequently tasked with the responsibility to estimate the post-mortem interval (PMI), or time since death, from human remains in an advanced stage of decomposition. A forensic entomologist may utilise the developmental data and/or pattern of successional communities of several carrion-feeding arthropods to assist in the PMI estimation of the decedent (Wells & Lamotte, 2010). Calliphoridae (blow flies), in the order Diptera are amongst the insects of forensic importance and have been extensively researched (Byrd & Castner, 2010). Their forensic significance is derived from their ability to rapidly, and in large masses, colonise a body after death (Hall, n.d.). Greenberg (as cited in Monthei, 2009) has suggested that the development data of blow flies provides the most accurate means of estimating the minimum PMI (Grassberger & Reiter, 2001).

As previously mentioned, there are currently two common approaches for the use of insect material to estimate the time since death. The first method is based on the analysis of the pattern of colonisation by succeeding waves of arthropods on the carrion (Verma & Reject, 2013). The second is based on the development of mainly fly larvae following ovi- or larviposition (Verma & Reject, 2013). Both approaches are dependent on several factors

inclusive of, but not limited to, the location of the corpse (Verma & Reject, 2013), blow fly accessibility (Hall, n.d.), season and several climatic conditions (Verma & Reject, 2013; Mullany *et al.*, 2014).

Estimating the PMI from larval development is a measure of the larvae size, which is a correlative function of age (Hall, n.d.; Donovan *et al.*, 2006). Therefore, until the post-feeding stage is reached, an increase in size directly correlates with an increase in age (Hall, n.d.; Donovan *et al.*, 2006). Other factors may however affect the growth rate of larvae such as: changes in temperature (Ames & Turner, 2003; Donovan *et al.*, 2006), geographic location (Donovan *et al.*, 2006), the amount of carrion (Monthei, 2009), and more recently identified, the transmission of chemical substances from the carrion during food intake (Hall, n.d.; Williams, 2003; Verma & Reject, 2013; Zou *et al.*, 2013; Mullany *et al.*, 2014).

Fluctuating larval growth rates have far reaching consequences as they may lead to inaccurate measurement of the PMI. For example, an over-estimation of the PMI, frequently seen in favourable conditions such as longer sustained temperatures higher than 20°C, may occur (Ames & Turner, 2003; Donovan *et al.*, 2006). Similarly, temperatures below 20°C may cause under-estimations in the PMI (Donovan *et al.*, 2006). To limit this error, numerous studies have investigated the effect of various temperatures on several blow fly species (Grassberger & Reiter, 2001; Ames & Turner, 2003; Donovan *et al.*, 2006; Niederegger, Pastuschek & Mall, 2010; Verma, 2013). As a result, isomegalen diagrams with developmental data at different temperatures have been published as standardised data for some blow fly species (Grassberger & Reiter, 2001). According to Grassberger and

Reiter (as cited in Madea, 2014) isomegalen diagrams combine larval lengths as a function of time and mean ambient temperature (Ames & Turner, 2003). However, studies on the influence of chemical substance transmission during larval food intake have not resulted in standardised data.

Entomotoxicology is a branch of forensic entomology that applies toxicological analyses for the detection of chemical substances in carrion-feeding insects and investigates the effect of chemical substances on the development of arthropods (Introna, Campobasso & Goff, 2001) Entomotoxicology may be relied upon when conventional samples for toxicological analysis cannot be used due to the condition of the remains (Introna *et al.*, 2001). However, due to increases in both therapeutic and recreational drug-related incidents resulting in death, entomotoxicology has aroused increased interest in the discipline of forensic investigation (Introna *et al.*, 2001).

Research trends have been directed towards elucidating the effects of drugs of use and abuse on blow fly larval development. A study by George *et al*. (2009) showed that pure morphine had no effect on certain blow fly species. Furthermore, a study by O'Brien and Turner (2004) on the effects of paracetamol indicated only a slight acceleration in larval growth rate, although not statistically significant. Additionally, Rezende *et al.* (2014) suggested that the presence of MPH (Ritalin) and/or phenobarbital in human remains may result in an under-estimation of the PMI based on blow fly development. Few attempts have been made to determine the influence of human hormones on blow fly larval development. However, research by da Silva and Villet (2006) indicated that prophylactic progesterone does not affect blow fly larval development to an extent that would alter a PMI estimate.

Several studies have suggested more pronounced alterations in blow fly development, particularly growth rate, resulting from drug ingestion by blow fly larvae. A study by de Carvalho, Linhares and Badan Palhares (2012) investigated the effects of cocaine on blow fly larvae. The results indicated an expedited larval development by approximately 2 days. Research has also shown that ante-mortem intake of both cannabis and ethanol yields an increased blow fly larval growth rate (Verma, 2013). Furthermore, a study by Mullany *et al*. (2014) suggests that methamphetamine results in an accelerated larval development and thus estimates of the minimum PMI may be over-estimated by approximately 44 hours.

In South Africa several forensically significant blow flies of the Calliphoridae family are prevalent (Villet, 2000; Williams, 2003; Richards, Williams & Villet, 2009). Little research has been conducted on the effects of therapeutic and recreational drugs on blow fly development prevalent in the Western Cape Province. MPH constitutes the active ingredient for attention deficit hyperactivity disorder (ADHD) medication such as Ritalin and Concerta. ADHD is a psychiatric disorder that has been estimated to affect as much as 10% of the children in the South African population (Muthukrishna, in Lloyd, Stead & Cohen as cited in Perold, Louw & Kleynhans, 2010). The Department of Social Development: Western Cape Substance Abuse Unit (2011) indicated Ritalin as one of the most common prescription stimulants of potential use and abuse. Given that previous research has indicated an association between the occurrence of drugs in human remains and inaccurate PMI estimations, it is clear that a greater understanding regarding the influence of drugs on blow fly larval development is required in order to provide a more accurate estimation of the PMI. The importance of an accurate PMI cannot be overstated as it may lead to the inclusion or exclusion of perpetrators.

1.3. Methodology

1.3.1. *Study design*

The proposed study will follow an experimental design. Bait (pig offal donated by Winelands Pork) will be placed at the Medical Research Council (MRC) in Delft to lure Calliphoridae (blow flies) species and facilitate oviposition. Resulting eggs will be collected from the bait and transported to the entomology laboratory at the UCT Health Sciences Faculty, Division of Forensic Medicine and Toxicology, Falmouth Building. The eggs and subsequent larvae will be reared on a diet consisting of pig liver in a temperature, humidity and photoperiod controlled chamber. The larvae will be divided into control and test groups. Test groups will be reared on a pig liver diet spiked with MPH. Control groups will be maintained on a pig liver diet without the drug. Selected larvae from both groups will be sequentially removed from the food source at the same specific time intervals and killed (details under "Killing of blow fly larvae" section). Once all the selected larvae have been killed, they will be examined in terms of stage of development and length. Subsequently, larvae from each group will be compared as a means to determine the effect of drug exposure.

1.3.2. *Characteristics of the study population*

A single blow fly species, *Lucilia sericata*, will be used during the proposed study. The research will be conducted at the University of Cape Town, Faculty of Health Sciences.

1.3.3. *Spiking of the food substrate*

MPH, in the form of Ritalin, will be purchased and used to prepare a concentration that is similar to a typical recreational dose in the Western Cape. The MPH will be spiked into pig

livers and homogenised. This will be achieved by diluting Ritalin tablets (ground with mortar and pestle) in saline, to the appropriate concentration required, and adding the solution to pig liver (also donated by Winelands Pork). Following this, the mixture will be homogenised, using a blender, to ensure equal drug distribution throughout the substrate. This test substrate will be made in sufficient quantity to last the entire experiment and stored by freezing. It is estimated that this will be for approximately 1 month.

1.3.4. *Rearing of blow fly larvae*

Eggs collected from the bait at MRC will be placed on pig livers and in plastic containers. The containers will be maintained under insectary conditions, at approximately 30°C, ambient relative humidity and 14:10 hour light/dark cycles. Following hatching, the larvae will be equally divided into respective control and test groups and transferred to separate plastic containers. Each container will be appropriately labelled and contain pig liver as food source and vermiculite as moisture retentive medium at the bottom of the container. The containers will contain blended pig liver with or without MPH respectively. These containers will be maintained under the same insectary conditions as previously described.

1.3.5. *Sampling of larvae*

Larvae will be sampled every 6 hours in the first 24 hours after hatching, every 12 hours in the second day after hatching and every 24 hours after that until pupariation. Sampling will be performed by randomly removing ten (or as the population allows) larvae from the feeding substrate at respective sampling times and killing them.

1.3.6. *Killing of blow fly larvae*

Blow fly larvae collected at specific time frames from the test and control groups will be killed by placing the larvae in near boiling water for at least 30 seconds. The respective larvae will subsequently be preserved in 70% ethanol at 4°C.

1.3.7. *Data analysis and measurement*

From the time when the eggs hatch and the larvae are exposed to the respective food sources, measurements of selected larvae from each group will be taken at specific time intervals. The individual lengths of the larvae will be measured. The lengths of each batch of larvae will be averaged respectively for the specific time frame. Furthermore, the average pupation time range will be recorded for each group. Subsequently, the corresponding calculated averages of the test and control groups will be compared. The length measurements will be collected using a calliper. Furthermore, length measurements will be entered onto length/time graphs as a means to visually represent progression. Pupation duration will be recorded for each group from the start of pupation to adult emergence.

1.3.8. *Quantitation of MPH from sampled larvae*

Following the morphological examination and measurement, the sampled larvae of each sampling time will be used to quantitate the concentration of MPH ingested during feeding. Quantitation will be performed by liquid chromatography - mass spectrometry (LC-MS/MS).

All instruments to be used are validated, standardised and in routine use by the University of Cape Town. All experiments will be conducted in duplicate and collection of measurements will strictly adhere to a time schedule.

1.3.9. *Disposal of end-products*

Any remaining offal, treated/untreated pig liver mixtures and insect remains will be disposed of in accordance with the Division of Forensic Medicine and Toxicology waste management protocol i.e. animal waste (in this case offal, treated/untreated pig liver mixtures and insect remains) will be disposed of in green veterinary bags, sealed and delivered to the building opposite the Chris Barnard Building (Cape Heart Centre) at the back, the waste management building, during their operational hours (08:00-09:30 Mon, Wed, Fri). Animal waste collected outside of these operational hours will be stored in freezers in the laboratory until the next available time for drop-off.

1.4. *Statistical analysis*

A multiple means comparison will be performed using ANOVA or the Kruskal-Wallis test depending on the distribution of the data.

1.5. Work plan

Duration 10/10/2015 – 30/12/2015										
Weeks										
Task	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Proposal										
Lit Review										
Ethics										
Experiment										
Write-up										

1.6. Ethics

Larvae containers will be of adequate size to allow free movement. Furthermore, larvae will have constant access to a food source. Additionally, the cages will be kept in a secure temperature-controlled area with the appropriate light/dark cycles, and distant from distresses inclusive of loud noises and vibrations. Blow flies and blow fly larvae will be killed by ethyl acetate gassing and near boiling water respectively.

1.7. Benefits of the study

The data resulting from the study may assist in establishing reference material regarding the influence of MPH on blow fly larval development in the Western Cape. This may prove invaluable as a means to accurately estimate the PMI, and thus assist law enforcement efforts in the resolution of crime.

1.8. References

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1.9. <u>Addendum to proposal</u>

Therefore, the objective of this study is to determine the influence of MPH on the development rate…

As a result of technical shortcomings, adequate assessment of development rate could not be achieved, therefore this study also investigated the influence of MPH on the duration of larval and pupal development stages, in addition to the entomotoxicological feasibility of *C. chloropyga* larvae in the detection of MPH.

A single blow fly species, Lucilia sericata, will be used during the proposed study.

L. sericata blow flies failed to oviposit in several laboratory rearing attempts, therefore eggs were collected from placed bait. Blow fly eggs collected at Delft, and early larval instars could not be identified due to their homogeneous morphologies. Thus sampling populations contained a mixture of *Chrysomya chloropyga, Luciliinae* and *Chrysomya albiceps.* Once larvae reached the third instar, different species could be distinguished. *C. chloropyga* were the most consistently present among sampling populations and were thus selected as the subject of investigation.

Once all the larvae have been killed, they will be examined in terms of stage of development and length.

Weight measures were also collected as a second size parameter to investigate larval growth.

Methylphenidate hydrochloride, in the form of Ritalin, will be purchased and used to prepare a concentration that is similar to a typical recreational dose in the Western Cape.

Reliable recreational dosage information is not available for the Western Cape. Therefore 100 times the recommended therapeutic dose for adults was selected, based on the only available study on the effects of MPH on blow flies.

Following this, the mixture will by homogenised, using a blender…

A blender reduced the pig liver to a sub-optimal consistency. Therefore after initial blending, the liver was manually mixed for 5 minutes.

Following hatching, the larvae will be equally divided into respective control and test groups…

Collected egg masses were approximately equally divided between test and control groups.

Sampling will be performed by randomly removing ten (or as the population allows) larvae…

Five larvae were randomly sampled as the population sizes were too small to facilitate higher sampling numbers for the duration of the sampling period.

…test and control groups will be killed by placing the larvae in near boiling water.

Given the labile nature of MPH and recommendations by the literature, in addition to boiling, some larvae collected for toxicological analysis were killed and preserved by freezing at - 20°C.

…the sampled larvae of each sampling time will be used to quantitate the concentration of MPH…

Due to instrument limitations only selected sampled larvae could be analysed for MPH.

Quantitation will be performed by liquid chromatography - mass spectrometry (LC-MS/MS).

Time constraints did not allow the acquisition of a MPH standard in time for a quantitative analysis. Therefore a qualitative approach was followed as the aim of this project was not reliant on quantitation.

All experiments will be conducted in duplicate

The influence of confounding factors introduced variability between the experimental groups and could therefore not be constituted as duplicates. Furthermore, time and logistical constraints did not allow the experiment to be replicated.

A multiple means comparison will be performed using ANOVA or the Kruskal-Wallis test…

The data resulting from the experiments did not fulfill the requirements of using a multiple means comparison (≥ 3 groups). Therefore a Mann-Whitney U test was used.

Blow flies and blow fly larvae will be killed by ethyl acetate gassing and near-boiling water respectively.

No blow flies were killed during this project.

Chapter 2: Literature Review

2.1. <u>Background</u>

Forensic investigators are frequently tasked with the responsibility to estimate the post-mortem interval (PMI), or time since death, from human remains in an advanced stage of decomposition. Medical manifestations of death are of limited use beyond 72 hours after death, in terms of PMI estimations (Amendt *et al.,* 2011). Therefore, forensic entomology may be employed as PMI estimates can be derived from entomological evidence before, and well beyond 72 hours after death (Amendt *et al.*, 2011). A forensic entomologist may utilise the developmental data and/or pattern of successional communities of several carrion-feeding arthropods, to assist in the PMI estimation of the decedent (Monthei, 2009; Higley & Haskell, 2010; Wells & Lamotte, 2010). Calliphoridae (blow flies) in the order Diptera are amongst the insects of greatest forensic importance (Amendt *et al.*, 2007) and have been extensively researched (Byrd & Castner, 2010). Their forensic significance in estimating the PMI may, in part, be attributed to their ability to rapidly locate and colonise a body after death (De Jong, 1994; Amendt *et al.*, 2011). Greenberg (as cited in Monthei, 2009) has suggested that development data of blow flies provide the most accurate means of estimating the minimum PMI when using entomofauna. This may likely be attributed to blow flies being recognised as the first wave of faunal succession (Grassberger & Reiter, 2001; Amendt *et al.,* 2007). Therefore, this review will primarily focus on the use of Calliphoridae species in forensic entomology and entomotoxicology as forensic indicators of the time since death.

There are currently two common approaches for the use of insect material to estimate the time since death. The first method is based on the analysis of the pattern of colonisation by succeeding waves of arthropods on the carrion (Verma & Reject, 2013). The second is

based on the development of mainly fly larvae following ovi- or larviposition (Verma & Reject, 2013). Either approach may be affected by several factors inclusive of, but not limited to, the species type, temperature (Hall & Brandt, 2006; Mullany *et al.*, 2014), season (Hall & Brandt, 2006; Verma & Reject, 2013), presence of xenobiotics in or on carrion (Hall & Brandt, 2006; Verma & Reject, 2013), maggot mass formation (Mullany *et al.*, 2014) and the geographic region (Hall & Brandt, 2006; Wells & Lamotte, 2010).

Estimating the PMI from larval development is frequently a measure of larvae size, which is a correlative function of age (Baqué *et al.*, 2015b) and modulated by time and temperature (Donovan *et al*., 2006; Sharma, Garg & Gaur, 2015). Therefore, until the post-feeding stage is reached where size measures are no longer useful criterions of age (Grassberger & Reiter, 2001; Baqué *et al*., 2015a), larval size positively correlates with age (Donovan *et al.*, 2006; Baqué *et al.*, 2015b). Several size parameters may be used as measures of larval age, including length, width and weight. Larval length however, is the preferred measure (Richards, Rowlinson & Hall, 2013).

Size parameters may be affected by alterations in larval growth rate, caused by external factors related to those previously mentioned, including changes in temperature (Ames & Turner, 2003; Donovan *et al.,* 2006), food type (Clark, Evans & Wall, 2006), the amount of carrion (Monthei, 2009) and competing larvae (Clark *et al*., 2006), and more recently identified, the transmission of chemical substances from the carrion to larvae during feeding (Williams, 2003; Hall & Brandt, 2006; Verma & Reject, 2013; Zou *et al.*, 2013; Mullany *et al.*, 2014).

Fluctuating larval growth rates may have far reaching consequences as it may detract from the reliability of a PMI estimate. Temperature is the foremost external factor affecting larval development, given the poikilothermic nature of blow flies (Ames & Turner, 2003; Byrd *et al.*, 2010). Larval development rate is positively correlated with temperature within the minimum and maximum temperature thresholds of development (Hall & Brandt, 2006; Byrd *et al.,* 2010). Therefore, PMI estimates based on larval development may be erroneous if temperature is not accounted for. For example, Dononvan *et al.* (2006) investigated the growth rate of *Calliphora vicina* larvae at temperatures ranging between 4°C and 30°C. The authors reported an under-estimation of larval growth at temperatures above 20°C, thereby alluding to the potential for PMI over-estimations. Conversely the authors reported an over-estimation of larval growth at temperatures below 20°C, thereby alluding to the potential for PMI under-estimations.

To limit this error, numerous studies have investigated the effect of various temperatures on several blow fly species (Grassberger & Reiter, 2001; Ames & Turner, 2003; Donovan *et al.*, 2006; Niederegger, Pastuschek & Mall, 2010; Verma, 2013). As a result, developmental models such as isomegalen and isomorphen diagrams, which contain developmental data at different temperatures, have been published as standardised data for certain blow fly species (Grassberger & Reiter, 2001; Grassberger, Friedrich & Reiter, 2003). Isomegalen diagrams combine larval lengths from hatching to peak-feeding as a function of time and temperature to calculate the PMI (Grassberger & Reiter, 2001; Madea, 2014). Similarly isomorphen diagrams combine the developmental stages of larvae from oviposition to eclosion (emergence of adult fly) (Gennard, 2007) as a function of time and temperature (Grassberger & Reiter, 2001; Gennard, 2007; Madea, 2014). Another method of estimating

the PMI from insect development is the thermal summation model, commonly referred to as the accumulated degree hour/day (ADH/ADD) model (Amendt *et al*., 2011). ADH/ADD values may be regarded as the summation of thermal energy units expended by a specific insect species over time and temperature to reach a certain developmental stage (Gennard, 2007; Madea, 2014). This linear regression model assumes a proportional relationship between insect development rate and temperature between a species-specific minimum and maximum threshold (Gennard, 2007; Amendt *et al.*, 2011; Madea, 2014). Furthermore, the model is based on the assumption that below the minimum threshold temperature, development ceases (Amendt *et al*., 2011). These insect development models account for the influence of temperature and allow a minimum PMI to be extrapolated. However, developmental models accounting for the influence of chemical substance transmission during larval food intake have not been devised.

Entomotoxicology is a branch of forensic entomology that applies toxicological analyses for the detection of chemical substances in carrion-feeding insects and investigates the effect of such chemical substances on the development of arthropods in order to assist PMI estimates (Introna, Campobasso & Goff, 2001). Entomotoxicology may be relied upon when conventional samples for toxicological analysis cannot be used due to the condition of the remains, such as skeletonisation or advanced decomposition (Introna *et al.*, 2001). However, due to increases in drug-related incidents resulting in death, entomotoxicology has stimulated interest in forensic investigations (Introna *et al*., 2001; International Overdose Awareness Day, 2015; United Nations Office on Drugs and Crime, 2015).

Research trends have been directed towards elucidating the effects of drugs of use and abuse on blow fly development. A study by George *et al.* (2009) showed that pure morphine had no effect on the growth rate of *Calliphora stygia* at the concentrations investigated. Furthermore, a study by O'Brien and Turner (2004) on the effects of paracetamol indicated only a slight acceleration in the larval growth rate of *C. vicina* which may over-estimate the PMI by approximately 12 hours. Moreover, Rezende *et al.* (2014) suggested that the presence of methylphenidate hydrochloride (Ritalin) and/or phenobarbital in human remains may result in an under-estimation of the PMI, based on observations of the development of *Chrysomya albiceps, Chrysomya putoria* and *Chrysomya megacephala*. Few attempts have been made to determine the influence of human hormones on blow fly development. However, research by da Silva and Villet (2006) on *Chrysomya chloropyga* indicated that prophylactic progesterone does not affect larval development to an extent that would alter a PMI estimate.

Several studies have suggested more pronounced alterations in blow fly development, particularly growth rate, resulting from drug ingestion by blow fly larvae. A study by de Carvalho, Linhares and Badan Palhares (2012) investigated the effects of cocaine on the development rate of *C. albiceps* and *C. putoria*. The results indicated an expedited larval and pupal development thereby reducing the total development time by more than 2 days. Research on *Chrysomya rufifacies* has also suggested that ante-mortem intake of both cannabis and ethanol by the decedent may increase the blow fly larval growth rate (Verma, 2013). Furthermore, a study by Mullany *et al.* (2014) on the development of *C. stygia* suggests that methamphetamine results in an accelerated larval development and thus, estimates of the minimum PMI may be over-estimated by up to 44 hours. Conversely, the

same treatment prolonged pupal development by up to 34 hours, thereby offsetting PMI estimations based on this stage by as much as 78 hours.

South Africa is host to several forensically significant blow flies (Williams, 2003). Little research has been conducted on the effects of therapeutic and recreational drugs on the development of blow flies prevalent in the Western Cape. Methylphenidate hydrochloride (MPH), commonly used to treat attention deficit hyperactivity disorder (ADHD) (Robison *et al*., 1999; Pelham *et al.*, 2001), is available as a prescription drug in several formulations and is commonly recognised by its commercial names such as Ritalin and Concerta (Pelham *et al.*, 2001). ADHD is a psychiatric disorder that has been estimated to affect as much as 10% of the children in the South African population (Muthukrishna, in Lloyd, Stead & Cohen as cited in Perold, Louw & Kleynhans, 2010). The Department of Social Development: Western Cape Substance Abuse Unit (2011) indicated Ritalin as one of the most common prescription stimulants of potential use and abuse. Given that previous research has indicated an association between the occurrence of drugs in human remains and erroneous PMI estimations, it is clear that a greater understanding regarding the influence of drugs on blow fly larval development is required in order to provide a reliable estimation of the PMI. The importance of a reliable PMI in forensic investigations cannot be overstated as it may lead to the inclusion or exclusion of suspects and the identification of the decedent (Buchan & Anderson, 2001; Wells & Lamotte, 2010).

2.2. <u>Literature review</u>

2.2.1. Historic foundation

This brief historic account of the progression of forensic entomology is described by Benecke (2001). Since the 19th century modern forensic entomology has evolved from what seemed to be a scientific curiosity, to a recognised scientific discipline. The obvious, but not yet understood relationship between insects and corpses has been observed for centuries, with one of the earliest accounts being that of death investigator and lawyer Sung Tźu in the 13th century book "*Hsi yüan chi lu*" translated as "*The Washing Away of Wrongs*". In this book he describes a murder that had occurred near a rice field. The day following the murder, the farm workers in the area were assembled and instructed to lay down their sickles in front of them. Blow flies were attracted to a single sickle, possibly due to residual blood traces. When confronted, the owner of the sickle confessed to the murder.

One of the first modern cases to employ forensic entomology to establish a PMI was reported by the French physician Bergeret d'Arbois (Haskell, 2006) in 1855. The case involved the discovery of the mummified remains of a child in a home near Paris (Haskell, 2006) undergoing renovation (Gennard, 2007), in 1850. Through crude, yet conceptually correct assumptions regarding insect development and successional patterns of colonisation, Bergeret determined that the murder had to have occurred around 1848. In light of this, Bergeret contested the involvement of the current occupants and suspicion fell to the previous tenants that had vacated the flat in 1848 (Haskell, 2006). Bergeret's conclusion was based on the assumption that insect metamorphosis requires a full year for completion. Both flesh fly and moth pupae were recovered from the remains. Therefore,

given that two generations of insects were present, resulting from flesh fly colonisation presumably when the corpse was still fresh, and moth colonisation presumed to have occurred after the corpse had dried, two years were suggested to have passed since death.

In the late 19th century, army veterinarian Jean Pierre Mégnin recognised that necrophagous arthropods visits a corpse in predictable waves and in 1894 he published the book "*La Faune des Cadavres: Application de l'entomologie a la médicine légale*" or as it is commonly referred to "*The Fauna of Cadavers*" (Haskell, 2006). He described eight successional waves of insects for exposed corpses and two waves for buried corpses, which served as a milestone for modern forensic entomology (Benecke, 2001; Amendt, Krettek & Zehner, 2004; Haskell , 2006).

2.2.2. Post-mortem interval (PMI)

The fundamental principle of estimating the PMI is the determination of a quantifiable period along a time-dependant curve to suggest the time since death (Henssge & Madea, 2007). Estimating the PMI plays an important role during death investigations. The PMI can provide valuable information that may lead to the apprehension of the perpetrator, or the identification of the decedent by comparing the PMI to the time when individuals were reported missing or last seen (Buchan & Anderson, 2001; Wells & Lamotte, 2010). PMI estimations are performed by forensic pathologists or other medico-legal investigators based on post-mortem changes in a corpse (Campobasso, Di Vella & Introna, 2001; Henssge & Madea, 2004; Saukko & Knight, 2004; Amendt *et al.*, 2007; Goff, 2009). Typical early post-mortem changes that may guide PMI estimations include, but is not limited to, algor-, livor-, and rigor-mortis (Amendt *et al*., 2004; Saukko & Knight, 2004; Amendt *et al*.,

2007; Goff, 2009). These stages of post-mortem changes are characterised by a gradual decrease in body temperature, gravitational blood pooling, and muscle stiffening respectively (Amendt *et al.*, 2004; Saukko & Knight, 2004; Amendt *et al*., 2007; Goff, 2009). Studies regarding the pathophysiology of these and other post-mortem changes have been directed toward improving PMI estimations. However, the variability and numerous influencing factors on these post-mortem indicators have, for the most part, only led to the development of rough approximations of the PMI, and requires further exploration (Buchan & Anderson, 2001; Campobasso *et al*., 2001; Amendt *et al.*, 2004; Saukko & Knight, 2004; Amendt *et al*., 2007; Donaldson & Lamont, 2013). As the time since death increases and later post-mortem stages are reached, estimating the PMI becomes more difficult using traditional post-mortem indicators (Buchan & Anderson, 2001; Amendt *et al.*, 2004; Wells & Lamotte, 2010; Sharma *et al*., 2015).

2.2.3. Forensic entomology

Reliable estimations of the PMI may be derived from ecological information when physical and biochemical indicators of the PMI become less useful, as the time since death increases (Amendt *et al.*, 2004; Amendt *et al*., 2007; Wells & Lamotte, 2010). This presents a basis for the use of complementary techniques when estimating the PMI. In this regard forensic entomology has broadened the forensic arsenal. Forensic entomology has been considered an accurate means of estimating the PMI, and is particularly useful when the time since death exceeds 72 hours (Buchan & Anderson, 2001; Sharma *et al*., 2015). Forensic entomology may also be useful in extrapolating information regarding the

circumstances surrounding the time of death, the peri-mortem interval (Ubero-Pascal *et al*., 2010).

It has long been observed that insects are associated with corpses and display time-dependent processes that can be related to the PMI (Wells & Lamotte, 2010). Among these processes are the development of necrophagous insects and their larval offspring, and the occurrence of successional waves of necrophagous insects (Wells & Lamotte, 2010). The underlying assumption for the use of insect development to estimate the PMI is that developmental data of immature insects can be used to determine insect age, which can be related to the time of colonisation, from which the PMI can be inferred (Amendt *et al.*, 2011). However, it should be noted that estimations derived from immature necrophagous insects represent the minimum PMI as the timing between death and ovi-or larviposition is variable (Wells & Lamotte, 2010).

The pattern of necrophagous insect succession is another time-dependent process used to estimate the PMI (Anderson, 2010; Wells & Lamotte, 2010). However, unlike the use of development data, analysis of the pattern of colonisation by succeeding waves of necrophagous insects provides an indication of both the mini- and maximum PMI (Wells & Lamotte, 2010; Sharma *et al.*, 2015). This is possible because each successional wave inherently provides information about the time elapsed between death and the arrival of a specific insect species, in addition to allowing extrapolation of the age of the immature insects (Wells & Lamotte, 2010). The value of insect succession in PMI estimations is underpinned by the predictable occurrence of succeeding waves (Anderson, 2010; Sharma

et al., 2015), specific to the geographic region (Buchan & Anderson, 2001; Anderson, 2010; Sharma *et al.*, 2015).

It has been reported that among insects, Diptera (flies) and Coleoptera (beetles) are of forensic interest (Lord & Rodriguez, 1989; Campobasso *et al.*, 2001) with Diptera being of the greatest forensic importance given their high levels of activity and frequent occurrence on corpses (Campobasso *et al.*, 2001). Diptera and Coleoptera comprise approximately 60% of the total necrophagous arthropods (de Souza & Linhares, 1997). Dipteran species are usually the earliest colonisers of human remains and colonisation can occur as early as minutes to 2-3 hours after death (Lord & Rodriguez, 1989; Campobasso *et al.*, 2001; Amendt *et al*., 2011). Among Diptera, the families Calliphoridae, Sarcophagidae and Muscidae are considered the most important decomposers (de Souza & Linhares, 1997) and are therefore the most relevant to forensic practice (Ubero-Pascal *et al.*, 2010).

Calliphoridae have been recognised amongst the first wave of faunal succession on human remains (Grassberger *et al*., 2003; Amendt *et al.*, 2004; Amendt *et al*., 2007; Amendt *et al*., 2011). Therefore they constitute the most accurate forensic indicators of the PMI when using entomofuana (Grassberger *et al.*, 2003; Amendt *et al*., 2007). This family of flies is easily recognised by their metallic green, blue, bronze or black appearance and occurrence in the vicinity of organic waste (Byrd & Castner, 2010). The family contains more than a thousand species and has a worldwide distribution (Byrd & Castner, 2010). In addition to their rapid colonisation of a corpse, it is conceivable that the importance of blow flies in forensic practice is derived from their predictable larval development at known

environmental conditions (Greenberg as cited in De Jong, 1994; Clark *et al.*, 2006; Cooper & Cooper, 2013; James, Nordby & Bell, 2014; Sharma *et al.*, 2015).

Blow flies are able to detect both human and animal remains through semiochemical stimulation of their olfactory receptors (Wall & Fisher, 2001; Byrd & Castner, 2010). This is followed by a visual search of the area to locate the exact position of the remains (Wall & Fisher, 2001; Byrd & Castner, 2010). Upon arrival at a corpse, blow flies will either immediately engage in oviposition in natural orifices (eyes, ears, nose and mouth) or wounds (Greenberg & Kunich, 2002; Amendt *et al.*, 2004), or first feed on protein-rich fluids purged from the decomposing corpse (Lord & Rodriguez, 1989). Blow fly eggs typically hatch within 1 – 3 days after oviposition and develop into larvae (Lord & Rodriguez, 1989). Blow fly larvae are typically soft-bodied, white to cream-coloured, legless (Byrd & Castner, 2010) and tapered anteriorly (Lord & Rodriguez, 1989). Larvae also contain spinose bands (girdles) around the body on most segments (Robinson, 2005). Anteriorly, larvae have spiracles (Robinson, 2005) and a cephalopharyngeal structure (mouthpiece) with a pair of mouth hooks (Wallman, 2002), for feeding and locomotion (Lord & Rodriguez, 1989). Posteriorly, larvae possess a pair of flattened spiracles for breathing (Lord & Rodriguez, 1989; Byrd & Castner, 2010), along with cone-shaped tubercles surrounding the terminal posterior segment (Robinson, 2005; Byrd & Castner, 2010). Variations of these morphological features contribute toward blow fly identification (Szpila, n.d.; Prins, 1982; Wallman, 2002; Byrd & Castner, 2010). Larvae will pass through three developmental stages (instars), continuously feeding until the third stage (Lord & Rodriguez, 1989; Greenberg & Kunich, 2002; James *et al.*, 2014). During the third development stage, after reaching a programmed size (Greenberg & Kunich, 2002), the larvae will stop feeding and

enter a “wandering stage” or “post-feeding stage” during which they will move away from the corpse/food source to find a suitable area for pupariation and subsequent pupation (Lord & Rodriguez, 1989; Greenberg & Kunich, 2002; James *et al.*, 2014). Blow fly puparia are typically reddish-brown to black in colour and football-shaped (Lord & Rodriguez, 1989; Greenberg & Kunich, 2002). Blow fly pupae undergo a complete metamorphosis (James *et al.*, 2014) and following pupation, an adult blow fly ecloses from the puparium thereby completing the life cycle (Lord & Rodriguez, 1989; James *et al.*, 2014).

2.2.4. *Chrysomya chloropyga*

C. chloropyga, or the green-tailed blow fly, is very common to the southern parts of Africa (Zumpt, 1965). As the name suggests *C. chloropyga* can be readily recognised in the adult stage as a metallic green-blue fly with the last two segments of the abdomen having a green-yellow colour (Appendix H) (Zumpt, 1965; Prins, 1982). A more definitive characteristic is the ┘└ shaped vittae on the presutural region of the mesonotum (Appendix H) (Zumpt, 1965; Prins, 1982). *C. chloropyga* are heliophilic, necrophagous blow flies (Zumpt, 1965) with a predominantly spring to early summer distribution in South Africa (Williams, 2003). The ubiquitous distribution of *C. chloropyga* throughout South Africa (Williams, 2003; Picker, Griffiths & Weaving, 2004) and its association with carrion (Zumpt, 1965) makes this species forensically important. Among this species a sexual size dimorphism exists with the females being larger than males (Williams, 2003). *C. chloropyga* females oviposit, on average, 450 white-cream coloured eggs which hatch within 12 hours - 3 days (Zumpt, 1965). These hatchlings ultimately develop into mature third instar larvae that can be identified by morphological features such as, an incomplete

peritreme around the posterior spiracles (Appendix H), obviously complete spinose girdles on segments 2-7 and sometimes 8 (Appendix H), anterior spiracles with 10-12 branches (Appendix H), and the absence of an accessory oral sclerite (Prins, 1982). Identification of blow flies based on first and second instar larvae has traditionally been challenging due to their small size and morphological similarity (Szpila & Villet, 2011; Samarakoon *et al*., 2012), however continuing research of both morphological (Brink, 2009; Szpila & Villet, 2011) and molecular indicators (Rajagopal *et al*., 2012) of a species has assisted in remedying this.

2.2.5. Entomotoxicology

The development of various blow fly species has been extensively researched at different environmental conditions, particularly temperature, to serve as reference data. For example Grassberger and Reiter (2001) investigated the development of *Lucilia sericata* at ten different temperatures and constructed an isomegalen and isomorphen diagram. Similarly, Richards, Crous and Villet (2009) established developmental curves for *C. chloropyga* and *C. putoria* at eight and ten different constant temperatures respectively, in addition to constructing isomegalen and isomorphen diagrams. Furthermore the authors calculated the thermal summation constants and developmental threshold for five developmental events in both species. Byrd and Butler (1997) established growth curves for *C. rufifacies* at a constant and four mean cyclic temperatures and emphasised the importance of *C. rufifacies* as a forensic indicator of the PMI due to their predictable development time and minimal larval length and cohort variation.

In addition to understanding the influence that variable environmental conditions has on the development of entomological forensic indicators, chemical transmission from carrion to arthropod demands consideration as an external factor influencing arthropod development. Ever since Beyer, Enos and Stajić (1980) published the use of blow fly larvae as alternative toxicological samples, interests were stimulated regarding chemical transmission from carrion to arthropods and the associated effects thereof. Subsequently, researchers began testing various substances on insects (mainly Calliphoridae and Sarcophagidae) with the purpose of identifying their use as alternative samples for toxicological analysis or elucidating its effects on larval and pupal development. These substances have primarily included commonly prescribed pharmaceutical and illicit drugs. According to the United Nations Office on Drugs and Crime (UNODC) (2015), 1 out of 20 people (~ 5.2%) globally, between the ages of 15-64 years used an illicit drug in 2013. Substances such as morphine (Bourel *et al.*, 1999; Bourel *et al.*, 2001; George *et al.*, 2009), cocaine (de Carvalho *et al.*, 2012), methamphetamine (Goff, Brown & Omori, 1992; Magni *et al.*, 2014; Mullany *et al.*, 2014), ketamine (Zou *et al.*, 2013; Lü *et al.*, 2014), heroin (Goff *et al.*, 1991), methylphenidate hydrochloride and phenobarbitol (Rezende *et al.*, 2014), 3,4-methylenedioxymethamphetamine (Goff *et al.*, 1997), amitriptyline (Goff *et al.*, 1993), methadone (Gosselin *et al.*, 2011a) and paracetamol (O'Brien & Turner, 2004) have been investigated presumably because of their prevalence or incidence in the respective populations. The advent of toxicological analyses from entomological samples led to the formation of a new branch of forensic science termed forensic entomotoxicology that is concerned with the detection of toxicological substances and determining their associated

effects on forensic entomological fauna development, and possible alterations in PMI estimates (Introna *et al.*, 2001).

Goff *et al.* (1991) investigated the influence of heroin in carrion on the development rate of *Boettcherisca peregrina* (Sarcophagidae) larvae and the subsequent implications thereof on PMI estimates based on insect development. Domestic rabbits were respectively treated with one of four heroin doses, 6 mg, 12 mg, 18 mg and 24 mg prepared in a saline solution and administered by cardiac puncture. The rabbits were euthanised 20 minutes after dose administration after which the livers were harvested and exposed to stock colonies of *B. peregrina* for larviposition. Heroin administered intravenously in humans reaches peak plasma concentrations between 1-5 minutes, after which it is rapidly metabolised to 6-monoacetyl morphine and subsequently morphine which has a half-life of approximately 20 minutes (Iversen *et al.*, 2009). However, heroin metabolism in humans and animals may not be comparable and therefore these findings must be interpreted with caution. Subsequent analysis of development indicated an accelerated larval development and conversely, a decelerated pupal development. The results suggest that the presence of heroin in carrion, primarily as morphine, may lead to a PMI over-estimation of up to 29 hours when based on larval development, and a PMI under-estimation of between 18 – 38 hours when based on pupal development.

Bourel *et al.* (1999) investigated the influence of morphine on the development rate of *L. sericata.* Similar to Goff *et al.* (1991) domestic rabbits were used and treated with either 12.5 mg, 25 mg or 50 mg of morphine hydrochloride/hour for three hours, prepared in a saline solution and administered through ear artery perfusion. These doses were

administered to simulate fatal morphine overdoses. The rabbits were subsequently euthanised and *L. sericata* eggs were placed in the eyes, nostrils and mouth of each rabbit. This is in contrast to many other studies, such as those by Goff *et al.* (1991) during which larval test colonies were reared on excised liver from treated animals. Placing test colonies in the natural orifices of a carcass, rather than on excised treated tissues, may provide a more reliable representation of the conditions at a death scene (Gosselin *et al*., 2011b). This relates to the study by Clark *et al.* (2006) that suggests that the type of tissue on which larvae feed can alter their development. Moreover, this relates to the variable distribution of drugs or toxins causing tissue specific accumulation (Gad, 2008). Therefore, it may be argued that exclusively rearing larvae on drug-containing substrates may incorporate an inherent bias as the drug would only be intermittently present in decomposing remains depending on the tissue type and location on the body. In contrast to the findings by Goff *et al.* (1991) the authors determined that the presence of morphine in tissues actively fed on by *L. sericata* larvae resulted in a dose dependant decelerated larval development rate to an extent that could cause PMI under-estimations of up to 24 hours.

Another study by Goff *et al.* (1993) investigated the influence of amitriptyline on the development of *Parasarcophaga ruficornis* (Sarcophagidae). Domestic rabbits were respectively treated with amitriptyline doses, 300 mg, 600 mg and 1000 mg prepared in a saline solution and administered by ear vein perfusion. These doses represented the sub-lethal, median lethal and two times median lethal dosages as calculated by body weight. Following treatment, the liver of each rabbit was harvested and exposed to *P. ruficornis* larvae. Subsequent analysis of development suggested a prolonged larval and pupal development. From the results it may be concluded that such prolonged development could

result in a PMI under-estimation of up to 30 hours when based on total larval development, and up to 47 hours when based on total pupal development. Therefore a total deviation in the PMI estimation of up to 77 hours was reported. In all of the abovementioned studies the error in PMI estimations is potentially 24 hours or more. This emphasises the importance of considering the influence of drugs and toxins when estimating the PMI.

Introna, Campobasso and Di Fazio (1998) describe three forensic cases in which entomological evidence was used to estimate the PMI. The first case involved the discovery of a 20-year-old man in a moderately advanced stage of decomposition. Consequently livor-, rigor-, and algor-mortis were no longer useful as a reliable means of estimating the PMI. Third instar larvae of *C. albiceps* and *Sarcophaga carnaria* were recovered from the remains and reared to adulthood for confirmative identification. Laboratory rearing of both species from hatching to third instar resulted in a PMI estimation of 5-8 days. The victim was last seen eight days before the body was discovered. The second case involved the discovery of the charred remains of a 25-year-old male. Second and third instar *Protophormia terraenovae* and *Sarcophaga haemorrhoidalis* larvae respectively, were recovered and reared to adulthood for confirmative identification. The PMI was estimated to be 3-4 days based on the time required by the two dipteran taxa to reach the second and third instar respectively, during laboratory rearing. The victim was last seen four days prior to discovery of the body. Similar to the previous case, the third case involved the discovery of charred remains of a man and woman both 25 years of age. The only entomological fauna present on the remains were second instar *C. vicina* larvae which were recovered during the post-mortem examination and reared to adulthood for confirmative identification. Laboratory rearing of *C. vicina* to the second instar suggested a

PMI estimate of 36-48 hours. The PMI was later confirmed when a suspect confessed to the murders. In each of these cases, conclusions regarding the PMI were based, in part or wholly, on entomological evidence through the reconstruction of life cycles under environmental conditions similar to that of the death scene. Although anamnestic witness testimonials are of questionable reliability, the apparent overlap between the calculated PMI and times when victims were reportedly last seen corresponded well between the independent cases. These cases indicate the utility of forensic entomology in the absence of, or in addition to other post-mortem indicators of the PMI. However, in the absence of concurrent toxicological and subsequent developmental studies, the influence of potentially occurring drugs or toxins on the development of entomological fauna is undetermined and therefore contributes to the uncertainty of a PMI estimate.

Larval size measures become of particular importance when PMI estimates are derived from life cycle reconstructions, during which blow fly species are experimentally reared to the stage of development observed at the crime scene (Introna *et al*., 1989; Introna *et al*., 1998; Sharma *et al.*, 2015). Furthermore, larval size measures or the stage of development are incorporated into the developmental models, isomorphen, isomegalen (Introna *et al.*, 1989) and thermal summation models, as a means to estimate the minimum PMI (Amendt *et al.*, 2011). Thus there are two aspects of larval development utilised in PMI estimates, the first being the developmental event (e.g. first, second or third instar) and the second pertains to size measures (Amendt *et al.*, 2011). The limitations of using larval developmental data in PMI estimates have been apparent for some time, with particular reference to fluctuating environmental conditions causing altered development (Introna *et al.*, 1989). Environmental influences such as temperature, humidity and photoperiod can

largely be accounted for through comprehensive data collection of these external factors at a death scene and subsequent comparison with developmental models or incorporation to life cycle reconstructions (Introna *et al.*, 1989). However, comprehending and accounting for the influence of chemical substances on blow fly larval development is limited. Despite the progression of ongoing research, the influence of drugs on the development of blow flies has been determined in relatively few species and geographic regions worldwide. Consequently this has led to the use of reference data that are not specific for the local species or geographic region. Several authors have alluded to Calliphoridae development being dependent on the geographic region and may thus vary even among members of the same species (Grassberger & Reiter, 2001; Donovan *et al.*, 2006; VanLaerhoven, 2008; Niederegger *et al.*, 2010; Baqué *et al*., 2015b). Therefore the generation of reference data specific to the region and local species is required.

2.2.6. Methodological inconsistencies

Despite the use of reference data not specific to a species and region, other variables that may draw scrutiny to forensic entomotoxicology include methodological inconsistencies. The primary implication of such inconsistencies is the generation of data that is not comparable between studies. A common inconsistency is the method of substance administration, as some studies prefer the use of animal models and others, treated substrates. Further still, the choice of rearing substrate also differs, with some preferring a natural diet (e.g. porcine liver) and others, artificial substrates. For example, George *et al.* (2009) investigated the influence of morphine on the development rate of *C. stygia.* The authors reared *C. stygia* larvae on pet mince spiked with different concentrations of

morphine. Following analysis, the authors concluded that morphine did not have any significant influence on the development rate of *C. stygia*. Similarly, Bourel *et al.* (1999) investigated the influence of morphine on the development rate of *L. sericata.* The authors treated domestic rabbits with different concentrations of morphine via ear artery perfusion. Following treatment, the rabbits were euthanised and used as rearing substrate for *L. sericata* larvae*.* After analysis, the authors concluded that the presence of morphine can cause a PMI under-estimation of up to 24 hours. The contradictory findings between these authors introduce a potential ambiguous interpretation regarding the effect of morphine on blow fly development. This may be a consequence of several factors, including different rearing substrates used (Clark *et al*., 2006), species-specific responses (Gosselin *et al.,* 2011b), animal metabolism of morphine (George *et al.*, 2009), or concentrations used. Thus the possibility exists that in such studies the observed effect may be erroneously attributed to the substance administered rather than the numerous influencing variables that should be controlled to achieve a level of consistency between studies in order to ensure comparability.

This has led to discussions regarding the validity of entomotoxicological data in the context of altered insect development. For example, data resulting from the use of animal models in entomotoxicological developmental studies may not be comparable to that obtained through non-living systems, as possible inherent drug/toxin metabolic mechanisms may alter substance availability (Oliveira *et al*., 2009; Gosselin *et al.*, 2011b) and produce secondary metabolites (George *et al.*, 2009) that may alter larval development. Other inconsistencies arise from variable rearing conditions such as temperature, humidity and photoperiod (Table A). Although these conditions require some variation between species

to accommodate species-specific environmental preferences, rearing conditions need to match that of their natural environment in the case of reference data, or the location of a corpse in the case of forensic investigations. This is to facilitate the generation of data that accurately portray blow fly developmental patterns during specific conditions. Non-conformance of rearing conditions with environmental conditions can lead to the generation of erroneous reference data or PMI estimates. In spite of this, many studies prefer optimal to actual environmental rearing conditions to facilitate expedited development and fecundity. Resultantly, not all generated reference data of blow fly development are accurate representations of that of the blow fly populations in the region. Lamentably, the methodological inconsistencies between authors complicate efforts to attribute the observed effects to a specific root cause, such as chemical substances. It should however, be noted that the control of certain variables is subject to the aim of the investigation. For example, if the aim of a study is to detect a drug from larvae, the control of variables influencing development may be less important, whereas in a study aimed at determining the influence of a drug on development, variables with the potential to affect development must be controlled.

Table A. Synthesis of methodological aspects and findings between studies

Author(s)	Dipteran	Drug	Dose	Rearing Temp, Humidity & Photoperiod	Drug Administration and Rearing Substrate	Possible PMI Modification
Fathy *et al.* (2008)	*C. albiceps*	Codeine	28 mg/Kg	42-45°C, Ambient, Natural light	Domestic rabbits (ear vein infusion)	Larval stage – up to 24h over-estimation Pupal stage – up to 48h over-estimation
Kharbouche *et al.* (2008)	*L. sericata*	Codeine	0.05, 0.25, 2, 30 mg/Kg	20°C, - , 15:9 LD	Pig liver homogenate	Larval stage – up to 29h over-estimation Total development (egg – imago) – up to 21h over-estimation
Monthei, (2009)	*Phormia regina*	Ethanol	BAC – 0.01, 0.04, 0.08% w/v	23°C, - , 24:0 LD	Ground pork loin	None
Tabor *et al.* (2005)	*P. regina*	Ethanol	BAC > 0.08%	20.2°C, >80%, Natural light	Pigs (excised loin) (oral & ear vein infusion)	Yes. Larval stage – up to 11.9h under-estimation
Lü *et al.* (2014)	*C. megacephala*	Ketamine	25, 50, 100 ug/g	24°C, 28°C, 32°C, 75%±10, 12:12 LD	Pig meat (Agar supplemented)	Yes. Larval stage - Under-estimation
Zou *et al.* (2013)	*L. sericata*	Ketamine	37.5, 75, 150 , 300 mg/Kg	28°C, 60-70%, 12:12 LD	New Zealand white rabbits (excised liver and muscles) (ear vein infusion)	Yes. Larval stage – Over-estimation
Goff *et al.* (1992)	*P. ruficornis*	Methamp hetamine	37.5, 71.4, 142.9 mg	26°C, - , -	Domestic rabbits (excised liver) (ear vein infusion)	Larval stage – up to 18h over-estimation Pupal stage – up to 48h over-estimation
Mullany *et al.* (2014)	*C. stygia*	Methamp hetamine (MA), p-hydroxy methamp hetamine	(MA) – 0.1, 1, 10 mg/Kg. (pM) – 0.015, 0.15, 1.5 mg/Kg (MA+pM) - 0.1	23°C, - , 12:12 LD	Kangaroo mince	Larval stage – up to 44h over-estimation. Pupal stage –up to 78h deviation

		(pM)	mg/Kg MA: 0.015 pM, 1 mg/Kg MA: 0.15 mg/Kg pM, 10 mg/Kg MA: 1.5 mg/Kg pM			
Bourel *et al.* (1999)	*L. sericata*	Morphine	12.5, 25, 50 mg/h for 3 hours	20-22°C, - , Natural light	Domestic rabbits (ear artery infusion)	Larval stage – up to 24h under-estimation
George *et al*. (2009)	*C. stygia*	Morphine	2,10, 20 ug/g	22°C, - , 12:12 LD	Pet mince (kangaroo mince, lambs fry & heart)	None

A brief overview of past studies indicates the varying methodological approaches to entomotoxicological studies. It is not uncommon for scientific methodology to vary between studies, however research with possible medico-legal applications may require standardisation. Relating to Table A, some methodological aspects that may require standardisation include the method of drug administration, choice of rearing substrate, and rearing conditions.

Method of drug administration

The use of animal models in research is a frequent debate among researchers, particularly due to ethical considerations. Animal models are arguably more suitable than non-living models to investigate the influence of xenobiotics on insect development (Gosselin *et al*., 2011b). This may be because a living system accommodates some form of metabolism of xenobiotics (George *et al*., 2009, Oliveira *et al*., 2009; Gosselin *et al*., 2011b) which may be related to that of humans. However, this motive is also a deterrent against the use of animal models as the absorption, distribution, metabolism and elimination (ADME) of different animals, treated with different compounds may not be clearly understood. This is

particularly relevant in studies where the influence of parent compounds on insect development is investigated, while attempting to avoid cumulative effects of known, or unknown secondary metabolites. In this regard non-living systems may be preferred. As can be seen from Table A, half the studies used animal models and when compared to studies on the same drug, varying results were observed. Notwithstanding other differences between the studies, the observed discrepancies may, in part, be due to the use of a living system, or not.

Rearing substrate

Natural diets such as liver (e.g. porcine or beef) or artificial diets such as a combination of whole milk, wheat germ, cellulose, agar, yeast and propionic acid (Tachibana & Numata, 2001), or a combination of natural and artificial diets are commonly used for rearing dipteran species (Table A). Natural and artificial diets have been demonstrated to have different effects on blow fly larval development (Tachibana & Numata, 2001). Therefore, despite other methodological differences, it is conceivable that the seemingly opposite effects of ketamine on larval development observed by Lü *et al.* (2014) and Zou *et al.* (2013) may have been the result of using agar supplemented pig meat as opposed to rabbit liver and muscle tissue respectively. Discrepancies may also be related to the species and type of tissue used as part of a natural or artificial diet, as Clark *et al.* (2006) postulated that this may also significantly alter insect growth rate.

Rearing conditions

Temperature (Anderson, 2000), humidity (James *et al*., 2014) and photoperiod (Nabity, Higley & Heng-Moss, 2007) have been known to influence blow fly development. Table A

indicates that temperature, humidity and photoperiod vary between studies which may relate to the species used and geographic region. However, in several studies, no indication was given that all of these factors were controlled. For example, Fathy *et al.* (2008) and Tabor *et al.* (2005) were field studies with fluctuating conditions. Moreover, it is not apparent whether Kharbouche *et al.* (2008), Monthei (2009), Goff *et al.* (1992), Mullany *et al.* (2014), Bourel *et al.* (1999), and George *et al.* (2009) controlled for humidity. Regarding photoperiod, with the exception of Goff *et al.* (1992) where photoperiod was not reported, cyclic regimes ranged between 12:12 hour L:D cycles, 24:0 hour L:D cycles, natural lighting and the study by Kharbouche *et al.* (2008) that relied on a 15:9 hour L:D regime which simulated that of the season.

Standardisation of all environmental conditions, particularly temperature and humidity, may not be feasible as these may naturally vary between the regions where studies are conducted. However, the application of standardised conditions between studies in the same region may improve the comparability of findings. For example, Tabor *et al*. (2005) and Monthei (2009) both investigated the influence of ethanol on *P. regina* at the Virginia Polytechnic Institute and State University, USA. Monthei (2009) investigated concentrations to expand on the data collected by Tabor *et al*. (2005). However, as can be seen from Table A, different methodologies were followed. Therefore, concluding on the influence of ethanol on *P. regina* development becomes difficult as the opposing findings may be a result of different concentrations used, or a consequence of different methodologies followed.

Independently the studies from Table A contribute to the knowledge base of entomotoxicology and may be relevant to the respective regions. However, the methodological variation underpins the care that must be taken when comparing between studies especially when inferences are made regarding the PMI. Moreover, the need for baseline data specific to a region is reaffirmed as data between geographic regions may inherently vary.

2.2.7. Methylphenidate hydrochloride

The Western Cape, South Africa, currently relies on blow fly developmental data published elsewhere to estimate the PMI or account for the influence of external factors on PMI estimates. In addition to the lack of research on the development of the local blow fly populations, a need exists to determine the effects of ante-mortem drug use by the decedent on blow fly development. Drug-related deaths are likely to go unnoticed for extended periods of time allowing advanced decomposition to set in (Goff *et al*., 1992). Consequently, physiological post-mortem changes may no longer be a viable means of estimating the PMI, thereby further emphasising the need for reference entomotoxicological data. The Western Cape has over the past decade consistently contributed more than a third of the national drug-related crime (Department of Community Safety, 2014). Methamphetamine (“tik”), cannabis (“dagga”) and heroin (“smack”) are amongst the most common illicit drugs of choice in the Western Cape (Department of Community Safety, 2014). Though these illicit drugs have historically been among the most common substances of abuse, the abuse of prescription drugs, such as Ritalin, has become

commonplace, especially among students (Babcock & Byrne, 2000; Klein-Schwartz, 2002; DuPont *et al*., 2008; Forlini & Racine, 2009; Health24, 2015).

ADHD is a psychiatric disorder that has been estimated to affect as much as 10% of the children in the South African population (Muthukrishna, in Lloyd, Stead & Cohen as cited in Perold *et al*., 2010). It has generally been the belief that ADHD is a childrens' disorder that does not persist in adolescence. However, it has been reported that there are increased trends of ADHD continuing into adolescence and adulthood (Truter, 2009). A meta-analysis has suggested a 15% persistence rate of ADHD into adolescence (Faraone, Biederman & Mick as cited in Walker *et al.*, 2011). Moreover, according to McGough (as cited in Barlow & Durand, 2015) approximately half of children with ADHD have persisted symptomology in adulthood. In the Western Cape, the prevalence of ADHD among children and adolescents are estimated at approximately 5% (Kleintjies *et al.*, 2006).

MPH is the first line treatment for those diagnosed with ADHD (Truter & van W Kotze, 2005; Truter, 2009; Rezende *et al.*, 2014). Truter (2009) demonstrated through a retrospective cohort drug utilisation study that the Western Cape has the highest average percentage of MPH prescriptions in South Africa. Additionally the Department of Social Development: Western Cape Substance Abuse Unit (2011) indicated Ritalin as one of the most common prescription stimulants of potential use and abuse. Several reports have indicated the misuse of Ritalin by students as a "study aid" (Dupont *et al.*, 2008; Forlini & Racine, 2009) or as a recreational drug (Dupont *et al.*, 2008; Health24, 2015). The prevalence of ADHD, together with the reported misuse and abuse of MPH, primarily in the form of Ritalin, in conjunction with the high rate of drug related crime (Department of

Community Safety, 2014) makes Ritalin a likely drug to be detected post-mortem among deaths occurring in the Western Cape.

MPH is a psychoactive stimulant that acts on the brain stem and cerebral cortex, increasing sympathomimetic activity (DrugBank, 2013). It serves to elevate dopamine and nor-adrenaline levels through the inhibition of transporters responsible for their re-uptake (Hysek *et al.*, 2014). MPH is structurally similar to amphetamine (Massello & Carpenter, 1999; Rush *et al*., 2001) and is hepatically metabolised to ritalinic acid (DrugBank, 2013). In South Africa, MPH is a schedule 6 drug available as pills or capsules. Misuse or abuse of MPH is associated with, among other, agitation, psychosis, tachycardia and hypertension, and severe intoxication may cause seizures (Klein-Schwartz, 2002). Some stimulant medications, such as Ritalin, are considered drugs of abuse owing to their dependence-forming liability and thus potential to be abused (Truter & van W Kotze, 2005). The misuse and abuse of Ritalin, also known as “Smarties”, “Rit”, or “Kiddie Cocaine” has become rife among students (Health24, 2015). Typically Ritalin is taken orally, however the popularised use of Ritalin as a “legal fix” has led to the exploration of alternative routes of administration such as intranasal usage (Massello & Carpenter, 1999; Babcock & Byrne, 2000; Klein-Schwartz, 2002) and parenteral routes including intravenous usage (Lundquest, Young & Edland, 1987; Massello & Carpenter, 1999; Klein-Schwartz, 2002).

Rezende *et al.* (2014) demonstrated that *Chrysomya* spp. larvae exposed to MPH results in altered development. Larvae of *C. putoria*, *C. albiceps* and *C. megacephala* were reared on artificial diets spiked with MPH. Three concentrations were used; 2.9 mg/Kg, 14.5 mg/Kg and 29 mg/Kg, to simulate drug abuse. These concentrations were based on the

recommended therapeutic dose of 0.29 mg/Kg for adults. Treated *C. putoria* and *C. albiceps* larvae exhibited decelerated development, thereby prolonging total development times. The total development time for *C. albiceps* was delayed by 24 hours, whereas that of *C. putoria* was delayed by 12 hours for all concentrations of MPH investigated. *C. megacephala* larvae were affected at certain growth stages without affecting the total development time at any concentration of MPH. This suggests a possible species-specific response to MPH. The authors postulated that the observed deceleration is sufficient to cause PMI under-estimations if the influence of MPH is not considered.

2.3. Summary

When reviewing the literature the utility and limitations of forensic entomology becomes clear. While serving an important purpose in assisting a PMI estimate, it may be hampered by external factors. Toxicological analysis of entomofauna has been a research pursuit since 1980 (Beyer *et al.*, 1980) and gave rise to forensic entomotoxicology. Discovery of the relationship between altered insect development and succession, and the presence of xenobiotics in a corpse, stimulated research interests. However, research findings are frequently not comparable due to the methodological diversity and developmental plasticity of insects in different geographic regions.

Forensic entomological/toxicological research in the Western Cape, South Africa is lacking. The reported misuse and abuse of MPH, along with the prevalence of ADHD in the Western Cape makes MPH a suitable compound for investigation. The influence of MPH on the development of forensically significant entomological fauna has not been well documented. To the best of this author's knowledge, with the exception of Rezende *et al.*

(2014) no research has been conducted on the influence of MPH on Calliphoridae development. Moreover, no research has been done on the influence of MPH on the Calliphoridae species local to the Western Cape. The need for research elucidating the influence of MPH on the development of blow flies is supported by the literature, reporting MPH as a possible external factor that can alter blow fly development and consequently PMI estimates based on development (Rezende *et al*., 2014) Therefore, this study investigated the hypothesis that MPH will impede the larval development of *C. chloropyga*. Thus, the aim of this study was to determine the influence of MPH on *C. chloropyga* larvae development prevalent in the Western Cape, with the objective of determining alterations in the growth rate. An additional objective was to assess the entomotoxicological value of *C. chloropyga* larvae in terms of the detection of MPH.

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Chapter 3: Manuscript

Preliminary observations of the effects of methylphenidate hydrochloride on the development of *Chrysomya chloropyga*: a stepping stone for the Western Cape, South Africa

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3.1. Abstract

Forensic entomologists rely on insect development and successional data to estimate the post-mortem interval (PMI). Ante-mortem drug use prior to an individual's death may result in drug transmission to feeding insects and subsequent alteration of their development, thereby altering PMI estimates. This study investigated the influence of Ritalin (methylphenidate hydrochloride) (MPH) on the development of *Chrysomya chloropyga*. *C. chloropyga* larvae were reared on pig liver treated with MPH and exhibited a trend of expedited larval development and prolonged pupal development. Conservatively, the results suggest that MPH may expedite the larval stage by up to 17 hours and prolong the pupal stage by up to 16 hours. These preliminary findings suggest that, at the concentration investigated, MPH may alter the duration of *C. chloropyga* developmental stages, and consequently PMI estimates if MPH is not detected or its effects not considered. Furthermore, MPH was detected in both frozen and ethanol preserved specimens. MPH could still be detected from treated larvae, after 3.5 days incubation at ~30°C. This may suggest an improved stability of MPH in insects. Moreover, detection of MPH from ethanol preserved specimens suggests the qualitative toxicological utility of specimens maintained in this preservation liquid, despite stability and self-extraction concerns. These are the first entomotoxicological data on MPH generated for blow flies local to the Western Cape, South Africa.

Keywords: Forensic entomology, Entomotoxicology, *Chrysomya chloropyga*, Post-mortem interval, Methylphenidate hydrochloride

3.2. Introduction

Forensic investigators are frequently tasked with the responsibility to estimate the post-mortem interval (PMI), or time since death, from human remains in an advanced stage of decomposition. However, traditional indicators of the PMI such as algor-, livor-, and rigor-mortis [1] can only reliably estimate the PMI within the first 72 hours after death [2-4]. This presents a basis for the use of complementary techniques when estimating the PMI. In this regard forensic entomology has broadened the forensic arsenal.

Forensic entomology has been considered to be an accurate means of estimating the PMI [5] and is useful before, and well beyond 72 hours after death [2-4, 6]. It has long been observed that insects are associated with corpses and display time-dependent processes that can be related to the PMI [7]. A forensic entomologist may utilise the developmental data and/or pattern of successional communities of several carrion-feeding arthropods, to assist in PMI estimations [7-9]. The former is based on the development of mainly fly larvae following ovi- or larviposition [10]. The latter is based on the analysis of the pattern of colonisation by succeeding waves of arthropods on the carrion [10]. However, it should be noted that estimations derived from immature necrophagous insects represent the minimum PMI as the timing between death and ovi- or larviposition is variable [7].

Calliphoridae or “blow flies”, have been recognised among the first wave of faunal succession on human remains [2, 4, 6, 11] and are thus accurate indicators of a minimum time since death [11]. Their forensic importance may, in part, be attributed to their ability to rapidly locate and colonise a body after death [4]. The use of blow fly development data has been suggested to provide the most accurate means of estimating the PMI when using

entomofauna (Greenberg as cited in [8]). Developmental data is derived from the duration of the immature developmental stages as it relates to specific temperatures [4]. This data can be used to construct developmental models such as isomegalen and isomorphen diagrams, and thermal summation models [4]. These models incorporate the size (length, weight or width) [12] or developmental event (e.g. first, second or third instar) to estimate the age of insects and thereby the PMI [4]. The underlying assumption for the use of insect development to estimate the PMI, is that developmental data of immature insects can be used to determine insect age, which can be related to the time of colonization, from which the PMI can be inferred [4]. However, PMI estimates derived from insects require careful interpretation as several factors such as temperature, season, maggot masses, xenobiotics and geographic region have been suggested to influence insect development and succession [7].

Forensic entomotoxicology is concerned with the detection of toxicological substances from forensic entomological fauna [13]. Moreover, it investigates the associated effects of such substances on the development of forensic entomological fauna as a means to assist PMI estimations [13]. This is a field that may be regarded as an intersection between forensic entomology and forensic toxicology. Entomotoxicology may be relied upon when conventional samples for toxicological analysis cannot be used due to the condition of the remains, such as skeletonisation or advanced decomposition [13]. The importance of determining the influence of xenobiotics on necrophagous first responders, particularly Calliphoridae, is underpinned by its association with altered blow fly development. Previous studies have investigated the effects of morphine [14, 15], cocaine [16], methamphetamine

[17], ketamine [18, 19], methylphenidate hydrochloride and phenobarbitol [20], methadone [21], paracetamol [22], codeine [23, 24] and ethanol [25] on blow fly development. Understanding the influence of chemical substances on blow fly development may therefore improve the reliability of PMI estimates.

Methylphenidate hydrochloride (MPH) is widely used to treat attention deficit hyperactivity disorder (ADHD) [26, 27] and is commonly recognised by its commercial names such as Ritalin and Concerta [26, 28]. MPH is a psychoactive stimulant that acts on the brain stem and cerebral cortex, increasing sympathomimetic activity [29] by elevating dopamine and nor-adrenaline levels through the inhibition of transporters responsible for their re-uptake [30]. MPH is structurally similar to amphetamine [31, 32] and is hepatically metabolised to ritalinic acid [29]. Some stimulant medications, such as Ritalin, are considered drugs of abuse owing to its dependence-forming liability and thus potential to be misused or abused [33]. Several reports have indicated the misuse of Ritalin by students as a "study aid" [28, 34, 35] or as a recreational drug [28, 35], and it has been reported as one of the most common prescription stimulants of potential use and abuse in the Western Cape, South Africa [36]. Therefore, investigating the influence of MPH on blow fly development may be of forensic importance.

Rezende *et al.* [20] demonstrated that *Chrysomya* subspecies (spp.) larvae exposed to MPH may result in prolonged development. The results indicated that the total development time for *Chrysomya albiceps* and *Chrysomya putoria* was delayed by 24 hours and 12 hours respectively, at each MPH concentration investigated. The authors postulated that

the observed delay is sufficient to cause PMI under-estimations if the influence of MPH is not considered.

To the best of the authors' knowledge, with the exception of Rezende *et al.* [20], no research has been conducted on the influence of MPH on the development of Calliphoridae. Moreover, no research has been done on the influence of MPH on the development of Calliphoridae species local to the Western Cape, South Africa. The need for research elucidating the influence of MPH on the development of blow flies is supported by the literature, reporting MPH as a possible external factor that can alter blow fly development and consequently PMI estimates [20].

Therefore, this study investigated the hypothesis that MPH will impede the larval development of *Chrysomya chloropyga*. Thus, the aim of this study was to determine the influence of MPH on larvae development of *C. chloropyga*, a species prevalent in the Western Cape, with the objective of determining alterations in the growth rate. An additional objective was to assess the entomotoxicological value of *C. chloropyga* larvae in terms of the detection of MPH.

3.3. Methodology

3.3.1. Study population

Bait in the form of pig offal (Winelands Pork, South Africa) was placed in an open field at the Medical Research Council (MRC) in Delft, Western Cape, to lure blow flies and facilitate oviposition. Egg masses were collected 24 and 48 hours later and were approximately equally divided into test and control groups from both collection times. Eggs from the first collection were divided into two test groups (T1 and T2) and two control

groups (C1 and C2) collectively designated “Collection A”. Eggs from the second collection were divided between one test group (T3) and one control group (C3) collectively designated “Collection B”.

3.3.2. Spiking of the food substrate

Sixteen tablets of Ritalin (Novartis, USA), each containing 10 mg of MPH were ground to powder and dissolved in 100 ml of a 0.9% sodium chloride (NaCl) solution, and mixed into 5 Kg of macerated pig liver to prepare a final concentration of 31.37 mg/Kg MPH treated pig liver (approximately 100 times the therapeutic dose [20]). The treated liver was manually mixed for 5 minutes to promote even drug distribution. The mixture was then divided into 300 g portions and stored at - 20°C until needed. Pig livers used for the control groups received 100 ml of a 0.9% NaCl solution and were macerated, mixed, and stored in the same manner as the treated livers. To avoid contamination, control livers were prepared and stored before treated livers.

3.3.3. Rearing of blow flies and larvae

Each egg mass collected from the bait site was placed on 150 g of macerated pig liver, with or without Ritalin, in 3 L clear plastic lidded containers containing vermiculite at the base, a perforated foil layer and the pig liver on top. Stockings were placed between the container and perforated lid to prevent larvae from escaping. The liver of each group was replenished as needed, and maintained at a ratio of at least 1 g liver/2 larvae, except for treated larvae from Collection B where the number of larvae were under-estimated. The vermiculite of each container was also replaced as needed. Each group was reared in an incubator at (29.92 ± 0.56°C) (mean ± SD), ambient relative humidity (36.75 ± 5.82%)

(mean ± SD) and a 14:10 hour light/dark (L:D) cycle, in accordance with the aestival natural rhythm [37]. Blow flies resulting from the experimental groups were transferred to separate insectary cages and maintained at the same conditions described above, but without any treatment. The flies were maintained until they naturally expired.

3.3.4. Sampling of larvae

Developmental analysis

Starting from when larval hatchlings were first observed, larvae were sampled every 6 hours in the first 24 hours, every 12 hours on the second day after hatching and every 24 hours after that until pupariation. Sampling was performed by randomly removing five larvae (or as the population allowed) from each group at the respective sampling times and killing them through immersion in near boiling water (> 90°C) for at least 30 seconds.

Toxicological analysis

Larvae sampled for developmental analysis were also used for toxicological analysis. Samples for toxicological analysis were selected from the approximate middle (72 h) and end (144 h and 168 h) of the total sampling period to assist in determining the continuous presence of MPH. Moreover, it was decided that sampling would not continue beyond 168 hours after hatching, in anticipation of pupariation.

To improve the likelihood of MPH detection and overcome foreseeable processing complications, twenty mature larvae were randomly sampled from a test and control group in both Collection A and B. These larvae were thoroughly rinsed with water and killed and preserved by freezing at – 20°C.

3.3.5. Larval identification and size measurement

Following killing, third instar larvae were identified using a Zeiss Stereo Discovery V8 stereo microscope (Oberkochen, Germany), and based on the key for identification described by Prins [38]. Species identification was confirmed through the identification of adult blow flies resulting from experimental rearing, through a morphological analysis based on characteristics described by Grassberger *et al*. [11]; Prins [38]; Szpila [39]; and Whitworth [40]. Identified larvae and adults were counted to estimate their percentage composition among the sampling populations. The blow fly species that was the most consistently present in all experimental groups was selected as the subject of investigation. For size measurements, larvae were rinsed with water and left to dry for 30 seconds on paper towel. Following this, the individual lengths of the sampled larvae were measured using a digital calliper. The individual weights of the larvae were also measured using an analytical balance. The size measures of third instar larvae were used to assess changes in larval growth rates as determined by the rate of change in larval length and weight over time. Following measurement, sampled larvae were preserved in plastic specimen containers with 70% ethanol, and stored at 4°C until toxicological analysis was performed.

3.3.6. Duration of developmental stages

Larvae from each sampling time were assessed for stage of development until pupariation, after which pupae were observed daily until adult emergence. The onset of a development stage was recorded, to the nearest hour, as the time when the stage was first observed.

3.3.7. Qualitative analysis of MPH from sampled larvae

Sample preparation

Liquid-liquid extraction

All reagents were purchased from Merck (Merck Millipore, Darmstadt, Germany) and were of LC-grade. Larvae from selected sampling times were analysed for MPH. Moreover, to assess the influence of different killing and preservation methods used in this study on MPH detection, approximately same aged larvae from those killed with boiling water (> 90°C) and preserved in 70% ethanol at 4°C, and those killed and preserved by freezing at – 20°C were selected and analysed for MPH. Sampled larvae were placed in respective 2 ml tubes along with ceramic beads and 800 µl of LC-grade water. Each tube contained no more than five larvae from a specific sampling interval, and was homogenised at 6.45 m/s for four cycles of 1 minute each with a 30 second rest between cycles, using a Bead Ruptor 24 (OMNI International, Georgia, USA) system. Samples were prepared by adding 500 µl of the larval homogenate, 500 µl of sodium carbonate buffer (0.75 M, pH 10) and 1 ml of hexane: dichloromethane (9:1 v/v) together. Subsequently, the liquid-liquid extraction (LLE) was performed by vortexing the solutions for 30 seconds followed by centrifugation (Heraeus Labofuge 300, Thermo Fisher Scientific, Massachusetts, USA) at 4000 rpm for 7 minutes. Following this, all of the organic phase (200 µl - 600 µl depending on the sample size) was transferred to glass vials and evaporated with nitrogen. Evaporated samples were stored at - 80°C and reconstituted in 200 µl mobile phase consisting of: 50% 10 mM ammonium formate in water (mobile phase A), and 50% acetonitrile:methanol (50:50 v/v), (mobile phase B). The mixture was vortexed and centrifuged for 5 minutes at 13000 rpm and 20 µl of the supernatant was diluted in mobile phase (1/10 v/v), after which 10 µl was

injected into a liquid chromatography mass spectrometry-mass spectrometry system (LC-MS/MS).

Liquid chromatography mass spectrometry-mass spectrometry

LC-MS/MS was performed on an AB Sciex 3200 Q-trap mass spectrometer (Sciex, Massachusetts, USA) fitted with a turbo spray ion interface, coupled with a Shimadzu Prominence ultra-high pressure liquid chromatography (UHPLC) system (Kyoto, Japan). Data acquisition was accomplished using MasterView^{TM} software. Retention time, ion transitions (234.100 to 84.300) and a 5:1 signal-to-noise ratio cut-off applied to the average peak intensities of the test and controls were used as criteria for positive detection of MPH. Chromatography separation of MPH was performed with a Phenomenex Kinetics C18 EVO column (50 x 3 mm, 5 µm) (California, USA) and using a linear gradient elution. The linear gradient ranged from 5% mobile phase B at the start to 95% mobile phase B over 5 minutes, and was held at 95% from 5 – 15 minutes. The temperature of the column was set at 40°C and the flow rate was 0.6 ml/minute.

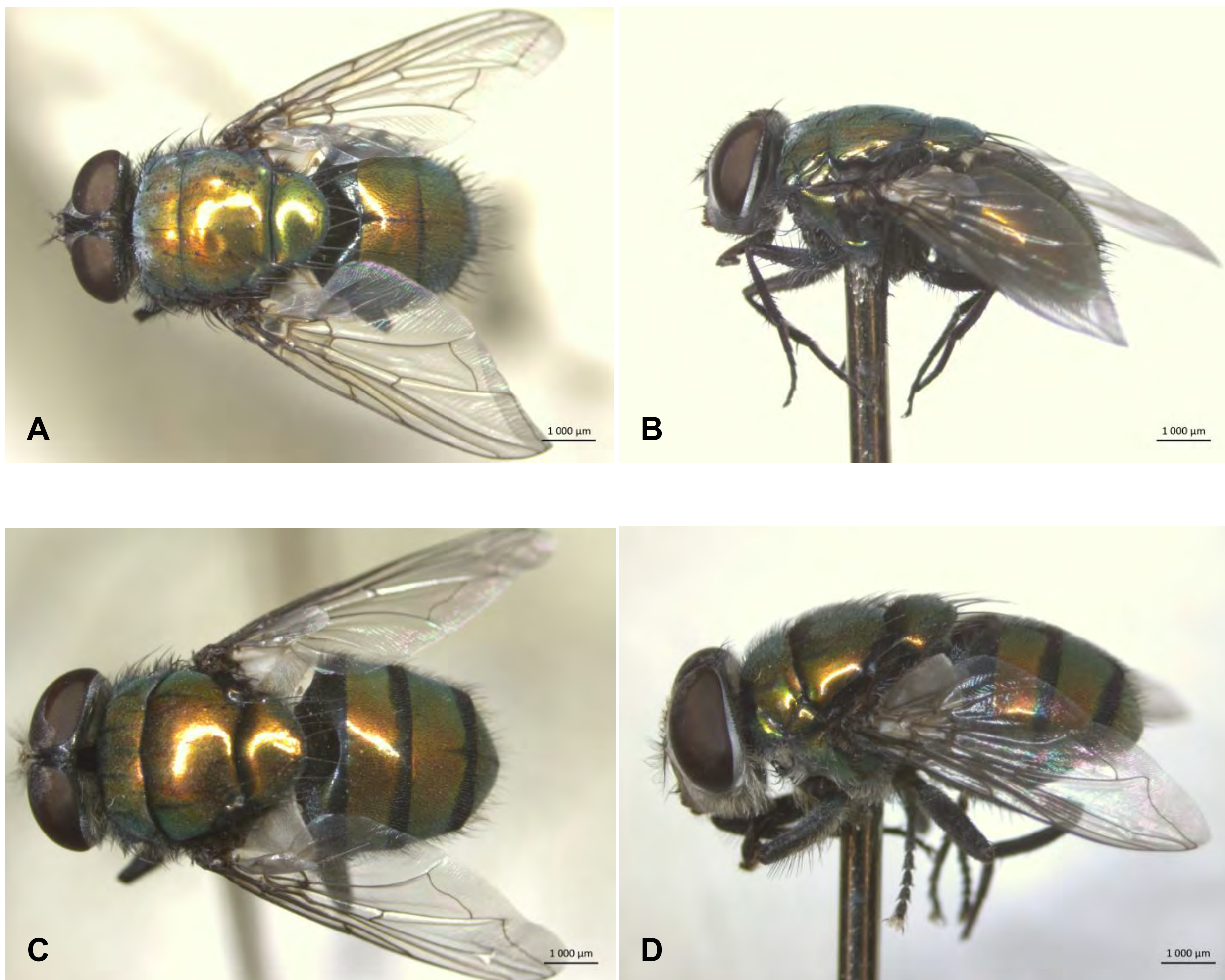
3.3.8. Statistical analysis

All statistical analyses were performed with MedCalc statistical software. Length and weight measurements were compared between the respective test and control groups using the Mann-Whitney U test. Data resulting from the test (T1 and T2) and control (C1 and C2) groups in Collection A were respectively pooled as these were performed at the same time.

3.4. Results

3.4.1. Species composition

Blow flies identified among the sampling populations were *C. chloropyga*, *C. albiceps,* and *Luciliinae* and can be seen in Fig. 1. *C. chloropyga* was the most consistently present in all experimental groups and was therefore selected as the subject of investigation. Only in T1 (94.3%) of Collection A and, T3 (86.7%) and C3 (61.5%) of Collection B did *C. chloropyga* constitute the majority of blow flies. In the remaining groups *C. chloropyga* was the minority (Table 1).



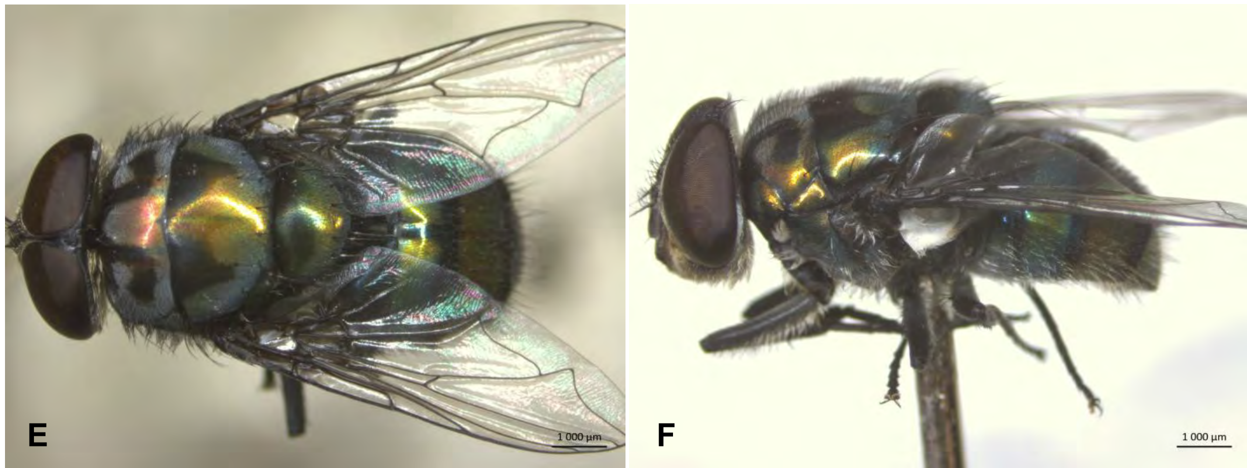


Figure 1. Species identified among the sampling populations. Dorsal (left) and lateral (right) view. *Luciliinae* (A and B); *C. albiceps* (C and D); *C. chloropyga* (E and F).

Table 1. Comparison of the estimated percentage composition (%) of *C. chloropyga* among sampling populations

Collection A	***C. chloropyga***	**Other**
Control 1 (C1)	25.5	74.5
Control 2 (C2)	14	86
Test 1 (T1)	94.3	5.7
Test 2 (T2)	20	80
Collection B		
Control 3 (C3)	61.5	38.5
Test 3 (T3)	86.7	13.3

3.4.2. Larval growth patterns

Growth rates of larvae

C. chloropyga growth rates yielded variable results between Collection A and B. Regarding the mean length of treated larvae from Collection A, the most notable changes were between 36 – 48 hours and 120 – 168 hours during which the growth rate was apparently accelerated, and between 48 – 72 hours during which a decelerated rate was observed

(Fig. 2.1). Regarding the mean weight, the apparent pattern of acceleration and deceleration was similar to that observed with treated larvae length (Fig. 2.2). Treated larvae in Collection B exhibited similar trends in growth rate to that of the controls, with only the growth rate determined by mean weight suggesting a modest acceleration between 96 – 144 hours (Figs. 3.1 and 3.2).

Larval sizes

In Collection A, statistically significant differences were observed in the median lengths of MPH treated larvae at 48, 72 and 168 hours, exhibiting a fluctuating trend of increasing and decreasing length when compared to control larvae at the respective time intervals (Table 2.1). A similar fluctuating trend, showing statistically significant differences, was observed when comparing the median weight of treated larvae in Collection A to that of the controls at respective time intervals (Table 2.2). In Collection B, the median lengths and weights of treated larvae respectively, were consistently shorter and less when compared to control larvae, showing statistically significant differences at 36, 96 and 120 hours for length (Table 3.1), and 36, 72, 96 and 120 hours for weight (Table 3.2).

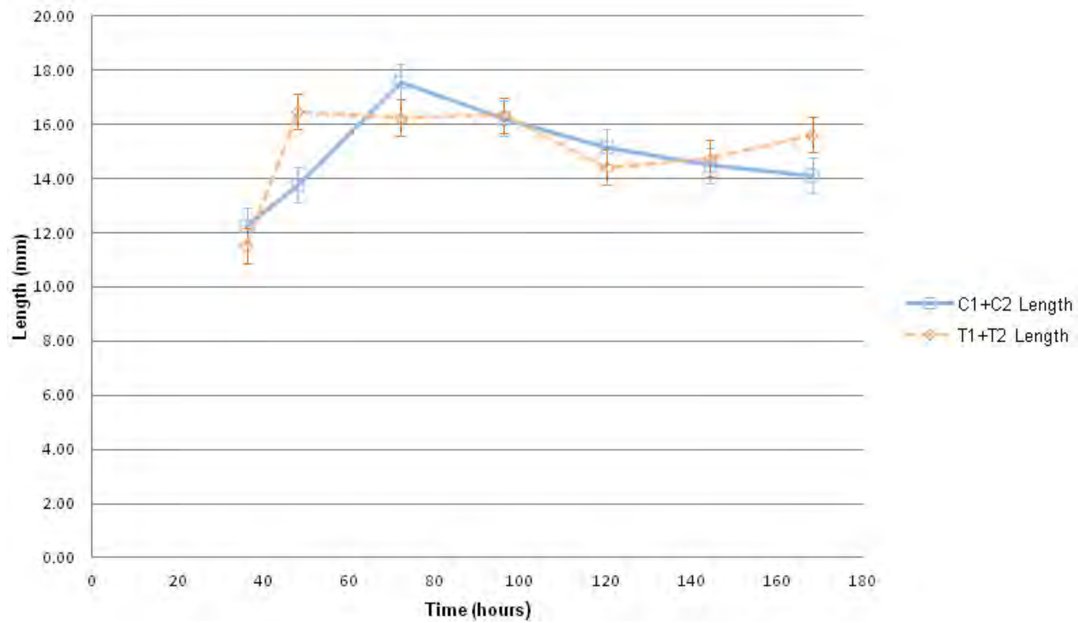


Figure 2.1. Comparison of the mean lengths of third instar *C. chloropyga* larvae sampled (n=5) at 36, 48, 72, 96, 120, 144 and 168 hours after hatching. T1 and T2 represent the mean of the pooled larval lengths of test groups in Collection A (24 hours) treated with 31.37 mg/Kg MPH. C1 and C2 represent the mean of the pooled larval lengths of control groups in Collection A. Data presented as mean ± SE.

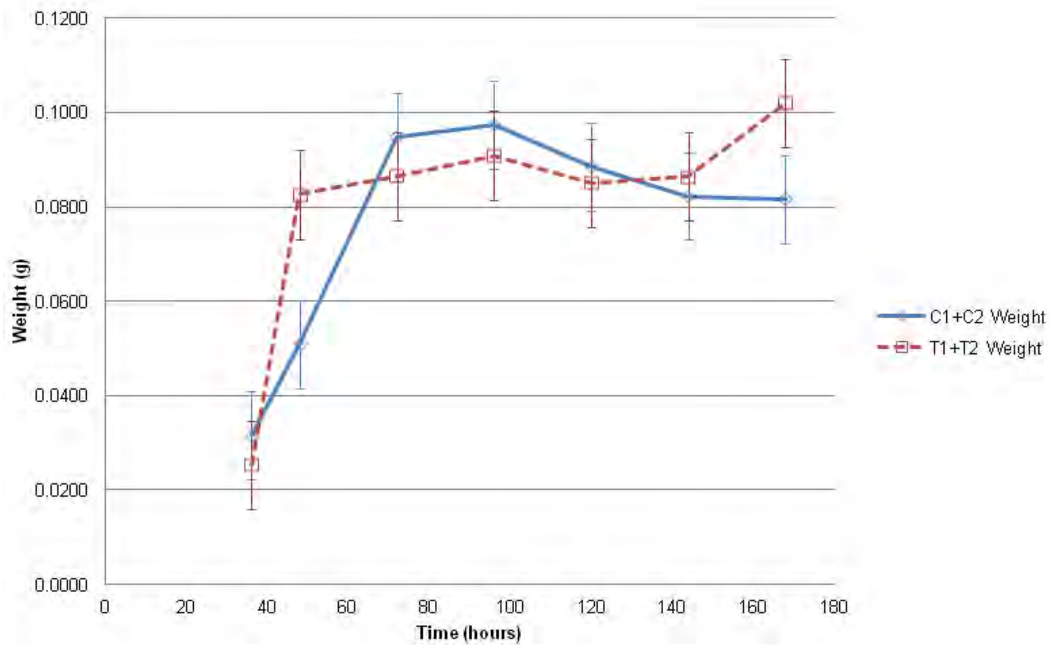


Figure 2.2. Comparison of the mean weights of third instar *C. chloropyga* larvae sampled (n=5) at 36, 48, 72, 96, 120, 144 and 168 hours after hatching. T1 and T2 represent the mean of the pooled larval weights of test groups in Collection A (24 hours) treated with 31.37 mg/Kg MPH. C1 and C2 represent the mean of the pooled larval weights from control groups in Collection A. Data presented as mean ± SE.

Table 2.1 Comparison of larval body length (mm) from Collection A							
Time since hatching (hours)							
	36	48	72	96	120	144	168
C1 and C2							
Mean±SE	12.255± 1.035	13.7733± 0.3845	17.564± 0.1534	16.23± 0.3818	15.157± 0.2621	14.489± 0.3402	14.126± 0.2813
Median	12.255[a]	13.73[a]	17.57[a]	16.205[a]	15.39[a]	14.44[a]	13.995[a]
T1 and T2							
Mean±SE	11.53± 0.7996	16.468± 0.1524	16.2575± 0.3819	16.3367± 0.685	14.425± 0.7373	14.7438± 0.6985	15.616± 0.5038
Median	11.06[a]	16.56[b]	16.37[b]	16.83[a]	14.31[a]	15.475[a]	15.8[b]
P-value	0.4386	0.0253	0.0192	0.6644	0.5967	0.5052	0.0275

Medians in the same column followed by the same letter are not significantly different ($P > 0.05$), according to the Mann-Whitney U test.

Table 2.2 Comparison of larval body weight (g) from Collection A							
Time since hatching (hours)							
	36	48	72	96	120	144	168
C1 and C2							
Mean±SE	0.03155 ± 0.00365	0.05097 ± 0.005616	0.09478 ± 0.001332	0.09737 ± 0.003269	0.08855 ± 0.001838	0.08229 ± 0.003338	0.08166 ± 0.002307
Median	0.03155[a]	0.0555[a]	0.0962[a]	0.0933[a]	0.0898[a]	0.08235[a]	0.0803[a]
T1 and T2							
Mean±SE	0.02536 ± 0.004821	0.0826 ± 0.001648	0.08655 ± 0.006573	0.0909 ± 0.008842	0.08511 ± 0.004724	0.08639 ± 0.004108	0.102 ± 0.003149
Median	0.021[a]	0.0827[b]	0.09145[a]	0.09705[a]	0.08305[a]	0.08965[a]	0.0994[b]
P-value	0.6985	0.0253	0.3798	1	0.5453	0.4772	0.0022

Medians in the same column followed by the same letter are not significantly different ($P > 0.05$), according to the Mann-Whitney U test.

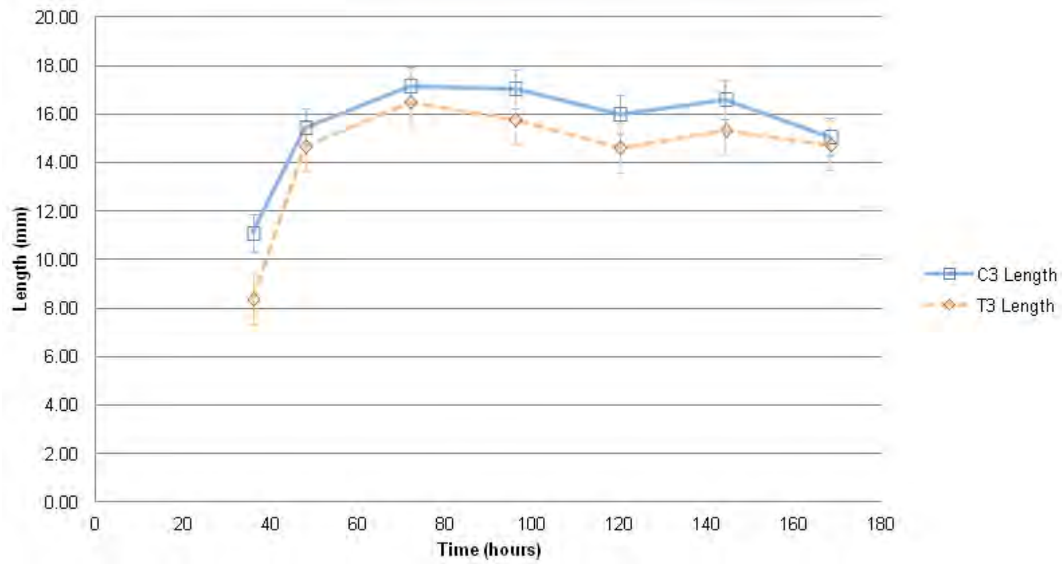


Figure 3.1. Comparison of the mean lengths of third instar *C. chloropyga* larvae sampled (n=5) at 36, 48, 72, 96, 120, 144 and 168 hours after hatching. T3 represents the mean larval lengths of the test group in Collection B (48 hours) treated with 31.37 mg/Kg MPH. C3 represents the mean larval lengths of the control group in Collection B. Data presented as mean ± SE.

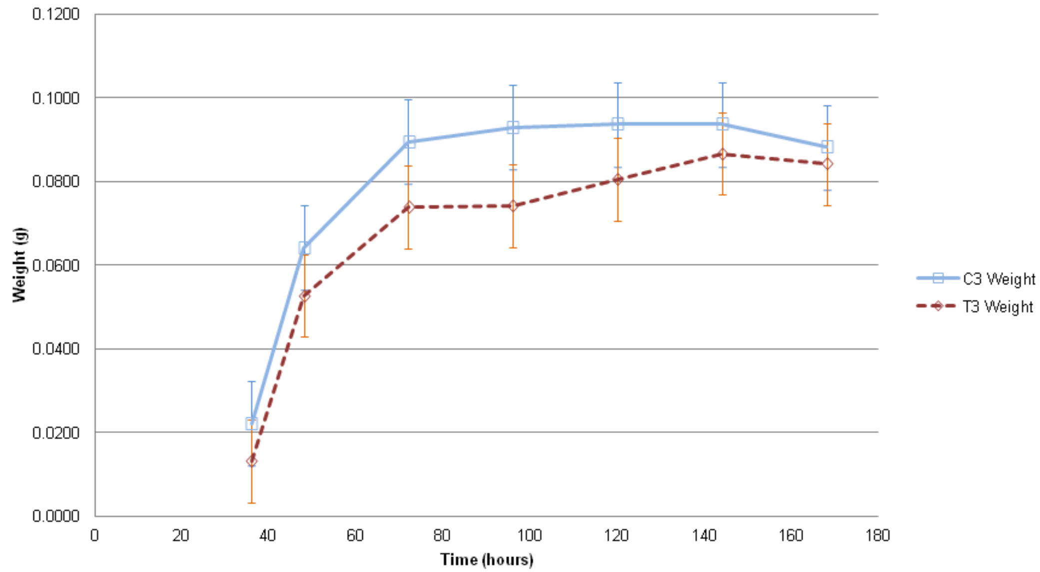


Figure 3.2. Comparison of the mean weights of third instar *C. chloropyga* larvae sampled (n=5) at 36, 48, 72, 96, 120, 144 and 168 hours after hatching. T3 represents the mean larval weights of the test group in Collection B (48 hours) treated with 31.37 mg/Kg MPH. C3 represents the mean larval weights of the control group in Collection B. Data presented as mean ± SE.

Table 3.1 Comparison of larval body length (mm) from Collection B

Time since hatching (hours)							
	36	48	72	96	120	144	168
C3							
Mean±SE	11.102± 0.3442	15.45 -	17.1733± 0.2118	17.026± 0.1315	15.992± 0.3214	16.598± 0.3885	15.068± 0.7247
Median	11.29[a]	15.45	17.3[a]	17.06[a]	15.99[a]	17.01[a]	15.53[a]
T3							
Mean±SE	8.35± 0.2897	14.685± 0.2779	16.476± 0.1589	15.788± 0.2775	14.594± 0.3339	15.322± 0.2178	14.72± 0.2261
Median	8.37[b]	14.625	16.37[a]	15.75[b]	14.69[b]	15.34[a]	14.78[a]
P-value	0.009	-	0.0526	0.0163	0.0283	0.0758	0.6015

Medians in the same column followed by the same letter are not significantly different ($P > 0.05$), according to the Mann-Whitney U test.

- Sample size too small for calculation

Table 3.2 Comparison of larval body weight (g) from Collection B							
Time since hatching (hours)							
	36	48	72	96	120	144	168
C3							
Mean±SE	0.02216±	0.0642±	0.0895±	0.09292±	0.0937±	0.0936±	0.08816±
	0.001228	-	0.003219	0.002597	0.001083	0.002886	0.001995
Median	0.0217[a]	0.0642	0.0879[a]	0.0896[a]	0.0931[a]	0.0919[a]	0.0867[a]
T3							
Mean±SE	0.01316±	0.0527±	0.07388±	0.07416±	0.08046±	0.08658±	0.08412±
	0.0007954	0.001826	0.001401	0.003137	0.001561	0.002406	0.002767
Median	0.0128[b]	0.05335	0.0736[b]	0.0725[b]	0.0826[b]	0.0852[a]	0.0824[a]
P-value	0.009	-	0.0253	0.009	0.009	0.0758	0.2506

Medians in the same column followed by the same letter are not significantly different ($P > 0.05$), according to the Mann-Whitney U test.

- Sample size too small for calculation

3.4.3. Duration of developmental stages

Treated larvae from Collection A and B displayed a shortened larval stage and prolonged pupal stage. Moreover, the total development time of treated larvae from both collections were expedited (Table 4). A timeline of *C. chloropyga* development can be seen in Fig. 4. T2 of Collection A became depleted before the pupal stage was reached and could not be used in the calculation of the duration of developmental stages.

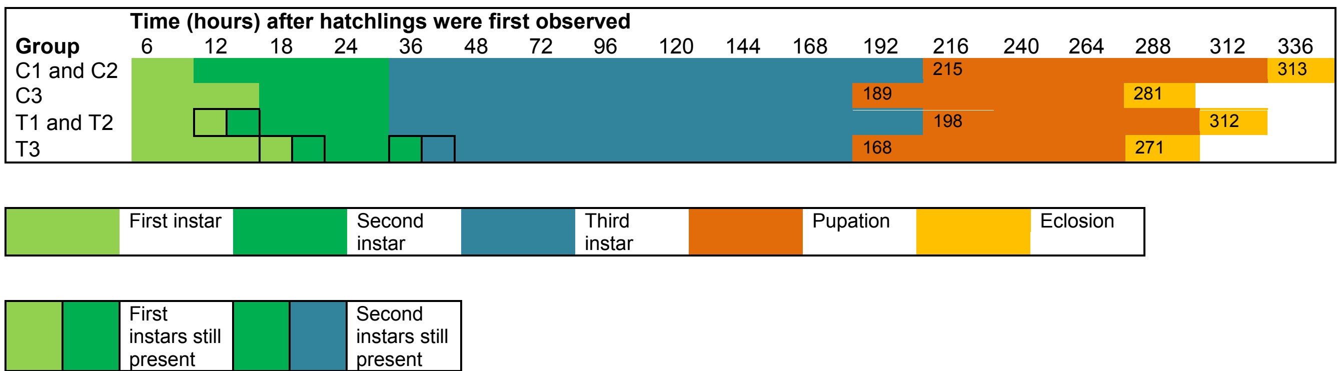


Figure 4. Comparison of the duration of the developmental stages of *C. chloropyga* between test and control groups of Collection A (24 hours) and B (48 hours). *C. chloropyga* larvae could only be reliably identified from the third instar, thus from 6-36 hours the development patterns are representative of a mixed population of larvae.

Table 4. Comparison of the duration (hours) of *C. chloropyga* development stages

	Larval stage	Difference	Pupal stage	Difference	Total Development	Difference
Collection A						
C1 and C2	215	17	98	16	313	1
T1 and T2	198		114		312	
Collection B						
C3	189	21	92	11	281	10
T3	168		103		271	

3.4.4. MPH detection

MPH was qualitatively detected in treated samples and liver, but not in the controls. An example of a typical total ion chromatogram (TIC) indicating an MPH positive sample can be seen in Fig. 5.1. MPH was detected in larvae from both killing and preservation

methods, however lower levels were observed in ethanol preserved samples. An example of these results may be seen in Figs. 5.1 and 5.2.

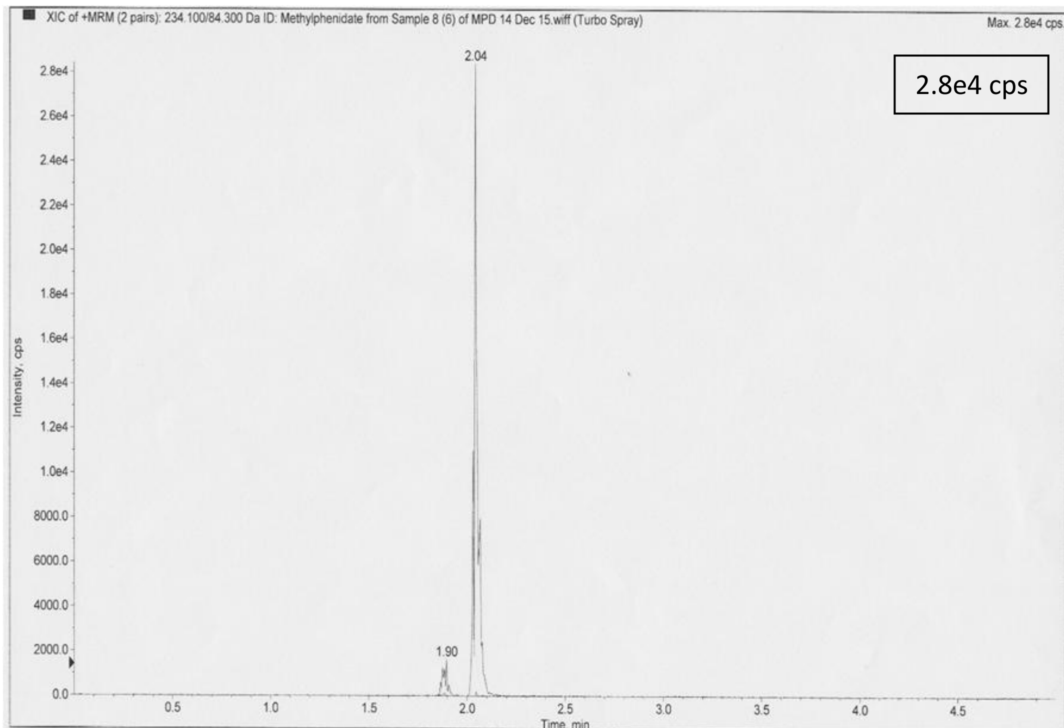


Figure 5.1. Total ion chromatogram (TIC) of MPH detected from treated third instar *C. chloropyga* larvae from Collection B (48 hours), killed in near boiling water (> 90°C) and preserved in 70% ethanol at 4°C.

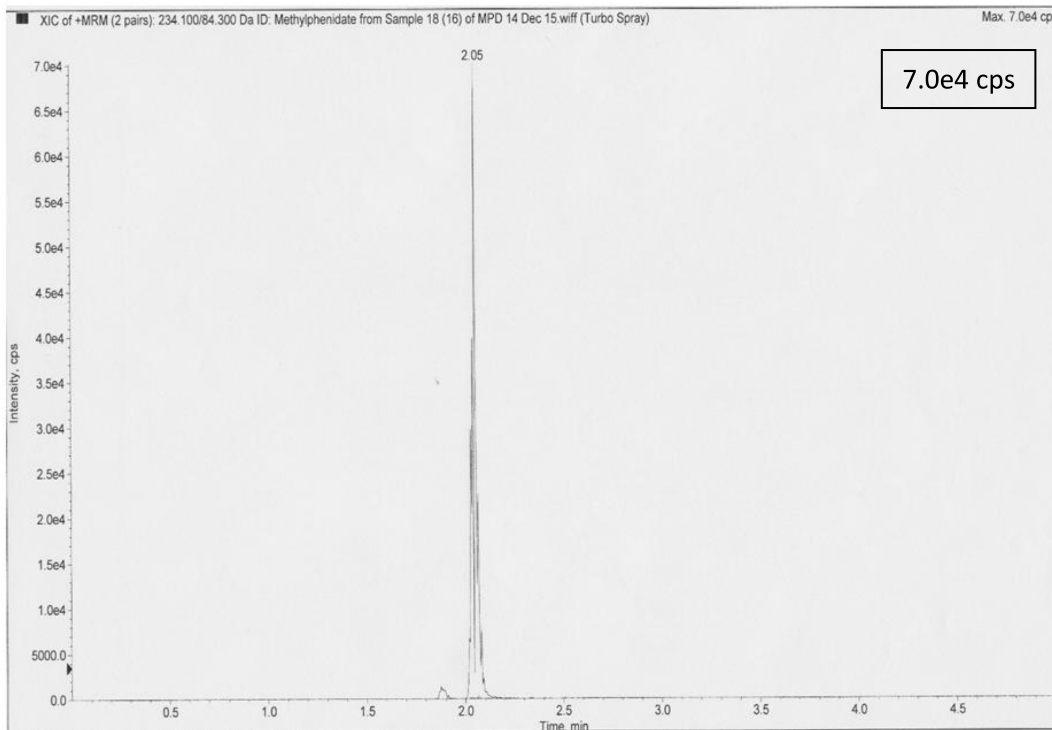


Figure 5.2. Total ion chromatogram (TIC) of MPH detected from treated third instar *C. chloropyga* larvae from Collection B (48 hours), killed and preserved by freezing at - 20°C.

3.5. Discussion

3.5.1. Mixed populations

In this study, blow fly eggs were collected from placed bait and resulted in a mixed population (Fig. 1). This was a consequence of not being able to establish a laboratory culture, due to poor blow fly fecundity despite changes in food and rearing conditions. Although more representative of real world conditions, these mixed populations complicate the investigation of a specific species as eggs and early instars have homogeneous morphologies [41, 42], thereby hindering species identification and sampling. Consequently, species identification was only achieved when larvae reached the third

instar. *C. chloropyga* was the most consistently present among sampling populations, occurring in every experimental group, wheras this was not the case for other occurring species (Table 1). Therefore *C.chloropyga* was selected as the subject of investigation.

3.5.2. The effect of MPH on larvae growth rates and sizes

Regarding the fluctuating growth pattern of treated larvae in Collection A, these findings may have been subject to statistical outliers given the small sample size (n < 10), thus emulating a fluctuating growth pattern (Figs. 2.1 and 2.2; Table 2.1 and 2.2). Regarding the apparent acceleration in growth rate of treated larvae between 120-168 hours in Collection A (Figs. 2.1 and 2.2), this may be indicative of a prolonged third instar considering that if the post-feeding stage had been reached, as is possible at this time interval [38, 43], it would be expected to observe a decrease in weight [44] as the crop is emptied, and gut content is digested [45]. Moreover, larval length also decreases during this period [46]. This may have been observed in the controls at the corresponding time interval as length and weight decreased (Figs. 2.1 and 2.2). However, given that a prolonged larval stage was not reflected in the total duration for the larval stages of treated larvae (Fig. 4), the generation of statistical outliers is supported. Alternatively, the possibility exists that an earlier larval instar may have been expedited, thereby undermining the effect that a possible prolonged third instar may have had on the duration of the larval stage. Rezende *et al*. [20] demonstrated that MPH treated *Chrysomya megacephala* larvae exhibit a prolonged second instar and prepupal stage by 12 hours respectively, and an expedited third instar by 24 hours, thereby causing no alteration in the duration of the larval stage.

However, it seems unlikely in this study, as the total larval duration was markedly shorter than that of the control (Fig. 4).

The trend of decreased length and weight in treated larvae from Collection B was not observed in the treated larvae of Collection A (Figs. 3.1 and 3.2; Table 3.1 and 3.2). Therefore, these findings may be suggestive of an external cause other than or in conjunction with MPH. A likely cause is larval crowding as the number of treated larvae in Collection B was under-estimated when collected eggs were initially placed on the treated pig liver. It has been reported that increased larval density results in undersized mature larvae [47] and puparia as measured by weight [48]. Saunders, Wheeler and Kerr [49] indicate 350 larvae/50 g meat (i.e. 7 larvae/g) as “over-crowding”. During this study, populations were initially maintained at less than 2 larvae/g liver, with the exception of the treated larvae from Collection B which were initially maintained at approximately 7 larvae/g liver (results not shown). Furthermore, it is reasonable to assume that the higher larval density may have resulted in competition for food. Competition and larval density has been reported to affect larval length and cause undersized larvae [6, 8]. Therefore, it may be postulated that the reduced length and weight of treated larvae in Collection B is a consequence of over-crowding and subsequent competition, either entirely, or in conjunction with a possible effect of MPH.

Important to remember is that Collection A and B were different cohorts, and blow flies, like many biological organisms, show some degree of developmental variation [50]. Notwithstanding overcrowding, the tenuous agreement between the responses of treated larvae from both collections may possibly be attributed to a confluence of influences

including that of, slow developing laggards [4], statistical outliers, and the variance in body size associated with post-feeding larvae [51]. Therefore, concluding on the possible influence of MPH on larval size and growth rate may be negligent. Moreover, this may be suggestive of the limited usefulness of post-feeding larvae in assessing the influence of xenobiotics on growth and thus future research should include earlier instars.

The findings of this study are dissimilar to those of Rezende *et al.* [20] at a similar MPH concentration (29 mg/Kg) and sampling times. Rezende *et al*. [20] demonstrated a possible species specific response with *C. megacephala* and *C. albiceps* displaying a trend of increased mean weight at the respective sampling times. *C. putoria* however, demonstrated a fluctuating trend with an inclination toward a decreased mean weight at the respective sampling times. Although the significantly increased weight observed in the treated larvae at 48 and 168 hours in Collection A show some similarity to the results observed from treated *C. megacephala* and *C. albiceps*, these findings should not be considered confirmative of an MPH induced effect in the *C. chloropyga* larvae used during this study, as two concordances in weight among trends of increasing weight are at best weakly suggestive of the same effect being observed. Moreover, regarding changes in larval weight, similarities between the findings of Rezende *et al*. [20] and this study are sporadic and therefore negligible.

3.5.3. <u>The effect of MPH on the duration of developmental stages</u>

Rezende *et al.* [20] also reported on the mean duration of each developmental stage for the three species investigated. Interpretations from their data suggest that MPH prolonged the total duration of the larval stage and subsequently, the total development time for *C.*

albiceps and *C. putoria*. Total development was prolonged by 24 hours for *C. albiceps* and 12 hours for *C. putoria*. Conversely, the total duration of the larval stage and total development time of *C. megacephala* was unaffected. Moreover, there was no indication that the duration of the pupal stage was affected. These findings contradict those observed in this study during which the total larval development was expedited and pupal development was prolonged (Fig. 4 and Table 4).

Treated larvae from Collection A reached the pupal stage 17 hours (198 h) before control larvae (215 h) and the duration of the pupal stage was prolonged by 16 hours. Treated larvae from Collection B reached the pupal stage 21 hours (168 h) before control larvae (189 h) and the duration of the pupal stage was prolonged by 11 hours (Table 4). A similar trend was observed by Mullany *et al*. [17] who demonstrated that methamphetamine, a psychostimulant with similar sympathomimetic effects to that of MPH, and p-hydroxymethamphetamine can expedite the larval stage of *Calliphora stygia* by up to 44 hours and prolong the pupal stage by up to 34 hours. Furthermore, the total development time of treated larvae in Collection A and B were expedited by 1 hour and 10 hours respectively. The disparity between these development times, caused by the shorter development time of treated larvae from Collection B, may also be attributed to larval density, as overcrowding has been suggested to shorten development time [48]. Furthermore, the difference in the duration of the larval stage of treated larvae between Collection A and B may similarly be attributed to overcrowding. Overcrowding may also be associated with metabolic heat generation from larval aggregations, known to influence development [52]. However, with the exception of treated larvae in Collection B, sampling populations within collections were maintained at relatable densities. Thus, any alteration

in development that may have occurred should still allow proportional comparisons to be made.

The differences between the duration of developmental stages and total development times of the controls from Collection A and B may be a consequence of inter-species competition. Several species were observed among the sampling populations (Fig. 1). The only noteworthy distinction between the control larvae of Collection A and B is the percentage composition of *C. chloropyga* in the sampling populations (Table 1). The sampling population was a mixed population of *C. chloropyga* and *Luciliinae* with the controls from Collection A having contained between 3 - 4 times more *Luciliinae* larvae than the control of Collection B. Moreover, the ratio of *Luciliinae* to *C.chloropyga* was 5 - 10 times greater in the controls from Collection A when compared to the ratio in the control of Collection B (results not shown). *Lucilia* spp. are avid competitors by being well adapted against adverse conditions [53]. Ullyett [53] suggested that the co-occurrence of *Luciliinae* and *C. chloropyga* on carrion may result in the reduction of the *C. chloropyga* population and survival of *Luciliinae*. This may be suggestive of competition between the species, and thus it is conceivable that the development time of *C. chloropyga* may have been prolonged due to possible adverse effects accompanying the competition. Interestingly, competition may not have been a result of an ephemeral food source as food was replaced as needed and larvae were maintained at less than 2 larvae/g liver.

Collection A required a longer development time compared to Collection B, despite uniform conditions being maintained between groups. Although the exact cause of the altered development cannot be speculated, it is likely a consequence of a complex interaction of

variables which require further investigation. Several experimental conditions have been suggested to influence blow fly development. These include food moisture, destructive sampling (permanent removal of larvae from the sampling population) [54], larval shelter and moisture [55]. The latter two conditions, in this instance, may relate to vermiculite depth which was not measured during this study. Wells and Kurahashi [55] postulated that pupariation may be delayed in cases of inadequate shelter or excess moisture. This suggests pupariation substrate depth as an independent variable. Furthermore, given that ambient humidity was relied upon, this likely contributed to variability owing to natural fluctuations and the different rearing times for Collection A and B. Regardless of the cause, it seems reasonable to assume that the test and control larvae from Collection A were proportionally affected, given the comparability of the duration of the developmental delay, when compared to the respective groups of Collection B (Table 4). Therefore, some measure of interpretative value is retained from Collection A.

Notwithstanding these possible confounding influences, the underlying trend of an expedited larval stage and prolonged pupal stage, and the similarity between the difference in the total duration of the larval (17 h and 21 h) and pupal (16 h and 11 h) stages, it is reasonable to conclude that there may be a possible communal cause of altered development that may be attributed to MPH.

3.5.4. The interaction of variables

Notwithstanding the differences between Rezende *et al.* [20] and this study already discussed, discrepancies may further be attributed to three possible causes namely: species specific responses to MPH, differences in the rearing substrate used, and

differences in the rearing conditions. Current knowledge of the absorption, distribution, metabolism and elimination (ADME) of xenobiotics in insects are limited [4, 56]. Consequently, it is difficult to conclude on the effects of a drug on insect development, or relate findings on the same drug between different species, due to possible inter-species pharmacokinetic differences [18, 56]. This may have been observed in the study by Rezende *et al.* [20] where varying results were obtained between the three species investigated. Therefore, it is conceivable that the *C. chloropyga* larvae used during this study may exhibit pharmacokinetic and/or pharmacodynamic processes different to those species already investigated.

Rezende *et al.* [20] reared *Chrysomya* spp. on artificial substrates spiked wih MPH. The artificial substrate contained powdered whole milk, beer yeast, Nipagin, casein, and agar [57]. During this study, MPH was spiked into macerated pig liver. Tachibana and Numata [58] demonstrated that the duration of the larval stage for *Lucilia sericata* may be prolonged when reared on artificial diets, compared to beef liver. Moreover, pupal weight was significantly less when reared on certain artificial diets. Therefore it is conceivable that larval weight may also be affected, thereby having a bearing on the findings of Rezende *et al.* [20], as the authors measured larval weight as a means to establish development curves.

C. chloropyga larvae were reared at 29.92 ± 0.56°C, ambient relative humidity (36.75 ± 5.82%) and a 14:10 hour L:D photoperiod. These conditions differ from those used by Rezende *et al.* [20], which reared larvae at 25 ± 1°C, 70 ± 10% humidity and a 12 hour photoperiod. Temperature [59], humidity [60] and photoperiod [61] have been known to

influence blow fly development. Moreover, environmental conditions have influences beyond developmental alterations. For example, the gut motility of insects which affects drug absorption and excretion is dependent on temperature [56]. Greenberg and Kunich [45] reported that the gut motility of larvae may be tripled at 31°C as opposed to 23°C. Furthermore, it has been suggested that the stability of drugs in tissues is dependent on, among others, temperature, pH and humidity [62]. Therefore, it is reasonable to conclude that variation of these conditions may alter the possible influence of a drug on insect development.

The methodological differences between this study and that of Rezende *et al.* [20], and the consequent reduced comparability of findings suggest that interpretations should be tentative. Future studies should compare blow fly populations with the same experimental set-up. This requires a standardisation of methodologies which may improve the reliability and confidence of inferences.

3.5.5. Detection of MPH

Treated livers and samples containing as few as three treated larvae screened positive for MPH. Pig liver was used in favour of animal models primarily because of ethical considerations and to isolate the effects of MPH as opposed to the metabolites potentially resulting from animal metabolism. Egger *et al.* (as cited in [63]) identified more than 20 metabolites in rats and 13 metabolites in dogs, resulting from MPH administration. Direct application of MPH to the pig liver also ensured that the larvae were exposed to a known concentration of MPH. Moreover, MPH was detected in approximately same aged samples killed and preserved by freezing at – 20°C and those killed with near boiling water (> 90°C)

and preserved in 70% ethanol and stored at 4°C (Figs. 5.1 and 5.2). MPH was detected in these samples to the same order of magnitude, albeit at a lower level in those preserved in ethanol. This may suggest that, despite concerns about drug stability and self-extraction from the matrix [56], insect specimens preserved in preservation liquid, such as ethanol, may be useful as qualitative toxicological specimens. Nevertheless, frozen samples are apparently more suitable for toxicological analysis [56], especially given the obvious limitation of ethanol preserved samples in deaths involving alcohol intoxication.

The concentration of MPH investigated may be an extreme example of Ritalin abuse not occurring frequently. It is possible that MPH may not be readily detectable at therapeutic doses given the labile nature of MPH. It has been reported that MPH only remains stable for up to 6 hours in whole blood when stored at room temperature [64]. However, given that MPH was detected from larvae incubated at approximately 30°C and sampled up to 3.5 days after replenishing the treated food source, may be indicative of an increased stability of MPH in larvae [56].

3.6. Limitations

3.6.1. Mixed population and laboratory cultures

This study demonstrates the importance of rearing laboratory stock cultures of blow fly species to conduct research. Blow fly eggs and early instars have homogeneous morphologies and are difficult, sometimes impossible, to distinguish between species [41, 42], without the appropriate key for identification and microscopic techniques. During this study the mixed populations complicated interpretations in the first 36 hours after hatching, before the third instar was reached and identification could be performed. Moreover, the

toxicological nature of this study prevented any dissection or chemical preparation of larvae, thereby eliminating the possibility of identification using histological techniques. Therefore, it is advisable to conduct such research with established laboratory cultures of a single, identified blow fly species to eliminate the need for early instar identification.

3.6.2. Sample size

During this study, the sample size (n=5) was, in part, a consequence of mixed populations. Data pooling was necessary to supplement the sample size as the random sampling did not always result in enough *C. chloropyga* larvae being sampled. In one instance only a single larva was collected and therefore the resulting length and weight measures were taken as the median. This was a consequence of blow flies that cannot readily be distinguished during early larval development based on macro-morphology, thereby resulting in an under-representation of *C. chloropyga*.

This reiterates the need for laboratory cultures of blow flies to establish sufficient population sizes. It is advisable to divide larval populations into several small populations to allow independent sampling for morphological identification and toxicological analysis. This will facilitate control over population sizes and eliminate the influence of metabolic heat production by larval aggregations. Furthermore, sufficient blow fly cultures will allow an increased sample size which will improve statistical power and thereby the reliability of inferences.

3.6.3. Data collection

The presence of mixed populations complicated efforts to ascribe observations from early instars to a specific species. Therefore, development times for each larval stage could not be established, but rather an estimate of the total duration of developmental stages. However, knowledge of the duration of each larval stage is of particular importance when investigating the influence of xenobiotics as it may cause an acceleration of development in one stage and deceleration in another, thereby having an effect that goes under-estimated, or unnoticed when only the total larval development is considered. Moreover, toxicological analysis of each development stage can provide insight as to drug excretion and identifying the most suitable specimens for toxicological analysis.

3.6.4. MPH concentration investigated

This study only investigated a single concentration, which may limit interpretations on the effect of MPH on blow fly development. However, the three concentrations of MPH investigated by Rezende *et al.* [20] indicated no dose-dependent effects among the three blow fly species investigated.

3.6.5. Toxicological analysis and drug distribution

The development of a validated LC-MS/MS protocol along with the investigation of possible matrix effects is recommended to improve the interpretive value of results. Moreover, during this study treated liver was manually mixed to achieve even drug distribution as an homogeniser was not available. However, the use of an homogeniser is recommended to ensure even drug distribution and thus increase the reliability of findings.

3.6.6. Replication of findings

During this study, confounding factors introduced too much variability between Collection A and B to be considered duplicates. This may be an inherent consequence of collecting blow fly eggs from placed bait. Therefore, laboratory rearing of blow fly cultures is recommended to facilitate the production of reliable replicates.

3.7. Conclusion

This study demonstrated the utility of *C. chloropyga* larvae as alternative toxicological specimens for the detection of MPH. MPH was detected from treated larvae preserved in ethanol and by freezing. This provides insight for the use of insects preserved in ethanol as toxicological specimens for qualitative analysis, except in death investigations involving alcohol intoxication. Furthermore, MPH could be detected from samples containing as few as three larvae, after 3.5 days incubation at ~30°C. This may be indicative of an improved preservation and stability of MPH in insect specimens, especially given the labile nature of MPH. Confounding factors complicated inferences regarding the possible influence of MPH on the size and growth rate of *C. chloropyga* larvae. However, the influence of MPH on the duration of developmental stages was evident. The most conservative estimation of the effects of MPH on the development of *C. chloropyga* is an expedited larval stage by up to 17 hours, and a prolonged pupal stage by up to 16 hours. These findings may vary, given the possible influence of confounding factors. However, the underlying trend of an expedited larval stage and prolonged pupal stage was observed in both collections. Therewith, our hypothesis is rejected as no impediment in larval development was apparent. This study suggests that, at the concentration investigated, MPH may alter *C.*

chloropyga development and consequently PMI estimates if MPH is not detected, or its effects not considered. These findings are preliminary in nature and require methodological refinement, and confirmation to assess its probative value and application to forensic investigations. Future studies should investigate the influence and detection of MPH at all developmental stages, particularly to expand the current understanding of drug excretion and accumulation at different stages. Moreover, different concentrations of MPH should be investigated to determine any dose-dependent effects. Quantitative detection of MPH and possible metabolites may provide insight to blow fly metabolism of MPH and should thus be investigated. This study underpins the importance of understanding the interaction of several variables on blow fly development. Thus, further investigation is required to establish, not only the implications of variable interaction on blow fly development, but also the interactions of xenobiotics with variables, and the subsequent influences on development and PMI estimates.

3.8. References

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Chapter 4. Appendices

Appendix A - Highlights

- *C. chloropyga* larvae are suitable for MPH detection.
- MPH shortens the larval stage and prolongs the pupal stage.
- Ethanol preserved specimens retain qualitative toxicological utility.

Appendix B - Acknowledgements: Manuscript

We would like to thank Dr Cameron Richards for his valued opinion on the identification of *C. chloropyga.*

We wish to extend our gratitude to Mr Calvin Gerald Mole for his valued consultation regarding statistical analyses.

We extend our deepest gratitude to Professor Peter Smith and the Division of Clinical Pharmacology for their assistance in performing toxicological analyses.

We extend our gratitude to Winelands Pork for the supply of pig liver and offal without which this study would not be possible.

Appendix C - Author contributions

Hartwig Visser – Conceived, designed and performed the experiments. Furthermore, he performed the data analysis, interpretation and wrote the main article.

Dr Marise Heyns – Supervised the experiments and managed logistical aspects such as the budget and acquisition of required materials. Furthermore, she reviewed written drafts and contributed, particularly, to forensic entomological aspects.

Ms Bronwen Davies – Co-supervised the project and contributed to the management of logistical aspects. Furthermore, she served as liaison to the Division of Clinical Pharmacology. She also reviewed written drafts and contributed, particularly, to forensic toxicological aspects.

All authors have read and approved the final manuscript.

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- Aging adult skeletal remains

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Reference to a chapter in an edited book:

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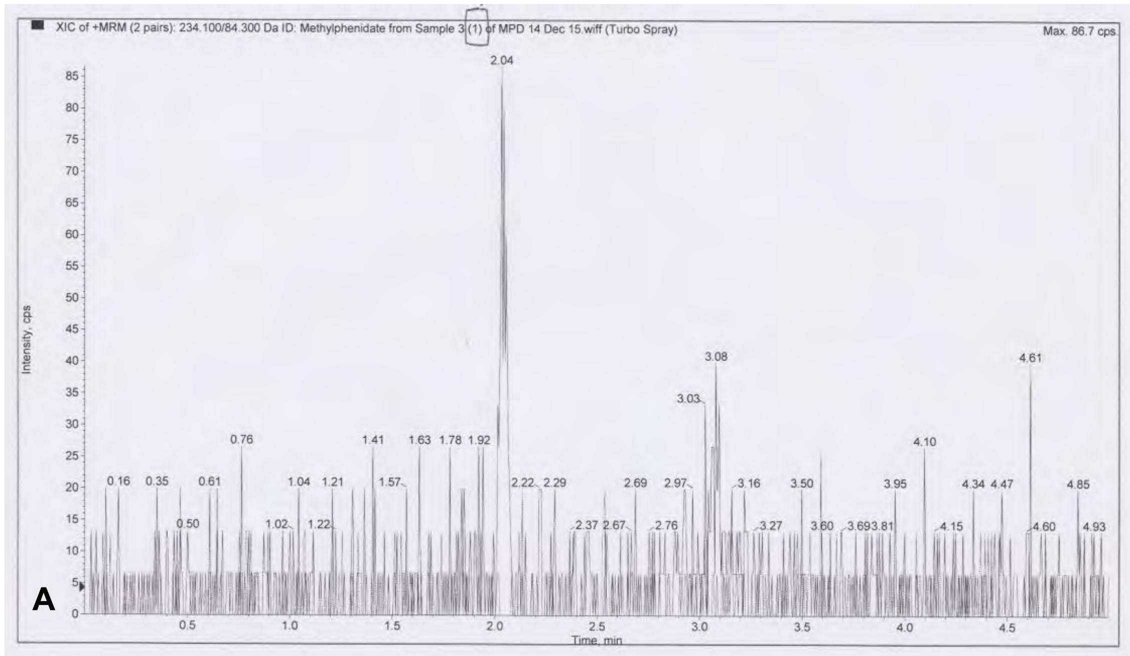
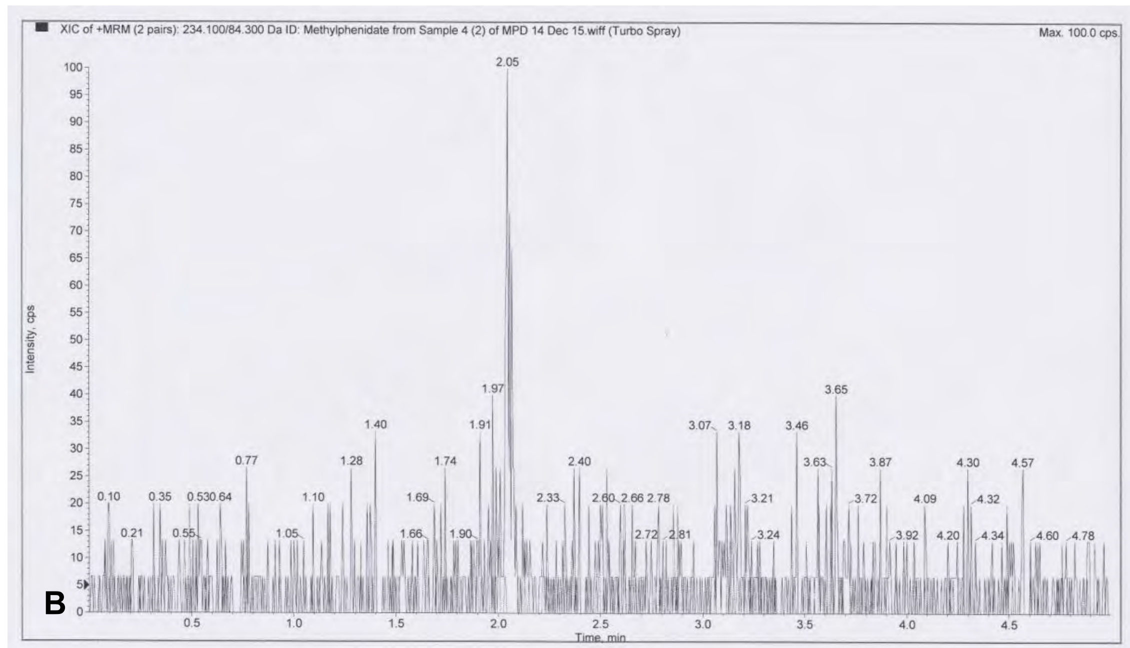
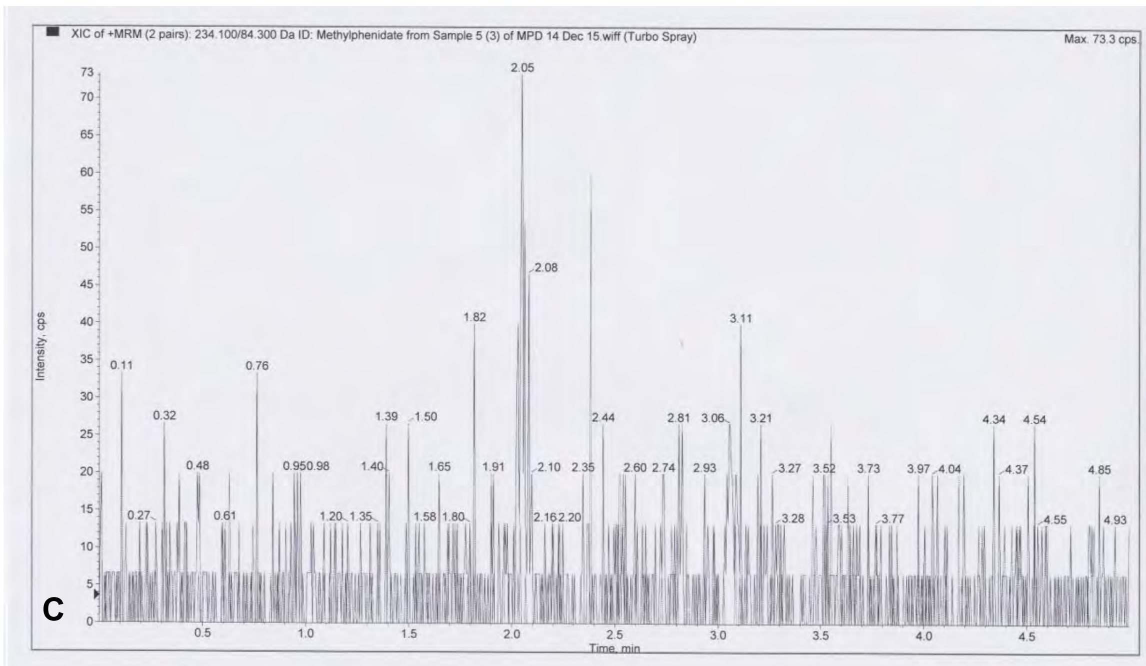
Appendix F - Total ion chromatograms (TIC) for control and MPH treated larvae



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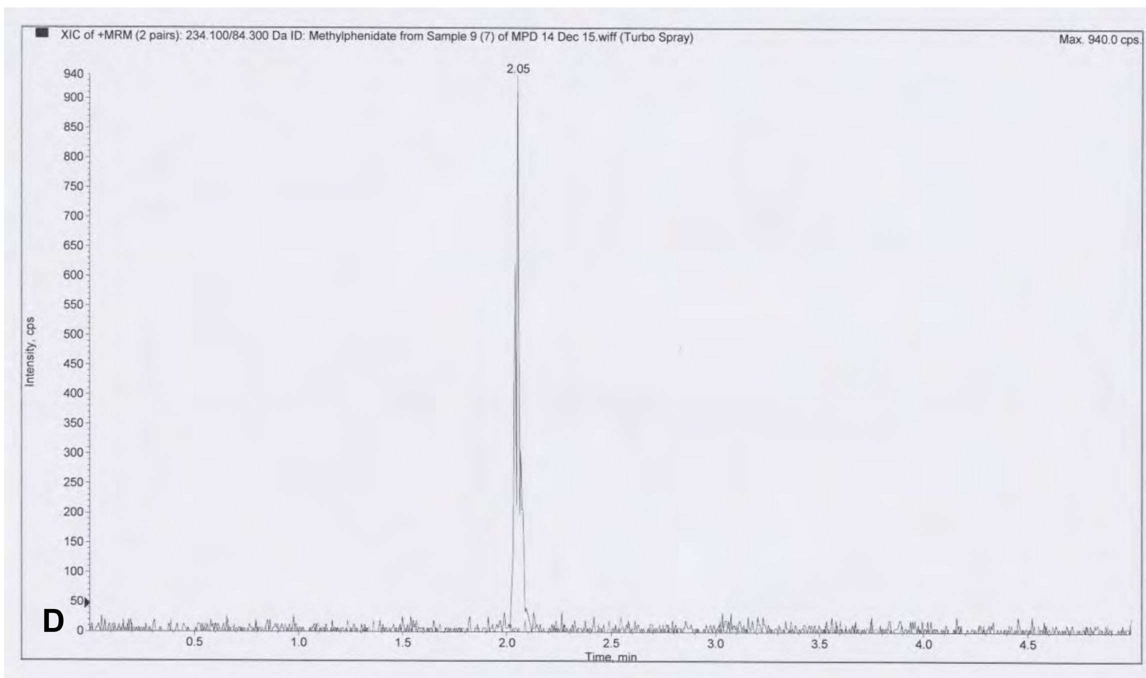
Intensity, cps
Time, min
C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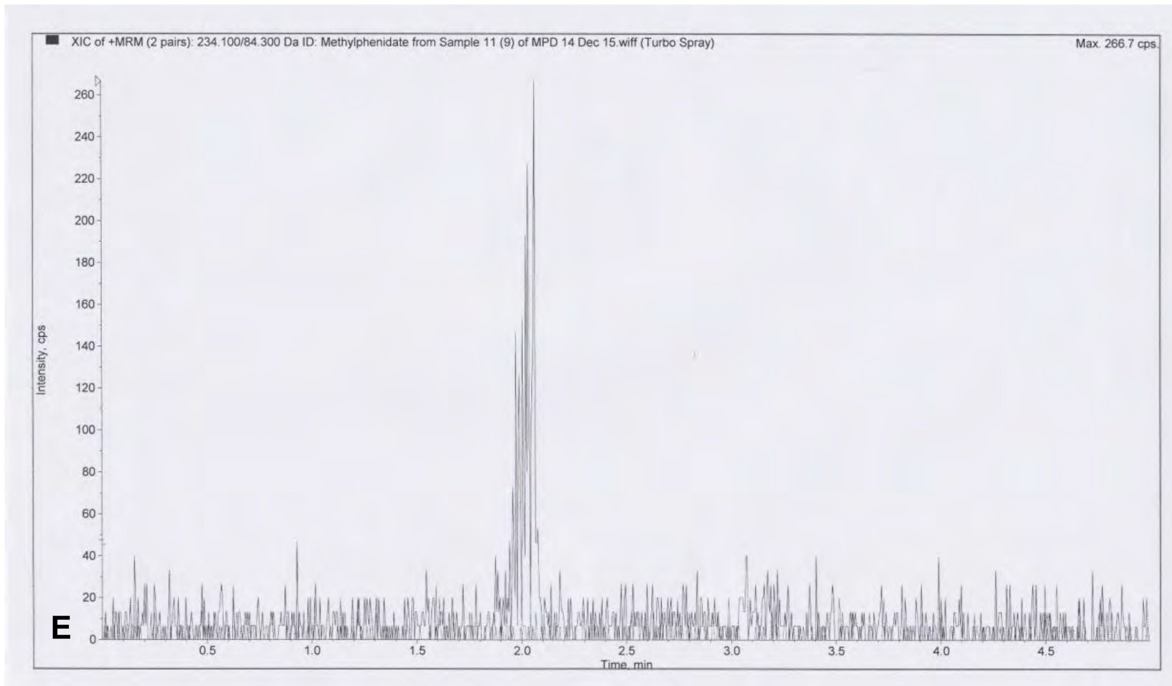
D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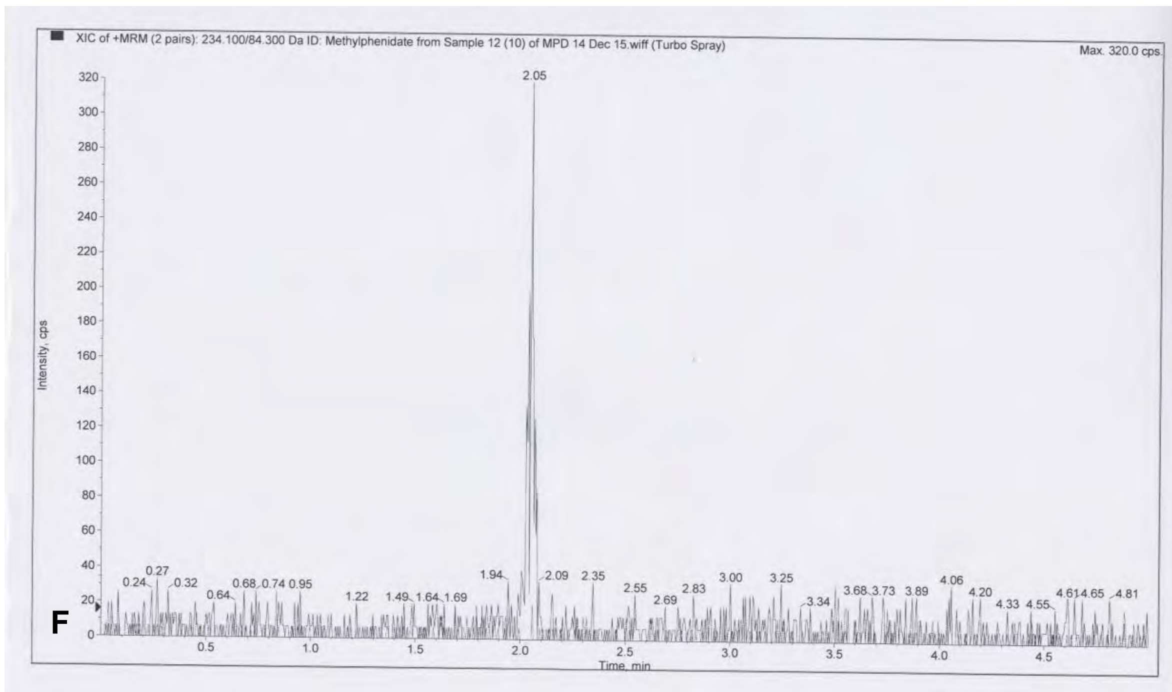
Time, min
E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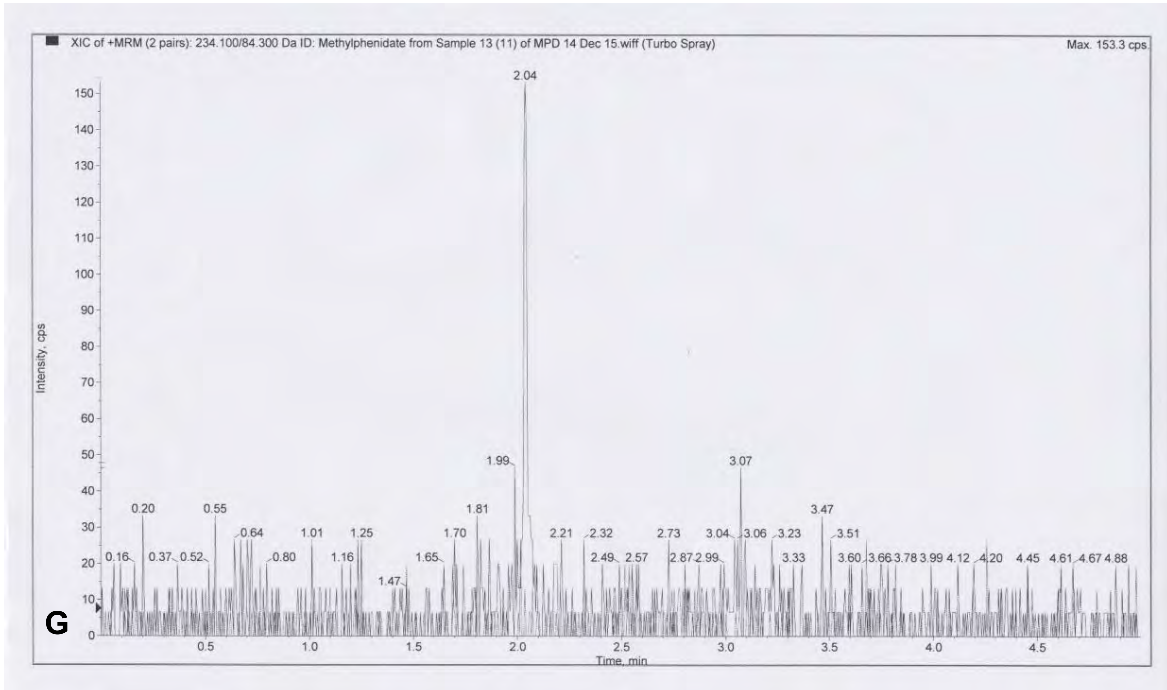
Time, min
F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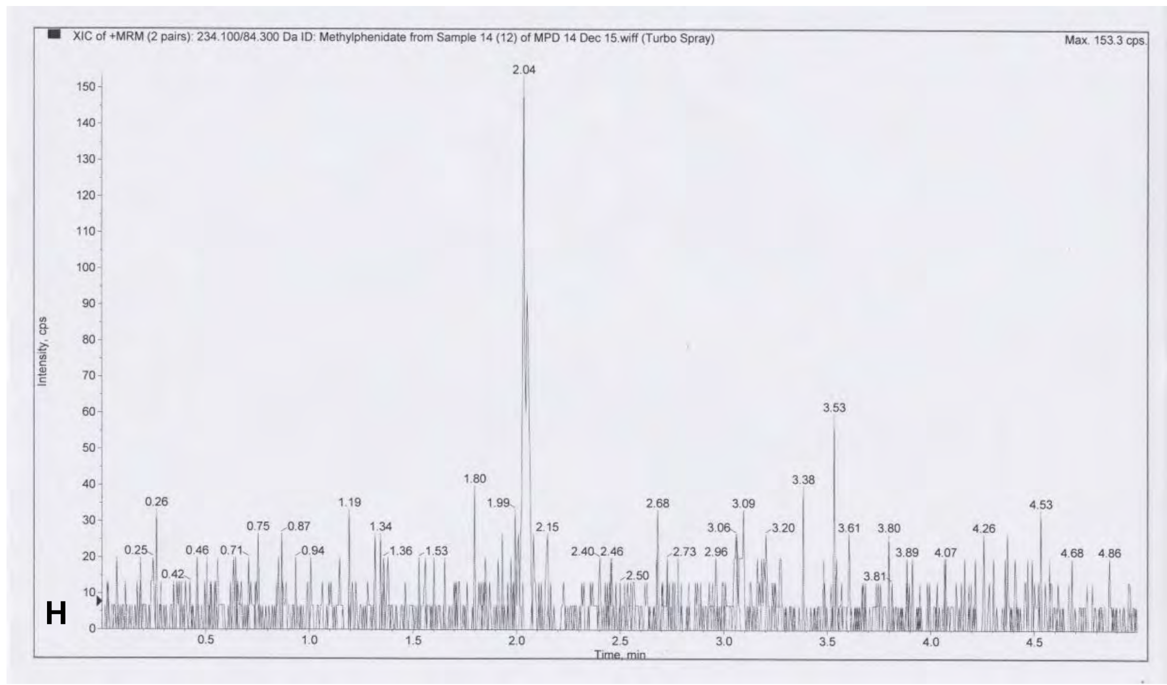
Max. 153.3 cps.
Intensity, cps
Time, min
G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Intensity, cps
Time, min
H

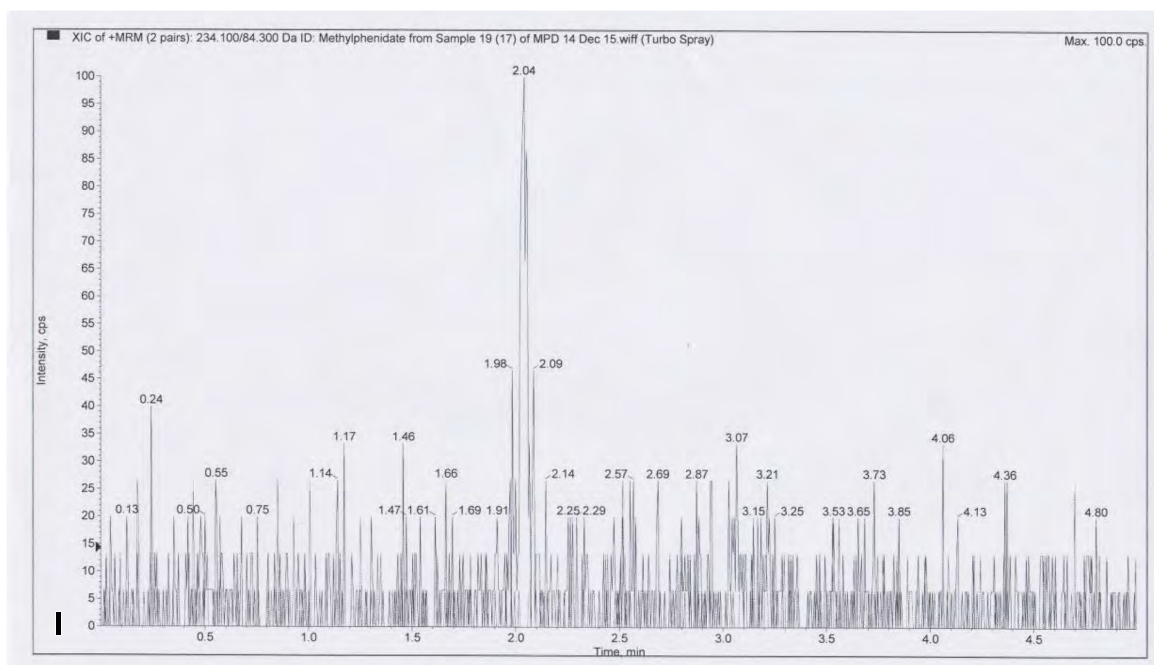
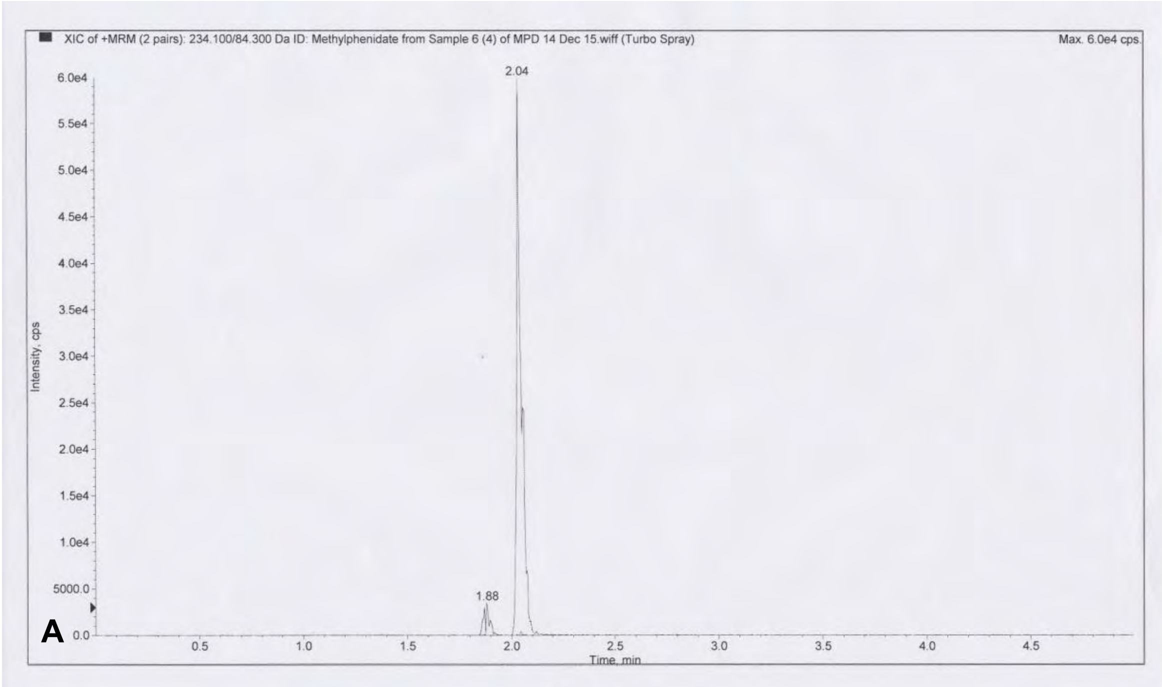


Figure 6.1. Total ion chromatograms (TIC): A-C and E and F – Selected *C. chloropyga* control larvae from Collection A (24 hours) and B (48 hours) killed in near boiling water (> 90°C) and preserved in 70% ethanol at 4°C; G and H- Control *C. chloropyga* larvae killed and preserved by freezing at – 20°C; D and I – Control pig liver.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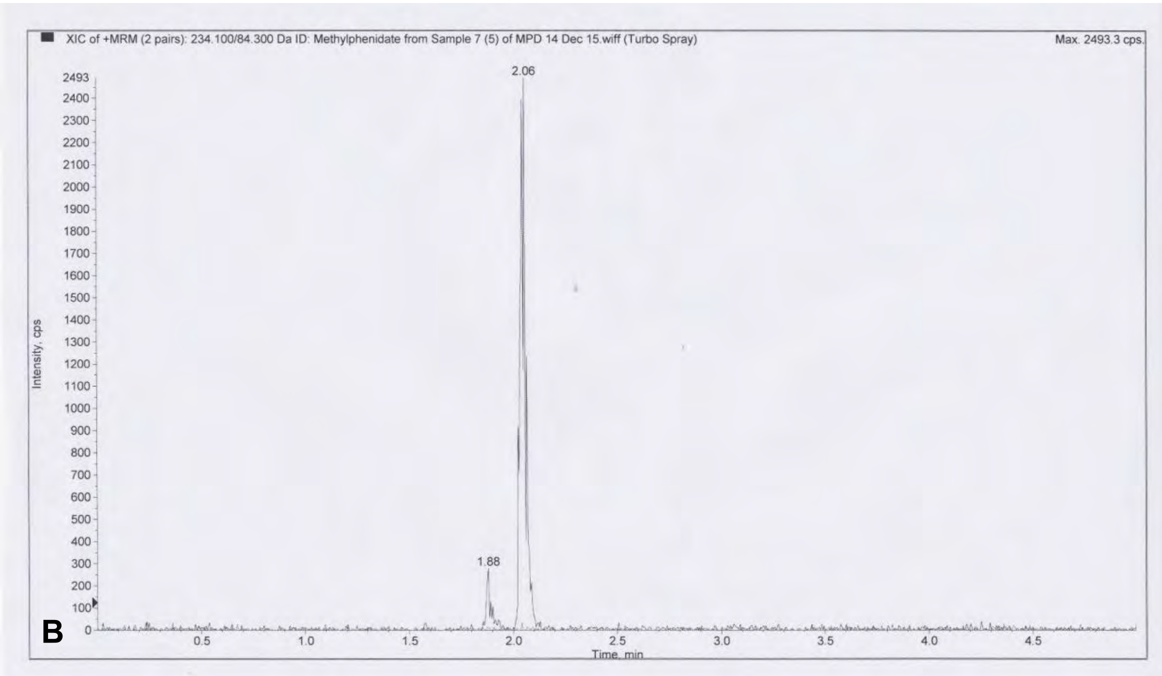
2.04
1.88
Intensity, cps
Time, min
A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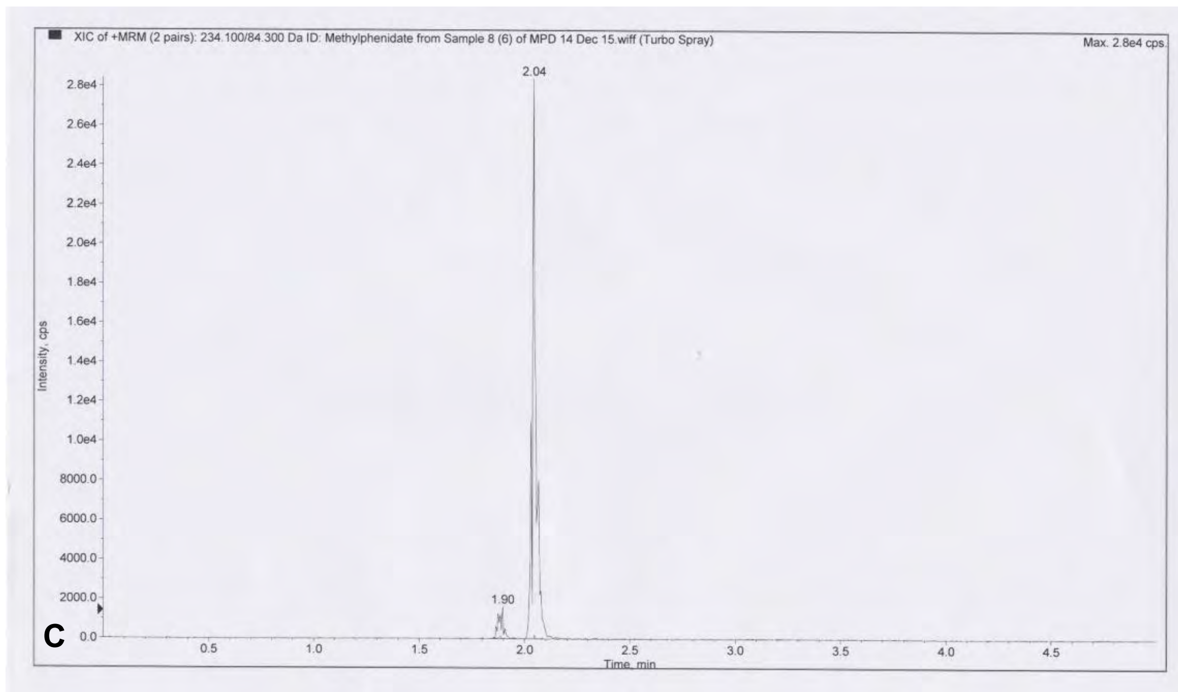
1.88
Intensity, cps
Time, min
B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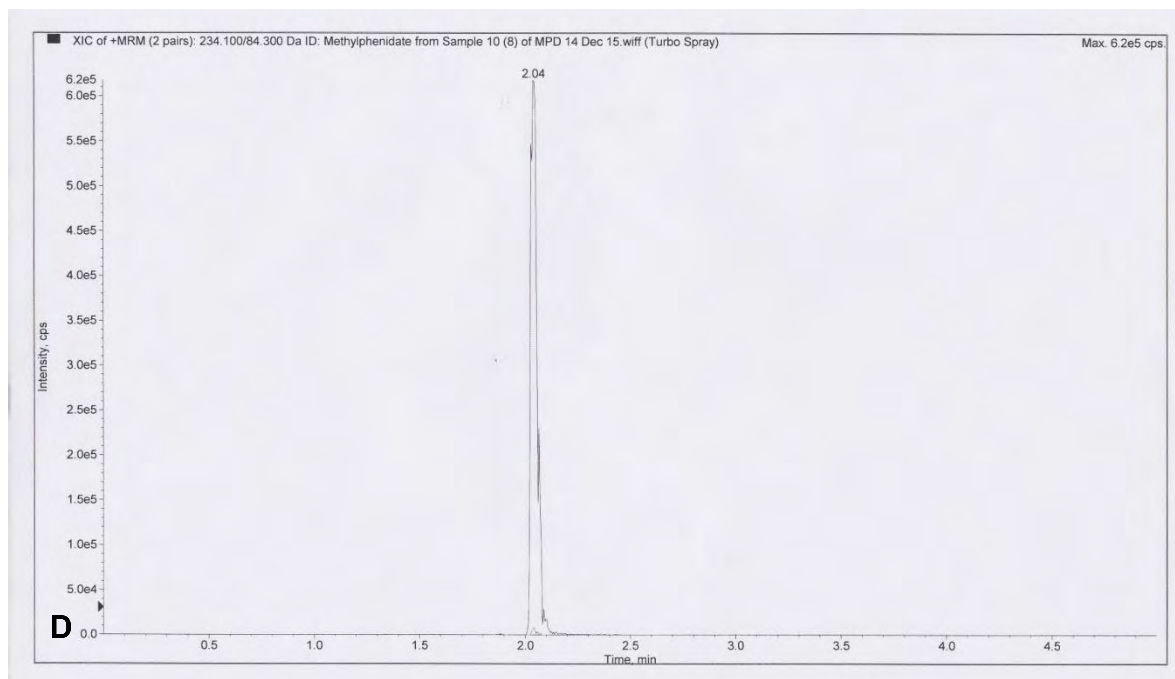
1.90
Intensity, cps
Time, min
C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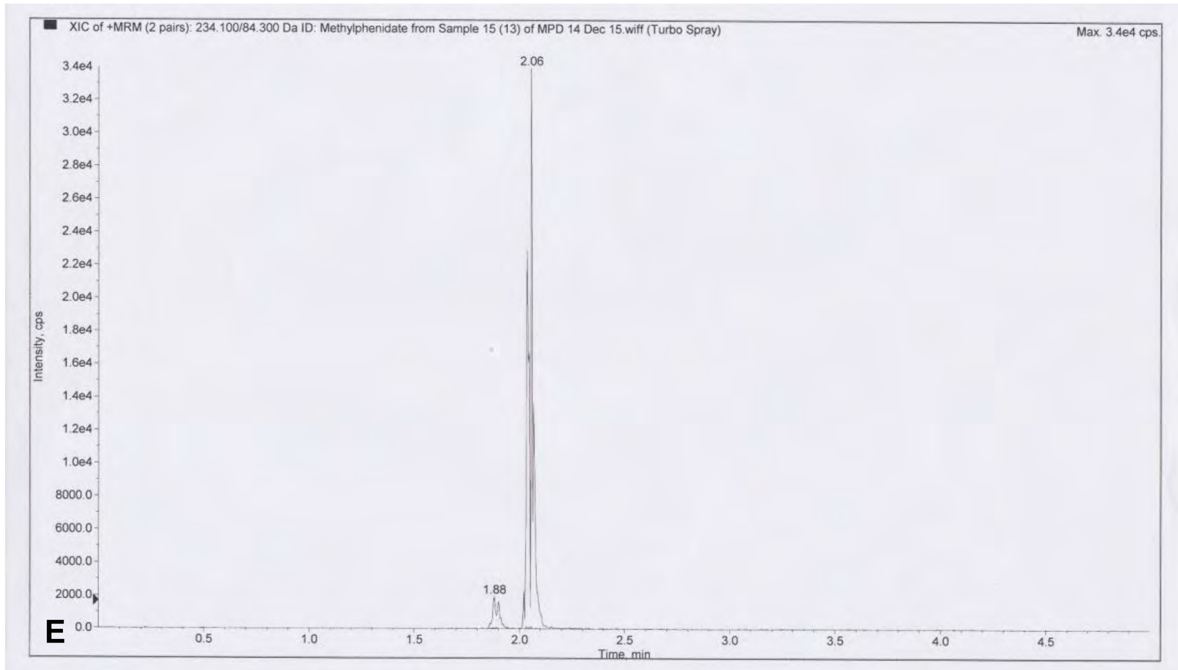
Intensity, cps
Time, min
D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Intensity, cps
Time, min
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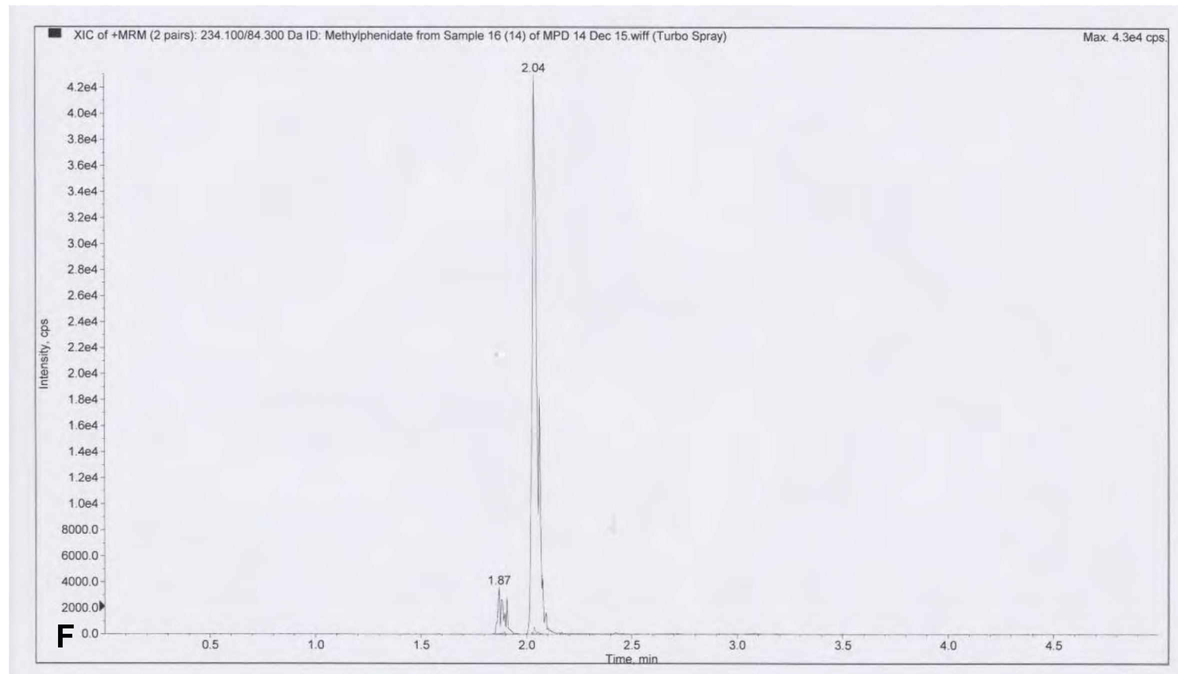
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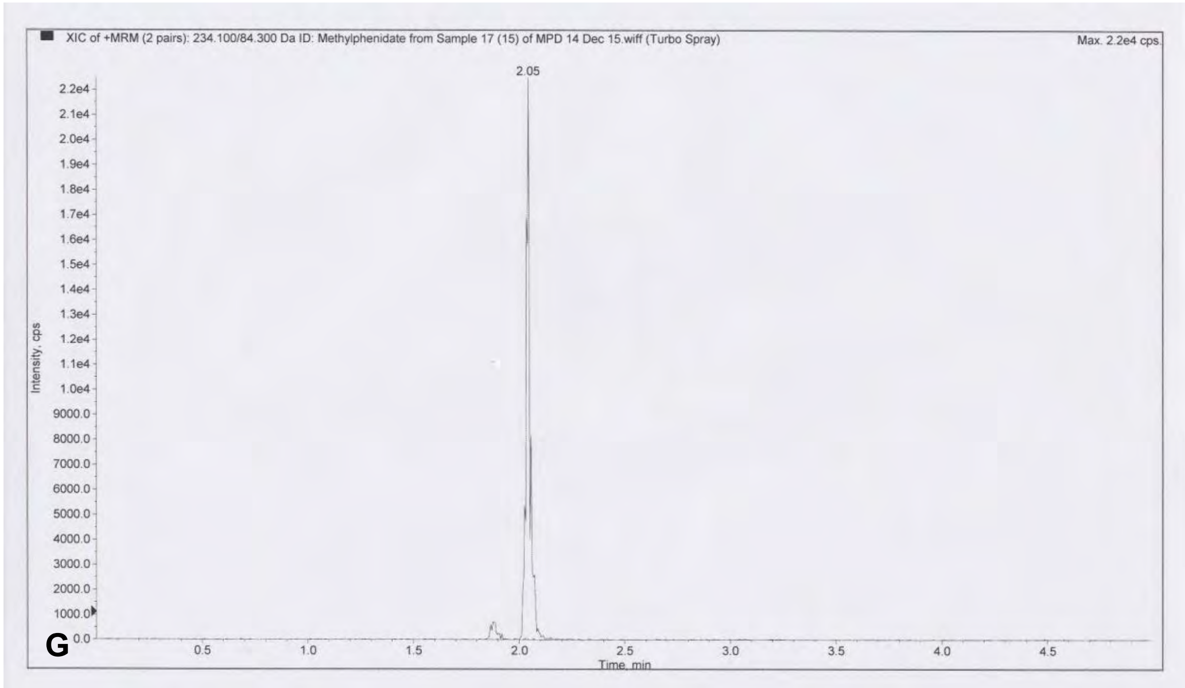
Time, min
F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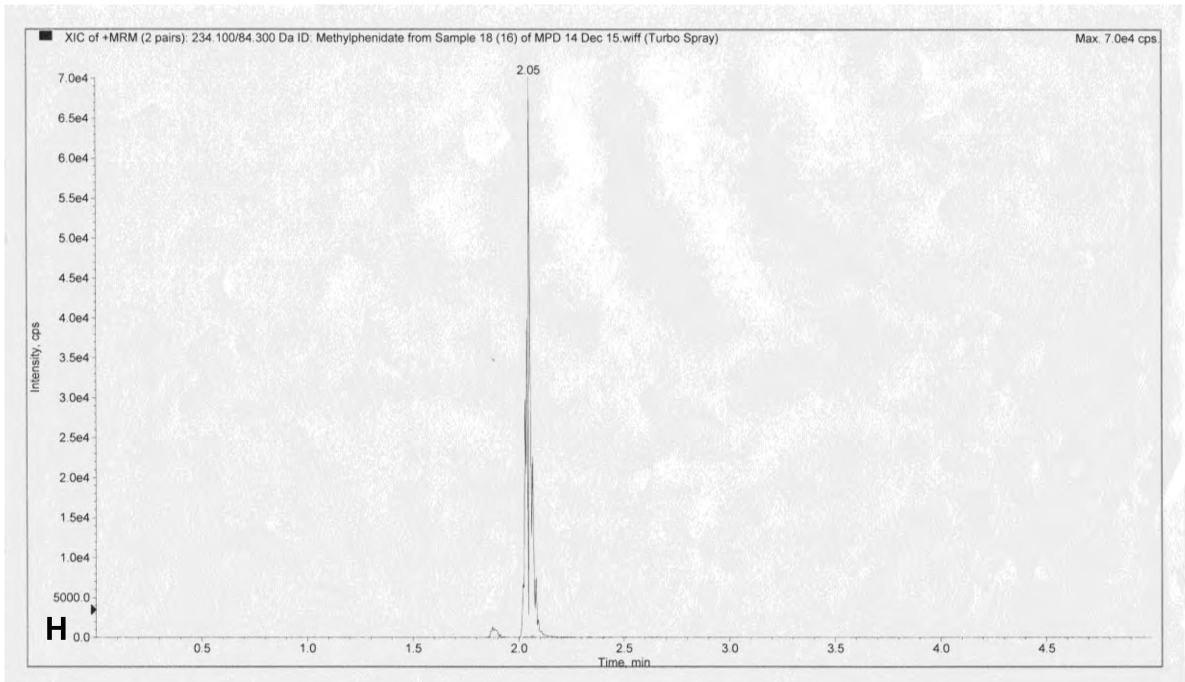
Intensity, cps
Time, min
G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Intensity, cps
Time, min
H

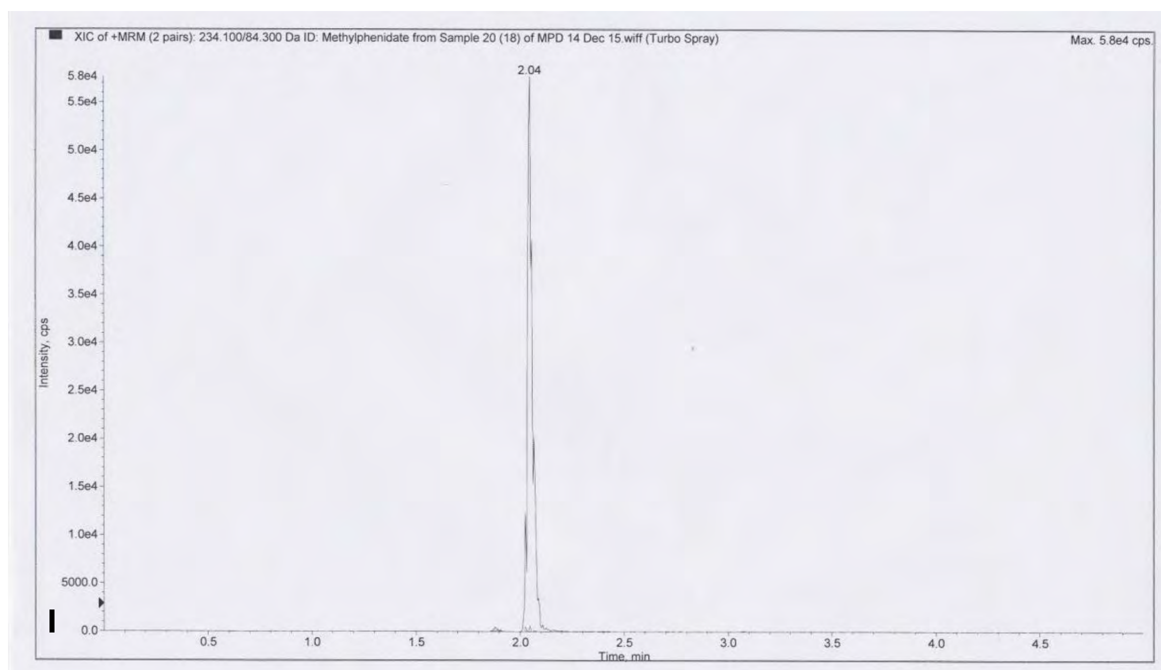
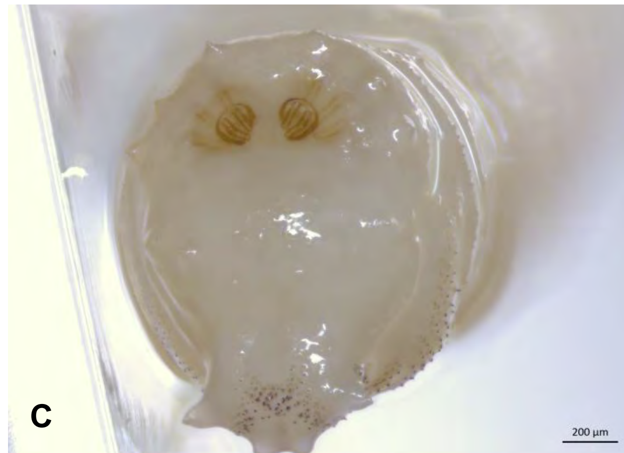
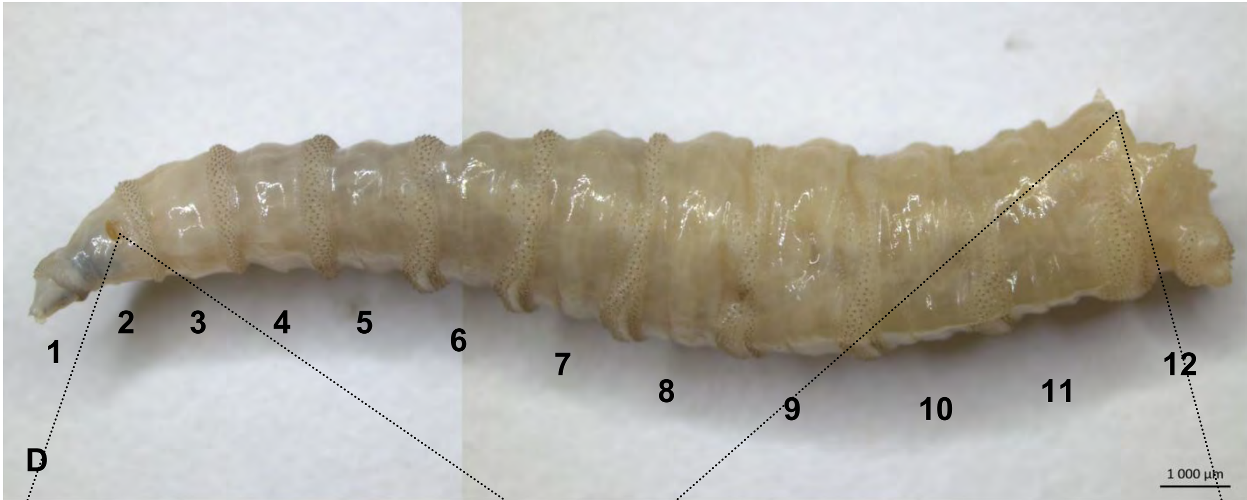


Figure 6.2. Total ion chromatograms (TIC): A-C and E and F – Selected *C. chloropyga* treated larvae from Collection A (24 hours) and B (48 hours) killed in near boiling water (> 90°C) and preserved in 70% ethanol at 4°C; G and H- Treated *C. chloropyga* larvae killed and preserved by freezing at – 20°C; D and I – Treated pig liver.

Appendix G – *C. chloropyga* life stages




1
2
3
4
5
6
7
8
9
10
11
12
D
1 000 µm

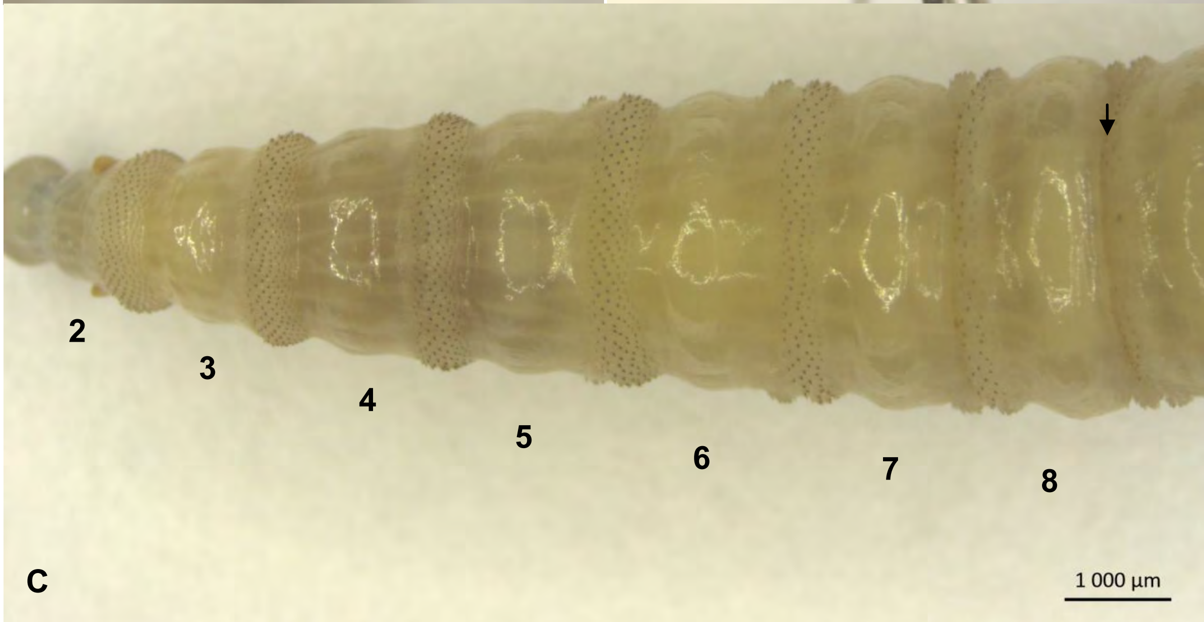
E
100 µm

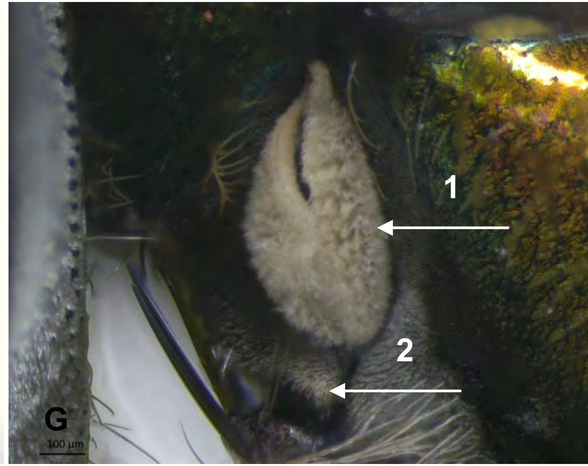
F
500 µm

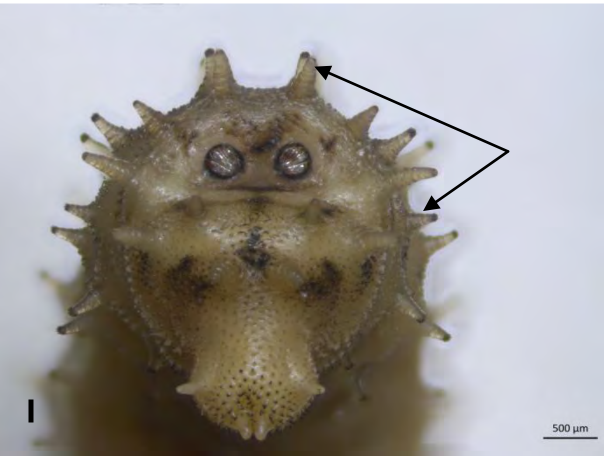
G

Figure 7. *C. chloropyga* life stages: A- first instar larva; B- second instar larva; C- transitioning second instar larva showing the underlying posterior spiracle with three respiratory slits; D- segments of mature third instar larva, lateral view; E- anterior spiracle showing 11 branches; F- heavy sclerotised, incomplete peritreme of the posterior spiracle; G- progressive darkening of puparium with age; H- newly emerged, unpigmented fly; **I**- adult fly with hardened, pigmented exoskeleton.

Appendix H – Identifying features of blow flies from the sampling population



F
1 000 µm

1
2
G
100 µm

H
1 000 µm

I
500 µm

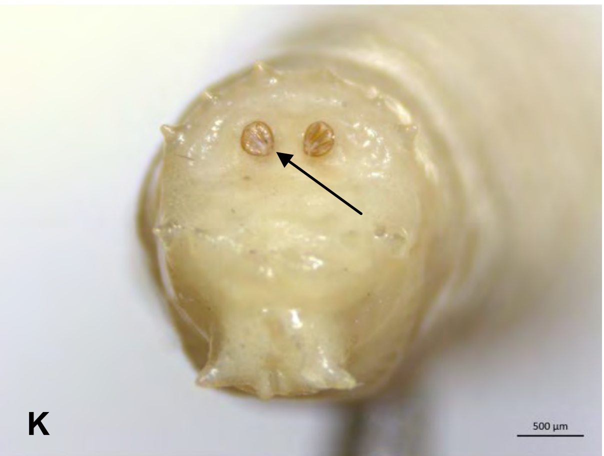
J
1 000 µm

K
500 µm

Figure 8. Identifying characteristics: A- *C. chloropyga*, black ┘└ pattern on the presutural region of the mesonotum; B- *C. chloropyga*, green-yellow discolouration of last two abdominal segments; C- *C. chloropyga*, complete spinose girdles between segments 2-7 and start of incomplete girdles; D- *C. chloropyga*, heavy sclerotised, incomplete peritreme; E- *C. chloropyga,* anterior spiracle showing 11 branches; F- *C. albiceps*, thick, dark bands on the terminal edges of the abdominal segments; G1- *C. albiceps*, pale yellow anterior thoracic spiracle, G2- absence of pro-episternal seta; H and I- *C. albiceps*, fleshy protrusions "spikes" across larval body; J- *Luciliinae*, three pairs of postsutural, acrostichal bristles; K- *Luciliinae*, complete peritreme; L- *Luciliinae,* bare lower calypter; M- *Luciliinae*, yellow basicosta.

Appendix I - Acknowledgements: Dissertation

I am indebted to my supervisors Dr Marise Heyns and Ms Bronwen Davies without whom I would be lost. Their tireless support and optimistic approach to presenting problems greatly assisted in its resolution.

I would also like to thank Mr Calvin Gerald Mole for his assistance, beyond statistical analyses, and sharing his experience in academic writing.

I would like to thank the National Research Foundation (NRF) for providing the necessary funding that allowed the pursuit of this course.

I would also like to thank my loving wife-to-be for her continuous support and understanding, and invaluable role as a soundboard through countless one-sided debates. Your support and understanding was tested beyond measure and I have not yet encountered, nor do expect to, a person more capable of gracefully accepting difficult circumstances particularly as this included my absence or pre-occupation with this project over the festive season, Valentine's day, and your birthday.

Appendix J – Ethics approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Animal Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 404 7682 • **Facsimile** [021] 406 6411
Email: nosi.tsama@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/animalethics/forms

23 November 2015

Mr H Visser
C/o Dr M Heyns
Forensic Medicine & Toxicology
Falmouth Building

Dear Mr Visser

PROTOCOL TITLE: THE INFLUENCE OF METHYLPHENIDATE ON THE DEVELOPMENT OF A FORENSICALLY SIGNIFICANT BLOWFLY (DIPTERA: CALLIPHORIDAE) IN THE WESTERN CAPE PROVINCE

FHS AEC REF NO: 015/020

Thank you for submitting your protocol to the Faculty of Health Sciences (FHS) Animal Ethics Committee (AEC) for review.

I am pleased to inform you that the FHS AEC has **authorised** your protocol, this authorisation is of limited duration and will terminate on 30 November 2018. If the project is to continue beyond that date, it must be reviewed not less than on annual basis and in accordance with the AEC policy.

Any modification to the study that affects or alters the use of animals or otherwise departs from the approved version of the protocol must receive prior approval from the AEC as an amendment of protocol.

Number of animals & species: Diptera

Please quote the FHS AEC REF NO (above) in all future correspondence.

Please note that the approval of this protocol imposes the following obligations on the principal investigator (PI):

1. To submit an annual mandatory progress report. The first annual report for this protocol is due on **29 February 2016**. The forms can be accessed from http://www.health.uct.ac.za/fhs/research/animalethics/forms

AEC REF# 015/020

2. To submit a final mandatory report on the **30 November 2018**, please access the final report form from: http://www.health.uct.ac.za/fhs/research/animalethics/forms
3. To ensure that all study participants perform within the confines of the procedures and experimental design of the protocol as authorised, or as amended.
4. Ensuring that all study participants comply with all applicable national legislation, UCT policies, FHS AEC policies and standard operating procedures (SOPs) and national standards (SANS 10386: 2008).
5. To ensure in your capacity as the PI (principal investigator) that you immediately alert the FHS AEC to any event involving the welfare of the animals which has occurred during the course of the study, as well as the actions that were taken to respond to these events.
6. To ensure in your capacity as the PI (principal investigator) that you alert the FHS AEC to any new or unexpected ethical issues that arose during the course of the study, and how these issues were addressed.
7. To ensure that research is conducted in duly registered facilities in accordance with the South African Veterniary Council Rule 32 (as applicable) and that all key personnel are registered with and/or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of SAVC-registered veterinary professionals or SAVC-registered para-veterinary professionals.
8. To report any instance of an animal discovered to be dead to the RAF on the appropriate form: http://www.health.uct.ac.za/fhs/research/animalethics/forms
9. To report any instance of an animal found in distress to the RAF on the appropriate form.
10. To consult with the AEC in regard to any confusion or uncertainty about how to respond to any of the obligations mentioned herein, how to deal with any of the issues mentioned herein, or otherwise conduct animal research responsibly and in a manner consistent with applicable UCT policies.

My best wishes for a successful research and /or teaching endeavour.

Yours sincerely

PROF PJ COMMERFORD
CHAIR, FHS AEC

FHSAEC 015/022

Appendix K - Project budget

Item	Cost/item	Nr required	Total cost	Justification	Cost incurred
Pig liver	R 12/ kg excl vat	10	R 136.8 incl vat	Rearing food	None - donation
Offal	R 12/ kg excl vat	10	R 136.8 incl vat	Bait	None - donation
Plastic containers	R 50	6	R 300	Three replicates	R 300
Vermiculite	R 199.64/8 kg	1	R 199.64	Pupariation substrate	None - in stock
Foil	R 22.99/ 5 m	1	R 22.99	Food placement surface	None - in stock
Sample containers	R 623.34/ 500	1	R 623.34	Sample preservation	None - in stock
Petrol	~R 12/L	-	~R 216	At 10 km/l: MRC Delft (research site) is ~30 km from Cape Town, and a maximum of 3 days would be required for sufficient specimen collection. Thus (30 km x 2 – round trip x 3 = 180 km/ 10 km/l = 18 L petrol) ~R 216	R 350
Methylphenidate	R 631.62/ month supply (10 mg)	2	R 1263.24	Drug of investigation	R 1263.24
Methanol	R 695.44/ L	1	R 695.44	LCMS (Mobile phase)	None - done by Pharmacology
Ethanol	R 4211.27/ 2.5 L	2	R 8422.54	Sample preservation & general use	None - in stock
Dichloromethane	R 1314.03/ L	1	R 1314.03	Drug extraction	None - done by Pharmacology
Hexane	R 1426.67/L	1	R 1426.67	Drug extraction	None - done by Pharmacology
Sodium carbonate	R 558.05/ kg	1	R 558.05	Drug extraction	None - done by Pharmacology
Ammonium formate	R 951.22/ 100 mL	1	R 951.22	LCMS (Mobile phase)	None - done by Pharmacology
Acetonitrile	R 2638.14/ L	1	R 2638.14	LCMS (Mobile phase)	None - done by Pharmacology
Waste removal Bag	R 20.57/ bag	5	R 102.85	Animal and general waste	R 41.14
Cable tie	60 c ea	5	R 3		R 1.20
Waste	R 19.47/ kg	15	R 292.05		R 97.35
Disposable gloves	R 617.23/ 100	2	R 1234.46	General use	None - in stock
		Total projected cost	**R 20537.26**	**Total cost incurred**	**R 2052.93**