

**ENTOMOLOGICAL EXAMINATION OF THE RELATIONSHIP  
BETWEEN ANTE-MORTEM AND  
POST MORTEM AMITRIPTYLINE CONCENTRATIONS  
IN INSECTS**

By

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MPhil (Biomedical Forensic Science)

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## ABSTRACT

When the death of an individual has occurred, the body of the deceased is not always discovered immediately and at times the body may be discovered after a long period of time. The consequence of discovering a body after a long period of time is that the body may be found severely decomposed or skeletonized. As a result no viable blood, urine or tissue samples may exist that can be collected and utilized in toxicological analyses. Entomotoxicology offers a supplementary method to detect and analyse the presence of drugs post-mortem, especially in cases where viable toxicological specimens such as human tissue cannot be obtained. In South Africa and globally, standardised methodology is required to perform entomological examinations accurately, however due to the large variation of experimental set-up no such standardised methods yet exist. The main aim of the research was to analyse the effect of Amitriptyline on the development and growth rate of forensically important blow flies, and to investigate the potential in using blow fly larvae of the Western Cape, South Africa in forensic entomotoxicological analyses and future implementation. To achieve this blow fly species *C. chloropyga* and *L. sericata* were reared on homogenised pig liver containing 1000 mg/kg Amitriptyline until emergence of imago. The duration of time taken by the blow fly larvae species to reach 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, pupae and imago growth stages in the presence and absence of Amitriptyline was noted. The presence of drugs in larvae was investigated using high-pressure liquid chromatography coupled with dual mass spectrometry (HPLC/MS-MS). Amitriptyline was detected in all experimental larvae samples and was found to delay pupation by at least 26 hours and imago emergence by at least 72 hours. Amitriptyline however showed no distinct effect on *C. chloropyga* length and mass but was associated with a smaller length and mass in *L. sericata* compared to controls. Results indicate that entomotoxicology can be of use for qualitative analysis for the presence of Amitriptyline in forensic cases. Results also highlight the unpredictability of research using biological specimens.

## TABLE OF CONTENTS

<b>DECLARATION</b> .....	<b>I</b>
<b>TURNITIN REPORT</b> .....	<b>III</b>
<b>ABSTRACT</b> .....	<b>VII</b>
<b>TABLE OF CONTENTS</b> .....	<b>VIII</b>
<b>LIST OF FIGURES</b> .....	<b>X</b>
<b>LIST OF TABLES</b> .....	<b>XI</b>
<b>LIST OF ABBREVIATIONS</b> .....	<b>XII</b>
<b>DEDICATION</b> .....	<b>XIII</b>
<b>CHAPTER ONE: PROJECT PROPOSAL</b> .....	<b>1</b>
<b>Introduction</b> .....	<b>2</b>
<b>Entomotoxicology</b> .....	<b>2</b>
<b>Entomotoxicology Research</b> .....	<b>5</b>
<b>Research Problem Statement</b> .....	<b>5</b>
<b>Aim</b> .....	<b>6</b>
Objectives: .....	<b>6</b>
<b>Methodology</b> .....	<b>6</b>
Rearing of larvae on spiked food sources .....	<b>6</b>
Development studies .....	<b>7</b>
Instrumental analysis.....	<b>7</b>
<b>References</b> .....	<b>9</b>
<b>PROPOSAL ADJUSTMENTS</b> .....	<b>11</b>
<b>CHAPTER TWO: REVIEW OF LITERATURE</b> .....	<b>12</b>
<b>Introduction</b> .....	<b>13</b>
<b>Amitriptyline</b> .....	<b>13</b>
<b>Mechanism of action of Amitriptyline</b> .....	<b>14</b>
<b>Depression and suicide</b> .....	<b>17</b>
<b>Blow flies and Forensic Entomology</b> .....	<b>17</b>
<b>Entomotoxicology</b> .....	<b>21</b>
<b>Rearing of blow flies</b> .....	<b>24</b>
<b>Blow fly larvae sample preparation and toxicological analysis</b> .....	<b>26</b>
<b>Concluding remarks on Entomotoxicology Research</b> .....	<b>27</b>

<b>Research Problem Statement.....</b>	<b>28</b>
<b>References.....</b>	<b>29</b>
<b>CHAPTER THREE: PUBLICATION READY MANUSCRIPT .....</b>	<b>34</b>
<b>Abstract.....</b>	<b>35</b>
Keywords: .....	35
<b>1. Introduction.....</b>	<b>35</b>
<b>2. Methodology .....</b>	<b>37</b>
2.1. Reagents .....	37
2.2. Rearing of larvae for Amitriptyline analysis.....	37
2.3. Sampling of larvae .....	38
2.4. Identification of maggot species and developmental stage.....	38
2.5. Statistical analysis .....	38
2.6. Amitriptyline extraction .....	38
2.7. Instrumental analysis.....	39
<b>3. Results and Discussion.....</b>	<b>39</b>
3.1. Effect of Amitriptyline on blow fly development.....	39
3.2. Effect of Amitriptyline on blow fly mass .....	41
3.3. Effect of Amitriptyline on blow fly length .....	43
3.3. Amitriptyline toxicological analysis .....	47
3.4. Limitations .....	48
<b>4. Conclusion .....</b>	<b>48</b>
<b>Acknowledgements .....</b>	<b>49</b>
<b>Conflict of Interest .....</b>	<b>49</b>
<b>Ethical approval.....</b>	<b>49</b>
<b>References.....</b>	<b>50</b>
<b>APPENDICES .....</b>	<b>52</b>
<b>APPENDIX A: ETHICAL APPROVAL LETTER.....</b>	<b>53</b>
<b>APPENDIX B: ACKNOWLEDGEMENTS .....</b>	<b>55</b>
<b>APPENDIX C: AUTHOR GUIDELINES .....</b>	<b>56</b>

## LIST OF FIGURES

<b>CHAPTER ONE: PROJECT PROPOSAL .....</b>	<b>1</b>
<b>CHAPTER TWO: REVIEW OF LITERATURE .....</b>	<b>12</b>
Figure 1: Chemical structure of Amitriptyline and its primary metabolite nortriptyline.....	14
Figure 2: Spiracle slits associated with larval developmental stages first instar, second instar and third instar. ....	18
<b>CHAPTER THREE: PUBLICATION READY MANUSCRIPT .....</b>	<b>34</b>
Figure 1: Length comparison of blow fly larvae in the absence of Amitriptyline. ....	44
Figure 2: Length comparison of blow fly larvae in the presence of Amitriptyline.....	45
Figure 3: Comparison of average blow fly larvae length between Control 1 and Treatment 1 .....	45
Figure 4: Length comparison of blow fly larvae between Control 2 and Treatment 2 .....	46
Figure 5: Length comparison of blow fly larvae between Control 3 and Treatment 3 .....	47
Figure 6: Selected example of HPLC chromatogram showing the presence of Amitriptyline.....	48
<b>APPENDICES .....</b>	<b>52</b>

## LIST OF TABLES

<b>CHAPTER ONE: PROJECT PROPOSAL .....</b>	<b>1</b>
<b>CHAPTER TWO: REVIEW OF LITERATURE .....</b>	<b>12</b>
<b>CHAPTER THREE: PUBLICATION READY MANUSCRIPT .....</b>	<b>34</b>
Table 1: Comparison of the time taken to reach pupae and imago between Control and Treatment samples. ....	40
Table 2: Mass /g of the blow fly species in the presence and absence of Amitriptyline over 200 hours .....	41
Table 3: Percentage increase and decrease of blow fly larval mass over a period of 200 hours .....	42
<b>APPENDICES .....</b>	<b>52</b>

## LIST OF ABBREVIATIONS

- ACN – Acetonitrile
  - ANOVA – Analysis of variance
  - *C. albiceps* – *Chrysomya albiceps*
  - *C. chloropyga* – *Chrysomya chloropyga*
  - CA – California
  - CNS – Central nervous system
  - CRH – Corticotropin releasing hormone
  - CYP1A2 – Cytochrome p450 enzyme isoform 1A2
  - CYP3A4 – Cytochrome p450 enzyme isoform 3A4
  - CYP2C19 – Cytochrome p450 enzyme isoform 2C19
  - CYP2D6 – Cytochrome p450 enzyme isoform 2D6
  - HPLC – High pressure liquid chromatography
  - *L. sericata* – *Lucilia sericata*
  - LTD – Limited
  - MeOH – Methanol
  - MI – Michigan
  - NaCl – Sodium chloride
  - NaOH – Sodium hydroxide
  - OTC – Over the counter
  - PMI – Post-mortem interval
  - SE – Standard error
  - SSRI – Selective serotonin re-uptake inhibitors
  - TCA – Tricyclic antidepressant
  - WHO – World Health Organisation
  - WV – West Virginia
- 
- °C – degrees celsius
  - µl – microliter
  - av – average
  - cm – centimetre
  - g – grams
  - kg – kilogram
  - mg – milligram
  - ml – millilitre
  - mm – millimetre
  - rpm – revolutions per minute
  - t – time
  - % – percent

## DEDICATION

*My constant motivation and support... Number 29*

**CHAPTER ONE:  
PROJECT PROPOSAL**

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# **Entomological examination of the relationship between ante-mortem and post-mortem Amitriptyline concentrations in insects belonging to orders Coleoptera and Diptera**

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## **Introduction**

Amitriptyline is a tricyclic antidepressant that also produces anti-nociceptive (pain stimulus inhibition) effects [1, 2]. When comparing the reaction of individuals to an applied pain stimulus, pain threshold has been proven to not differ greatly physiologically, but to be greatly dependent on the psychological state of the individual [2, 3]. In the management of nociceptive injuries, the combination of psychotropic drugs together with analgesics, has been associated with a reduction in drug dependence and an increase in pain threshold especially in the instance of chronic pain medication [3]. Though there are decreases in drug dependency, the prolonged administration of medication can result in behavioural changes, tolerance and drug dependence [4]. When tachyphylaxis (rapidly diminishing response to a drug, rendering it less effective) presents in individuals on chronic medication, a greater dose of the drug is required to produce the desired effect. The increase in the dosage taken can potentially result in an overdose of the drug and lead to death of the individual.

## **Entomotxicology**

Deaths arising from the use of drugs can be seen as intentional, accidental or suicidal. In some cases where a death has occurred, bodies are not discovered immediately, and may be discovered after a period of time whereby the body is extremely decomposed or skeletonized. Extreme decomposition makes the determination of post-mortem interval (PMI) (or time since death) difficult [5].

Furthermore, the limited availability of tissues, organs, urine or viable blood in the body due to extreme decomposition or skeletonization reduces the chance of successful toxicological analysis which may be of aid in the determination of cause of death [6].

A possible solution to the lack of viable tissues, organs, urine or blood for forensic toxicological analysis, would be the use of entomological specimens for toxicological analyses. Entomotoxicology is the application of toxicological analysis on carrion feeding insects to test and identify the presence of drugs or poisons that may have been present in the body ante-mortem, as a possible cause of death. The potential for entomotoxicology in forensics was first discovered in 1980 when phenobarbital was discovered in fly larvae present on skeletonized remains, marking its potential for implementation in forensics [7, 8]. Forensic entomological studies have previously focused on abuse and neglect cases especially in children [9], possible location of death, season, possible body movement, suspect identification and sexual molestation in both deceased and non deceased individuals [10]. More research has now shifted into the application of forensic entomology in PMI estimation [11] and this creates an opportunity for entomotoxicology.

The effect of different drugs on the entomological determination of post-mortem interval has previously been studied by Verma and Paul [12]. This study found that the growth rate of insects is affected by the type of drug present in the food source, and that the drugs are detectable in the sampled larvae or pupae. The effect of post-mortem drug concentrations present in decedents and their effect on insect growth has also previously been investigated in 2011 by Gosselin *et al.*, [13] through the use of Methadone-feeding Diptera. Results suggested that an increase in methadone led to an increase in the number emerged adults; however retardation in the developmental time of these specimens was noted. In another study by Mullany *et al.*, [14] it was discovered that the presence of methamphetamine accelerated development of larvae and improved their size compared to the controls, however insects remained as pupae for longer. Entomological growth rate is a major factor utilised in the determination of PMI [12, 15]. The increase in size and growth rate observed in 2014 by Mullany *et al.*, [14], suggest that understanding of the phenomenon of drug

concentration and its effect upon PMI estimation using insects is necessary to avoid over-estimation of the minimum PMI. With regard to the overestimation and underestimation of PMI, in 1993 Goff *et al.*, [15] analysed the effect of Amitriptyline on the development of insects and results showed that the presence of the drug altered the PMI by as much as 77 hours.

The effects of one drug on the development of insects cannot be used as a baseline for all drugs and resultant development, as different classes of drugs may affect insects differently. For example, some stimulants (for example methamphetamine and cocaine) have been found to be responsible for accelerating development of larvae [14, 16, 17] unlike an opioid (for example methadone) which reduces the developmental time [13]. Methamphetamine has been associated with weight loss and loss of appetite in human individuals [18, 19] and the same effect is anticipated in insects, although the pharmacokinetics of the drug in insects is not well understood.

The sampling of insects for entomotoxicological analysis has been shown to reveal important information and is dependent upon the sequence of colonisation of the insects. The sequence of colonisation on the decomposing body is mainly dependent upon the current stage of decomposition, with insects belonging to either Diptera order such as blow flies and flesh flies being the first to arrive at a body post-mortem [20, 21]. Therefore the first insects to arrive are more likely to have detectable concentrations of drugs present ante-mortem and should be sampled first, as opposed to insects that arrive later in decomposition [22]. Larvae are most often sampled due to their ease in being collected and due to their presence in high numbers on a body [23]. In the absence of larvae, pupae and empty pupae cases, have also been analysed and had drugs detected in them [13].

Compared to the toxicological analysis of post-mortem samples, entomotoxicology also has a number of advantages including: a greater sensitivity of drug analysis of insect larvae compared to putrefied specimens, and more rapid sampling and less emulsification during extractions [24-26]. Research has further shown that the detection of drugs in insects is more accurately conducted on larvae and pupae, and not on adult flies as drugs do not bioaccumulate in insects [26]. A

disadvantage however is the difficulty in determining a trend or relationship between the concentration of drug found in insects and the drug concentration found in the body of the deceased. Highly variable experimental set up also affects the accuracy of the results, and a lack of pharmacokinetics research of drugs, reduces the reliability of the interpretation without further research [26, 27], as there is no standard experimental set up in either *in vitro* or *in vivo* studies. The aforementioned disadvantages show large research gaps present within the field of forensic entomotoxicology, indicating the need for more entomotoxicological research to improve knowledge as the field has a lot of potential for further research, and more research should result in the improvement of forensic science as a whole.

### **Entomotoxicology Research**

Most of the research pertaining to the entomotoxicology of drugs is centered around illicit substances with little research covering prescription or over the counter (OTC) medication. This reveals a large research area that should be analysed to advance the discipline of forensic entomotoxicology. Previous studies have mostly involved American and European standards. Africa has a different climate and different species of insects compared to those present in these regions, and therefore corresponding research in Africa is required so as to develop appropriate standards for the insect populations and climate.

### **Research Problem Statement**

Amitriptyline is a prescription drug in South Africa used for the management of chronic depression and pain. Due to its multiple uses, especially in chronic conditions, there is a higher potential for drug dependence and tachyphylaxis to occur, which could result in drug overdoses. In drug-related deaths, standard toxicology protocols are available for the analysis of drug concentrations in post-mortem samples such as blood, urine, tissues and organs. In the absence of these samples, forensic entomotoxicology offers an alternative method of analysis. Due to the lack of knowledge of insect pharmacokinetics, it is thought that different classes of drugs should be analysed individually.

## **Aim**

The primary aim of the study is to characterise the effect of a suggested human lethal concentration dose of Amitriptyline in a spiked food source on the rate of development of Coleoptera and Diptera larvae. The secondary aim is to determine a relationship between Amitriptyline concentrations present in food sources and the concentration of drug present in larvae feeding on the food source at a specific developmental stage.

## **Objectives:**

1. Establishment of Coleoptera and Diptera insect colonies for scientific research.
2. Impregnation of food source with Amitriptyline and rearing of insects on this spiked food source.
3. Determination of effect of Amitriptyline on insect development and calculation of drug concentration present in insects at 1st instar, 2nd instar and 3rd instar development stages.
4. Statistical analysis and determination of mathematical relationship between initial Amitriptyline concentrations present in food source and concentrations present in insects.

## **Methodology**

Due to the absence of Coleoptera and Diptera culture labs in South Africa, insect colony cultures will have to be established prior to analysis on a non-Amitriptyline containing food source. A license to work with and procure controlled substances legally will be required from the Department of Health, South Africa, to conduct research on Amitriptyline.

## **Rearing of larvae on spiked food sources**

The analysis of the effect of Amitriptyline concentrations on the development of Coleoptera and Diptera larvae will be performed by a combination of methodologies according to Mullany *et al.*, [14] and Gosselin *et al.*, [13] with slight modification. A 20 mg/mL Amitriptyline stock solution will be prepared by dissolving 1000 mg of crushed Amitriptyline tablets (from prescription) into 50 ml deionised water. A mortar and pestle will be used to crush the tablets. Amitriptyline will then be

added to five food sources. Four food sources each will be spiked with Amitriptyline 14 mg/mL (lethal dose) respectively by diluting the Amitriptyline stock solution and mixing it into the minced pig liver. The remaining food source will be used as a negative control (no Amitriptyline).

Of the blow fly colony eggs, 10 will then be added to each food source and flies grown for not more than 5 generations at 25 °C in transparent plastic cages containing vermiculite to dry the environment and to encourage pupation, while irradiating daily with white fluorescent light over 12 hour light and 12 hour dark cycles.

A known amount of drug has been added to the food source hence drug concentration in the food source prior to analyses will not be determined. Fresh pig liver will be added *ad libitum* to promote oviposition in the adults and to decrease competition between the larvae, so as to increase the survival likelihood. To control the growth and numbers of the larvae, batches of eggs will be removed after every 6 hours and to also aid in competition reduction between the larvae. The development time of the larvae will be monitored by measuring the hatching time and time to reach next developmental stage during the 12 hour daylight cycle.

#### Development studies

From the developing larvae, five will be randomly sampled from each developmental stage (1st, 2nd and 3rd instar larvae) for Amitriptyline concentration and development analysis. The larvae developing on the different food sources will be weighed and have length measured for comparison to the control samples, to observe the effect of Amitriptyline on growth.

The duration of development between the control and the larvae treated with the Amitriptyline spiked diet will be analysed using one way analysis of variance (ANOVA) to determine if there is significant difference between growth rates.

#### Instrumental analysis

Sample preparation will be conducted according to modified methodology by Duke [28], High Performance Liquid Chromatography tandem mass spectrometry (HPLC-MS/MS) analysis will be carried out according to Mullany *et al.*, [14] with modifications. Briefly, the five randomly sampled

larvae will have their length and mass measured, followed by freezing to kill the larvae. Upon drying, the larvae will be washed using deionised water and homogenised by grinding with a mortar and pestle. Cellular debris will be removed via methanol precipitation followed by paper filtration.

A sample of the food source will also be collected and homogenised using a mortar and pestle and prepared for analysis. Cellular debris present on the food source will be removed by methanol precipitation followed by centrifugation and filtration to remove any pupae cases that might be present.

Analysis of Amitriptyline present in the larvae will then be determined using HPLC/MS to determine the ratio of the drugs concentration present in the insects, compared to that present in the food source. To allow for the identification of Amitriptyline present in the insects, standards of Amitriptyline and its metabolite nortriptyline will be purchased and prepared at a range of concentrations of 0-16 mg/mL for analysis using HPLC-MS/MS (AB SCIEX API 3200 Q-TRAP® HPLC/MS), and AB SCIEX MasterView™ software analysis performed to analyse the results. Solvents to be used for analysis will include acetonitrile, water and ethylamine. ANOVA analysis will then be used to determine whether there is any significant difference in concentration of Amitriptyline between the initial concentration, in food sources and developmental stage of the larvae.

The concentrations will then be compared to determine whether a mathematical relationship exists between the Amitriptyline concentration present in the food source and the Amitriptyline concentrations present in the larvae.

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## PROPOSAL ADJUSTMENTS

From the time the research proposal was written the following methodology has been amended:

- A 14 mg/ml concentration of Amitriptyline could not be added to the food substrate due to pharmacy restrictions on the amount of Amitriptyline that could be obtained an individual. A 1 mg/ml (1000 mg/Kg) concentration was used instead.
- As opposed to adding Amitriptyline to four containers and having one control, Three containers and three controls were used to check for any experimental variation as living studies involving living organisms have been deemed to be unpredictable.
- It was not possible to induce oviposition in adult flies or obtain pure larvae colonies, hence 300 blow fly eggs were collected from the fynbos biome and deemed a possible substitute that would still allow for sufficient sampling.
- Methanol was not used to extract Amitriptyline. Chlorobutane was preferred due to ease of evaporating before reconstitution for HPLC analysis.
- It was not possible to purchase nortriptyline standards; therefore comparison of Amitriptyline to nortriptyline was not conducted.
- Due to the lack of quantitative methodology to determine Amitriptyline concentrations, the research shifted to take a qualitative approach and concentrations could not be compared.

**CHAPTER TWO:  
REVIEW OF LITERATURE**

## **Introduction**

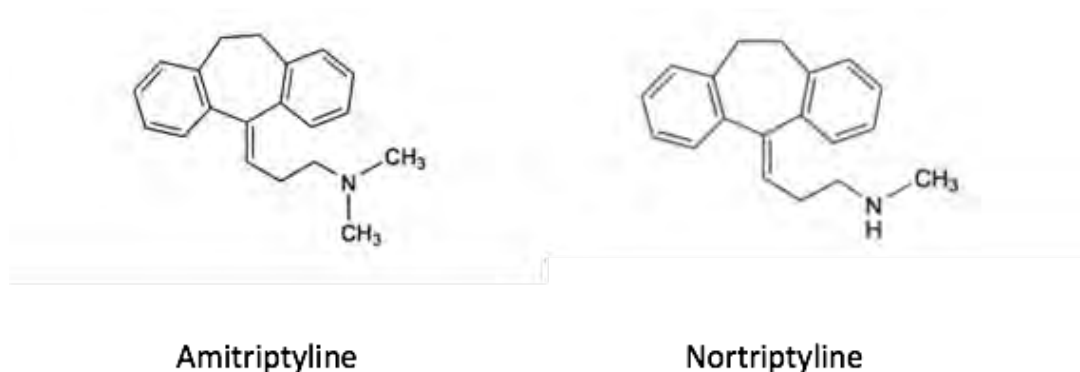
Major depressive disorders are identified as a mood disorder characterised as having more than two weeks of depression and a reduction in interest in pleasurable activities along with a loss in appetite, weight, sleep, energy and an increase in guilt. Diminished concentration and thoughts of death and suicide are also a symptom of depression [1, 2]. Depression has a lifetime prevalence of 15% in the population and in affected individuals, approximately 25% of cases of depression become chronic [2]. The World Health Organisation (WHO) states that approximately 121 million individuals worldwide live with depression [3], and with depression in communities on the rise research suggests that by the year 2020 depression could become the second most common disease worldwide after cardiovascular disease [4].

There is no definite cause of depression in individuals, however it is believed that depression occurs as a result of changes in the neural activity of areas of the brain responsible for the control of mood and stress, along with the limbic structures such as the amygdala, hippocampus and nucleus accumbens [2]. The interference of the limbic connection to the prefrontal cortex, affects neuronal activity feedback that regulates limbic activity. The negative emotions experienced by depressed individuals is processed in the amygdala, which is over-active in depressed individuals [2].

## **Amitriptyline**

Amitriptyline is a tricyclic antidepressant (TCA) that also produces anti-nociceptive (pain stimulus inhibition) effects [5, 6]. TCAs consist of a tricyclic ring structure with the central ring consisting of an alkyl amine substituent (Figure 1). Amitriptyline is used to treat major depressive disorders in individuals [1, 7]. In depressed individuals the drug is associated with an improvement in the mood and wellbeing of patients, whereas in non-depressed individuals Amitriptyline causes a sedative effect and does not affect mood or mental wellbeing [8, 9]. The sedative effect prompts the use of Amitriptyline before bedtime, to help patients sleep better. The tertiary amine group present in Amitriptyline makes it a more potent antidepressant than nortriptyline which is a secondary amine

(Figure 1) [8, 9]. The type of amine group determines the selectivity of the pharmacologic action in TCAs.



**Figure 1: Chemical structure of Amitriptyline and its primary metabolite nortriptyline**

Adapted from Sweetman, S. C., Martindal: The Complete Drug Reference, 36th Edition, 2009, Pharmaceutical Press, London [10].

Other than in the treatment of depression Amitriptyline can be used in the management of anxiety disorders, chronic pain, migraines and conditions such as shingles. Shingles is a painful vesicular rash that results from the reactivation of the varicella-zoster virus that causes chicken pox [1].

When comparing the reaction of individuals to an applied pain stimulus, pain threshold has been proven to not differ greatly physiologically, but to be greatly dependent on the psychological state of the individual [6, 11]. In the management of nociceptive injuries the combination of psychotropic drugs together with analgesics has been associated with a reduction in drug dependence, and an increase in pain threshold. This is of most importance especially in the treatment of chronic pain where, Amitriptyline maybe a choice of drug for pain where the psychotropic effect and analgesic effect reduce drug dependency [11].

### **Mechanism of action of Amitriptyline**

In individuals more susceptible to depression the remodelling and elimination of hippocampal circuits responsible for controlling mood, cognition and behaviour is initiated by stress. This is as a

result of the production of the stress hormone cortisol and the stress peptide corticotropin-releasing hormone (CRH) [2].

In the treatment of depression the various TCA dosage forms contain numerous properties including; serotonin re-uptake inhibition activity, norepinephrine re-uptake inhibition activity, anticholinergic-antimuscarinic activity, an alpha1-adrenergic antagonist activity and antihistamine activity [12, 13].

When an oral dose of Amitriptyline is administered, the drug is readily absorbed from the gastrointestinal tract with peak plasma concentrations occurring within 6 hours of administration [10]. Upon absorption, Amitriptyline is metabolised by the P450 enzyme isoforms CYP1A2, CYP3A4, CYP2C9, CYP2C19 and CYP2D6 in the liver and demethylated to form its active primary metabolite nortriptyline [10, 14-16] (Figure 1). After metabolism Amitriptyline achieves most of its therapeutic action via the inhibition of the re-uptake of serotonin and the inhibition of norepinephrine. Amitriptyline also has an effect on histamine, muscarinic and alpha1-adrenergic receptors, and these interactions have been associated with adverse effects [9, 12]. nortriptyline inhibits the re-uptake of norepinephrine to a greater extent compared to the re-uptake of serotonin, while Amitriptyline favours the reverse.

After absorption and metabolism, Amitriptyline and nortriptyline are distributed throughout the body where they have an elimination half-life of 9-25 hours, and are subsequently eliminated through the urine as free or conjugated molecules [10]. Other literature states that the half-life of Amitriptyline ranges from 18 to 70 hours [9]. While still present in the body, Amitriptyline and nortriptyline are found to be comprehensively bound to plasma and tissue proteins [10].

The British National Formulary has regarded Amitriptyline to be extremely dangerous during overdose [17-19], with the antimuscarinic and cardiotoxic effects of Amitriptyline limiting the use of Amitriptyline in patients. Cardiotoxic properties of Amitriptyline increase the risk of fatalities in patients taking overdoses of the medication [9, 10] and as a result the Department of Health in South Africa has marked this drug as being capable of causing fatalities during overdoses [1, 7].

During an overdose of Amitriptyline, cardiac arrhythmias and seizures occur due to inhibition of sodium channels [12]. Fatalities resulting from ingestion of Amitriptyline can be as a result of taking a high dose of the drug, or could be as a result of additive toxicity that can result from the co-administration of other drugs, and/or the altered metabolism of one drug to another or the conversion of Amitriptyline to nortriptyline. Drugs that hinder or incite the action of the P450 enzyme isoform CYP2D6 have an effect on plasma concentrations that could possible lead to severe drug toxicity [9, 10]. Central nervous system (CNS) depressants such as alcohol increase the metabolism and elimination of Amitriptyline therefore reducing efficacy, whereas antipsychotic drugs and calcium channel blockers reduce the metabolism and elimination of Amitriptyline leading to greater plasma concentrations and increase the likelihood of toxicity [10]. Apart from the ingestion of other drugs, the age of an individual and the effect on P450 microsomes affects the plasma drug concentration. In the elderly there is a reduction in first pass metabolism of Amitriptyline [16].

When tachyphylaxis (rapidly diminishing response to a drug, rendering it less effective) presents in individuals on chronic medication for either pain and/or depression, a greater dose of the drug is required to produce the desired effect. The increase in the dosage taken can potentially result in an accidental overdose of the drug and lead to death of the individual [1]. The overdose can also be intentional. Compared to other antidepressants such as selective serotonin re-uptake inhibitors (SSRI), TCAs have been shown to be associated with more fatalities during toxicity [20]. SSRIs were introduced as an alternative to the use of Amitriptyline due to their higher overall tolerability [21, 22]. However Amitriptyline is believed to have a higher efficiency especially in extremely ill individuals but the high potency has the potential to lead to mania in individuals [21, 22]. In a study in Australia involving drug related deaths in rehabilitated prisoners; illicit substances such as opioids, benzodiazepines, alcohol, cannabis and methamphetamine were found in the majority of cases. Toxicology results showing the presence of TCA's were only recorded in approximately 17% of deaths studied [23].

## **Depression and suicide**

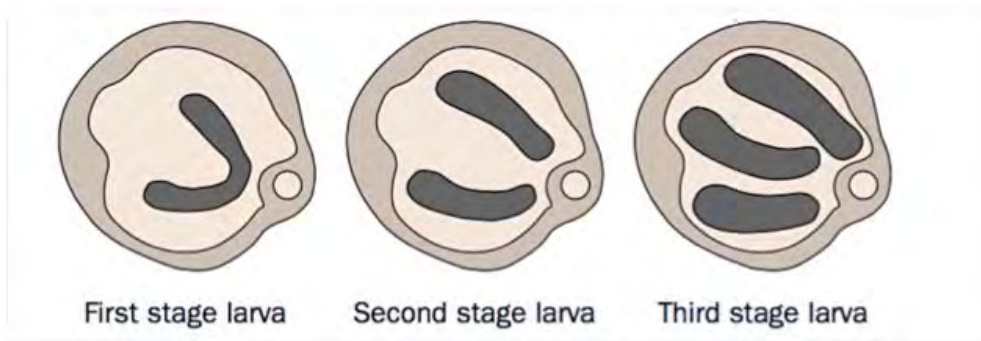
Prolonged use of antidepressants has been associated with suicidal thoughts as a side effect. The prevalence of suicide varies throughout the world, however WHO suggests a global prevalence of 14/100 000 population [24]. Approximately 10-25 individuals depending on the city, in every 100 000 in South Africa commit suicide [25-28]. Based on the current trend it is believed that by the year 2020, 1.53 million people will die as a result of suicide, with suicide being attempted 10-20 times more worldwide [29]. Previous research performed has already shown that the deaths with regard to intentional or accidental anti-depressant use have been gradually increasing over the past 10 years [30]. With antidepressants such as Amitriptyline common and inexpensive, this opens up the possibility of suicide being carried out by means of a drug overdose. Of the TCAs Amitriptyline is one of the most commonly prescribed. In Germany Amitriptyline is the third most prescribed antidepressants after Citalopram and Mirtazapine [31]. Compared to newer and safer antidepressants, Amitriptyline is commonly involved in suicide and suicide attempts in countries worldwide, and is the most commonly prescribed antidepressant that results in death [17, 20, 32]. A study conducted in Turkey data indicated that 59.7% of patients coming into the hospital as a result of a suicide attempt were currently taking Amitriptyline [33].

These results indicate the need for post-mortem toxicological analysis to be performed on bodies to detect the presence of xenobiotics. Where traditional toxicological specimens such as body tissues are not available, other technology in obtaining toxicological data is necessary and should be researched to obtain as much post-mortem information on bodies as possible.

## **Blow flies and Forensic Entomology**

Blow flies are flies belonging to the family Calliphoridae and reproduce through the action of laying eggs on meat and carcasses. After the death of an individual or animal, blow flies are attracted to the decaying remains via the production of two gases, putrescine and cadaverine that are produced during decomposition [34]. During growth and development blow flies have four distinct

stages of development namely; egg, larvae (maggot), pupae and adult [34]. The larval stages are further divided into three developmental stages (instar's), which are first, second and third instar respectively. The different stages of larvae are determined by the number of spiracle slits present on the maggot body (Figure 2). First instar, second instar and third instar larvae contain, one, two and three spiracle slits each respectively all enclosed within a peritreme ring (Figure 2).



**Figure 2: Spiracle slits associated with larval developmental stages first instar, second instar and third instar.**

Adapted from Bertino, A.J. Forensic Science: Fundamentals and Investigations 1st Edition, ed. J.W. Calhoun and K. Schmohe. 2008, United Kingdom: South-Western Cengage Learning [34].

The pupae stage serves as a transition to adulthood where the maggots move away from the body and cease feeding [34]. The maggots then form a puparium around their skins, which is hard and dark in texture, and from inside the pupae they develop into adults. The size of the maggot is directly proportional to growth stage, whereby the older maggots are generally larger in size compared to the younger ones [34]. The availability of food and the presence of substances in food also affects the size of the maggots and their developmental time.

In Africa, *Chrysomya chloropyga* and *Chrysomya putoria* are widespread [35, 36] and are both regarded to have application in medical, veterinary and forensic science [37, 38]. *Chrysomya chloropyga* is metallic greenish-bluish in colour with the most posterior part of the abdomen a green-yellow colour. This blow fly species is a first wave invader during the decomposition of bodies. *Chrysomya albiceps* is also a first wave invader but is very vulnerable to parasites and predators in the pupal stage. This blow fly species mainly occurs during colder months, and species

numbers are generally limited during summer [39]. The larvae of *C. chloropyga*, and *C. putoria* are conical in shape and white and/or cream in colour through all instars. *C. chloropyga* and *C. putoria* consist of an incomplete peritreme ring located on the posterior (which looks like a pair of eyes but are structures used for respiration).

Another common blow fly species in South Africa is *Lucilia sericata* which can be found in most areas of the world, especially the coastal areas. *L. sericata* has found uses in wound debridement and in forensic science [40, 41]. This blow fly species prefers warm moist climates [42, 43]. Although *L. sericata* is a member of Diptera, Calliphoridae it has been reported to be a second wave invader during deposition, however with regard to gutted animals it has been reported as a first wave invader [42]. The eggs of *Lucilia sericata* are white in colour and are normally laid in large masses or batches so as to avoid the desiccation of the eggs. The larvae of *L. sericata* are canonical in shape and white and/or cream in colour through all instars and contain a complete peritreme ring [44].

For use in forensic science, the blow fly life cycles are of interest because their life cycles have an effect on the determination of post mortem interval (PMI)(time since oviposition on bodies after death) as the developmental stage of the insect is used in its determination. Two main methods exist for the use of blow fly maggots in PMI estimation. One method involves observing the sequence of colonisation of blow flies and predicting the time since death [45, 46]. For example third wave invaders will only invade the body after a specific time since death has elapsed. The second method involves observation of the oldest maggot on the body and monitoring its growth. During PMI investigation, the age and developmental behaviour of the insect is firstly observed and the used in a reverse calculation to the likely time of oviposition on the body [47].

In the determination of PMI environmental conditions such as temperature and humidity have an effect on growth and have to be taken into account [47-49], as they all affect developmental time. Temperature governs the metabolic activity of maggots, which are cold blooded. The temperature is directly proportional to metabolic activity and the growth, and ultimately the size of the maggots

[50]. Knowing this relationship it is possible to adjust for the time of death by comparing the surrounding temperature to the growth stage of the maggots to more accurately determine the time of colonisation of the body by adult blow flies [51]. It should however be noted that the maggot internal temperature is also affected by metabolic activity. The metabolic activity and aggregation of maggots has been reported to be able to raise the internal temperatures by as much as 15-35 °C [52].

Although research stipulates that insects colonise bodies after a specific intervals of time, constant research in the field is necessary as nature will not always behave as expected as observed in *L. sericata* that has adapted to being a first wave invader on carrion. Different analyses of blow fly colonisation data is necessary for various climate regions to observe as to whether for example, second wave invading blow flies do not invade with first wave blow fly. The implication of this could affect post-mortem interval determination, and lead to a potential over estimation of the time since death.

Forensic entomology also finds use in the geographical mapping of crimes [34, 53, 54]. In such cases the taxonomic identification of maggots is important. Different species of maggots grow at different rates, seasons, geographical areas and different environmental conditions [34]. The identification can be performed using either DNA techniques or biological comparison charts through the identification of specific features present on the maggot bodies. For example at a crime scene if insects belonging to a specific geographical area are discovered on a body or piece of evidence, geographical mapping of the insects could be used to determine where the original crime took place and give extra information as to whether the body was moved or not. To improve the geographical mapping of crimes it is necessary to obtain information as to the regions where the blow flies are localised. The mapping of blow fly region localisation, and data collection is complicated however, as blow fly species can always spread, for example, in the case of the forensically important *L. sericata*, that originated in Europe and was introduced to other countries such as South Africa. The limitations in this would be that mapping of the blow fly to a specific

geographic regions may yield erroneous results as the flies could still be spreading the limits of its distribution [55].

### **Entomotoxicology**

Deaths arising from the use of drugs can be seen as homicidal, accidental or suicidal. In some cases where a death has occurred, bodies are not discovered immediately, and may be discovered after a period of time whereby the body is extremely decomposed or skeletonized. Extreme decomposition makes the determination of PMI difficult [56]. Furthermore, the limited availability of tissues, organs, urine or viable blood in the body due to extreme decomposition or skeletonization reduces the chance of successful toxicological analysis which may be of aid in the determination of cause of death [34].

A possible solution to the lack of viable tissues, organs, urine or blood for forensic toxicological analysis, would be the use of entomological specimens for toxicological analyses. Entomotoxicology is the application of toxicological analysis on carrion feeding insects to test and identify the presence of drugs or poisons that may have been present in the body ante-mortem.

The potential for entomotoxicology in forensics was first discovered in 1980 when phenobarbital was discovered in fly larvae present on skeletonized remains, marking its potential for implementation in forensics [57, 58]. Forensic entomological studies have previously focused on abuse and neglect cases especially in children, possible location of death, season, a possible body movement, suspect identification and sexual molestation in both deceased and non-deceased individuals [59, 60]. More research has now shifted into the application of forensic entomology in PMI estimation [61]. The effect of drugs (both medicinal and illicit) on growth rate and metabolism or carrion feeding insects is not fully understood. This may play a role in PMI estimation and this creates an opportunity for entomotoxicology.

The presence of xenobiotics on bodies has the potential to affect the size, feeding and the growth rate of blow fly maggots. The effect of different drugs on the entomological determination of post-

mortem interval has been previously studied by Verma and Paul [62]. This study found that the growth rate of insects is affected by the type of drug present in the food source and that the drugs are detectable in the sampled larvae or pupae. The effect of post-mortem drug concentrations present in decedents and their effect on insect growth has also previously been investigated in 2011 by Gosselin et al., [63] through the use of Methadone-feeding Diptera. Results suggested that an increase in methadone led to an increase in the number emerged adults; however retardation in the developmental time of these specimens was noted. In another study by Mullany *et al.*, [64] it was discovered that the presence of methamphetamine accelerated development of larvae and improved their size compared to the controls, however insects remained as pupae for longer. Entomological growth rate is a major factor utilised in the determination of PMI [62, 65]. The increase in size and growth rate observed in 2014 by Mullany *et al.*, [64], suggest that understanding of the phenomenon of drug concentration and its effect upon PMI is important. Therefore the appropriate corrections are required for insects feeding on tissue which may contain drugs or drug metabolites. With regard to the overestimation and underestimation of PMI, in 1993 Goff et al., [65] analysed the effect of Amitriptyline on the development of insects and results showed that the presence of the drug altered the PMI by as much as 77 hours.

The effects of one drug on the development of insects cannot be used as a baseline for all drugs and resultant development, as different classes of drugs may affect insects differently [66]. When blow flies are exposed to a drug, different drugs have been associated with different effects in maggots such as size increases or decreases. In some cases these effects are accompanied by a high mortality in the maggots [64, 67, 68].

An interesting finding in studied literature relating to the effect of drugs on development is that growth may be affected at different stages during development. For example although cocaine and methamphetamine are both associated in increases in the growth rates of blow fly maggots it has been demonstrated that cocaine is associated with an increase in larval development rate whereas methamphetamine is associated with an increase in pupal developmental rate [62]. Another effect,

which drugs may have is associated with delaying oviposition on bodies. Toxins like Malathion have been shown to delay oviposition on bodies by up to three days [69].

With regard to the sequence of colonisation it is expected that the first insects to arrive are more likely to have detectable concentrations of drugs present ante-mortem and should be sampled first, as opposed to insects that arrive later in decomposition [70]. Larvae are most often sampled due to their ease in being collected and due to their presence in high numbers on a body [71]. In the absence of larvae, pupae and empty pupae cases, have also been analysed and had drugs detected in them [63]. However drugs are not always detectable in pupae [72] which suggests that for more accurate detection and determination blow fly larvae are more ideal to use [73].

Compared to the toxicological analysis of post-mortem samples, entomotoxicology also has a number of advantages including: a greater sensitivity of drug analysis of insect larvae compared to putrefied specimens, and more rapid sampling and less emulsification during extractions [74-76]. Research has further shown that the detection of drugs in insects is more accurately conducted on larvae and pupae, and not on adult flies as drugs do not bioaccumulate in insects [76, 77], therefore drugs are more likely to be detected in first instar larvae as opposed to third instar larvae, pupae or adult flies [77]. With this in mind, previously conducted research has suggested that the first colonising insects during decomposition are more likely to have detectable drug concentrations present.

Disadvantages however include: difficulties in determining a trend or relationship between the concentration of drug found in insects and the drug concentration found in the body of the deceased. Also highly variable experimental set up affects the accuracy of the results, as well as reproducibility and a lack of pharmacokinetics research of drugs, reduces the reliability of the interpretation without further research [76, 78, 79], as there is no definite standardised experimental set up for either *in vitro* or *in vivo* studies. Upon analysing previous research it is notable that some studies have reported a direct correlation between food source or substrate and larvae in quantitative analyses [75, 80]. However other studies have reported that a correlation does not exist [81, 82].

The aforementioned disadvantages show large research gaps present within the field of forensic entomotoxicology, indicating the need for more entomotoxicological research to improve knowledge as the field has a lot of potential for further research, and more research should result in the improvement of forensic science as a whole.

### **Rearing of blow flies**

In the wilderness blow flies obtain protein as a nutrient from decaying animal bodies but it has been suggested that they use nectar and honeydew as a carbohydrate source [83]. Some research involves the creation of synthetic media rich in nutrients such as proteins for the growth of blow flies. For use in toxicological analysis on the effect of xenobiotics in blow flies, the drug is administered to the blow fly larvae in either one of two methods. One method involves the mixing of the drug into the food source such as kangaroo mince, pig liver or cow heart and placing the blow fly eggs on the substrate to feed [64, 72]. The other method involves injection of the drug into an animal such as a rabbit, mouse or pig and the using organs from the autopsied animal to rear the blow fly larvae on [68]. Although both methodologies have had success in the detection of drugs in blow flies, the use of live specimens has been noted to be better for forensic entomotoxicology analyses.

A draw back to performing studies on live animals is large variability of a drug and its metabolites. The final concentration of the drug and its metabolites may not be known and the metabolism of many drugs in animal models may not be fully understood. For example, in animals methamphetamine could either produce; *p*-hydroxynorephedrine and *p*-hydroxymethamphetamine (rats), norephedrine and benzoic acid conjugates (guinea pigs), and amphetamine (rabbits) as metabolites [84, 85]. However in humans *p*-hydroxymethamphetamine is the main metabolite.

Literature with regard to the feeding times of larvae are contradictory, some literature suggests that the larvae of blow flies prefer to feed at night in the darkness, while other research suggests that blow flies are heliotropic and remain inactive at night [86, 87]. Other research suggests that neither circadian rhythms nor light affect feeding of the larvae [88]. *L. sericata* has been shown to oviposit

during nocturnal conditions when the temperature is cool compared to other Diptera members which rarely oviposit under nocturnal conditions [53, 86]. When *L. sericata* oviposit eggs between 21 °C to 27 °C ambient temperature, the eggs normally take approximately 18 to 21 hours to hatch [89]. During the growth of maggots, conditions can either be favourable for growth or inhibit growth.

Research investigations as to the effect of environmental stress conditions or resource limitations have been conducted to observe their effect on the development in organisms that undergo a complete metamorphosis [50]. It is believed that under stressful conditions, organisms can employ one of two strategies to ensure survival. The first strategy involves an increase in developmental rate to adulthood (ensuring survival), as opposed to delaying maturation that which normally results in larger and more fit adult organisms [90]. A limitation to the first strategy is, however, that in some studies larger adult organisms have been observed and correlated to an increase in the developmental rate [91].

The second strategy involves organisms remaining dormant for long periods of time until such adverse conditions in the environment improve [92-94]. Blow flies such as *L. sericata* that depend on ephemeral food supplies such as biological waste and carrion for development, have evolved phenotypic plasticity (changes in the phenotype as a result of the environment) to balance the relationship between developmental time and blow fly average size [91]. The implications of this are that, the size and developmental duration can be controlled by changing laboratory conditions such as food source, moisture and temperature [49, 95].

*Chrysomya albiceps* has been recorded to having predatory habits against other maggot species feeding on carrion, when developing under adverse conditions. This allows them to reduce the competition for resources ultimately increasing the probability of survival [39]. The implications of this is that where *C. albiceps* is present on a body at a crime scene, their predatory behaviour can ultimately affect the presence of other blow fly species present at the crime scene and as a result affect PMI estimations.

It should however be noted that although the rate of development may be affected by temperature different blow fly species from different areas and regions behave differently. For example in 2011 Tarone *et al.*, [50] analysed the effect of temperature on the developmental rates of different blow fly species obtained from different regions in the United States of America namely, California (CA), Michigan (MI) and West Virginia (WV). CA had the shortest developmental time at 20 °C followed by MI and WV. This data however, contrasted at 33.5 °C where CA had the longest developmental time followed by WV and MI. These results suggest that a generalised developmental pattern for blow fly species cannot be calibrated and that blow fly tests and analyses with respect to PMI estimation need to be standardised to the appropriate flies of interest under specific conditions [50]. However to perform this, a blow fly general population analysis has to be performed to identify the forensically important blow fly species in the community. With respect to forensic science this adds a limitation in that every geographic region where forensic entomology is performed will be required to have a database of the growth standards at varying conditions for the blow fly species relevant to that area. Currently blow flies can only be used to measure the minimum time since death, however with improved research methodology blow flies could find use in the future with predicting maximum time since death [50]. This presents a research gap that needs to be studied to improve the accuracy of forensic entomology applications with regard to crimes.

### **Blow fly larvae sample preparation and toxicological analysis**

When sampling for maggots, the selected maggots are collected and then boiled in water for 15 seconds, followed by storage in 80% ethanol. The boiling of larvae prior to adding to ethanol reduces the likelihood that the maggots would shrink during storage thus reducing the chance that the maggots have their age mistaken [96]. However, for the purposes of toxicological analyses blow fly larvae are usually killed via freezing at -20 °C [72, 82]. Preservation in ethanol is not common as some drugs such as Amitriptyline are soluble in alcohols. Therefore in order to reduce the chance of

a premature drug extraction occurring, alcohols or organic solvents are only added when drug extraction is to be performed.

The sampled maggots are often homogenised using mechanical grinding. In the studied literature there has been no evidence of chemical methods being employed to homogenise blow fly larvae. The only chemicals employed during the sample preparation include addition of bases or buffers to control and raise the pH of the homogenate to facilitate drug extraction into organic solvents [96]. With blow flies containing a chitin exoskeleton, addition of bases such as sodium hydroxide can serve to hydrolyse the chitin releasing any drug that might be bound to it, as well as releasing the drugs that may be bound to the proteins in maggots [10].

It is possible to check for the presence of Amitriptyline using spectrophotometry, capillary electrophoresis and chromatography. Spectrophotometric analysis of Amitriptyline is usually carried out when analysing pharmaceutical preparations where Amitriptyline reacts with methyl orange and bromocresol green to form 1,2 dichloroethane which is yellow coloured and absorbs light at 410 and 420 nm [97]. Capillary electrophoresis and chromatographic techniques such as high performance liquid chromatography (HPLC) are normally conducted on biological specimens [98]. HPLC and spectroscopy have been compared and shown to both have high accuracy and precision [99]. However due to the costs involved with conducting HPLC analysis and the expensive nature of the equipment, some laboratories do not have HPLC equipment and as a result cannot perform HPLC analysis. This indicates the need to investigate cheaper alternatives to conduct toxicological analysis.

### **Concluding remarks on Entomotoxicology Research**

The difference with respect to the growth stage of the blow fly larvae or pupae and the observation of drug effects, indicates a need for the thorough analysis and research of each and every drug prior to PMI estimation and drug detection. Furthermore, different effects of drugs on blow fly behaviour elucidates potential limitations of the implementation of forensic entomotoxicology, in that

scientific analysis involving live specimens shows high variability and could usher in some unexpected behaviour all affecting post mortem interval and the interpretation of the results of the presence of drugs in toxicological analysis. It is therefore necessary to control as many variables as possible during experimentation.

Most of the research pertaining to the toxicology of drugs is centred around illicit substances with little research covering prescription or over the counter (OTC) medication. Over the counter medication may have an effect on the development of larvae and such medication has also been known to be abused. This reveals a large research area that should be analysed to advance the discipline of forensic entomotoxicology.

Previous studies have mostly involved American and European standards. For forensic investigations, a population analysis of the presence of these blow fly species and the regions where they occur is necessary. South Africa has a different climate and different species of blow flies compared to America and Europe, and therefore corresponding research in South Africa is required so as to develop appropriate standards for the insect populations and climate.

### **Research Problem Statement**

Amitriptyline is a prescription drug in South Africa used for the management of chronic depression and pain. Due to its multiple uses and inexpensive nature there is a higher potential for drug dependence and tachyphylaxis to occur, which could result in drug overdoses. In drug-related deaths, standard toxicology protocols are available for the analysis of drug concentrations in post-mortem samples such as blood, urine, tissues and organs. In the absence of these samples, forensic entomotoxicology offers an alternative method of analysis. Due to the lack of knowledge of insect pharmacokinetics, it is thought that different classes of drugs should be analysed individually.

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**CHAPTER THREE:  
PUBLICATION READY MANUSCRIPT**

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## South African blow fly *Chrysomya chloropyga* and *Lucilia sericata* growth kinetics, and effect of Amitriptyline presence on larval development – A pilot study

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### Abstract

Entomotoxicology offers a supplementary method to analyse for the presence of drugs post-mortem, especially in cases where viable toxicological specimens such as human tissue cannot be obtained. In South Africa and globally, standardised methodology is required to perform entomological examinations accurately so as to improve the reproducibility and accuracy of results. The aim of the current study was to analyse the effect of Amitriptyline on the developmental growth rate of forensically important blow flies, and to investigate the potential for blow fly larvae (present in the Western Cape South Africa) in forensic entomotoxicological implementation. To achieve this blow fly species *C. chloropyga* and *L. sericata* were reared on homogenised pig liver containing 1000 mg/kg Amitriptyline until emergence of imago. The presence of drugs in larvae was investigated using high-pressure liquid chromatography coupled with dual mass spectrometry (HPLC/MS-MS). Amitriptyline was detected in all experimental larvae samples and was found to delay pupation by at least 26 hours and imago emergence by at least 72 hours. There was no distinct effect observed for the effect of Amitriptyline on *C. chloropyga* however Amitriptyline was associated with a smaller length and mass in *L. sericata*. Results indicate the potential for qualitative analysis for the presence of Amitriptyline in entomotoxicology.

### Keywords:

*Chrysomya chloropyga*; *Lucilia sericata*; Entomotoxicology; Amitriptyline; Post-mortem interval; Tricyclic antidepressants.

### 1. Introduction

Deaths arising from the use of drugs can be seen as homicidal, accidental or suicidal. In some cases where a death has occurred, bodies are not discovered immediately, and may be discovered after a period of time whereby the body may

be decomposed or skeletonized. Extreme decomposition may make the estimation of post-mortem interval (PMI) difficult [1]. The deterioration of the body may result in the limited availability of tissues, urine or viable blood for toxicological analysis which may hinder the determination of cause of death in forensic investigations [2].

A possible solution to the lack of viable tissue, urine or blood for forensic toxicological analysis, may be the use of entomological specimens for toxicological analyses. Entomotoxicology is the application of traditional toxicological analysis on carrion feeding insects to test and identify the presence of drugs or poisons that may have been present in the body ante-mortem, as a possible cause of or contributing factor to death. When feeding, these insects ingest the drug containing tissues and thus allow for an alternative sample of analysis for drug detection.

Compared to the toxicological analysis of decomposed post-mortem samples, entomotoxicology has a number of advantages including: a greater sensitivity of drug analysis of insect larvae compared to putrefied specimens, and more rapid sampling and less emulsification during extractions [3-5]. Previous research has demonstrated that the detection of drugs in insects is more accurately conducted on larvae and pupae, than on adult flies [5, 6]. Disadvantages however include: difficulties in determining a trend or relationship between the concentration of drug found in insects and the drug concentration found in the body of the deceased [7-10], highly variable experimental set up affects the accuracy, as well as reproducibility of the results and a lack of pharmacokinetics research of drugs reduces the reliability of the interpretation without further research [5, 10-13], as there is no definite standardised experimental set up in either *in vitro* or *in vivo* studies.

Major depressive disorders are identified as the lack of interest in pleasurable activities, as well as increased thoughts of death and suicide [14, 15]. Depression has a lifetime prevalence of 15%, and the condition may become chronic in 25% of patients [15]. Amitriptyline is a tricyclic antidepressant (TCA) containing anti-nociceptive properties [16, 17] and is commonly used in the management of depression. Prolonged use of antidepressants has been associated with suicidal thoughts as a side effect. Approximately 10-25 in every 100 000 in South Africa commit suicide [18-21]. With the ever increasing suicide rates annually it is believed that by the year 2020, 1.53 million people will die as a result of suicide, with 10-20 times more suicides being attempted worldwide [22].

Most of the research pertaining to the entomotoxicology of drugs is centred on illicit substances with little research covering prescription or over the counter (OTC) medication. This reveals a large research area that should be analysed to advance the discipline of forensic entomotoxicology. Previous studies have also mostly involved American and European climates. South Africa has a different climate and blow fly species populations therefore corresponding research in South Africa is necessary to develop appropriate South African standard for forensic entomotoxicology.

The aim of the current study was to analyse the effect of Amitriptyline on the developmental growth rate of forensically important blow flies in the Western Cape region of South Africa, and to investigate the potential of these blow fly larvae for the implementation of entomotoxicological analysis in forensic investigations.

## **2. Methodology**

### 2.1. Reagents

Amitriptyline hydrochloride tablets 50 mg were obtained from a local pharmacy. Amitriptyline reference standards 1mg/ml were provided by the Department of Pharmacology, University of Cape Town situated at Groote Schuur, Cape Town. Winelands Pork, Cape Town, donated pig offal used to cultivate eggs and pig livers used as food substrate for larvae.

All chemicals used were of the highest analytical grade. Ethanol, sodium hydroxide and chlorobutane were purchased from Merck PTY Ltd (Johannesburg, South Africa). Sodium chloride was obtained from Kimix Chemicals (Cape Town, South Africa).

### 2.2. Rearing of larvae for Amitriptyline analysis

Rearing of larvae was performed according to methodology of Kharbouche *et al.*, [6] with modification. Diptera eggs used in this study were obtained by placing pig offal in the fynbos biome at the Medical Research Centre (M.R.C) Delft Cape Town. Blow fly eggs to be used for analysis were collected and allocated into batches depending on the location of the egg mass on the offal.

Approximately 300 Diptera eggs specific to location on the offal were added to homogenised pig liver (200 g). A mixture of 300 Diptera eggs from different egg batches was also added to homogenised pig liver. Each liver homogenate sample was previously spiked with 15 ml of 0.9% sodium chloride solution containing 1000 mg/kg (lethal dose) of Amitriptyline hydrochloride mixed in a blender for 5 minutes at room temperature.

Negative controls containing 0 mg/kg Amitriptyline on 200 g homogenised pig liver were also used.

Larval rearing samples were first placed onto a sheet of aluminium foil paper (11 x 8 cm), then inside plastic containers (22 x 16 x 8 cm) containing vermiculite (3 cm height in container) to absorb excessive moisture in the container. Containers were covered with a lace stocking to prevent larval escape and subsequently sealed.

During the study, the containers were maintained in an incubator at  $30 \pm 0.5$  °C and ambient humidity. Larvae were reared under the irradiance of incandescent lighting, consisting of light/dark cycling of 14/10 hours. Light cycling was related to the current seasonal day/night durations.

### 2.3. Sampling of larvae

The effect of Amitriptyline concentration on the development of Diptera larvae and sampling was performed according to methodology by Mullany *et al.*, [23] and Gosselin *et al.*, [24] with slight modification. Briefly, during larval growth fresh pig liver was added *ad libitum* to decrease competition between larvae.

To control the growth and numbers of the larvae as well as to reduce competition, batches of 20 maggots each were sampled from each container every 6 hours up to 18 hours, then every 12 hours up to 78 hours, then every 24 hours up to the appearance of pupae and stored in plastic bottles. Pre-pupae were defined as non-feeding inactive stage between the larval growth stage and the pupal growth stage.

The sampled larvae were washed using deionised water followed by 0.9% NaCl solution to remove any drug traces that could be present on the surface of the larvae and improve analysis accuracy. The larvae were dried using paper towel, killed by freezing at -20 °C and stored at this temperature for future analysis. The weight and length of a random sample of 10 maggots was measured using an analytical balance and vernier callipers respectively and the results noted. For toxicological analysis approximately 2 g of pig liver homogenate were sampled at t = 0 and t = 78 hours and stored at -20 °C.

### 2.4. Identification of maggot species and developmental stage

The growth stage and identity of the maggot was determined using a Carl Zeiss (Zeiss, Cape Town, South Africa) SteREO Discovery.V8 stereo microscope in conjunction with a larval identification key provided by Prins, [25].

### 2.5. Statistical analysis

Statistical analysis of the growth data was performed using analysis of variance (ANOVA) and a t-test for parametric data, and the Kuskall-Wallis and Mann-Whitney U test for non-parametric data. Statistical analysis and figure generation was performed using Statistica version 12 software (Statsoft, Tulsa, OK, USA) and Microsoft® Office Excel® 2011 (Microsoft, Redmond, Washington, US).

### 2.6. Amitriptyline extraction

Amitriptyline extraction was performed on homogenised samples according to a combination of methodology by Sadler *et al.*, [8] and Duke [26] with slight modification. A 4 ml solution of the homogenate in 0.9% NaCl was adjusted to pH 12 using 0.5 N NaOH. The solution was then vortexed to re-suspend cellular material and debris. An equal volume of chlorobutane was added and the solution incubated at room temperature for 30 minutes. Aqueous and organic phase were separated via centrifugation at 4000 rpm for 15 minutes. The organic phase at the top of the tube was recovered. The remaining aqueous phase at the bottom of the tube was re-extracted using chlorobutane.

The organic layers were then concentrated together and evaporated under a constant stream of nitrogen gas at 50 °C until complete dryness. The dry samples were then covered in parafilm and stored at -80°C until analysis.

### 2.7. Instrumental analysis

Amitriptyline was reconstituted in 200 µl 10 mM ammonium formate in 50% ACN:MeOH (acetonitrile:methanol) solution and vortexed. This solution was then vortexed again and centrifuged at 13000 rpm for 5 minutes. The supernatant (20 µl) was then extracted and added to 180 µl of reconstitution reagent. Of this solution 10 µl was injected onto a C18 EVO column and the samples analysed using HPLC-MS/MS (AB SCIEX API 3200 Q-TRAP® HPLC/MS), and AB SCIEX MasterView™ software analysis was used to process and analyse the results.

## **3. Results and Discussion**

In this study the effect of Amitriptyline on the development of blow flies was examined for developmental time comparison between a negative control comprising of larvae not exposed to Amitriptyline and a treatment group comprising of larvae exposed to an Amitriptyline containing food source. Amitriptyline toxicological analysis was performed to determine the presence of Amitriptyline in 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> instar blow fly larvae.

Diptera larvae species depending on offal collection sites were divided into three samples namely; Control 1 and Treatment 1, Control 2 and Treatment 2, Control 3 and Treatment 3. Control 2 and Treatment 2 refer to blow fly eggs obtained on the intestines of the offal, while Control 3 and Treatment 3 refer to blow fly eggs obtained on the underside of the offal. Control 1 and Treatment 1 were a mixture of the eggs obtained from the intestines and the underside of the offal. The Control 1, 2 and 3 refer to larvae not exposed to Amitriptyline. Treatments 1, 2 and 3 refer to larvae exposed to 1000 mg/kg Amitriptyline.

During the 2<sup>nd</sup> and 3<sup>rd</sup> instar growth stages the blow fly larval species were identified as follows, Control 1 and Treatment 1: a mixture of *Chrysomya chloropyga* and *Lucilia sericata*; Control 2 and Treatment 2: *Chrysomya chloropyga*; Control 3 and Treatment 3: *Lucilia sericata*. Evidence of this classification was supported by further characterisation of emerged adult blow flies.

### 3.1. Effect of Amitriptyline on blow fly development

The time taken to reach a specific developmental stage for larvae was measured and results were as follows, Control 1, 2 and 3 reached 2<sup>nd</sup> instar after approximately 12 ± 6 hours, while 3<sup>rd</sup> instar larvae were noted after approximately 18 ± 6 hours. Pupae and imago in all controls were observed after 152 ± 12 hours and 248 ± 12 hours respectively. A similarity in growth durations with respect to all controls was expected, and this similarity indicates no unexpected experimental variance occurred within the controls.

Results in the controls with regard to the growth rate of the larvae show similarity to those obtained in Treatments 1, 2 and 3. In all the treatment samples 2<sup>nd</sup> instar and 3<sup>rd</sup> instar larvae were observed after approximately 12 ± 6 hours and 18 ± 6 hours respectively and results showed no observable effect of Amitriptyline on the growth durations. The effect of Amitriptyline only became evident in the time taken to reach pupae and imago (Table 1).

Table 1: Comparison of the time taken to reach pupae and imago between Control and Treatment samples.

Growth Stage	Control 1 (Hours ± SE)	Treatment 1 (Hours ± SE)	Control 2 (Hours ± SE)	Treatment 2 (Hours ± SE)	Control 3 (Hours ± SE)	Treatment 3 (Hours ± SE)
Pupae	152 ± 12	178 ± 2	152 ± 12	178 ± 2	152 ± 12	192 ± 2
Imago	248 ± 12	320 ± 12	248 ± 12	392 ± 12	248 ± 12	-

- no imago observed even at 536 hours

Data in Table 1 shows that Treatment 1 and Treatment 2 reached pupation quicker than Treatment 3 suggesting more rapid growth in these treatments. Treatment 1 however reached imago before Treatment 2. No imago was observed emerging from Treatment sample 3 even after 536 hours. With this in mind results suggest that the approximate rates of development for the larval treatment samples can be ordered as follows; Treatment 1 > Treatment 2 > Treatment 3. Comparison of data in Treatment samples to Control 1 show that the difference in the time taken to reach pupae was 26 ± 12 hours. The same time difference was noted between Treatment 2 and Control 2. Comparison of Treatment 3 to Control 3 suggests that the difference in time taken to reach pupae was 40 ± 12 hours.

Difference in the time taken to observe imago between Treatment 1 and Control 1 is 72 ± 17 hours. With respect to Treatment 2 and Control 2 the time difference in the emergence of imago is 144 ± 17 hours. With Treatment 1 containing an approximately equal amount of blow fly eggs from Treatment 2 and Treatment 3 added to it, upon imago emergence via eye inspection it was observed that there were a higher number of *C. chloropyga* adults as opposed to *L. sericata* adults. This can however be attributed to the possibility of *C. chloropyga* being the more dominant species. However, no ratios of species proportion numbers in comparison to total blow fly population were calculated.

The data in Table 1 gives strong indication that the effect of Amitriptyline is mostly observable in the late 3<sup>rd</sup> instar, pupae and imago growth stages. Previously the effect of Amitriptyline on blow fly development had been studied by Goff *et al.*, [27] who found a difference of 77 hours between the appearance of imago between control and treatment samples of *Parasarcophaga ruficornis*. The effect of Amitriptyline on *C. chloropyga* and *L. sericata* has not been studied yet.

During growth, fatalities were noted in all treatment samples between 102 and 150 (126 hours average) hours post Amitriptyline exposure. These fatalities were attributed to a potential drug overdoses. Fatalities in larva arising from ingestion of drug laden food source have however been documented in previous studies [27]. During this time period

larvae began to appear sluggish in movement and it is worth noting that drowsiness or sluggish behaviour is a symptom of Amitriptyline overdose in humans. Drug pharmacokinetics in blow fly larvae research is minimal, however previous studies have shown possibility of drugs in larvae having the same effect as that in humans [23, 28, 29].

### 3.2. Effect of Amitriptyline on blow fly mass

During development the largest larvae were sampled with respect to growth time for mass determination and analysis. Results are indicated in Table 2.

Table 2: Mass /g of the blow fly species in the presence and absence of Amitriptyline over 200 hours

<b>Time /hours</b>	<b>Control 1 (grams)</b>	<b>Treatment 1 (grams)</b>	<b>Control 2 (grams)</b>	<b>Treatment 2 (grams)</b>	<b>Control 3 (grams)</b>	<b>Treatment 3 (grams)</b>
6	0,0054	0,0054	0,0054	0,0054	0,0054	0,0073
12	0,016	0,0163	0,0105	0,0158	0,0201	0,0149
18	0,0332	0,0385	0,0459	0,0439	0,0377	0,0379
30	0,207	0,2008	0,2363	0,1475	0,1602	0,1552
42	0,3099	0,3444	0,1888	0,2346	0,2823	0,1989
54	0,3846	0,4798	0,2311	0,2594	0,338	0,2789
66	0,374	0,5015	0,2317	0,2954	0,3164	0,2873
78	0,4133	0,4768	0,2271	0,2756	0,3295	0,2951
102	0,7952	0,7963	0,6359	0,669	0,7202	0,6593
128	0,8412	0,8442	0,6725	0,6827	0,7736	0,6956
152	0,8273	0,7415	0,7117	0,6388	0,7453	0,6577
176	0,8147	0,6981	0,6687	0,6525	0,6953	0,6348
200	0,7668	0,5761	0,5921	0,5191	0,6587	0,4965

Data from Table 2 shows the average larvae mass of 10 larvae at a specific time interval. Table 3 indicates the percentage increase or decrease of blow fly larvae mass over the period of 200 hours.

Results and data in Table 2 show that on average from 42 hours onwards Control 3 has a larger mass than Control 2 (difference 0.07913 g). Control 1 has a larger average size larger than Controls 2 and 3 (difference 0.149 g and 0.06995 g respectively). With regard to the treatment samples, comparison of Treatment 2 and Treatment 3 does not give any indication as to which sample contains larvae with a heavier mass (difference 0.00005 g respectively). Each sample has heavier larvae at a different time period. Treatment 1 however appears to have a larger average mass than Treatments 2 and 3 (difference 0.1468 g and 0.146875 g respectively). Results in Table 1 suggest that the blow fly larvae in this

experiment have a larger average mass when different species are added together (Control 1 and Treatment 1) as opposed to individual growth. This adds on to the results in section 3.1 where better growth was noted when the different blow fly populations were added together.

Comparing Control 1 to Treatment 1 data in Table 2, during the initial growth period shows that Treatment 1 has a larger mass than Control 1 until 152 hours, where an overall decrease in the mass of all the larval samples was noted. Interestingly this decrease in mass at 152 hours coincides with the appearance of pupae in the control samples noted in section 3.1. From data in Table 3 for 152 hours to 200 hours, Treatment 1 has more pronounced decreases in mass compared to Control 1 evidenced by the larger percentage decreases.

Control 2 is similar in size to Treatment 2 up to 42 hours. From 42 hours to 128 hours Treatment 2 maintains a larger average size than Control 2. The mass in Control 2 only starts decreasing at 200 hours unlike Control 1 and Control 3 that begin decreasing at 152 hours. The late decrease in mass can be attributed to species variation. An anomaly in the results is however noted in Control 2 at 42 hours where there was an unexpected drop in mass. A possible reason for the anomaly could be due to the sampling of the larvae. Treatment 2 also shows an unexpected result between 152 and 176 hours. The mass decreases at 152 hours and increases at 176 hours before finally decreasing at 200 hours. This may again be due to to the sampling of larvae.

Results in Table 2 with respect to Control 3 and Treatment 3 show that from 30 to 200 hours the control larvae have a larger average mass than the treatment. The result is different to that observed in Control 1 and Treatment 1, and Control 2 and Treatment 2. In these experiments the treatment larvae had an average mass larger than the controls up-till 152 hours. Although Control 3 had a larger average mass than Treatment 3, results in Table 3 show the treatment sample had a more pronounced mass decrease at 152 hours and 200 hours.

During the experimental steps the second food substrate was added at 78 hours. This explains the low mass increases in Table 3 observed at 66 hours where Control 1 and Control 3 larvae lost mass and Control 2 and Treatments 1, 2 and 3 gained mass. This also explains the sharp increases in percentage mass that occurred at 102 hours. It is however notable that at 78 hours the Treatment samples 1 and 2 have a larger percentage mass decrease compared to their corresponding controls, and Treatment 3 has a lower percentage mass increase compared to Control 3.

Table 3: Percentage increase and decrease of blow fly larval mass over a period of 200 hours

<b>Time /hours</b>	<b>Control 1 (%grams)</b>	<b>Treatment 1 (%grams)</b>	<b>Control 2 (%grams)</b>	<b>Treatment 2 (%grams)</b>	<b>Control 3 (%grams)</b>	<b>Treatment 3 (%grams)</b>
6	0,00	0,00	0,00	0,00	0,00	0,00
12	196,30	201,85	94,44	192,59	272,22	104,11

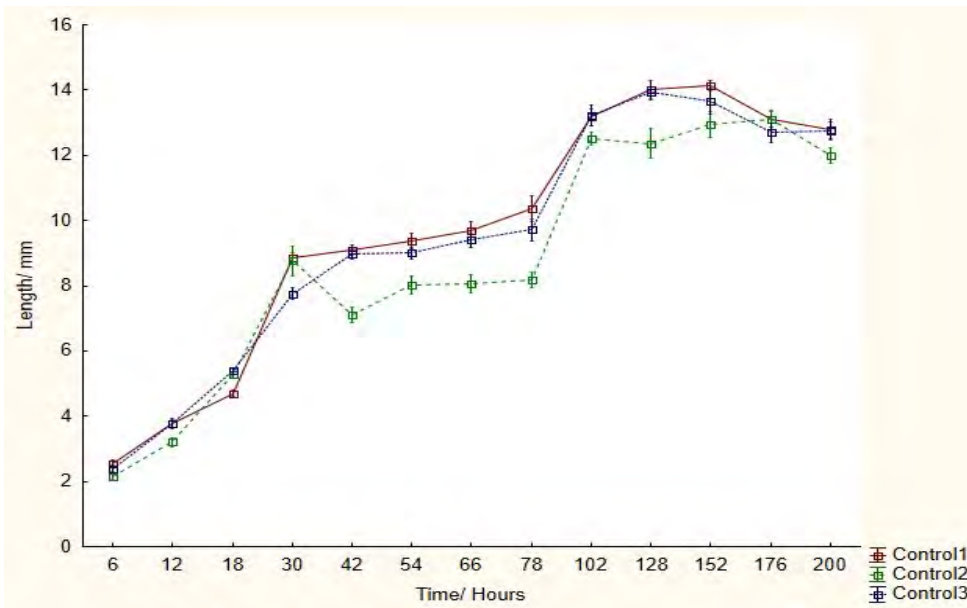
18	107,50	136,20	337,14	177,85	87,56	154,36
30	523,49	421,56	414,81	235,99	324,93	309,50
42	49,71	71,51	-20,10	59,05	76,22	28,16
54	24,10	39,31	22,40	10,57	19,73	40,22
66	-2,76	4,52	0,26	13,88	-6,39	3,01
78	10,51	-4,93	-1,99	-6,70	4,14	2,71
102	92,40	67,01	180,01	142,74	118,57	123,42
128	5,78	6,02	5,76	2,05	7,41	5,51
152	-1,65	-12,17	5,83	-6,43	-3,66	-5,45
176	-1,52	-5,85	-6,04	2,14	-6,71	-3,48
200	-5,88	-17,48	-11,46	-20,44	-5,26	-21,79

Overall from the data in Table 3, there is no clear trend as to the effect of Amitriptyline on the percentage increase in mass. Results vary at different time intervals. With respect to mass decreases in Treatment 1 a trend emerges. Data at 152, 176 and 200 hours show that Treatment 1 larvae lose mass faster than Control 1. A slight trend is noted with respect to Control 2 and 3 and Treatments 2 and 3. At 176 hours however the Controls 2 and 3 have larger percentage decreases in mass than the treatment samples. The treatment samples all exhibit a larger percentage mass decrease at 152 and 200 hours. Factoring in the mass percentage values obtained at 78 hours, results may suggest that Amitriptyline causes a low mass retention in blow fly larvae

The consequence of low mass retention could possibly have an effect on imago emergence and/or potential size. The significant loss of larval mass could explain the longer time taken to reach pupation and imago in Table 1 section 3.1 [30]. It is worth noting that at 200 hours Treatment 3 is associated with having the greatest percentage mass loss and could explain the lack of imago observed in section 3.1. Likewise of all the treatment samples Treatment 1 has the lowest percentage mass decrease and as a result this could explain the better growth observed in Table 1 compared to Treatments 2 and 3.

### 3.3. Effect of Amitriptyline on blow fly length

The effect of Amitriptyline on the length of blow fly larvae over a period of 200 hours was measured, and statistics ( $\alpha=0.05$ ) used to analyse results.

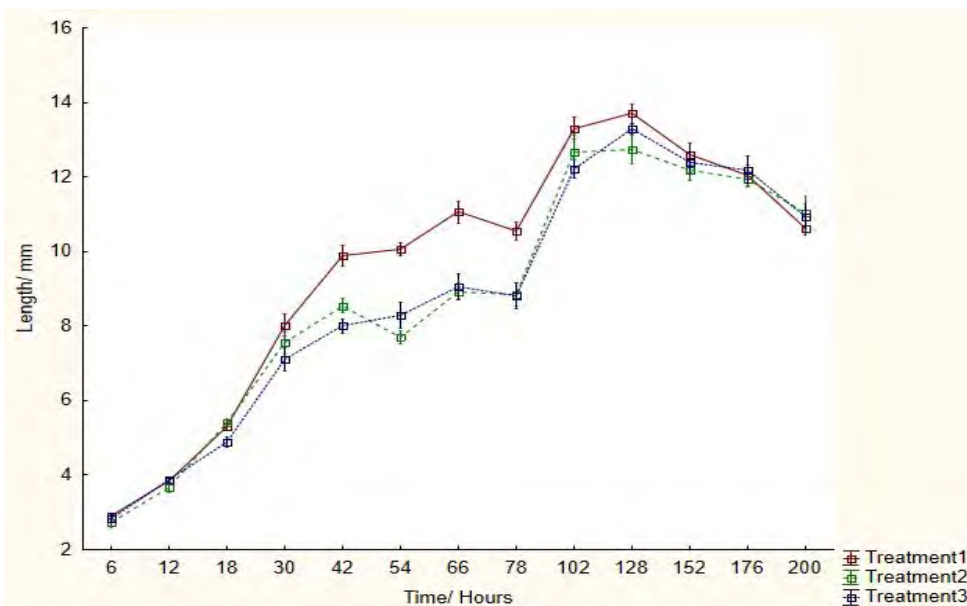


**Figure 1: Length comparison of blow fly larvae in the absence of Amitriptyline.**

Data from Figure 1 shows that on average Control 3 has a larger length (P-value < 0.05) as opposed to Control 2. Results in Figure 1 also indicate that the length of Control 1 is similar to that of Control 3 (P value > 0.05).

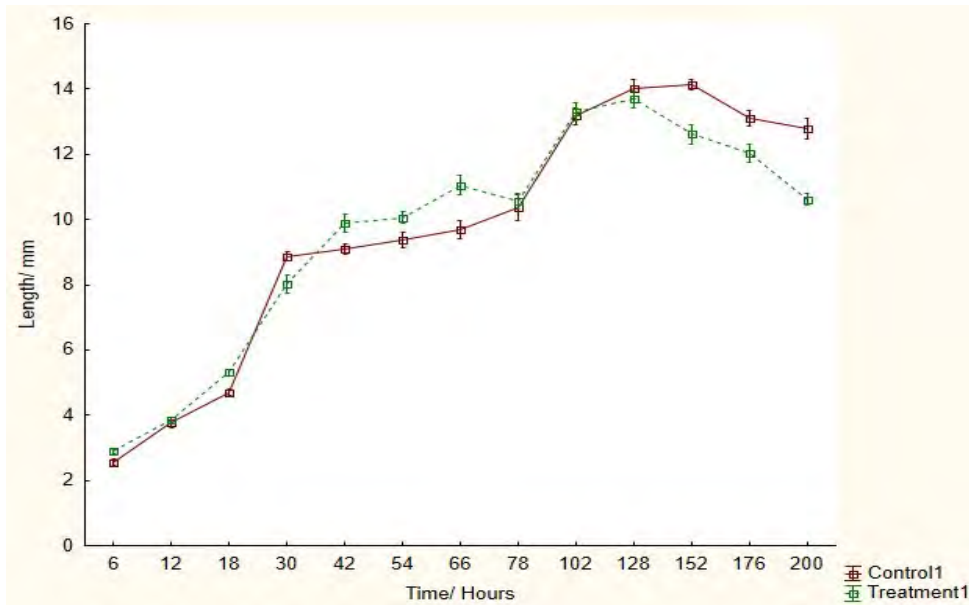
Figure 1 also shows that the length of the larvae is directly proportional to duration of growth. The only anomaly observed in the results is the decrease in length in Control 2 between 30 and 42 hour. This decrease in length coincides with the decrease in larval mass in Table 2.

Comparing Figure 1 to Figure 2 there is a distinct difference in the behaviour of Treatment 3. In the control studies Control 1 and Control 3 had similar lengths, however data in Figure 2 shows that Treatment 1 is significantly larger than both Treatments 2 and 3. It is also observable that the length of Treatment 2 is now similar to that of Treatment 3 (P-value > 0.05).



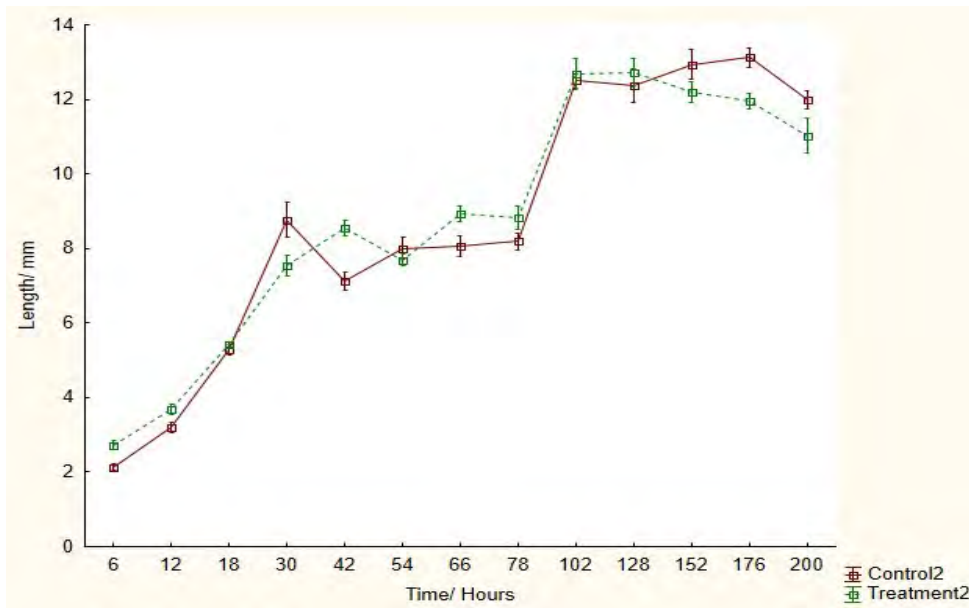
**Figure 2: Length comparison of blow fly larvae in the presence of Amitriptyline.**

Results strongly suggest that the presence of Amitriptyline has had an effect on the behaviour of Treatment 3 larvae and reduced their length significantly. An interesting observation in the results is that all Treatment 1 samples have been observed to have a larger length, mass and quicker growth.



**Figure 3: Comparison of average blow fly larvae length between Control 1 and Treatment 1**

Figure 3 compares Treatment 1 to Control 1. From the results in Figure 3 there is no observable trend on the effect of Amitriptyline on blow fly larvae length. The lengths of the control and treatment larvae are similar at 12, 78, 102 and 128 hours (P-values = 0.668, 0.683, 0.714 and 0.443 respectively). With the mass of Treatment 1 being larger than Control 1 (Table 2), it was expected that the treatment larvae would have a larger length as opposed to the control. With the exception of the result at 30 hours where the control was larger than treatment samples, Treatment 1 larvae are associated with larger lengths. From 152 hours onwards larvae exposed to Amitriptyline lost length faster than the control.

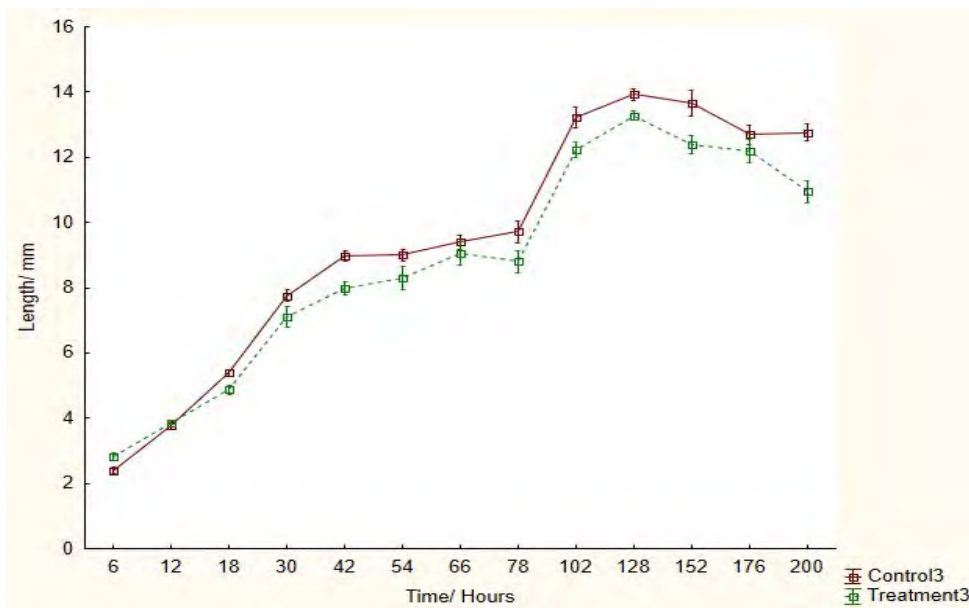


**Figure 4: Length comparison of blow fly larvae between Control 2 and Treatment 2**

As previously observed in Figure 3 there is no definite trend observable as to the effect of Amitriptyline on blow fly larval length from the data in Figure 4. This is evidenced by the two graphs intersecting each other at 18, 36, 52, 56 and 140 hours. The only observable trait in Figures 3 and 4 is the more pronounced decrease in larval length from 152 hours onwards.

Analysis of the results Figure 5 gives evidence to the possibility of a trend between larval length comparison between treatment and control larvae. The graphs only intersect at 12 hours and remain separate up to 200 hours. With the exception of 12 hours, control larvae have a greater length as opposed to the treatment larvae up to 54 hours. At 54, 66, and 78 hours the lengths of the control and treatment larvae are similar (P-value = 0.0908, 0.424 and 0.0766 respectively). As observed in the Figures 3 and 4, the presence of Amitriptyline is associated with a more rapid decline in the length of larvae.

The lack of a definite trend as to the effect of Amitriptyline on the length and mass of blow fly larvae highlight the complications associated with the use of entomotoxicology in PMI estimation. The biological organisms are not predictable. With Control 3 and Treatment 3 consisting of *L. sericata*, the more defined behaviour that has been observed in Table 2 and Figure 5 where Treatment 3 has had a lower length and mass compared to Control 3 could be as a result of the phenotypic plasticity of *L. sericata* as a species and coping with adverse environmental conditions [30], where the growth rate and larvae size are balanced to ensure survival. However, in this case imago was not observed and this is probably due to the lethal concentrations of Amitriptyline.



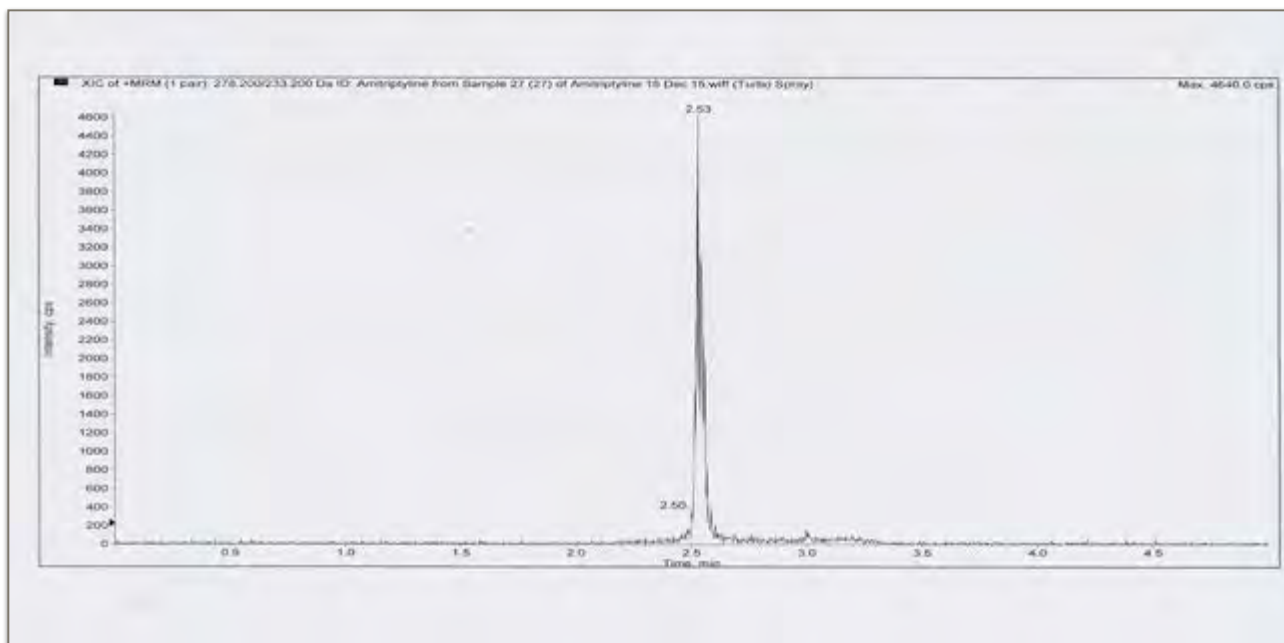
**Figure 5: Length comparison of blow fly larvae between Control 3 and Treatment 3**

### 3.3. Amitriptyline toxicological analysis

Toxicological analysis of the concentration of Amitriptyline showed the presence drug in the larvae samples up till 200 hours of development. Previous research into drug detection in blow fly larvae has been performed with mixed results, with some drugs not being detected in larvae being fed on heavily drug laden muscle [31].

Unfortunately negative controls in the analysis also showed evidence of trace levels of Amitriptyline that could have been attributed to either contamination during the mechanical homogenisation process or contamination during the nitrogen drying of the samples. Care must ne taken to ensure that all instruments are thoroughly cleaned before use. Another solution, which may assist in eliminating cross contamination in future, is to prepare all control samples before preparing treatment samples. A further and possibly best solution for this problem would be to adopt bead vortexing or chemical treatment as method to macerate the blow fly larvae. Due to the high stability of Amitriptyline (8 weeks at room temperature) [32] it is not recommended to use the same containers to process treatment controls and samples even if they are washed, as there is still a possibility of cross contamination.

Data in Figure 6 shows an example of the expected chromatogram during HPLC showing the presence of Amitriptyline. Amitriptyline has a retention time of approximately 2.5 minutes.



**Figure 6: Selected example of HPLC chromatogram showing the presence of Amitriptyline**

### 3.4. Limitations

The limitations for the analysis of Amitriptyline include; with no standardised experimental set-up results are difficult to compare due to the high variation of protocols. Any effects of Amitriptyline observed on the development of blow fly larvae can be noted, however the effect of standard or experimental variation cannot be conclusively ruled out. Although the qualitative analysis showed the possibility to detect Amitriptyline, a quantitative analysis protocol is required to improve the overall accuracy of the results, provided contamination issues can be addressed. Due to the inability to obtain a pure colony of blowfly larvae these results only present a brief overview of the effect of amitriptyline on blowfly growth. The accuracy of the results can be better improved through the obtainment of pure blowfly larvae colonies and increasing the number of larvae sampled and repeats.

The extraction process also yields some emulsifications, and more research is required into researching more methods with fewer emulsifications. HPLC is expensive to run and a more sustainable and cheaper method is required for drug analysis such as spectrophotometric analysis.

### **4. Conclusion**

In conclusion the effect of Amitriptyline on the development of blow fly larvae was studied for potential implementation in post-mortem estimation and toxicological analysis. Results indicate that Amitriptyline delays pupation in blow fly larvae by at least 26 hours and the emergence of imago by at least 72 hours. Due to the unpredictable behaviour of biological organisms, the presence of a trend as to the effect of Amitriptyline on blow fly

larvae and length could not be established for *C. chloropyga* however Amitriptyline was associated with a smaller length and mass in *L. sericata*. Amitriptyline could be detected in all treated blow fly larvae, however the negative controls tested positive for Amitriptyline. Possible future work could include the optimisation of the Amitriptyline extraction to reduce cross contamination and the possible quantification of the drug present in the larvae.

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### **Conflict of Interest**

The authors declare that there is no conflict of interest.

### **Ethical approval**

Ethical clearance was obtained from the university of Cape Town Animal Research Ethics Committee reference: *AEC 015/022*. All applicable institutional guideline for the care and use of animals were followed.

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## **APPENDICES**

## APPENDIX A: ETHICAL APPROVAL LETTER



UNIVERSITY OF CAPE TOWN

**Faculty of Health Sciences Animal Ethics Committee**  
Room E53-24 Groote Schuur Hospital Old Main Building  
Observatory 7925  
Telephone [021] 404 7682 • Facsimile [021] 406 6411  
e-mail: [nosi.tsama@uct.ac.za](mailto:nosi.tsama@uct.ac.za)  
<http://www.health.uct.ac.za/fhs/research/animalethics/forms>

**23 November 2015**

**Mr TA Sanyanga**  
C/o Dr M Heyns  
Forensic Medicine & Toxicology  
Clinical Lab Sciences  
Falmouth Building

Dear Mr Sanyanga

**PROTOCOL TITLE: ENTOMOLOGICAL EXAMINATION OF THE RELATIONSHIP BETWEEN ANTE-MORTEM- AND POST-MORTEM AMITRIPTYLINE CONCENTRATIONS IN INSECTS BELONGING TO ORDERS COLEOPTERA AND DIPTERA**

**FHS AEC REF NO: 015/022**

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 authorization 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 an annual basis and in accordance with 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: Blowflies & Maggots

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

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

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>

FHSAEC 015/022

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 Veterinary 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**

## APPENDIX B: ACKNOWLEDGEMENTS

My sincerest thanks to:

- My supervisor, Dr Marise Heyns for the constant support and guidance over the past year full of trials and tribulations.
- My co-supervisor, Mr Calvin Gerald Mole for his patience and the extra work he took upon himself to ensure the success of the project.
- Ms Bronwen Davies, for guidance and project support
- Professor Peter Smith and the University of Cape Town Pharmacology Department, for technical assistance and toxicological analysis.
- Winelands Pork, for donating porcine specimens fundamental to the success of the research project.
- Medical Research Centre (M.R.C) Cape Town, the location of the fynbos biome used to collect sample specimens
- National Research Foundation, for providing the scholarship and funding that ensured this research was a success. It was been an honour being supported by such a reputable institution.
- My parents, for their continued unwavering love and support throughout my entire career and love. I would not have made it this far without you!
- Almighty God for opening up doors and a path for me that no man can ever close.
- The “#Professional” group, my second family. It was a pleasure having you as colleagues and knowing you all.

## APPENDIX C: AUTHOR GUIDELINES



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In 1997 Prof. Bernd Brinkmann formulated guidelines for the submission of manuscripts on short tandem repeat (STR) population data (Brinkmann 1997). These earlier guidelines have now been extended to include haploid DNA markers, i.e. mitochondrial DNA (mtDNA) and Y-chromosomal polymorphisms.

For specific information, see the Short Communication "Publication of population data of linearly inherited DNA markers in the International Journal of Legal Medicine" (Parson and Roewer 2010; DOI 10.1007/s00414-010-0492-y) published online in Int J Legal Med in July 2010.

All forensic population genetics papers should always contain information on the description of the population, ethical requirements and quality control. For mtDNA papers, previous acceptance of the dataset in EMPOP is required; for YSTR and YSNP data, previous inclusion of the data in the YSTR/YSNP database is required.

EMPOP database

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Please provide 4 to 6 keywords which can be used for indexing purposes.

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LaTeX macro package (zip, 182 kB)

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Always use footnotes instead of endnotes.

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Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

## REFERENCES

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Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
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The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

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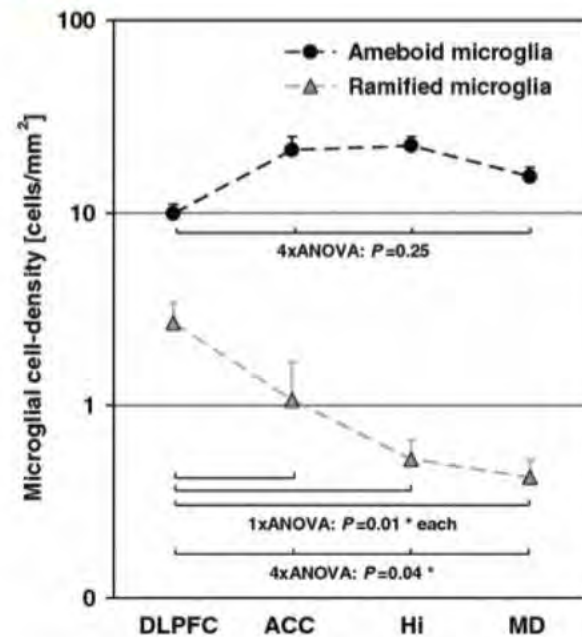
asterisks for significance values and other statistical data) and included beneath the table body.

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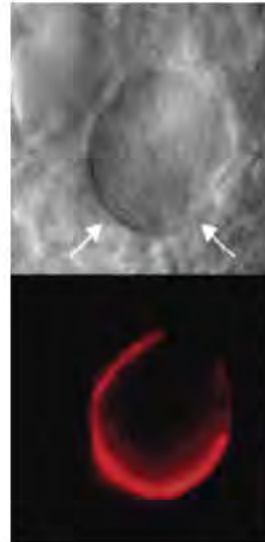
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- ☐ Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.
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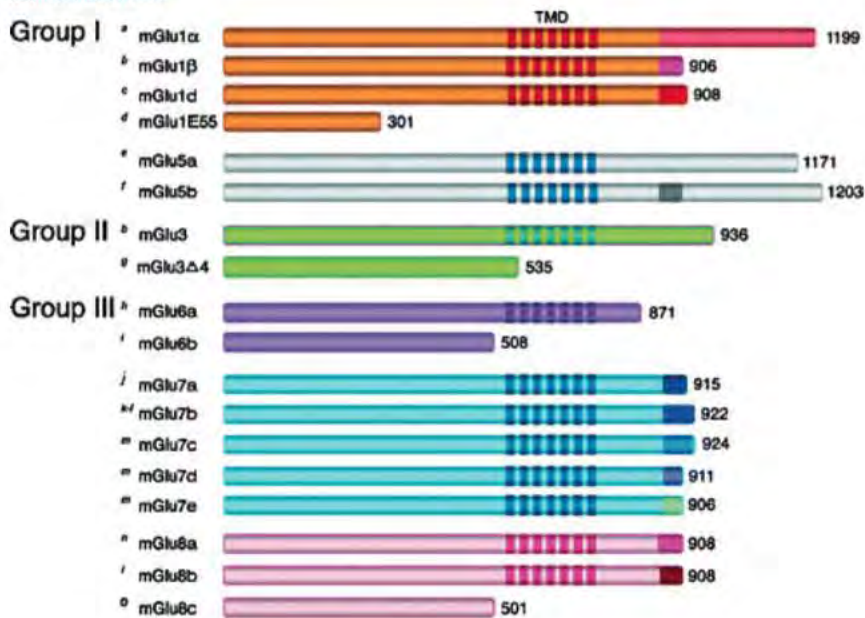
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Authors should include the following statements (if applicable) in a separate section entitled "Compliance with Ethical Standards" when submitting a paper:

- Disclosure of potential conflicts of interest
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"This article does not contain any studies with human participants or animals performed by any of the authors."

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