The evaluation of potential dietary media, measurement parameters and storage techniques for use in forensic entomotoxicology

A thesis submitted in fulfilment of the requirements for the MSc (Entomology) degree in the department of Zoology and Entomology of Rhodes University

By

Erica Isabel Tavares Da Silva Mbatha

December 2017

Abstract

The term *forensic entomotoxicology* was coined by Pounder and is used to describe the process of using insects to determine the presence or absence of toxicants in decomposing corpses. Forensic entomotoxicology is most applicable when the orthodox sources of evidence (i.e. blood and urine) are no longer available for testing due to the degree of putrefaction as a result of the decomposition process. As the field is relatively new, various authors have conducted studies to determine the effects of different toxicants on different insects. These studies have all been conducted in the absence of a standardised protocol and we hypothesise that this has led to conflicting results (i.e. two different authors will conduct a study using the same toxicant and model insect and the effects on the insects will differ significantly). The aim of this thesis was to identify the areas which might have led to the artefacts in the results and identify ways in which to standardise them. The three areas selected were the feeding substrates and the measures taken to quantify growth rate, as well as the preservation techniques that should be used for preserving larval flies.

The recommendation from the literature review was that artificial diets would be the most appropriate dietary media to use for entomotoxicological studies. An artificial diet was selected and modified for potential used in entomotoxicological studies. Four different diets (no meat treatment, fish, beef and pork artificial diets) were used to rear *Chrysomya chloropyga* larvae and their growth rates were measured using length and width. The fly larvae reared on the fish and no meat treatment diets did not reach pupation stage. The beef and pork diets produced the largest larvae and the flies in these treatments reached adult stage. The recommendation was that the beef and pork treatments be tested with various toxicants to establish their stability in the matrix and the diet that provides the toxicants with the most stability should be used for future entomotoxicological studies.

The two other factors selected for standardisation were the parameters used to quantify growth rate, as well as the preservation techniques used to store empty *Chrysomya chloropyga* pupal casings and *Calliphora croceipalpis* third instar larvae. Previous authors have suggested that width be used as an alternative to length to quantify growth rate. The results from this thesis show that length should continue to be used as the standard parameter because the incremental change in length is much larger than the change in width, and these larger increments allow for greater resolution when estimating the age of the larvae. Various

authors have also suggested that pupal casings should be stored without any preservative, whereas fly larvae should be stored in concentrations of ethanol >70%. The results in this thesis have shown that the concentration of ethanol does not make any significant difference to the proportional change of length and width of the empty pupal casings and the third instar larvae. The recommendation is that when selecting the preservation technique, the integrity of the specimen for examination of other evidence (i.e. DNA or toxicological extraction) should take precedence.

Although this thesis has not completely standardised the protocol for forensic entomotoxicology, it has indicated the areas that need to be focused on in order for standardisation to occur. Future studies should focus on standardisation, as this makes studies more comparable and ultimately makes entomotoxicological evidence admissible in the court of law.

Table of Contents

Abstract ii

Table of Contents iv

List of tables vi

List of figures vii

List of abbreviations viii

Preface ix

Acknowledgements x

Declaration xii

Chapter 1: Forensic entomotoxicology revisited: towards professional standardisation of study designs 1

- Abstract 1
- Introduction 1
  - The scope of entomotoxicology 15
  - Qualitative toxicant detection 15
  - Quantitative toxicant detection 16
  - Postmortem interval estimation 20
  - Toxicant source localization 21
  - Measurement parameters and storage techniques 21
- Analytical review of the available literature 22
  - Insect taxa 22
  - Feeding substrates 23
  - Toxicants 26
  - Toxicological analysis 27
  - Statistical analysis 28
- Experimental vs. Applied: the application of entomotoxicology 28
- The Future: a basic protocol for future entomotoxicological studies 29

Research objectives 30

Chapter 2: Comparison of different dietary media on the development of *Chrysomya chloropyga* 31

- Abstract 31
- Introduction 31
- Materials and methods 39
  - Insect collections and rearing 39
  - Dietary treatments 39
  - Larval growth rate 40
  - Statistical analysis 41

Results 42
Diet, age and cup effects 43
Comparison of the body lengths and widths of the Chrysomya chloropyga larvae reared on the different diets. 43
Costs of producing the dietary media 44
Odour of the dietary media 44
Discussion 45
Nutrient content of the feeding substrate 45
Completion of life stages 45
Overall successes and failures of the dietary media 45
Conclusion 46
Chapter 3: Standardising methods for quantifying growth and preservation techniques of larval flies of forensic relevance 47
Abstract 47
Introduction 47
Materials and methods 51
Length and width 51
Assessing preservation techniques 52
Results 54
Length vs. width 54
Chrysomya chloropyga pupal casing preservation 55
Calliphora croceipalpis third instar larvae preservation 60
Discussion 64
Pupal casing and third instar larvae preservation 65
Conclusion 66
Chapter 4: General discussion 67
References 70

List of tables

Table 1.1. List of literature examined for this review, with the toxicants, insect species, and research goal of each study……3 - 13

Table 2.1. The different donor organisms and donor organs used in entomotoxicological studies from 1980 – 2016 ……32 - 36

Table 2.2. The composition of the diets used for the experiments……39

Table 2.3. The mean body lengths and widths of the *Chrysomya chloropyga* larvae reared on the NMT, beef and pork treatments……41

Table 2.4. The ANCOVA results for the effects of age, diet and cups on the *Chrysomya chloropyga* body lengths and widths……42

Table 2.5. Tukey HSD results of the ANCOVA models showing the comparison of the body length and width of *Chrysomya chloropyga* reared on the different artificial diets……..42

Table 2.6. The cost of making 600ml of each dietary medium……43

Table 3.1. Mean differences between sampling periods in body length and width of *Chrysomya chloropyga* reared on NMT, beef and pork artificial diets……54

Table 3.2. GLM results for the effects of the preservation times and treatments on the proportional changes in lengths of the empty *Chrysomya chloropyga* pupal casings……..55

Table 3.3. GLM results for the effects of the preservation times and treatments on the proportional changes in widths of the empty *Chrysomya chloropyga* pupal casings ……56

Table 3.4. GLM results for the effects of the preservation times and treatments on the proportional changes in length of the *Calliphora croceipalpis* third instar larvae……59

Table 3.5. GLM results for the effects of the preservation times and treatments on the proportional changes in width of the *Calliphora croceipalpis* third instar larvae ……60

List of figures

Fig. 1.1. Conceptual model of the nested processes of transport, storage and modification that affect the distribution of toxicants in the environment, insects and tissues……..16

Fig. 3.1. The proportional changes in length of the *Chrysomya chloropyga* empty pupal casings preserved in no preservative, 100%, 70% and 50% ethanol……57

Fig. 3.2. The proportional changes in width of the *Chrysomya chloropyga* empty pupal casings preserved in no preservative, 100%, 70% and 50% ethanol……58

Fig. 3.3. The proportional length change of the *Calliphora croceipalpis* third instar larvae preserved in 100%, 70% and 50% ethanol solutions……61

Fig. 3.4. The proportional width change of the *Calliphora croceipalpis* third instar larvae preserved in 100%, 70% and 50% ethanol solutions……62

List of abbreviations

AD	Artificial diet
AAFS	American Academy of Forensic Science
AIC	Akaike's information criterion
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
DNA	Deoxyribonucleic acid
GC/MS	Gas chromatography-mass spectrometry
GLM	General linear model
HPLC	High performance liquid chromatography
HSD	Honestly significant difference (Tukey HSD)
HWK	Hot-water-killed
IEC	International Electrotechnical Commission
ISO	International Organisation for Standardisation
LCL	Lower confidence limit
LSD	Least significant different (Tukey LSD)
MS	Mean square error
NMT	No meat treatment
PMI_{min}	Minimum post mortem interval
PMR	Post mortem redistribution
RIA	Radioimmunoassay
SOFT	Society of Forensic Toxicologists
SOP	Standard Operating Procedure
SS	Sum of squares
THC	Tetrahydrocannabinol
UCL	Upper confidence limit

Preface

This thesis consists of four chapters. The first chapter is a review of all of the publications within the field of forensic entomotoxicology, highlighting the shortfalls within the literature and the need for standardisation within the field. The second chapter aims to select a pre-existing dietary medium and modify it for use in future entomotoxicological studies. The third chapter aims to address the issue of growth rate quantification and identify whether length or width is a better parameter to quantify growth rate. The third chapter also aims to address the effects of different concentrations of ethanol in the preservation of the morphometric integrity of empty *Chrysomya chloropyga* pupal casings and *Calliphora croceipsalpis* third instar larvae. The fourth chapter synthesises the first three chapters and highlights the importance of shifting the direction of future studies towards standardisation as opposed to the testing of different toxicants on different species using different protocols, which is the case with the current literature. A combined reference list is included at the end so as to avoid reference repetition. The following paper has been published from this thesis:

Da Silva, E.I.T., Wilhelmi, B., Villet, M.H., 2017. Forensic entomotoxicology revisited – towards professional standardisation of study designs. Int. J. Legal Med. 131, 1399–1412. https://doi.org/10.1007/s00414-017-1603-9

Acknowledgements

First, I would like to start off by thanking my supervisors Prof Martin Villet and Dr Brendan Wilhelmi. From the initiation of this project, it was very clear that no part of it would be easy. Thanks to their hard work and dedication, I was able to complete this masters and I am forever indebted to them. Thank you for your patience through the years and for pushing me to finish.

A special thanks to Dr Claudia Tocco, Dr Sydney Moyo, Dr Lenin Chari and Dr Tatenda Dalu for not only helping me with the technical aspects of my work but also, for being good friends who motivated me throughout this degree. Thank you for all of the laughs and for the shoulders when I cried and for just being amazing people. I appreciate you all so much.

Thank you to Liam Yell, my extra pair of hands in the lab. Thank you for helping with the fly husbandry and for the enthusiasm. Your extra pair of hands, good conversation and sense of humour made everything much easier so thank you for that.

To all of my office mates over the years, Dr Megan Riddin, Ryan Van Zeeventer, Dr Terence Bellingan and Carmen Wormald, thank you for always being around and reminding me that I wasn't doing this alone. Thank you for all the laughs and serious conversations and making the work experience pleasant. To my colleagues, Sandiso Mnguni, Evans Mauda, Bahia Bradley, thank you so much for the moral support and input towards my thesis. I truly appreciate all of the help that you provided.

To all of the technical staff that helped with all of my experimental work, particularly Andre Van Rooyen, Pendrick Kotelo, Thabisa Mdlangu (Department of Zoology and Entomology) and Dr Sagaran Aboo (Department of Biochemistry), thank you for working tirelessly to ensure that I had all the necessary equipment to conduct my experiments.

A special thanks to the National Research Foundation for parts of my studies. Thank you to the Department of Zoology and Entomology and all of the students and staff and to Rhodes University.

Finally, I would like to thank all of my friends and family. I would like to especially thank Yakira Bahadur, Ntebogeng Kgokong, Faatimah Mansoor, Mazvita Sachikonye, Megan Reid, Daniela Maia and Wenzile Ndlovu. Your friendships have kept me going through it all. Thank you for all of the lunches and suppers and general conversations that were all about me venting my

frustrations. Thank you for all of the laughs and happy times and for making this process so much more bearable. To my grandmother, brother and mother thank you so much for all the love. Living away from home was never going to be an easy process, but having an amazing family made it easier. Even though we were apart, I felt your love every single day, and I dedicate this thesis to you.

Declaration

I, Erica Isabel Tavares Da Silva Mbatha, hereby declare that this thesis is being submitted for a degree at Rhodes University (Grahamstown, South Africa), under the supervision of Prof Martin Villet and Dr Brendan Wilhelmi. The various components of the thesis comprise of original work done by the author unless otherwise stated, and has not been submitted to any other university.

_____

Erica Da Silva

15 December 2017

Chapter 1: Forensic entomotoxicology revisited: towards professional standardisation of study designs

The following paper has been published from this chapter:

Da Silva, E.I.T., Wilhelmi, B., Villet, M.H., 2017. Forensic entomotoxicology revisited – towards professional standardisation of study designs. Int. J. Legal Med. 131, 1399–1412. https://doi.org/10.1007/s00414-017-1603-9

Abstract

Forensic entomotoxicology is the use of insects as evidence of whether a toxicant is present in an environment such as a corpse, river or landscape. The earliest overtly forensic study was published in 1977 and since then at least 63 papers have been published, most of them focused on the detection of environmental toxicants in insects or on effects of environmental toxicants on diverse insect indicator taxa. A comprehensive review of the published literature revealed various inconsistencies between studies that could be addressed by introducing standard protocols for such studies. These protocols could include selecting widespread and common model organisms (such as *Chrysomya megacephala* and *Dermestes maculatus*) and model toxicants (e.g. morphine and amitriptyline) to build up comparative databases; developing a standard matrix for use as a feeding substrate; setting guidelines for statistically adequate sample sizes; and deploying more sophisticated analytical methods from the general field of toxicology. Future studies should then be aimed at refining standardised protocols to improve experimental results, and make these results more comparable between studies.

Keywords: Forensic, Entomotoxicology, Ecotoxicology, Standardisation

Introduction

The term *forensic entomo-toxicology* was coined by Derrick Pounder (Pounder, 1991). Forensic entomotoxicology is concerned with the use of insect specimens as an indirect source of toxicological evidence in the absence of direct forensic matrices, such as blood,

urine, soil or water, in determining the presence of a toxicant in those insects' environments, which may be a dead body, a river or even an entire landscape.

Environmental forensic entomotoxicology has emphasised the use of organisms (specifically insects) as bioindicators of environmental toxicants like pollutants (Azam et al., 2015; Moriarty, 1988; Nuorteva, 1977; Richmond et al., 2016) , while medicolegal forensic entomology has tended to focus on using insects as surrogate or proxy samples when bodies are too decomposed to provide toxicological samples (Beyer et al., 1980; Gosselin et al., 2011b). Secondary applications of forensic entomotoxicology are best developed in medicolegal forensic entomology, where knowledge about toxicants in bodies may have implications for the estimation of post mortem intervals (Beyer et al., 1980; Gosselin et al., 2011b).

The first publications in environmental entomotoxicology appeared about 40 years ago (Nuorteva, 1977), and were soon followed by work in medicolegal entomotoxicology (Beyer et al., 1980). Over 60 primary studies are now published. The literature of medicolegal entomotoxicology has been reviewed intermittently (Gagliano-Candela and Aventaggiato, 2001; Goff and Lord, 2010; Gosselin et al., 2011b; Introna et al., 2001) and its goals critiqued (Tracqui et al., 2004). Like the rest of forensic science (National Research Council Committee on Identifying the Needs of the Forensic Sciences Community [NRCCINFSC], 2009), forensic entomotoxicology faces both academic and practical challenges to its validity as a source of forensic evidence and these have not been evaluated. Toxicology laboratories are required to have well-documented and consistently applied Standard Operating Procedures (SOPs) (Cooper et al., 2010; Lentini, 2009; Penders and Verstraete, 2006), and there is a clear disciplinary understanding of the need for standards in forensic entomology (Amendt et al., 2015, 2007; Villet and Amendt, 2011), but few standard experimental protocols and procedures have been transferred from the general field of toxicology to ensure that the results of academic entomotoxicological studies are fit-for-purpose in forensic settings. Because of the lack of standard experimental protocols, research results in forensic entomotoxicology are often hard to compare or generalise (National Research Council Committee on Identifying the Needs of the Forensic Sciences Community [NRCCINFSC], 2009), let alone theorise.

This chapter aims to review practically all of the primary research literature (Table 1.1), published in several languages (see reference list), that is currently available on forensic entomotoxicology. From this basis, we evaluate potential challenges to the validity of this source of evidence and outline components of a standardised methodology for future studies in forensic entomotoxicology.

Table 1.1. List of literature examined for this review, with the toxicants, insect species, and research goal of each study. 'Method' refers to the paper focusing on method development; 'Detection' refers to the paper focusing on the detection of toxicants; 'Effect' refers to the paper focusing on the effects of toxicants on insects; and 'Case study' refers to the paper using a forensic case as an example in which forensic entomotoxicology was used.

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case Study	
Calliphora dubia	[1]	Gun-shot residue		X			(Roeterdink et al., 2004)
Calliphora stygia	[1]	Methamphetamine		X	X		(Mullany et al., 2014)
Calliphora stygia	[1]	Morphine		X			(Gunn et al., 2006)
Calliphora stygia	[1]	Morphine		X			(Parry et al., 2011)
Calliphora stygia	[1]	Morphine			X		(George et al., 2009)
Calliphora vicina	[1]	Alprazolam	X	X			(Wood et al., 2003)
Calliphora vicina	[1]	Clonazepam	X	X			(Wood et al., 2003)
Calliphora vicina	[1]	Diazepam	X	X			(Wood et al., 2003)
Calliphora vicina	[1]	Flunitrazepam	X	X			(Wood et al., 2003)
Calliphora vicina	[1]	Lorazepam	X	X			(Wood et al., 2003)
Calliphora vicina	[1]	Nordiazepam	X	X			(Wood et al., 2003)
Calliphora vicina	[1]	Oxazepam	X	X			(Wood et al., 2003)
Calliphora vicina	[1]	Prazepam	X	X			(Wood et al., 2003)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case Study	
Calliphora vicina	[1]	Temazepam	X	X			(Wood et al., 2003)
Calliphora vicina	[1]	Triazolam	X	X			(Wood et al., 2003)
Calliphora vicina	[1]	Nordiazepam		X	X		(Pien et al., 2004)
Calliphora vicina	[1]	Morphine hydrochloride		X			(Hédouin et al., 2001)
Calliphora vicina	[1]	Morphine		X		X	(Introna et al., 1990)
Calliphora vicina	[1]	Amitriptyline		X		X	(Sadler et al., 1995)
Calliphora vicina	[1]	Temazepam		X		X	(Sadler et al., 1995)
Calliphora vicina	[1]	Trazodone		X		X	(Sadler et al., 1995)
Calliphora vicina	[1]	Trimipramine		X		X	(Sadler et al., 1995)
Calliphora vicina	[1]	Acetylsalicyclic acid		X			(Sadler et al., 1997)
Calliphora vicina	[1]	Sodium salicylate		X			(Sadler et al., 1997)
Calliphora vicina	[1]	Paracetamol		X			(Sadler et al., 1997)
Calliphora vicina	[1]	Aminohippuric acid		X			(Sadler et al., 1997)
Calliphora vicina	[1]	Amphetamine sulfate		X			(Sadler et al., 1997)
Calliphora vicina	[1]	Sodium amylobarbitone		X			(Sadler et al., 1997)
Calliphora vicina	[1]	Sodium phenobarbitone		X			(Sadler et al., 1997)
Calliphora vicina	[1]	Sodium thiopentone		X			(Sadler et al., 1997)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case Study	
Calliphora vicina	[1]	Sodium barbitone		X			(Sadler et al., 1997)
Calliphora vicina	[1]	Sodium brallobarbitone		X			(Sadler et al., 1997)
Calliphora vicina	[1]	Paracetamol			X		(O'Brien and Turner, 2004)
Calliphora vicina	[1]	Co-proxamol		X			(Wilson et al., 1993)
Calliphora vicina	[1]	Acetaminophen		X			(Wilson et al., 1993)
Calliphora vicina	[1]	Amitriptyline		X			(Wilson et al., 1993)
Calliphora vicina	[1]	Nortriptyline		X			(Wilson et al., 1993)
Calliphora vicina	[1]	Morphine hydrochloride		X		X	(Bourel et al., 2001c)
Calliphora vomitoria	[1]	Methamphetamine		X	X		(Magni et al., 2014)
Calliphora vomitoria	[1]	Morphine hydrochloride		X		X	(Bourel et al., 2001c)
Calliphora vomitoria	[1]	Morphine hydrochloride		X			(Bourel et al., 2001a)
Calliphoridae larvae (species unidentified)	[1]	Triazolam		X		X	(Kintz et al., 1990)
Calliphoridae larvae (species unidentified)	[1]	Phenobarbital		X		X	(Kintz et al., 1990)
Calliphoridae larvae (species unidentified)	[1]	Alimemazine		X		X	(Kintz et al., 1990)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case Study	
Calliphoridae larvae (species unidentified)	[1]	Clomipramine		X		X	(Kintz et al., 1990)
Calliphoridae larvae (species unidentified)	[1]	Oxazepam		X		X	(Kintz et al., 1990)
Chrysomya albiceps	[1]	Cadmium			X		(Al-Misned, 2001)
Chrysomya albiceps	[1]	Cadmium			X		(Al-Misned, 2003)
Chrysomya albiceps	[1]	Diazepam			X		(Carvalho et al., 2001)
Chrysomya albiceps	[1]	Cocaine			X		(de Carvalho et al., 2012)
Chrysomya albiceps	[1]	Codeine			X		(Fathy et al., 2008)
Chrysomya albiceps	[1]	Methylphenidate chloride			X		(Rezende et al., 2014)
Chrysomya albiceps	[1]	Methylphenidate hydrochloride			X		(Rezende et al., 2014)
Chrysomya albiceps	[1]	Phenobarbital			X		(Rezende et al., 2014)
Chrysomya albiceps	[1]	Nandrolone decanoate			X		(Souza et al., 2011)
Chrysomya chloropyga	[1]	Medroxyprogestrone acetate			X		(Da Silva and Villet, 2006)
Chrysomya chloropyga	[1]	Norethisterone enanthate			X		(Da Silva and Villet, 2006)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case Study	
Chrysomya chloropyga	1	Hydrocortisone			X		(Williams and Villet, 2014)
Chrysomya chloropyga	1	Sodium methohexital (hydrocortisone sodium succinate)			X		(Williams and Villet, 2014)
Chrysomya megacephala	1	Buscopan® (Butylscopolamine bromide)		X	X		(Oliveira et al., 2009)
Chrysomya megacephala	1	Malathion	X	X	X		(Rashid et al., 2008)
Chrysomya megacephala	1	Methylphenidate chloride			X		(Rezende et al., 2014)
Chrysomya megacephala	1	Methylphenidate hydrochloride			X		(Rezende et al., 2014)
Chrysomya megacephala	1	Phenobarbital			X		(Rezende et al., 2014)
Chrysomya megacephala	1	Nandrolone decanoate			X		(Souza et al., 2011)
Chrysomya megacephala	1	Malathion			X		(Bakr et al., 2012)
Chrysomya megacephala	1	Flunitrazepam	X	X			(Oliveira et al., 2014)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case Study	
Chrysomya megacephala	1	Flunitrazepam	X	X			(Baia et al., 2016)
Chrysomya megacephala	1	Malathion		X		X	(Gunatilake and Goff, 1989)
Chrysomya putoria	1	Gentamicin			X		(Ferraz et al., 2014)
Chrysomya putoria	1	Gentamicin sulphate (Hyatamicina)			X		(Ferraz et al., 2014)
Chrysomya putoria	1	Methylphenidate chloride			X		(Rezende et al., 2014)
Chrysomya putoria	1	Methylphenidate hydrochloride			X		(Rezende et al., 2014)
Chrysomya putoria	1	Phenobarbital			X		(Rezende et al., 2014)
Chrysomya putoria	1	Nandrolone decanoate			X		(Souza et al., 2011)
Chrysomya rufifacies	1	Malathion		X		X	(Gunatilake and Goff, 1989)
Chrysomya putoria	1	Cocaine			X		(de Carvalho et al., 2012)
Cochliomyia macellaria	1	Flunitrazepam	X	X			(Baia et al., 2016)
Cochliomyia macellaria	1	Phenobarbital		X		X	(Beyer et al., 1980)
Creophilus maxillosus	6	Methyl mercury		X			(Nuorteva and Nuorteva, 1982)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case Study	
Crocothemis servilia	[9]	Cadmium		X			(Azam et al., 2015)
Crocothemis servilia	[9]	Chrome		X			(Azam et al., 2015)
Crocothemis servilia	[9]	Nickel		X			(Azam et al., 2015)
Crocothemis servilia	[9]	Zinc		X			(Azam et al., 2015)
Crocothemis servilia	[9]	Copper		X			(Azam et al., 2015)
Danaus chrysippus	[8]	Cadmium		X			(Azam et al., 2015)
Danaus chrysippus	[8]	Chrome		X			(Azam et al., 2015)
Danaus chrysippus	[8]	Nickel		X			(Azam et al., 2015)
Danaus chrysippus	[8]	Zinc		X			(Azam et al., 2015)
Danaus chrysippus	[8]	Copper		X			(Azam et al., 2015)
Dermestes frischi	[4]	Morphine hydrochloride		X			(Bourel et al., 2001b)
Dermestes frischi	[4]	Morphine hydrochloride		X		X	(Bourel et al., 2001c)
Dermestes maculatus	[4]	Amitriptyline		X		X	(Miller et al., 1994)
Dermestes maculatus	[4]	Nortriptyline		X		X	(Miller et al., 1994)
Lucilia sericata	[1]	Morphine hydrochloride		X		X	(Bourel et al., 2001c)
Lucilia sericata	[1]	Tramadol		X	X		(El - Samad et al., 2011)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case Study	
Lucilia sericata	[1]	Methadone		X	X		(Gosselin et al., 2011a)
Lucilia sericata	[1]	2-ethylidene-15-dimethyl-33-dipheylpyrrolidine (EDDP).		X	X		(Gosselin et al., 2011a)
Lucilia sericata	[1]	Cadmium		X	X		(Moe et al., 2001)
Lucilia sericata	[1]	Morphine hydrochloride			X		(Bourel et al., 1999a)
Lucilia sericata	[1]	Ampicillin			X		(Sherman et al., 1995)
Lucilia sericata	[1]	Mezlocillin			X		(Sherman et al., 1995)
Lucilia sericata	[1]	Cefazolin			X		(Sherman et al., 1995)
Lucilia sericata	[1]	Ceftizoxime			X		(Sherman et al., 1995)
Lucilia sericata	[1]	Gentamicin			X		(Sherman et al., 1995)
Lucilia sericata	[1]	Clindamycin			X		(Sherman et al., 1995)
Lucilia sericata	[1]	Vancomycin			X		(Sherman et al., 1995)
Lucilia sericata	[1]	Opiates		X		X	(Campobasso et al., 2004)
Lucilia sericata	[1]	Cocaine		X		X	(Campobasso et al., 2004)
Lucilia sericata	[1]	Barbiturates		X		X	(Campobasso et al., 2004)
Lucilia sericata	[1]	Clomipramine		X		X	(Campobasso et al., 2004)
Lucilia sericata	[1]	Amitriptyline		X		X	(Campobasso et al., 2004)
Lucilia sericata	[1]	Nortriptyline		X		X	(Campobasso et al., 2004)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case Study	
Lucilia sericata	[1]	Levomepromezine		X		X	(Campobasso et al., 2004)
Lucilia sericata	[1]	Tioridazine		X		X	(Campobasso et al., 2004)
Lucilia sericata	[1]	Phenobarbital		X		X	(Campobasso et al., 2004)
Lucilia sericata	[1]	Norcodeine		X	X		(Kharbouche et al., 2008)
Lucilia sericata	[1]	Codeine		X	X		(Kharbouche et al., 2008)
Lucilia sericata	[1]	Morphine		X	X		(Kharbouche et al., 2008)
Lucilia sericata	[1]	Ketamine		X	X		(Zou et al., 2013)
Lucilia sericata	[1]	Morphine hydrochloride		X		X	(Bourel et al., 2001c)
Megaselia scalaris	[2]	Amitriptyline		X		X	(Miller et al., 1994)
Megaselia scalaris	[2]	Nortriptyline		X		X	(Miller et al., 1994)
Oxya hyla hyla	[10]	Cadmium		X			(Azam et al., 2015)
Oxya hyla hyla	[10]	Chrome		X			(Azam et al., 2015)
Oxya hyla hyla	[10]	Nickel		X			(Azam et al., 2015)
Oxya hyla hyla	[10]	Zinc		X			(Azam et al., 2015)
Oxya hyla hyla	[10]	Copper		X			(Azam et al., 2015)
Phormia regina	[1]	Ethanol			X		(Tabor et al., 2005)
Protophormia terraenovae	[1]	Morphine hydrochloride		X		X	(Bourel et al., 2001c)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case Study	
Protophormia terraenovae	1	Morphine hydrochloride		X			(Hédouin et al., 2001)
Sarcophaga peregrine	3	Cocaine		X	X		(Goff et al., 1989)
Sarcophaga peregrine	3	Heroin		X	X		(Goff et al., 1991)
Sarcophaga ruficornis	3	MDMA		X	X		(Goff et al., 1997)
Sarcophaga ruficornis	3	Methamphetamine		X	X		(Goff et al., 1992)
Sarcophaga ruficornis	3	Amitriptyline			X		(Goff et al., 1993)
Sarcophaga ruficornis	3	Nortriptyline			X		(Goff et al., 1993)
Sarcophaga tibialis	3	Hydrocortisone			X		(Musvasva et al., 2001)
Sarcophaga tibialis	3	Sodium methohexital (hydrocortisone sodium succinate)			X		(Musvasva et al., 2001)
Tenebrio molitor	7	Methyl mercury		X			(Nuorteva and Nuorteva, 1982)
Thanatophilus sinuatus	5	Morphine hydrochloride		X			(Bourel et al., 2001b)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case Study	
Unknown		Paraquat		X			(Lawai et al., 2015)
Unknown		Amitriptyline	X	X			(de Aguiar França et al., 2014)
Unknown		Carbamazepine	X	X			(de Aguiar França et al., 2014)
Unknown		Bromazepam	X	X			(de Aguiar França et al., 2014)
Unknown		Clonazepam	X	X			(de Aguiar França et al., 2014)
Unknown		Diazepam	X	X			(de Aguiar França et al., 2014)
Unknown		Flunitrazepam	X	X			(de Aguiar França et al., 2014)
Unknown		Cocaine	X	X			(de Aguiar França et al., 2014)
Unknown		Benzoylecgonine	X	X			(de Aguiar França et al., 2014)
Unknown		Aldicarb sulfone and sulfoxide metabolites	X	X			(de Aguiar França et al., 2014)

[1] Diptera: Calliphoridae; [2] Diptera: Phoridae; [3] Diptera: Sarcophagidae; [4] Coleoptera: Dermestidae; [5] Coleoptera: Silphidae; [6] Coleoptera: Staphylinidae; [7] Coleoptera: Tenebrionidae; [8] Lepidoptera: Nymphalidae; [9] Odonata: Libellulidae; [10] Orthoptera: Acrididae.

The scope of entomotoxicology

Our current knowledge of forensic entomotoxicology is largely organised around the two central questions that medicolegal forensics must commonly address in practice: from what immediate cause did a human or animal body die, and when did it die? In the context of toxicology, the first of these questions is about direct and indirect evidence drawn from insects of whether a body was poisoned; the second question is about whether the presence of toxicants must be taken into account when using the presence and development of insects in estimating the post mortem interval. Other applications include assisting in identifying people by adding to profiling studies, e.g. by revealing chronic medication. Environmental entomotoxicology is concerned with very similar questions (what toxicant/s affected an environment and when?) and with a third issue: where did the toxicant/s enter the environment?

The primary literature on entomotoxicology currently (1977 – 2016) includes over 63 papers (Table 1.1), mostly focused on the detection of toxicants. Although the findings of medicolegal entomotoxicology have been reviewed (Gagliano-Candela and Aventaggiato, 2001; Gosselin et al., 2011b; Introna et al., 2001), they have not been put into the broader context of medical or environmental toxicology. In the following discussion, we examine entomotoxicological questions in the light of pharmacological and physiological mechanisms that affect toxicants in bodies, because this can serve as a model for the dynamics of toxicants in environments in general.

Qualitative toxicant detection

Insects can provide indirect samples to establish the presence of certain toxicants in their environment when processes like putrefaction or drainage have affected the media that are usually sampled (i.e. blood, urine, water, soil or air) (Goff and Lord, 2010). Insects may also bioaccumulate certain toxicants (especially through food chains) to levels where they can be more readily detected (Azam et al., 2015; Nuorteva, 1977; Nuorteva and Nuorteva, 1982). The improving sensitivity of analytical instrumentation is eroding the significance of bioaccumulation for toxicological detection, and even putrefactive attrition in primary samples is a diminishing technical concern (Tracqui et al., 2004). One exception where entomotoxicology remains relevant is that in the extreme case of totally skeletonised bodies, the only remaining traces of toxicants may be in the associated insect faeces (termed *frass*)

and cast exoskeletons of larvae that have fed on the corpse (Bourel et al., 2001c; Miller et al., 1994). Similarly, insects may contain traces of toxicants even if the toxicant in the environment has been broken down, washed downstream or blown away.

It is important to understand the metabolism of toxicants by insects because this could influence whether a toxicant is detected. Knowing how toxicants are metabolised allows one to use appropriate extraction techniques to target diagnostic metabolic products. For example, the prior presence of codeine is indicated by the current presence of its metabolites, norcodeine and morphine (Kharbouche et al., 2008). The detection of a toxicant in an insect sample is highly dependent on the extraction and detection efficiencies of an analytical method and if more is known about relevant metabolites, then appropriate protocols could be deployed. The literature comparing the metabolism of toxicants across species has focused on mammals and little research has included insects (Gosselin et al., 2011b), although some species have been tested fairly intensively (e.g. *Drosophila* (Herskowitz, 1951); *Calliphora* (Sadler et al., 1997, 1995)).

Quantitative toxicant detection

Can insects provide samples to establish the quantity of a toxicant present in their environment? The quantity of toxicant in the environment to which an insect is exposed depends on processes of transportation, catalysis and sequestration of the toxicant (Figure 1.1), and on the insect's ecological function within that environment. Once the insect encounters it, the toxicant undergoes tropism (preferential movement to certain tissues) through pharmacokinetic processes such as absorption, distribution and excretion, metabolism, and sequestration (Figure 1.1), which is why the insect is considered an indirect sample. The degree to which an indirect sample represents a primary matrix depends on the magnitude of these complex processes.

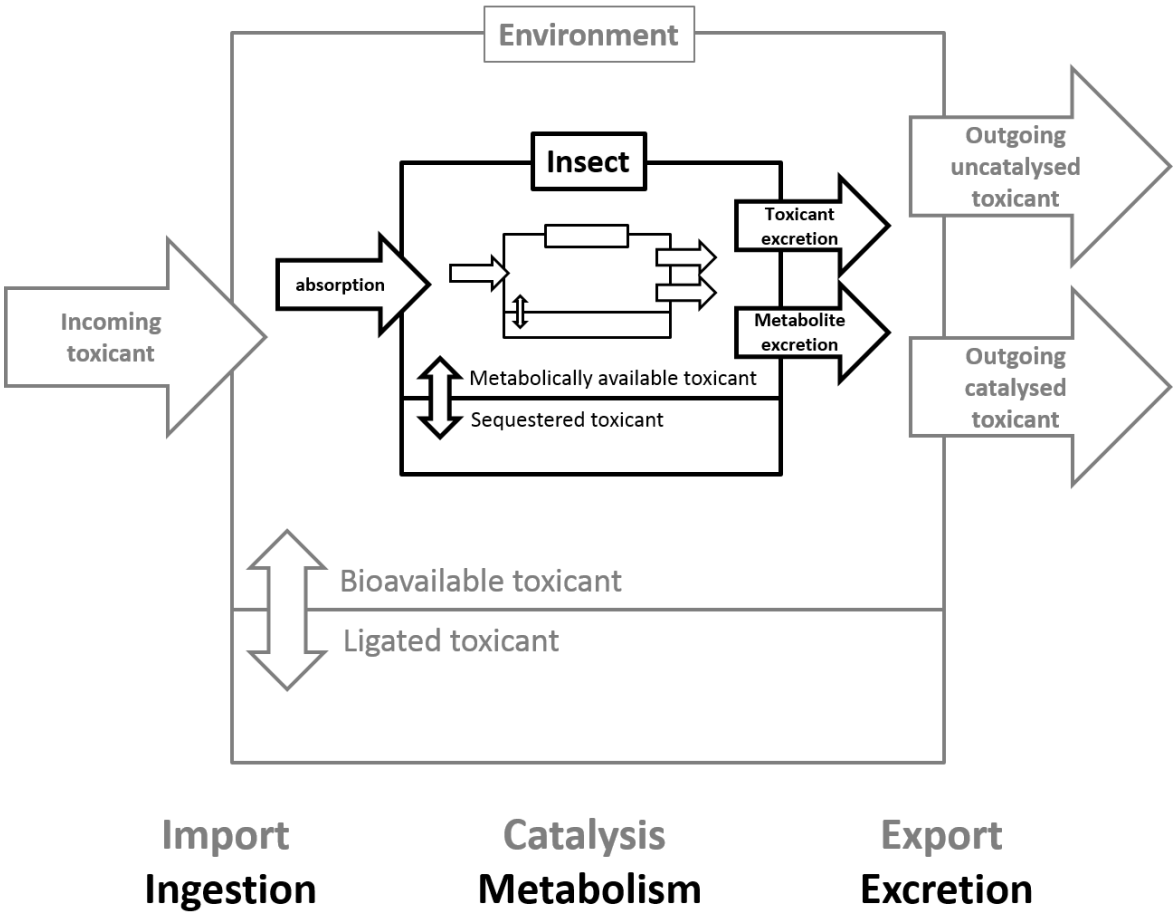

Fig. 1.1. Conceptual model of the nested processes of transport, storage and modification that affect the distribution of toxicants in the environment (grey), insects (bold black) and tissues (feint black).

Absorption, distribution and excretion may occur at different rates so that even when no other processes affect the quantity of toxicant in an insect, it may be at a different concentration from that in the environment. The environmental concentrations to which insects are exposed are affected by similar processes (Figure 1.1). In corpses, for example, the manner in which the toxicant is ingested by a human being can affect the rate at which it is metabolised and distributed in the body (Langer, 1998). A toxicant administered intravenously bypasses the absorption phase (unlike orally administered toxicants), reducing the lag time between administration and the appearance of detectable toxicant concentrations in blood (Drummer, 2008); analogously, toxicants may diffuse into an environment or be dumped.

The distribution of toxicants through particular tissues is also affected by blood flow within the tissues, ionisation characteristics of the toxicant, degree of protein binding and the affinity

of the toxicant for each tissue (for example, THC has a higher affinity to muscle and adipose tissue whereas alcohol is evenly distributed throughout the body (Drummer, 2008)). The processes may also not reach an equilibrium that can be related back to the original dose, particularly if the duration of exposure is not known. For instance, when absorption exceeds excretion, toxicants will bioaccumulate (Rainbow, 2007; Wang and Rainbow, 2008).

Catalysis of toxicants in the environment or their metabolism within a body will generally give them a characteristic half-life, which affects their recovery from forensic samples. Various metabolic processes modify toxicants within an animal's body, particularly in organs such as the vertebrate liver (Gordon Gibson and Skett, 2008a), which enhances tropism (Nassar et al., 2009). The metabolism of toxicants depends primarily on their physiochemical properties, including their physical state (solid, liquid or gaseous), lipophilicity and solubility (Gordon Gibson and Skett, 2008b; Hörter and Dressman, 2001). For instance, the metabolism of mercury by larvae of *Calliphora vicina* is affected by whether the mercury is methylated (Nuorteva and Nuorteva, 1982). The pathways and rates of metabolism are affected by intrinsic factors like species, genetics, sex, age, hormone activity, pregnancy and disease, and extrinsic factors like diet and environment (Gordon Gibson and Skett, 2008c; Nassar et al., 2009; O'Malley et al., 1971). Postmortem changes to toxicant concentrations also occur in bodies (Drummer, 2008; McIntyre and Escott, 2012). This phenomenon is known as postmortem drug redistribution (PMR) (Gerostamoulos et al., 2012). PMR occurs as a result of the rupturing of cell membranes, causing changes to the concentrations of toxicants as a result of diffusion through different tissues (Drummer and Gerostamoulos, 2002). This process may cause toxicant concentrations to increase *post mortem* in some tissues. Some of these toxicants include antipsychotics (such as amisulphide, palperidone, chlorpromazine, clozapine, haloperidol, olanzapine, promethazine, quetiapine, risperidone and zuclopenthixol) (Saar et al., 2012), THC and its metabolites (Holland et al., 2011), digoxin (Aderjan and Mattern, 1980; Drummer, 2008) and fentanyl (Olson et al., 2010).

Sequestration in insects' environments may involve binding of toxicants to sediments or ligands that can make them unavailable; in bodies this is a component of toxicant tropism (Rainbow, 2007; Wang and Rainbow, 2008). Each body tissue has its own unique chemical and physical properties (Shen et al., 2005), and these can have an effect on how a toxicant distributes and deposits in the body. For instance, hydrophobic molecules may deposit in

lipid-rich tissues, while hydrophilic molecules accumulate in more aqueous tissues (Hörter and Dressman, 2001). Patterns of tropism may vary with the age and sex of the body (O'Malley et al., 1971). Similar processes affect the distribution of toxicants in other environments (Figure 1), but tropism in the insect itself is usually not a forensic concern unless frass or exoskeletons are being used for toxicological analysis. Even so, blowfly larvae may accumulate toxicants that are then deposited into the intestine of the pupa and excreted with the meconium by the adult (Nuorteva and Nuorteva, 1982), leaving almost no trace of the toxicant in the adult insect.

In principle it may be possible to estimate the quantity of toxicant in a source by correcting for these metabolic processes empirically (e.g. using an empirical ensemble rate for the whole organism) provided that the duration of exposure is known, but in medicolegal cases this duration (i.e. the postmortem interval) is commonly the issue in question. Toxicants show unusual empirical patterns of transfer to insect samples (e.g. heavy metals), and these are typically specific to both the toxicant and the insect species (DeForest et al., 2007). In addition, insects feeding in particular parts of an environment or body may eat quantities of a toxicant that are uncharacteristic of the environment or body as a whole, thus providing quantitatively unrepresentative toxicological samples. In particular, fly larvae may wander about a body and feed from different sites on it, and if there is significant toxicant tropism in the body, it may require many replicated samples of insects to represent the general quantity of toxicant in the body accurately.

The implication of these processes (Figure 1.1) for medicolegal forensic entomotoxicology is that the concentrations of toxicants that are detected in insect specimens are affected by (a) toxicant tropism in the body, (b) pre- and postmortem changes, and (c) the extraction and detection efficiencies of the analytical techniques. This means that the quantities of toxicant detected in insects is best interpreted qualitatively or by using empirical experimental surrogates such as dead pigs. Environmental toxicology has made progress in refining the interpretation of indirect samples (e.g. (DeForest et al., 2007; Wang and Rainbow, 2008)), and approaches such as comparative modelling (Wang and Rainbow, 2008), biokinetic modelling (Wang and Rainbow, 2008) and saturable uptake kinetics modelling (Simkiss and Taylor, 1989) can be adopted and adapted by medicolegal entomotoxicology.

Postmortem interval estimation

Thirty-three studies have addressed the effects of different toxicants on the growth rates of different insect species of forensic interest (Table 1.1). However, with the lack of standardisation and replication in methods and conflicting outcomes of these studies, it is difficult to determine whether the results are drug effects or artefacts. There are indications that neuroactive toxicants have similar effects on insects and mammals. For instance, cocaine (McClung and Hirsh, 1998), caffeine (Hendricks et al., 2000; Shaw et al., 2000), modafinil (Hendricks et al., 2003) and methamphetamine (Andretic et al., 2005) are pharmacological stimulants for *Drosophila*, while antihistamines cause them to sleep (Shaw et al., 2000). It is therefore understandable that cocaine should cause accelerated feeding and growth in blow flies (de Carvalho et al., 2012; Goff et al., 1989). Vertebrate hormones like progesterone can be expected to have little effect on insects, and this appears to be the case (e.g. (Da Silva and Villet, 2006)). The effects of steroid hormones are less predictable (Sadler et al., 1997).

Toxicants that affect insect development and behaviour may have dose-dependent effects on growth, but because of our currently incipient understanding of insect pharmacokinetics (Figure 1.1), it is difficult to model these unless one knows the duration of exposure, which is the unknown post-mortem interval that many medicolegal entomotoxicological investigations need to estimate (Musvasva et al., 2001). Furthermore, the dose-dependence is also not necessarily linear, and may show hormesis (Musvasva et al., 2001), where initial dose-dependent trends reverse at even higher concentrations (Andretic et al., 2005).

Toxicant-induced variation in development may be confounded with variation in development caused by feeding on different tissues, particularly vertebrate liver (Beuter and Mendes, 2013; Ireland and Turner, 2006; Kaneshrajah and Turner, 2004; Niederegger et al., 2013; Richards et al., 2013). This effect can be problematic for medicolegal investigations if larvae have migrated around a body to any degree while feeding, and also needs to be taken into account in the selection of toxicant matrices for laboratory experiments.

This means that at present the effects of particular drugs on particular species feeding on particular tissues must be determined empirically, but the scope for developing a database based on a standard testing method is apparent.

Toxicant source localization

Toxicants in the environment may originate from point sources, such as a dumping site, or diffuse sources such as agricultural run-off. Point sources are typically traced by mapping the concentrations of the toxicant in samples and identifying gradients emanating from the source. In rivers, it can also be shown that toxicants are absent upstream of a point source. Diffuse sources have shallow gradients. Pioneering work in this direction was done on mercury in flies, fish and birds in Finland (Nuorteva, 1977).

Measurement parameters and storage techniques

The most common parameters used to quantify age have been the lengths and masses of the insect specimens (Knapp, 2012). Day and Wallman (2006b) have suggested that width could also be used as a parameter to quantify growth because there are limitations to using length and mass. The length is affected by an anomaly described by Day and Wallman (2006b) as "head curling" in which the head of the larva curls in as a result of the killing and preservation method. The mass would also be a problematic measure to use because dry mass would require destructive sampling and there are currently no models in place to convert wet mass to live mass (Day and Wallman, 2006a). It is important to use a standardised method of quantifying age because the use of different parameters could have an effect on the accuracy of the age determination because of the differences in resolution of the different parameters. Villet et al. (2010) highlight the importance of precision, bias and accuracy in obtaining measures to then use in the determination of age of the insect and ultimately the PMI_{min} of a corpse.

The collection, killing and storage techniques used to collect entomological samples will also have an effect on the morphometric measures taken from the specimens (Adams and Hall, 2003; Midgley and Villet, 2009). Day and Wallman (2008) and Brown et al. (2012) have both stated that shrinkage can occur as a result of the killing and preservation methods used. It is also important to consider the evidence that is to be examined from the specimen. Ideally, the preservation method used should preserve the morphometric, DNA (King and Porter, 2004; Bisanti et al., 2009) and toxicological (Amendt et al., 2015) integrity of the specimen so as to obtain as much evidence as possible in cases where there might not be an abundance of entomological evidence or specimens available. Chapter 3 deals with determining whether

length or width is a better parameter to estimate age, and which preservative solution is ideal for preserving the morphometric measures of pupal casings and third instar larvae.

Analytical review of the available literature

We reviewed the literature on entomotoxicology written in several languages to assess the status of the subject and to identify patterns in the published data that could put it on an explanatory and predictive footing. Of the 63 papers considered, six were excluded because they did not meet the following inclusion criteria: the research had to be relevant to the field of entomotoxicology; the data had to be original and not published elsewhere; and the methods had to describe the drug delivery matrix or feeding substrate of the insects.

Insect taxa

The insects that were studied in each of the papers were classified by family and species. This was of importance because the insect species played a role in the observed effects and whether there was higher interest in certain species of insects and to postulate reasons for the interest from an entomotoxicological perspective.

In total, ten families of carrion-feeding insects have been studied (Table 1.1), although the list of potential research targets is much larger (e.g. (Campobasso et al., 2001; Ortloff et al., 2012; Shi et al., 2009; Sukontason et al., 2007; Villet, 2011; Wang et al., 2008)). The most studied family was the blow flies (Diptera, Calliphoridae) which featured in 70% (43 papers) of the literature, followed by the flesh flies (Diptera, Sarcophagidae) (10%, 6 papers) and the hide beetles (Coleoptera, Dermestidae) (5%, 3 papers).

The blow flies *Lucilia sericata* and *Calliphora vicina* were studied the most (12%, 9 papers), followed by *Chrysomya albiceps* and *Chrysomya megacephala* (11%, 8 papers) and *Calliphora stygia* and *Chrysomya putoria* (5%, 4 papers). Although the previously mentioned species were forensically relevant, other species related to ecotoxicology were also studied (*Crocothemis servilia* [Odonata, Libellulidae], a dragonfly, *Danaus chrysippus* [Lepidoptera, Nymphalidae], a butterfly and *Oxya hyla hyla* [Orthoptera, Acrididae], a grasshopper: 1% or once each). Ideally, the model insects for these studies should be insects that fit the following criteria:

1. They should be associated with forensic cases and they should be insects that have a direct relationship with a decomposing corpse (i.e. feeding, oviposition, etc.). In the case of ecotoxicology; they should be in constant contact with the system that is being tested.
2. The species should be geographically widespread, common and abundant. This would allow many research groups to contribute comparative data, which entomotoxicology is currently short of.
3. Their husbandry should be relatively straightforward to facilitate research.

The early stages (fresh, bloated and active decay) of decomposition are colonised by the blow flies *L. sericata, C. vicina* and *C. megacephala*, which fulfil the abovementioned criteria and would therefore be ideal taxa in those stages. The hide beetle *Dermestes maculatus* (Coleoptera, Dermestidae) would be ideal as an indicator of toxicants later in the decomposition process (during the advanced decay and skeletonised stages) and this species also fulfils these criteria (Byrd and Castner, 2010; Voss et al., 2009; Wang et al., 2008).

Feeding substrates

The ideal feeding and rearing substrate or experimental toxicological matrix should fit the following criteria, which are not necessarily intended to also apply to oviposition media.

(1) The toxicant should be stable and homogenous throughout the matrix. Stability and homogeneity of the toxicant in the matrix allows the concentration of the toxicant to remain consistent throughout the duration of the study. Ideally, the toxicant should be evenly distributed throughout the feeding substrate to increase the chance of the insects ingesting the desired amount of toxicant, and ingesting it at the steady rate or dose anticipated by the experimental design. It also makes it possible to calculate the concentration of the toxicant being ingested by the insect, by measuring the amount of the matrix consumed.

(2) The matrix should not react significantly with the toxicant (i.e. there should be no metabolites present). Unreactive matrices help to ensure that the experimental insects are exposed to consistent doses of toxicants, and avoid the confounding effects of metabolites. Liver has been identified as an particularly poor matrix in this regard (Kharbouche et al., 2008; Richards et al., 2013).

(3) The matrix should be palatable, digestible and nutritious for the target animal. Insects should find their feeding substrate palatable because if they do not eat it, they will not grow normally and the toxicant will not be delivered to them. Along with palatability, it should also provide the insects with the nutrients necessary for growth and reproductive maturation.

It has been suggested that the matrix should closely represent matrices in forensic investigations, to ensure that experimental results can be applied to field investigations without criticism (Clements et al., 2013). Considering that the ultimate goal of the matrix is to deliver nutrients and the toxicant to the insect, the importance of its similarity to matrices found in forensic investigations is questionable. If the insects are provided with all of the necessary nutrients to reach the landmarks of its life (e.g. growth, ecdysis, reproduction, etc.) and there are no observable effects as a result of the feeding substrate (control), then it does not necessarily have to mimic the tissues of the human body.

(4) The rearing matrix should be easy to handle, have minimal to no odour and be disposed of easily. Handling properties of the matrix will be affected by its tendency to decay, produce a putrid odour, become unpleasant and potentially present a health hazard when handled. The ideal *feeding* or *rearing* substrate should have very little odour and preferably be easy to handle and dispose of. Unfortunately, an *oviposition* substrate cannot be completely odourless as the odour is what attracts insects to the matrix to lay eggs. The solution to this would be to restrict oviposition matrices to facilities with efficient air extraction, and to transfer eggs to the feeding matrix for the experiment.

(5) The production cost of the matrix should be economical. Production costs are a central concern for forensic laboratories, but if entomotoxicological studies are to be conducted in all regions of the world, the cost to make the ideal feeding substrate should be low enough for any laboratory to produce. Another criterion should be that all of the ingredients needed for the production of the substrate should be accessible in any region of the world.

The first step in the selection of the ideal feeding substrate should be to test criteria excluding the toxicological aspect in order to ensure that the substrate is viable for use in the husbandry of insects (Chapter 2). Once this has been established, studies should be done to determine the stability of toxicants within the selected substrate and furthermore, that the toxicant is being delivered efficiently to the model insect.

We classified the feeding substrates used in the literature by donor organism and the donor organ used in each study. Rabbit was used most often (25%, 15 papers), followed by cow (20%, 12 papers) and human (15%, 9 papers). When considering which donor organism to use for a study, it is important to consider the statistical analyses required because there needs to be a suitable number of replicates. More research needs to be done to determine the number of treatments, controls and replicates per treatment and control that would be required. Due to ethical constraints on using animals such as rabbits, it is seldom possible to get sufficient numbers of animals.

Human tissue (which would be the most realistic matrix for medicolegal entomotoxicology) was used in only nine studies (conducted in the USA, France, Italy, Scotland and Japan), none of which were experimental. This is understandable because human tissue is difficult to obtain in many countries for ethical and practical reasons. These studies obtained human tissues from decedents who were suspected of overdosing on toxicants. Although there are clinical average and lethal dosages, this can be problematic because the tolerance level for drugs in humans is dependent on variables which vary between individuals, as outlined earlier. This means that the dosage that could result in an overdose in one person could be sublethal in another.

Two studies used kangaroo meat, which is not easily available in most countries. Ideally, one would use a tissue type that is easily and widely accessible. Another factor which should be taken into consideration are religious constraints on using certain animals in different countries (e.g. the use of beef in a Hindu country or pork in a Muslim country). Taking all of these factors into consideration, the ideal donor organisms would be chicken and fish. These could be incorporated into standardised artificial diets.

In terms of the organs used, 31% (20 studies) used liver, followed by whole carcasses (17%, 10 studies) and muscle and mince (12%, 8 studies). We classified muscle and mince as being different because the fat content of mince is most likely to be variable across different studies. Liver is catalytically active (Kharbouche et al., 2008), which violates an important criterion for matrix selection. Most published studies did not consider the stability of the toxicant within the feeding substrate and so it is not known whether the concentration of the toxicant remained consistent throughout the duration of these studies. Kharbouche *et al*. (2008) noted that metabolism of codeine (into norcodeine and morphine) occurred in their study as a result

of enzymes that remained active after autolysis of the liver tissue that they used (Kharbouche et al., 2008). This shows that liver is not an inert tissue type, which violates our first criterion for matrix selection.

An alternative to using particular organs as a feeding substrate is the use of whole carcasses. This gives the insects a varied diet that counteracts the confounding effects of drug tropism, especially if the carcass is small. In many studies where whole carcasses were used, there was insufficient replication for confident statistical analysis. If there is variation among two or three carcasses, it is hard to know which carcass (if any) is an outlier. Such sample sizes are often insufficient to show that a sample is representative. There are also ethical constraints on how many whole organisms can defendably be used in a study.

In chapter 2, a pre-existing diet is selected and modified for potential use in entomotoxicological studies. The diet was tested in accordance with the abovementioned criteria.

Toxicants

A wide variety of toxicants have been reported in the literature. Most of them were of forensic relevance and not merely model toxicants because most of the literature was medicolegally or environmentally relevant. In total, 122 toxicants have been examined, including, analgesics (25%, 18 studies), depressants (23%, 16 studies), stimulants (14%, 10 studies) and antidepressants (10%, 7 studies). Toxicants that fall within these pharmacological action groups are usually also classified as drugs of abuse, associated with overdoses and other forensically relevant cases.

Toxicants most often reported in the literature were morphine (8%, 10 studies), amitriptyline (6%, 7 studies) and cocaine, cadmium, flunitrazepam, nortriptyline and phenobarbital (3%, 4 studies). Morphine is a metabolite of heroin (Rook et al., 2005) which is an abused drug in many regions of the world (United Nations Office on Drugs and Crime, 2015). Amitripyline is an antidepressant associated with overdose and suicide cases. Cocaine is a drug of abuse and, like heroin, is a problem in many countries (United Nations Office on Drugs and Crime, 2015).

Other toxicants examined such as heavy metals (cadmium, copper, nickel, zinc and chrome), insecticides and accelerants are relevant to the fields of ballistics, environmental ecotoxicology and arson. In these cases, the insects were successfully used as bioindicators

for potential toxicants in the environment (Al-Misned 2001, 2003; Azam et al., 2015; Moe et al., 2001; Nuorteva and Nuorteva, 1982) and gunshot residues (Roeterdink et al., 2004) (Table 1.1).

The presence of a target toxicant in the feeding substrate before and after the experiment, the stability of the toxicant within the feeding substrate, and the presence (and if possible the concentration) of the toxicant in the insect need to be validated by toxicological analyses. The results will validate whether the observed effects are a direct result of the toxicants that the insects ingest and also what processes the toxicant undergoes once ingested by the insect (i.e. whether the toxicant is metabolised, excreted or assimilated). As a result of this field being underdeveloped, information on the fate of the toxicant once it is ingested by an insect is generally not currently available.

Toxicological analysis

The preparation of a forensic sample for toxicant detection is highly dependent on the type of detection method that will be used. When developing an extraction technique, it is important that the extraction efficiency is known and that this known value will remain constant for all extractions. The extraction efficiency on its own needs to be tested prior to analysis to ensure that the toxicant will be extracted from the insects (SOFT & AAFS, 2006).

Each of the published studies was examined for its extraction technique and detection method. Most of the literature (57%, 31 studies) either did not mention or did not use an extraction technique. The extraction efficiency should be reported as this helps to validate the results, especially if quantities of toxicant are reported. In the literature where extraction techniques were reported, solid phase extraction was used in 31% (17 studies) and liquid-liquid extraction was used in 7% (4 studies).

After extraction, the matrices should be tested to confirm the presence and concentration of the toxicants, otherwise it is impossible to confirm that the observed effects are a direct result of the presence and amount of the toxicant. Of the 55 papers examined, 21% (12 studies) did not use any analytical methods to detect or quantify the toxicant in the insects. When analytical methods had been used, 18% (10 studies) used HPLC or GC/MS and 11% (6 studies) used RIA. Recently, Oliveira et al. (2014) and Baia et al. (2016) developed quantification methods which are not invasive or destructive to the insect evidence. From an entomological

perspective, this could be highly beneficial, especially if the insects are also needed to estimate a PMI or for DNA analysis.

Most analytical techniques can be expensive, technical and labour-intensive, and most of the papers that did use analytical methods only detected and did not quantify the toxicant in the insects. Quantification usually requires a mass spectrometry system, which is high-maintenance and demands technical experience. It is therefore understandable that it can be difficult to conduct comprehensive toxicological validation.

Statistical analysis

There was a high level of inconsistency in the numbers of controls, treatments and replicates amongst the studies. More studies should be conducted to determine what the appropriate number of the above mentioned parameters should be for the results to be statistically robust.

Experimental vs. Applied: the application of entomotoxicology

Most experiments conducted within the field of medicolegal forensic entomotoxicology have been done in the controlled settings of a laboratory, while ecotoxicological research usually draws samples from the environment. Although laboratory results are indicative of what could possibly happen in an actual case, there are confounding factors (such as weather conditions) that cannot be controlled accurately in the field. When using results from laboratory studies, it is crucial to determine how applicable they would be to an actual case by taking into account the processes summarised in Figure 1.1.

A factor to consider is the feasibility of conducting these tests in a mortuary or forensic laboratory. Unfortunately, due to the equipment and expertise required, and the challenge of meeting the SOPs for toxicological testing (e.g. routine calibration, logging protocols, interinstitutional benchmarking) (SOFT & AAFS, 2006), there is a high chance that many mortuaries and forensic laboratories would not be able to conduct these tests. The focus should also lean towards getting forensic laboratories ISO 17000 accredited as this would further validate the results being produced by these laboratories. In particular, ISO/IEC 17025 provides the general standard for competence of testing and calibration laboratories, and

most laboratories must hold such accreditation to be deemed technically competent by many suppliers and regulatory authorities.

The Future: a basic protocol for future entomotoxicological studies

Our review of the literature has shown a few inconsistencies in the field of entomotoxicology:

1. There is currently no standardisation in this field in terms of the methods which are used to conduct these studies. This has ultimately led to the inability to compare results between studies (comparison might still be possible but results might be of minimal value).
2. There is insufficient replication in most of studies, which increases the risk of both Type I and Type II errors of statistical inference.
3. The resources required to conduct a successful entomotoxicological study can be expensive and this leads to studies not being validated to broader toxicological standards.
4. The entomological and toxicological aspects of entomotoxicology are equally important. Some studies cover one aspect of the field well and completely omit the other aspect, e.g., an entomologist conducting a study without a toxicologist. This means that some vital information can be overlooked when considering the overall application and meaning of the results of the study.

The ideal protocol for entomotoxicological studies would address all of these issues. Ideally, the focus of future research should work towards developing a comprehensive protocol which could be convenient to people in most parts of the world and if all of the studies are conducted correctly using the same protocol, the results would be more comparable.

Ideally, the standard protocol would be cost-efficient and user-friendly. It would allow for sufficient replication and enough dose levels for dose-dependence and hormesis (or lack thereof) to be observed and validated. One of the most expensive parts of such research would be the analytical work and this is because it requires not only expertise but also high-end technology. In the case of institutions that lack these capacities, they could send samples

to other institutions and open gateways for collaborations, particularly where fields of expertise do not overlap.

Currently, the focus of the available research has been to determine the effects of different toxicants on various insect species. The hope with this review is that the gaps in the field are recognised and that the focus is then shifted from determining effects to standardising the field. Once the field has been standardised based on the abovementioned criteria, the hope would then be to carry on with the determination of the effects of different toxicants on insect species, bearing in mind the implications of these results. Ultimately, any results that are obtained in the laboratory setting should aim to aid scientists working on forensic cases.

Research objectives

This thesis aimed to address the following research objectives:

1. Identify the current issues with the experimental protocol (or lack thereof) within the field of forensic entomotoxicology
2. Select an already existing dietary medium and modify it for use in future entomotoxicological studies.
3. Quantify the growth rates of *Chrysomya chloropyga* using length and width and determine which of the two parameters is a more accurate representation of growth rate.
4. Determine the effects of different concentrations of ethanol on the lengths and widths of the empty pupal casings of *Chrysomya chloropyga* and the third instar larvae of *Calliphora croceipalpis.*

Chapter 2: Comparison of different dietary media on the development of *Chrysomya chloropyga*

Abstract

The use of insects in the detection of toxicants within a substrate is referred to as entomotoxicology, and there are numerous studies that have tested the effects of various toxicants on different species. Most of these studies have used different feeding substrates to deliver the toxicants to the insects. Using different feeding substrates to rear insects can affect their growth rates and this could result in artefactual effects and varying conclusions. The aim of the study was to investigate the effects of dietary media on insect development. Using body length and width as measures of insect development, an artificial dietary medium was selected. Four meat types (control (no meat treatment), fish, beef and pork) were added to these media and they were used to test effects of diet on development of *Chrysomya chloropyga* larvae. The lengths and widths were measured every 6 hours for 7 days to monitor their growth rates. The results showed that there was a statistical difference in the lengths and widths of the larvae between the treatments over time. The larvae that were reared on the fish and control media did not reach imago stage. The pork and beef diets were found to yield larvae which were not significantly different from one another in terms of their size. Future studies should focus on testing other artificial diets for entomotoxicological studies and should focus on the standardisation of experimental protocols.

Introduction

In medico-criminal forensic experimental work, it is not always possible to replicate conditions found at crime scenes. For example, due to ethical considerations and difficulties in availability of corpses, it is not always possible to use human corpses in decomposition studies. Therefore, substitutes such as pigs and rats are used as models (Byrd, 1998; Richards et al., 2013). In the case of forensic entomology, the idea has always been to use a model donor and model tissue which closely represents human tissue. The use of artificial diets has become a popular choice because of their reduced odour, and easier and cleaner handling in comparison to organic media such as whole animal carcasses and organs (Osuji, 1978; Leal et al., 1982; Estrada et al., 2009; Byrd and Tomberlin, 2010; Rueda et al., 2010; Rabêlo et al., 2011). The current literature presents a wide array of donor organisms and tissues which

have been used in place of human tissue (Table 2.1), but each model has presented its own set of shortcomings (Da Silva et al., 2017). Another issue to take note of is that the lack of standardisation with regards to the dietary media used amongst different laboratories could be resulting in artefactual effects within the produced results (i.e. the observed results are a product of the diet used rather than the toxicant being tested) (Rabêlo et al., 2011).

Table 2.1. The different donor organisms and donor organs used in entomotoxicological studies from 1980 – 2016.

Diet/Donor organism	Tissue	Reference
Artificial diet	Liver, agar, brewer's yeast	(Sherman et al., 1995)
Artificial diet	Muscle, powdered egg and powdered agar	(Sadler et al., 1997)
Artificial diet	Artificial diet	(Moe et al., 2001)
Artificial diet	Diet described by Leal et al (1982)	(Oliveira et al., 2009)
Artificial diet	Artificial diet	(Souza et al., 2011)
Artificial diet	Muscle, agar	(Ferraz et al., 2014)
Artificial diet	Bovine liver, raw muscle, stomach and chicken	(Rezende et al., 2014)
Cow	Mince	(Bourel et al., 2001a)
Cow	Mince	(Bourel et al., 2001b)
Cow	Mince	(Al-Misned, 2001)
Cow	Heart	(Wood et al., 2003)
Cow	Mince	(Al-Misned, 2003)
Cow	Heart	(Pien et al., 2004)
Cow	Muscle	(Roeterdink et al., 2004)
Cow	Mince	(Gunn et al., 2006)
Cow	Heart	(Gosselin et al., 2011a)
Cow	Liver	(Magni et al., 2014)
Cow	Heart	(Oliveira et al., 2014)

Diet/Donor organism	Tissue	Reference
Chicken	Liver	(Musvasva et al., 2001)
Chicken	Liver	(Da Silva and Villet, 2006)
Chicken	Liver	(Williams and Villet, 2014)
Dry adult flies	Flies	(Nuorteva and Nuorteva, 1982)
Human	Whole carcass	(Beyer et al., 1980)
Human	Whole carcass	(Gunatilake and Goff, 1989)
Human	Heart, Liver, Lung, Spleen, Kidney and Bile	(Kintz et al., 1990)
Human	Liver	(Introna et al., 1990)
Human	Muscle	(Wilson et al., 1993)
Human	Whole carcass	(Miller et al., 1994)
Human	Muscle	(Sadler et al., 1995)
Human	Muscle (psoas)	(Bourel et al., 2001b)
Human	Liver	(Campobasso et al., 2004)
Kangaroo	Mince	(Mullany et al., 2014)
Kangaroo and Lamb	Kangaroo mince, lamb fry and heart (pet mince)	(George et al., 2009)
Living blow fly larvae	Flies	(Nuorteva and Nuorteva, 1982)
N/A	N/A	(Azam et al., 2015)
Pet food	Pet food	(Parry et al., 2011)
Pig	Liver	(Kharbouche et al., 2008)

Diet/Donor organism	Tissue	Reference
Pig	Liver	(O'Brien and Turner, 2004)
Pig	Muscle	(Tabor et al., 2005)
Pig	Offal	(de Aguiar França et al., 2014)
Rabbit	Liver	(Goff et al., 1989)
Rabbit	Liver	(Goff et al., 1991)
Rabbit	Liver	(Goff et al., 1992)
Rabbit	Liver	(Goff et al., 1993)
Rabbit	Liver	(Goff et al., 1994)
Rabbit	Liver	(Goff et al., 1997)
Rabbit	Whole carcass	(Bourel et al., 1999b)
Rabbit	Whole carcass	(Bourel et al., 2001)
Rabbit	Whole carcass	(Hédouin et al., 2001)
Rabbit	Liver	(Carvalho et al., 2001)
Rabbit	Whole carcass	(Fathy et al., 2008)
Rabbit	Whole carcass	(El - Samad et al., 2011)
Rabbit	Liver	(de Carvalho et al., 2012)
Rabbit	Whole carcass	(Bakr et al., 2012)
Rabbit	Muscle	(Zou et al., 2013)
Rabbit	Whole carcass	(Lawai et al., 2015)

Diet/Donor organism	Tissue	Reference
Rat	Liver	(Rashid et al., 2008)
Rat	Whole carcass	(Baia et al., 2016)

When an organism dies, a variety of arthropods aggregate on the organism to make full use of the newly available resources (Denno and Cothran, 1976). Calliphorid adult females are usually the first insects to be attracted to a corpse as a result of the volatiles released from the corpse (Turner, 1991; Shi et al., 2009; Voss et al., 2011; Ortloff et al., 2012). The insects found on a corpse, as well as the age of the insects, and the degree of decomposition of the corpse are all indicators used by entomologists to estimate the minimum post mortem interval (PMI_{min}) (Baqué and Amendt, 2013).

When selecting an oviposition site, female insect adults tend towards areas on the decomposing corpse with putrefying liquids that are high in protein (Rivers et al., 2011). There is evidence that nutritional requirements in insects can vary for a number of reasons including sex and developmental changes (House, 1962). Even within a species, the nutritional requirements between the larval and adult stage can vary (House, 1962). The basic dietary needs of a growing larva include amino acids, carbohydrates, lipids, vitamins and minerals (House, 1962). These dietary requirements need to be provided to the insects in certain ratios as they all have different functions and are used in the insect body to perform various functions (e.g. sterols (which are found in fat) are necessary for cell membrane synthesis and are essential in the production of the hormones responsible for moulting and metamorphosis (Osuji, 1978)). When selecting an artificial diet for laboratory experiments, it is important to ensure that the insects are being provided with all of the necessary nutrition to complete all stages of growth, as well as ensure reproductive viability.

Apart from mimicking field conditions, there is a need to ensure unnatural toxicants/chemicals/substrates do not influence life stages of laboratory reared flies. A typical example is provided by Leal et al. (1982). In their study, an experiment was performed to determine the effects that artificial diets would have on larval growth of *Chrysomya putoria* in comparison to growth on a mouse carcass. Their results showed that the larvae grown on the mouse carcass developed faster than the larvae that were reared on any of the artificial diets used. It is very important to note that nipagin was used in all of the artificial diets. Nipagin is used in agar solutions as an antifungal agent (Rohlfs, 2006) and the literature has shown that nipagin can retard insect growth (Child, 1939). Ideally, for entomotoxicological studies, the diet used for rearing the insects should not have any effects on their growth, development and fecundity. The aim of the feeding substrate should be to provide the insects

with the necessary nutrition whilst efficiently delivering the toxicant to the insect. Currently, the literature available on forensic entomotoxicology varies in that different authors have all used different feeding substrates in their experiments (Table 1.1). Different feeding substrates can affect the growth rates of insects (Day and Wallman, 2006b). This is of concern because the question then becomes whether the observed growth effects are a result of the toxicant or the feeding substrate and it makes comparison between studies which have evaluated the effects of the same toxicant on insects difficult.

The deposition and concentration of a toxicant in different tissues within the body is highly dependent on its physical and chemical properties (Cook et al., 2000; Gerostamoulos et al., 2012; Han et al., 2012; McIntyre and Escott, 2012). This means that if the whole body of the organism was used, there is no way of knowing how much of the toxicant the insect consumed because there is no way of knowing which part of the body the insect was feeding on and therefore, what the sum total of the feeding is (Tracqui et al., 2004). One of the benefits of using an artificial diet to deliver the toxicant to the insect is that the concentration of the toxicant is known and can be monitored over the duration of the experiment.

The aim of this study was to investigate the effects of dietary media (in the absence of nipagin) on insect development. Therefore, the hypothesis of this study was that insect larvae growing in different artificial media will have varying growth rates because nutritional value determines growth. An additional aim was to select an artificial feeding substrate from the existing literature and modify it for entomotoxicological studies. The following criteria had to be met for the feeding substrate to be considered successful:

1. The substrate should be palatable to the insects.
2. The substrate should provide insects with the necessary nutrients for growth.
3. Production costs should be low.
4. Handling and disposal should be easy.
5. The substrate should preferably have as minimal an odour as possible.

Materials and methods

Insect collections and rearing

A sweep net with 0.5 m diameter and a 1 m handle was used to catch adult flies from a dead dog found on the Rhodes University campus (33°18'48.6"S 26°30'32.3"E; South Africa). Sweeping was carried out until more than 100 flies had been captured. It was assumed that since sweeping was random there would be a good representation of both male and female adult flies. Captured flies were stored in a fly cage made of a wooden frame (30 x 40 x 35 cm) and fabric mesh and placed in a constant environment room (24°C, 12 hour day/night cycle). They were given three Styrofoam cups each with 200 g of chicken livers for oviposition as well as three Petri dishes with cotton wool which was either soaked in water or a sugar and milk powder solution on which flies were fed *ad libitum*. Once eggs were seen on the chicken livers, they were removed and transferred into Styrofoam cups with 200 g of chicken liver and placed into separate cages. When the eggs hatched, the larvae were sustained on a chicken liver diet and allowed to reach adulthood to build up a colony. The third generation of eggs were used in the experiment. The adult flies were identified as *Chrysomya chloropyga* using the identification key by Irish et al. (2014).

Dietary treatments

Four types of diets were prepared (Table 2.2). Bearing the abovementioned criteria in mind, the diet tested by Rabêlo et al. (2011) was selected. The ratios of the different components of the diet were kept consistent. Nipagin was not included because it has been shown to retard insect growth (Child, 1939). The protein sources used in the modified diets were beef (*Bos taurus*), fish (*Merluccius capensis*) and pork (*Sus scrofa*). A no meat treatment (herein referred to as NMT) was included as a control. The NMT had all of the ingredients of the other treatments except that no meat was included in this treatment.

The muscle tissues for each respective diet were pureed using a food processor. The dry ingredients (Table 2.2) were then blended with the pureed muscle tissue and 533.34 ml of distilled water using a food processor. In order for the agar to dissolve into the solutions, the four diets were autoclaved at 120°C for 20 minutes. Once the different treatments had been autoclaved and allowed to cool to 70°C, an amount of 100 ml of the agar solution was

decanted into 250 ml Styrofoam cups. The agar solution was then left to cool to room temperature to set. A total of six cups of agar were prepared for each of the treatments (three experiment cups and three replacement cups). Each cup was labelled with the date when the agar solutions were made as well as the tissue type (or lack thereof) in the cup.

Table 2.2. Composition of the diets used for the experiments (Adapted from Rabêlo et al., 2011). AD refers to artificial diet, NMT refers to the no-meat treatment.

Ingredient	Beef AD	Pork AD	Fish AD	NMT AD
Distilled water (ml)	533,3	533,3	533,3	533,3
Milk powder (g)	60	60	60	60
Brewer's yeast (g)	60	60	60	60
Casein (g)	3,06	3,06	3,06	3,06
Agar (g)	4,26	4,26	4,26	4,26
Bos taurus muscle tissue (g)	120	-	-	-
Sus scrofa muscle tissue (g)	-	120	-	-
Merluccius capensis muscle tissue (g)	-	-	120	-

Larval growth rate

The eggs laid on the liver in the stock colony were removed, placed into a Petri dish with water, counted, and then transferred onto the artificial diets using a fine paintbrush. A total of 40 eggs were placed into each Styrofoam cup and these were then placed into four separate fly cages grouped according to feeding substrate. Each cage contained six cups. Of the six cups, three were labelled "replacement" cups and three were labelled "experiment" cups. The cages were placed in controlled environment rooms set at 24°C with 12 hour day/night cycle and no humidity control. The larvae were sampled from the "experiment" cups and to ensure that there were constantly 40 larvae in each "experiment" cup, one larva was taken from the corresponding "replacement" cup and placed into the "experiment" cup after each sampling event. Every six hours, one larva from each experiment cup was removed, weighed, placed into boiling hot water for approximately 30 seconds and then placed into

100% ethanol in a microcentrifuge tube so as to prevent shrinkage (Amendt et al., 2015). The insects were sampled until pupation had occurred. The length and width of the larvae was recorded using the gauge described by Villet (2007). The lengths of the larvae were measured from the head to the most distal part of the last abdominal segment. The widths of the larvae were measured at the junction of the fifth and sixth abdominal segments. These measurements were taken in accordance with (Day and Wallman, 2006a). The measures were taken once the experiment had concluded.

Statistical analysis

The hypothesis of this study was that the insect larvae being reared on the different artificial dietary media would have varying growth rates. To investigate the relationship between larval growth and dietary media, body lengths and widths of larvae grown on the different media were compared. A one-way mixed effects Analysis of Covariance (ANCOVA) was used to test these differences, with larval age as a covariate and diet/treatment as the fixed factor and either length or width as the response variable. To account for differences that may arise other than those as a result of the diet treatments, the three cups per treatment were nested in treatment. Pre-ANCOVA tests for normality and homogeneity of variance were carried out to determine the suitability of the data for ANCOVA. The normality of the residuals of the length and width data were tested using a Shapiro – Wilks test in order to determine whether an ANCOVA test would be appropriate to determine the effects of the age, diet and cups on the body lengths and widths of the larvae using R software. The results showed that the residuals of the body length ($W = 0.99$, $p>0.05$) and width ($W = 0.98$, $p>0.05$) data were normally distributed. The Levene's test was used to test for equality of variance among the response variables (length and width). The results showed that neither the length (d.f.=2; F. stat= 11.65 ; $p<0.05$) nor width data (d.f.=2; F. stat= 10.80, $p<0.05$) were homogenous. The data was then further truncated to the last 48 hours of the experiment when the larvae were between 85 and 133 hours old. This data was still found to not be homogenous. The data was then log transformed and both the length (d.f.=2; F. stat= 1.95; $p>0.05$) and width (d.f.= 2; F. stat= 0.48; $p>0.05$) were now homogenous. A Shapiro Wilk test was done on the residuals of the data set using R, to confirm whether the ANCOVA would be the appropriate model to use on this data set. The residuals were normally distributed for both loglength (W. stat = 0.99;

p>0.05) and logwidth (W. stat = 0.98; p>0.05), therefore this was the data set that was used for the statistical analyses

An ANCOVA test to determine the effects of diet, age and cups on the lengths and widths of the larvae, as well as the descriptive statistics for the respective treatments were done using Statistica version 10 (StatSoft Inc, 2011). A Tukey HSD post hoc test was also done to determine the differences in body length and width between the different diets using R software, using the glm2 package (R Core Team, 2017).

Results

The larvae reared on the fish and NMT diets did not reach pupation stage. The pork and beef treatment larvae both took 7 days to reach pupation stage. The NMT larvae were present in all three cups until the sixth day of the experiment and the fish larvae were present in all three cups until the fourth day of the experiment. As a result, the fish treatment was excluded from the statistical analysis and the data was truncated until the sixth day of the experiment when the larvae were 133 hours old (this was the last complete sample of all three cups of the NMT).

Once the data were truncated, each treatment had a total of 57 observations (Table 2.3). The mean body lengths and widths of the NMT larvae were lower than the pork and beef larvae, indicating that these individuals were smaller in size than the individuals reared on the beef and pork artificial diets. The mean body lengths and widths were similar for the larvae reared on the beef and pork artificial diets (Table 2.3).

Table 2.3. The mean body lengths and widths of the *Chrysomya chloropyga* larvae reared on the NMT, beef and pork treatments (NMT refers to no meat treatment and AD refers to artificial diet).

Substrate type	Number of terms	Length mean ± s.d. (mm)	Width mean ± s.d. (mm)
NMT AD	57	6.09 ± 1.88	1.48 ± 0.37
Beef AD	57	7.71 ± 3.28	1.87 ± 0.57
Pork AD	57	7.75 ± 3.48	1.90 ± 0.63

Diet, age and cup effects

An ANCOVA test was done to determine the effects of the diets, age and cups on the lengths and width of the larvae reared on each of the treatments. The results showed that the lengths and widths both significantly varied with diet and age of the larvae (Table 2.4). The results also showed that the larvae being reared in different cups (cages) within the same treatments did not differ significantly in body lengths and widths (Table 2.4)

Table 2.4. The ANCOVA results for the effects of age, diet and cups on the *Chrysomya chloropyga* body lengths and widths. Bolded p values were statistically different

Parameter	Effect	M.S.	S.S	F-stat	d.f.	p-value
Length	Age	0.19	0.19	29.91	1	**$1x10^{-6}$**
	Diet	0.22	0.43	34.72	2	**0.000**
	Cup	0.03	0.01	2.38	2	0.102
Width	Age	0.04	0.04	5.64	1	**0.021**
	Diet	0.24	0.48	31.08	2	**0.000**
	Cup	0.01	0.02	1.25	2	0.295

Comparison of the body lengths and widths of the Chrysomya chloropyga larvae reared on the different diets.

Post hoc Tukey HSD tests were done to determine the difference between the body lengths and widths of the treatments using the ANCOVA models. The results showed that the body lengths and widths of the beef- and pork-reared diets were significantly different from the NMT (Table 2.5). The body lengths and widths of the larvae reared on the beef and pork artificial diets did not differ significantly from one another (Table 2.5)

Table 2.5. Tukey HSD results of the ANCOVA models showing the comparison of the body length (white) and width (grey) of *Chrysomya chloropyga* reared on the different artificial diets. Bolded p values were statistically different

	NMT	Beef	Pork
NMT		**0.000**	**0.000**
Beef	**$7x10^{-7}$**		0.999
Pork	**0.000**	0.575	

Costs of producing the dietary media

All of the ingredients for the production of the dietary media were purchased from local supermarkets and a local butchery in Grahamstown, South Africa. The ingredients were relatively easy to obtain, except for the brewer's yeast which had to be bought from Dischem in Port Elizabeth, South Africa, which is approximately 120 km from Grahamstown. However in larger cities, brewer's yeast is far easier to come by in local grocery stores. In accordance with the cost of the ingredients as purchased at Pick n Pay Grahamstown, Tip Top Butchery in Grahamstown, and Dischem Pharmacy at Bay West Mall in Port Elizabeth, the cheapest dietary medium to make was the NMT (R51.62) and the most expensive dietary medium to make was the fish artificial diet (R66.61) (Table 2.6). Overall, the cost price for the production of the feeding substrates was relatively low and the most expensive ingredient was the agar (Table 2.6).

Table 2.6. The cost of making 600 ml of each dietary medium*. AD refers to artificial diet, NMT refers to the no meat treatment.

Ingredient	Beef AD	Pork AD	Fish AD	NMT AD
Milk powder	R 6.24	R 6.24	R 6.24	R 6.24
Brewer's yeast	R 17.99	R 17.99	R 17.99	R 17.99
Casein	R 7.23	R 7.23	R 7.23	R 7.23
Agar	R 20.16	R 20.16	R 20.16	R 20.16
Bos taurus muscle tissue	R11.99	-	-	-
Sus scrofa muscle tissue	-	R 9.59	-	-
Merluccius capensis muscle tissue	-	-	R14.99	-
Total	R63.61	R61.21	R66.61	R 51.62

*The conversion rate of South African Rands (ZAR) to the United States of America Dollar (USD) was R1 = $0.073 (as of the 13 December 2017)

Odour of the dietary media

The media produced an unpleasant smell for the duration of the experiment. The most unpleasant smelling medium was the fish medium as a result of the ammonia which was emanating from it. The least unpleasant smelling medium was the NMT which is

understandable considering that there was no meat in the substrate and the most likely cause of the odour in the other treatments was the putrefying meat in the media.

Discussion

Nutrient content of the feeding substrate

One of the most essential components of a diet which will ensure that large individuals are reared in a fly colony is protein (Leal et al., 1982). Hence, the diets with the highest protein content will most likely yield the largest individuals. This was the case with the larvae in this study as the larvae reared on the porcine and bovine diets were larger in size and these muscle types have been shown to have high protein content (Lan et al., 1995; Leal et al., 1982). The NMT diet yielded the smallest individuals which furthermore emphasises the importance of a protein source within the dietary medium.

Completion of life stages

An important factor to consider when judging the success of each diet is looking at which of the diets was able to rear individuals to the completion of their life stages. The larvae reared on the bovine and porcine diets were reared to adult stage whereas the fish treatment and NMT were not. A possible explanation for this is that there was a very distinct ammonia smell emanating from the fish diet media and this may have had lethal effects on the larvae (Day and Wallman, 2006a). The NMT diet did not have a rich protein source and this means that the larvae did not have sufficient nutrients to complete all the stages of their life cycle, making these diets unsuitable for the rearing of fly larvae.

Overall successes and failures of the dietary media

This experiment aimed to select an already existing diet and modify it to fit the five mentioned criteria. All of the dietary media were palatable to the insects as they were observed consuming the media in all of the cups. In terms of the nutritional content, all of the larvae showed positive growth which is indicative of them being provided with the necessary nutrients. The difference between the diets was the protein source provided, and the results showed that the porcine and bovine diets provided the larvae with better protein and thus yielded larger larvae. The production cost of the feeding substrates was relatively low, but one might argue that it would be cheaper to simply opt for using a muscle tissue on its own. Future studies could perhaps investigate the stability of different toxicants using muscle tissue

versus using the suggested dietary media in this study and perhaps this could be the deciding factor as to whether it would be better to use muscle tissue versus the suggested artificial dietary media. Other reasons why the use of raw meat might not be considered desirable include the fast rate at which it putrefies and the unpleasant handling and disposal once experiments have been completed, and the risk of contamination (Rabêlo et al., 2011). Considering that the dietary media was made in Styrofoam cups, the handling and disposal was easy because once the experiment had concluded, the Styrofoam cups and their contents were simply discarded in bio-safety bins. Unfortunately, the one criterion that the dietary media were unable to fulfil was the need for a minimal odour. However, it is plausible that the dietary medium does require an odour as this is one of the cues which alerts the larvae to the palatability of the feeding substrate (von Hoermann et al., 2012; Wallis, 1962).

Conclusion

Although the abovementioned dietary media have their shortfalls, the results are promising as they show that some artificial substrates have the ability to rear larvae to adulthood. Future studies should focus on looking at the comparisons between using muscle tissues versus using the artificial diets, as well as looking at which of the two work best at retaining the stability of a toxicant within the matrix. This study has shown that porcine and bovine meat yield the most robust larvae, but this could be an issue for certain regions of the world that do not use these particular meats due to religious reasons (e.g. India is predominantly Hindu and cows are sacred to the Hindu culture and Pakistan is predominantly Muslim and pork is considered Haraam or prohibited). It would thus be beneficial to explore other meat substitutes (e.g. chicken). As the focus of this study was to test the abovementioned media as controls for entomotoxicology experiments, future studies should test the stability of different toxicants using either the porcine or bovine substrates.

Chapter 3: Standardising methods for quantifying growth and preservation techniques of larval flies of forensic relevance

Abstract

There is a need for the standardisation of the experimental designs used in the field of forensic entomotoxicology. This is because it is a relatively new field of science and there are currently no standard protocols in place that researchers can ascribe to when conducting experiments within the field. Previous studies have advocated that body width of fly larvae be used as an alternative measure to body length to quantify growth rate. The accuracy of these measures is complicated by the storage technique selected to preserve entomological specimens collected as evidence from crime scenes. To address the lack of standardisation in the experimental design of entomotoxicological studies, this study determined whether length or width would be better suited to measure growth. The second aim was to assess the effect of different ethanol concentrations on the morphometric integrity of pupal casings and third instar larvae. The larvae used in the experiment in Chapter 2 were used to determine whether length or width would be a better measure to demonstrate growth over time. Pupal casings of *Chrysomya chloropyga* and third instar larvae of *Calliphora croceipalpis* were preserved in different concentrations of ethanol to determine whether varying concentrations of ethanol would have an effect on the structural integrity of the specimens over time. The results showed that length changed in larger increments over time in comparison to width, giving it more resolution and thus making it a more reliable indicator of age. There was no significant difference in the proportional changes in the lengths and widths of the *Chrysomya chloropyga* pupal casings and *Calliphora croceipalis* third instar larvae stored in various preservatives. These findings suggest that future studies should focus on testing more parameters within the experimental design and standardising them, to make entomotoxicological evidence more admissible in the court of law.

Introduction

The predictability of the insect assemblage on a corpse is one of the main features that forensic entomologists use to estimate time of death or the minimum post mortem interval (herein referred to as PMI_{min}) (Anderson, 2010). Insects that are most useful in determining PMI_{min} include flies (order Diptera) and beetles (order Coleoptera) (Byrd and Castner, 2010).

In the Diptera, the Calliphoridae are the first colonisers, followed by the Sarcophagidae. The beetles arrive a few days later (Archer and Elgar, 2003; Smith, 1986; Turner, 1991). Various parameters have been used to determine PMI_{min} from insects, including length (Adams and Hall, 2003; Amendt et al., 2011), width (Day and Wallman, 2006a) and mass (Amendt et al., 2011; Williams, 1984). Although these parameters may allow for a trend in growth rate to be recorded, the question remains as to which of the parameters would be most accurate and practical for use in forensic cases. Another possibility could be that the use of all three parameters (length, width and mass) in tandem could provide a more holistic reflection of the growth rates of the insects, but in most cases, it is not practical to obtain all three measures. This is because with mass specifically, dry masses would need to be obtained and this involves destructive sampling which could prevent species identifications (Day and Wallman, 2006a). Additionally, preservative solutions could have an effect on the mass of the preserved specimen and there are currently no available models to convert wet masses after specimen preservation back to live masses (Day and Wallman, 2006a).

The collection and storage protocols used when collecting entomological specimens are an important factor to consider as these can affect the measurement of the abovementioned parameters. Various authors (Adams and Hall, 2003; Midgley and Villet, 2009; Brown et al., 2012) have demonstrated that the manner in which an insect is killed and preserved has an effect on the parameters measured from the insect and that this is dependent on the amount of time that elapses between collection and measurement of the insect. This suggests that collection and storage methods should be re-examined and standardised to reduce the margin of error that occurs due to the use of different storage methods.

The estimation of the PMI_{min} is predicted on the assemblage of insects found on the corpse (Amendt et al., 2011), the time it would have taken for the insects to discover and inhabit the corpse (Amendt et al., 2007; Goff, 1993) and their ages (Amendt et al., 2011). The most well-known models include the isomorphen and isomegalen models (Amendt et al., 2011). The isomorphen model relies on the use of live larvae being reared to their next instar whereas the isomegalen model works by using measurements of dead larvae (Amendt et al., 2011). The isomegalen model relies on the size of the larvae and, for the result to be as accurate as possible, the measures taken need to be representative of the insect's age. Therefore, it is important to utilise a parameter that changes in relation to the insects' age.

The most common parameters used to quantify growth rate have been the change of length and mass (Knapp, 2012). Day and Wallman (2006) suggested that width might be better for predicting the ages of larvae. The storage of the maggots can sometimes result in the head of the maggot curling inwards and this can skew the length measurements taken. The use of mass also has its shortcomings because dry mass requires sample destruction and wet mass cannot be taken because sample preservation can alter the specimen's mass (Day and Wallman, 2006a). The second issue with using mass as a measure of age is that depending on the preservative used, the lipids might break down or dissolve into the preservative, and this will alter the mass of the larvae (Villet et al., 2010). For this reason Day and Wallman (2006) have suggested that perhaps the better parameter to use would be the width of the larvae. The one issue with using width as a parameter to measure age would be that it does not change to the same extent the length of the larvae over time, thus using width on its own might not provide an accurate estimate of the age of the larvae (Villet et al., 2010). Villet et al. (2010) highlighted the importance of precision, bias and accuracy in the measurement of the parameters that are ultimately used to calculate the PMI_{min}. As a result of the degree of uncertainty surrounding the parameter that should be used, there is a need to examine the use of length or width as a predictor of age. The current study seeks to determine whether there are differences in the changes between length and width. The ideal parameter should have larger increments, as this would provide greater resolution and make the estimation of the PMI_{min} more accurate.

Collection and storage of entomological specimens is important as incorrect handling of specimens could result in decreased accuracy in measurements and ultimately skew the estimated PMI_{min} (Amendt et al., 2015). With most forensic cases, the entomologist is not present to collect the entomological evidence and as a result, crime scene technicians are often left to collect these specimens (Byrd et al., 2010; Day and Wallman, 2008). Insects, particularly fly larvae, undergo very predictable stages in their life cycle (Boehme et al., 2013). Once the species is correctly identified, age can be determined using species – specific growth curves (Grassberger and Reiter, 2001; Richards et al., 2008). Length and width are used to estimate their ages and thus specimen preservation needs to be such that these parameters are not drastically altered as this could diminish the accuracy of the age estimation. The recommended technique for preserving fly larvae is to first place them in hot water (>80°)

and then placing them in 70 – 95% ethanol (Amendt et al., 2007). This is not always practical for personnel collecting entomological evidence in the field (Day and Wallman, 2008; Amendt et al., 2015) and what this means is that insects might have undergone physical changes (such as shrinkage) as a result of the preservation techniques used, thus creating a margin of error in the estimated PMI (Day and Wallman, 2008).

The use of the specimen should also be considered when picking a particular storage method (Amendt et al., 2015). Ideally, the method chosen should preserve the specimen for measurements but also potentially for molecular analysis of the specimen (Amendt et al., 2011; Dowell et al., 2011; Frampton et al., 2008). This is because factors such as the preservative, its concentration and storage time will affect the quantity and quality of the DNA extracted (Frampton et al., 2008). It is also important to select a technique that retains the morphometric integrity of the specimen, as features such as size and shape could be used in species identification, and it is not always possible for specimens to be identified in the field (Frampton et al., 2008). Bisanti et al. (2009) showed that 100% ethanol and acetone worked best for extracting insect DNA of a higher quality as opposed to weaker concentrations of ethanol. The reason for this was that the higher concentrations of these preservatives meant that the insect tissues were penetrated faster than preservatives of lower concentrations. When selecting the method of preservation for the insect specimen, one should be cognisant of which analyses will be conducted on the specimen and ensure that the preservation technique does not compromise the integrity of the specimen (Amendt et al., 2015).

The pupae and pupal casings are important pieces of evidence that is often overlooked (Davies and Harvey, 2013) and should also be collected at crime scenes (Amendt et al., 2015). Upon examination, the puparial casings can allude to the species that were present at the scene (Castner, 2010), as in some cases, there is no longer any insect activity and the only evidence left behind are the casings (Boehme et al., 2013; Byrd et al., 2010). Recent studies have also started to develop different techniques to age the pupae to make the estimation of the PMI_{min} more accurate (Boehme et al., 2013; Brown et al., 2012; Davies and Harvey, 2013). As these are still relatively new and underdeveloped methods, the authors have delved into the importance of a preservation technique for the pupae, but this has yet to be standardised. The puparial casings are also an indicator of presence or absence of a toxicant in the corpse of the deceased as toxicants accumulate in the exoskeleton of the larva and subsequently, in

the puparial casing (Goff et al., 1997; Gosselin et al., 2011a; Kharbouche et al., 2008; Miller et al., 1994; Pien et al., 2004; Wood et al., 2003). As with larvae, the collector of the puparial casings must be cognisant of the potential tests that will be done and thus pick a preservation technique which will cause minimal to no damage to the samples. Amendt et al. (2015) have recommended that any eclosed puparial casings be stored in tubes without any preservative solution.

To make all PMI_{min} measures comparable it would be ideal to have a standardised parameter for quantifying growth. This parameter would have to be one which would require a minimal amount of equipment as the financial aspects should be taken into consideration. It should also be one which is minimally affected by the preservation technique. Given that length and width likely have varying increments of change, the aim of this study was to determine whether larval body length or width is the best parameter for quantifying growth rate. The second aim was to determine which concentration of ethanol (if any) would retain the morphometric integrity of pupal casings and third instar larvae.

Materials and methods

Length and width

Insect collections and rearing

For the length and width of larvae and the pupal casing experiments, adult flies were collected using a sweep net with 0.5 m diameter and a 1 m long handle from a dead dog found on the Rhodes University campus (33°18’48.6”S 26°30’32.3”E; South Africa). Sweeping was carried out until more than 100 flies had been captured. It was assumed that since sweeping was random there would be a good representation of both male and female *Chrysomya chloropyga* adult necrophagic flies. Captured flies were stored in a fly cage made of a wooden frame (30 x 40 x 35 cm) and fabric mesh and taken back to a constant environment room (24°C, 12 hour day/night cycle). The adult flied were provided with three Styrofoam cups, each with 200 g of chicken livers for oviposition and three petri dishes with cotton wool which were either soaked in water or a sugar and milk powder solution. The flies were fed *ad libitum*. Once eggs were seen on the chicken livers, they were removed and placed into Styrofoam cups with 200 g of chicken liver and placed into separate cages. When the eggs hatched, they were then sustained on a chicken liver diet and allowed to reach adulthood to build up a

colony. The third generation of eggs were then used in the experiment. Using a key by Irish et al. (2014) the adult flies were identified as *Chrysomya chloropyga*.

For the third instar larvae experiment, a total of 45 third instar larvae were collected from rotting entrails of warthog (*Phacochoerus africanus*) located at a residence in Grahamstown (33°19”2.53”S, 26°31’34.38”E, South Africa). The larvae were collected using pliable forceps. They were placed into a vial and subsequently hot water killed (HWK) for use in the experiment.

Dietary treatments

The larvae reared for the experiments in Chapter 2 were used to determine which parameter (length or width) would better represent their growth rates. Their lengths and widths were recorded using the gauge described by Villet (2007).

Statistical analysis: Length vs. width

The hypothesis for this component of the current study was that the length and width would have varying increments of change over time. To determine the degree to which each parameter changed, the differences in length and width between each day of live age were calculated for each diet and an average rate of change was obtained for each diet and these were compared to one another to determine which increments were larger between the lengths and widths. This was done using the truncated dataset from Chapter 2 (having removed the fish treatment and truncated the data to the point where the larvae were 133 hours old). It was hypothesised that the parameter with the larger incremental change over time would provide greater resolution and would be the most appropriate parameter to use when calculating the age using the isomegalen or isomorphen models.

Assessing preservation techniques

Pupal casing preservation technique

To determine the effects of different preservation techniques on pupal casings, 15 pupal casings per treatment were sampled from an existing colony of *Chrysomya chloropyga*. The pupal casings were sampled and 15 were preserved dry (control), or in 1 ml of 100%, 70% or 50% ethanol solutions and stored at 4°C. Each of the pupal casings was stored in numbered and colour coded 1.5 ml microcentrifuge tubes to ensure that repeated measures were being taken. Once the pupal casings were placed in the respective treatments, their lengths and

widths were recorded every 12-hours for the first three days, and thereafter once a day every three days using the geometric gauge described by Villet (2007). The experiment ran for three weeks. This was done to determine whether there were any significant changes in their lengths and widths over time and whether the method of preservation influenced the parameters taken over time.

Third instar larvae preservation technique

The larvae collected from the warthog carcass were removed from the vial and hot water killed (HWK), and 15 larvae were preserved in 1ml of either 100%, 70% or 50% ethanol solutions and stored at 4°C. These larvae were then identified by Prof Martin Villet as *Calliphora croceipalpis* larvae using the key by Prins (1982). Each of the larvae was stored in a numbered and colour coded 1.5 ml microcentrifuge tubes to ensure that repeated measurements were taken. These larvae were measured every 48 hours and this experiment ran for two weeks. The lengths and widths of the larvae were measured using the geometric gauge described by Villet (2007).

Statistical analysis: Pupal casing and third instar larvae preservation

A repeated-measures general linear model (GLM) was built with either length or width as the dependent variables and treatment (no preservative [pupal casings only], 100%, 70% or 50% ethanol [pupal casings and third instar larvae]) as a fixed independent factor. The minimum adequate model was achieved through stepwise deletions, starting from the highest order non – significant interactions to the least, followed by model comparisons using Akaike's information criterion (AIC) and analysis of variance.

The proportional change per sampling period was calculated for each dataset. This was done by calculating the change per sampling period and dividing by the initial lengths/widths which were taken to obtain the proportional change per sampling period in relation to the initial reading. This data was then used in the models to determine the proportional change in length or width across treatments. For the pupal casings data, a Levene's test for homogeneity was done to determine whether the data was homogenous and hence suitable for a repeated-measures ANOVA. The results (Levene's test) showed that neither the length (d.f. = 3, F. stat. = 10.06, p= 0.032), nor width (d.f.= 3, F. stat= 10.67, p= 0.000) were homogenous, hence an ANOVA could not be used. Instead GLM models, fitted with a Gaussian distribution and an identity link function were used. The minimum adequate model was then

applied to this dataset and used to investigate the effects of treatment on the dependent variables (length and width).

For the third instar larvae dataset, a Levene's test for homogeneity was also done to determine whether the length and width data was homogenous and this would then determine whether a repeated-measures ANOVA would be the appropriate test for the data set. The results from the test showed that the length was not homogenous (d.f. = 2, F. stat. = 10.84, p= 0.000) but the width was homogenous (d.f. = 2, F. stat. = 1.133, p= 0.3234). This dataset was not severely skewed and thus the GLM minimum adequate model could be applied to the dataset to determine the effects of treatment on the dependent variables (length and width).

All of the abovementioned statistical tests were done using R software, version 3.3.2, using the agricolae and MASS packages (R Core Team, 2017). All of the box plots representing the spread of proportional data were done using Statistica version 10 (StatSoft Inc, 2011).

Results

Length vs. width

The aim was to determine whether the larval body length or width would better represent the growth of the larvae over time. The absolute incremental differences were calculated by obtaining the average changes of the body lengths and widths of the live larvae over time per treatment. On average, the change in length between sampling periods was greater than the change in width (Table 3.1). The standard deviations were larger than the means for these data because larvae of all ages and sizes were combined to obtain these means.

Table 3.1. Mean differences between sampling periods in body length and width of *Chrysomya chloropyga* reared on NMT, beef and pork artificial diets.

Treatment	Mean length difference ± SD (mm)	Mean width difference ± SD (mm)
NMT	0.30 ± 1.41	0.06 ± 0.35
Beef	0.56 ± 1.63	0.10 ± 0.45
Pork	0.56 ± 1.35	0.09 ± 0.44
All diets	0.47 ± 1.47	0.08 ± 0.41

Chrysomya chloropyga *pupal casing preservation*

The GLM results showed that the preservation time was not a significant factor, and did not affect the lengths and widths of the pupal casings, and that the treatments did not differ significantly from one another (Tables 3.2 and 3.3).

Table 3.2. The pupal casing length GLM results for the effects of the preservation times that the empty *Chrysomya chloropyga* pupal casings were in their respective preservatives and the effects of the different treatments on the proportional changes in the lengths of the pupal casings. Bolded values indicate significance, PT denotes preservation time, CT denotes control.

	Estimate	Std. Error	d.f.	M.S.	t-value	Pr(>|t|)
Intercept	6.596×10^{-3}	7.157×10^{-3}	-	-	0.922	0.357
PT	1.820×10^{-6}	2.963×10^{-5}	1	-	0.061	0.951
CT: 50%	2.436×10^{-3}	1.012×10^{-2}	3	-	0.241	0.810
CT: 70%	1.188×10^{-2}	1.012×10^{-2}	3	-	1.174	0.241
CTl: 100%	1.061×10^{-2}	1.012×10^{-2}	3	-	1.048	0.295
PT: 50%	4.496×10^{-5}	3.581×10^{-5}	3	-	1.256	0.210
PT: 70%	-1.189×10^{-6}	3.581×10^{-5}	3	-	-0.033	0.974
PT: 100%	-5.603×10^{-7}	3.581×10^{-5}	3	-	-0.016	0.988
PT: Specimen	-1.533×10^{-7}	1.923×10^{-6}	1	-	-0.081	0.936
Residuals	-	0.060	651	0.0036	-	-

Table 3.3. The pupal casing width GLM results for the effects of the preservation times that the empty *Chrysomya chloropyga* pupal casings were in their respective preservatives and the effects of the different treatments on the proportional changes in the widths of the pupal casings. Bolded values indicate significance, PT denotes preservation time, CT denotes control.

	Estimate	Std. Error	d.f.	M.S.	t-value	Pr(>|t|)
Intercept	-6.596×10^{-3}	7.157×10^{-3}		-	-0.922	0.357
PT	-1.820×10^{-6}	2.963×10^{-5}	1	-	-0.061	0.951
CT: 50%	-2.436×10^{-3}	1.012×10^{-2}	3	-	-0.241	0.810
CT: 70%	-1.188×10^{-2}	1.012×10^{-2}	3	-	-1.174	0.241
CTl: 100%	-1.061×10^{-2}	1.012×10^{-2}	3	-	-1.048	0.295
PT: 50%	-4.496×10^{-5}	3.581×10^{-5}	3	-	-1.256	0.210
PT: 70%	1.189×10^{-6}	3.581×10^{-5}	3	-	0.033	0.974
PT: 100%	5.603×10^{-7}	3.581×10^{-5}	3	-	0.016	0.988
PT: Specimen	1.533×10^{-7}	1.923×10^{-6}	1	-	0.081	0.936
Residuals	-	0.060	651	0.0036	-	-

Distributions of the proportional length and width data for the Chrysomya chloropyga *empty pupal casing data*

The box plots showed that there were no visible differences in the spreads of data between the treatments, validating the GLM results that there were no differences in the proportional changes in lengths and widths of the empty *Chrysomya chloropyga* pupal casings preserved in the different treatments (Fig. 3.1 and 3.2).

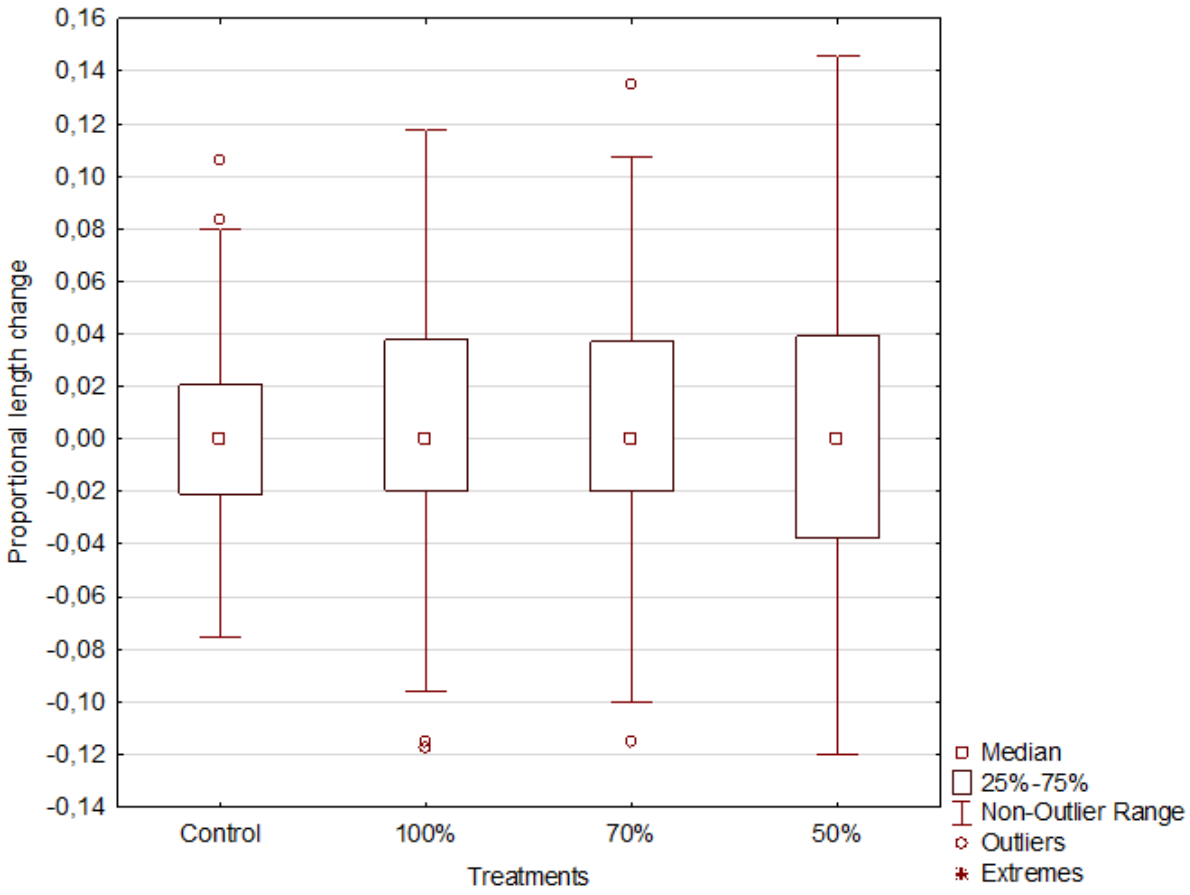

Fig. 3.1. The proportional changes in length of the *Chrysomya chloropyga* empty pupal casings preserved in no preservative, 100%, 70% and 50% ethanol.

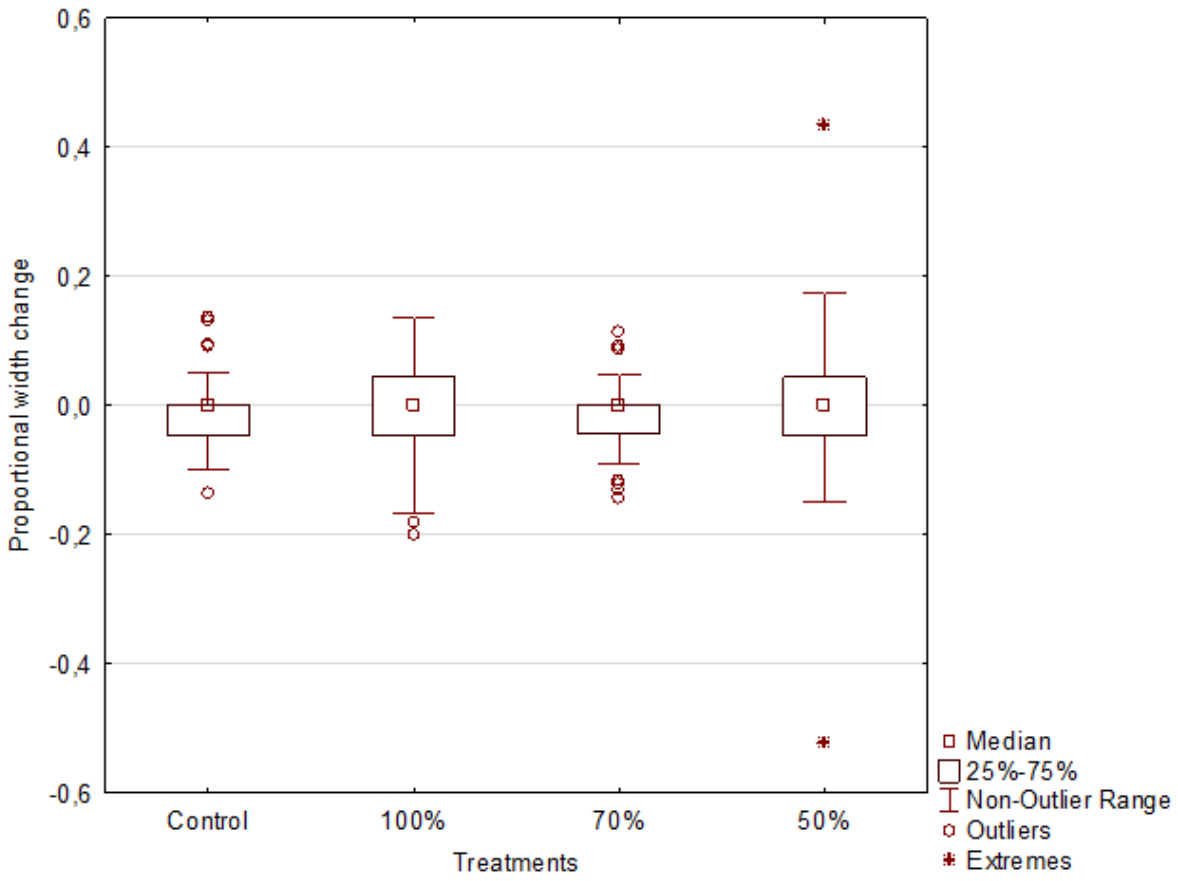

Fig. 3.2. The proportional changes in width of the *Chrysomya chloropyga* empty pupal casings preserved in no preservative, 100%, 70% and 50% ethanol.

Calliphora croceipalpis *third instar larvae preservation*

A GLM was done to determine whether the morphometric integrity (length and width) of the third instar larvae was affected by using different preservative solutions. The results showed that preservation time was significant in the model and that none of the treatments were significantly different from one another in terms of proportional changes in length or width. The preservation time estimate value also indicates that as preservation time increased, the lengths and widths of the specimens decreased (Tables 3.4 and 3.5).

Table 3.4. The length GLM for the effects of the preservation time on the lengths of the *Calliphora croceipalpis* third instar larvae in their respective preservatives and the effects of different treatments on the proportional changes in lengths of the third instar larvae. Bolded values indicate significance, PT denotes preservation time.

	Estimate	Std. Error	d.f.	M.S.	t-value	Pr(>|t|)
Intercept	9.741×10^{-2}	2.330×10^{-2}		-	4.180	**3.87×10^{-5}**
PT	-2.528×10^{-4}	1.109×10^{-4}	1	-	-2.279	**0.0234**
50%: 70%	4.042×10^{-2}	3.295×10^{-2}	2	-	1.226	0.2210
50%: 100%	4.934×10^{-2}	3.295×10^{-2}	2	-	1.497	0.1354
PT: 70%	-1.403×10^{-4}	1.405×10^{-4}	2	-	-0.999	0.3187
PT: 100%	-1.754×10^{-4}	1.405×10^{-4}	2	-	-1.248	0.2130
PT: Specimen	-3.109×10^{-6}	6.398×10^{-6}	1	-	-0.486	0.6274
Residuals	-	0.111	287	0.0124	-	-

Table 3.5. The width GLM for the effects of the preservation time on the widths of the *Calliphora croceipalpis* third instar larvae in their respective preservatives and the effects of different treatments on the proportional changes in widths of the third instar larvae. Bolded values indicate significance, PT denotes preservation time.

	Estimate	Std. Error	d.f.	M.S.	t-value	Pr(>|t|)
Intercept	-9.741×10^{-2}	2.330×10^{-2}		-	-4.180	**3.87×10^{-5}**
PT	-2.528×10^{-4}	1.109×10^{-4}	1	-	2.279	**0.0234**
50%: 70%	-4.042×10^{-2}	3.295×10^{-2}	2	-	-1.226	0.2210
50%: 100%	-4.934×10^{-2}	3.295×10^{-2}	2	-	-1.497	0.1354
PT: 70%	1.403×10^{-4}	1.405×10^{-4}	2	-	-0.999	0.3187
PT: 100%	1.754×10^{-4}	1.405×10^{-4}	2	-	1.248	0.2130
PT: Specimen	3.109×10^{-6}	6.398×10^{-6}	1	-	0.486	0.6274
Residuals	-	0.111	287	0.012	-	-

Distributions of the proportional length and width data for the Calliphora croceipalpis *third instar larvae data*

The box plots showed that there were no visible differences in the spreads of data between the treatments, validating the GLM results that there were no differences in the proportional changes in lengths and widths of the *Calliphora croceipalpis* third instar larvae preserved in the different treatments (Fig. 3.3 and 3.4).

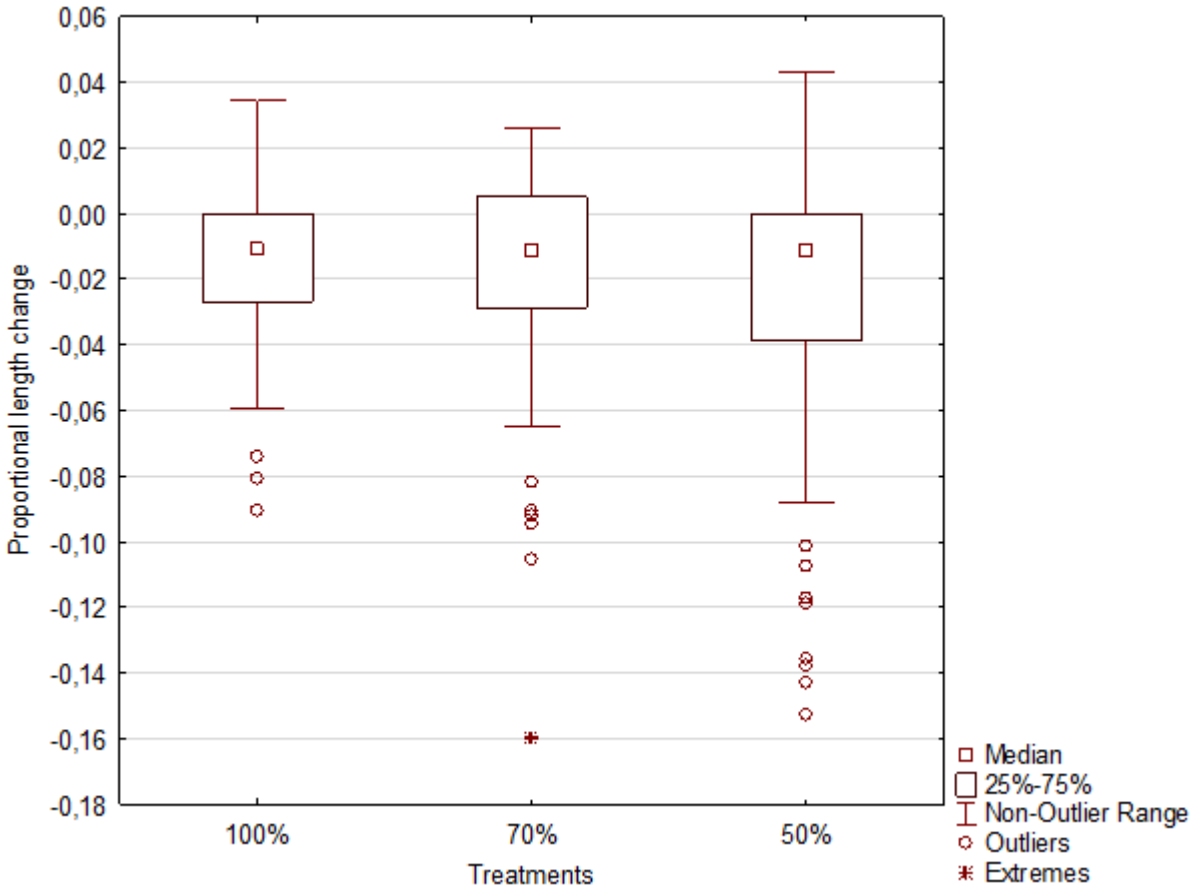

Fig. 3.3. The proportional length change of the *Calliphora croceipalpis* third instar larvae preserved in 100%, 70% and 50% ethanol solutions

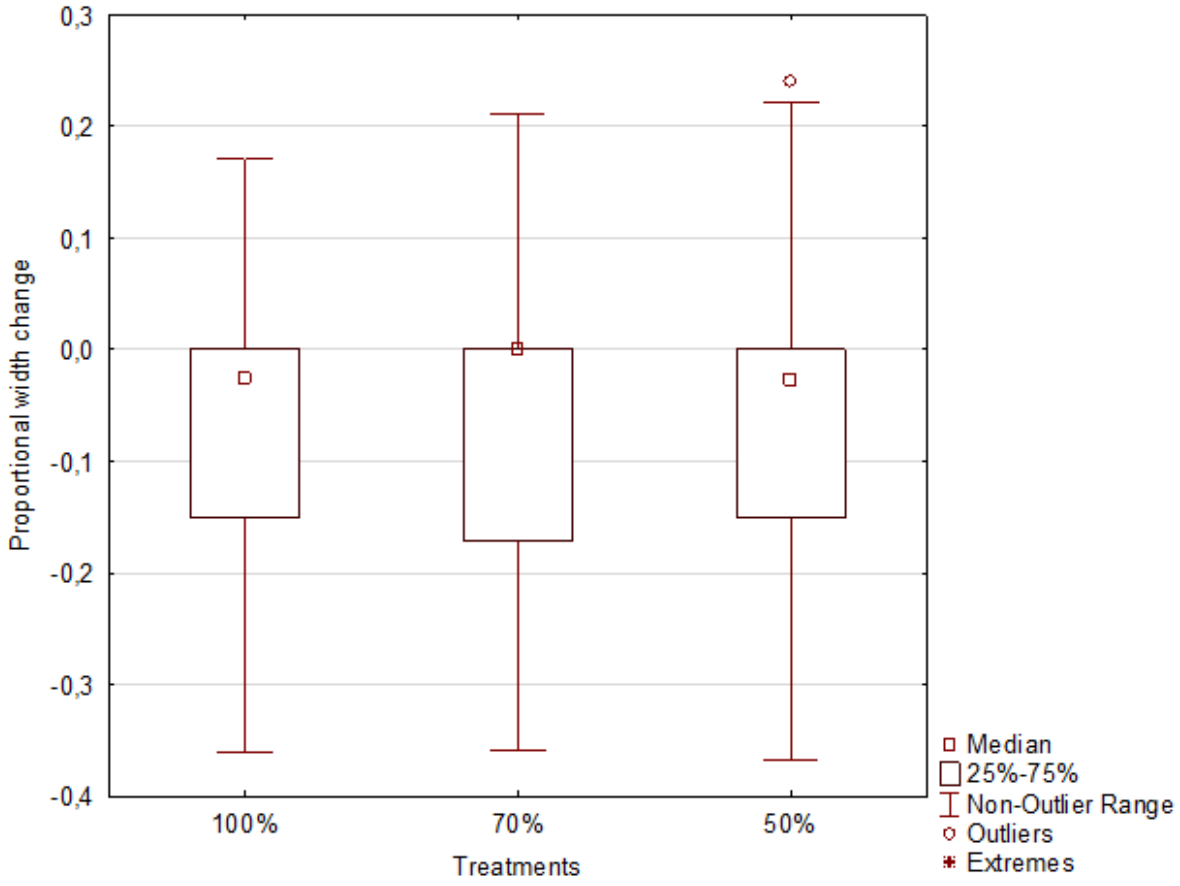

Fig. 3.4. The proportional width change of the *Calliphora croceipalpis* third instar larvae preserved in 100%, 70% and 50% ethanol solutions.

Discussion

The aims of this study were (1) to determine which parameter (length or width) is more suitable to quantify the growth rate of fly larvae, and (2) to determine whether the use of different preservation techniques would influence the morphometric integrity of pupal casings and third instar larvae. Although inferential statistics were not used, the results have shown that the widths of the larvae had less absolute variation but less relative variation over time as opposed to the lengths. This can result in the inaccurate determination of the age of the larvae and this can skew the PMI_{min}. The concentration of the ethanol used does not have an effect on the morphometric integrity of the empty *Chrysomya chloropyga* pupal casings or the *Calliphora croceipalpis* third instar larvae. However, the model did show that preservation time did have an effect on the length and width of the specimens, as these dimensions decreased with increased preservation time.

The ideal parameter to measure the age of an insect should preferably be one that shows a substantial amount of correlated variation over time as the insect grows. The reason for this is that if very little variation is shown between sampling periods, it lessens the resolution between different ages and thus reduces the degree of precision in the determination of the age. Throughout the results, there is a clear discrepancy in the ability with which the length and width data have been able to represent differences between treatments. Due to the larger and clearer differences in the lengths, it has been the parameter which has shown higher resolution and thus, provided clearer differences between treatments. Mass does not work in every situation because in most cases the larvae are HWK and preserved immediately and this process can affect the mass of the specimen (Day and Wallman, 2006a). Secondary to that is the fact that there are currently no models to convert preserved mass back to live mass and this would make calculating age from mass a tedious task (Day and Wallman, 2006a). Therefore, the recommendation is that length should be the parameter of choice when modelling the insects' growth and age, and thus estimating the PMI_{min}.

Day and Wallman (2006a) showed that the body length and width data of the *Calliphora augur* correlated well over time. They proposed that width be used as an alternative measurement to length because larvae undergo head curling during the preservation process, whereas the width of the larvae does not usually change severely and this would make it a more reliable parameter. Furthermore, by using a simple linear model, they were able to convert the width

measure to a length with 95% accuracy (Day and Wallman, 2006a). The results presented in the current study have shown that the length changes by much larger increments over time in comparison to the width. Although the conversion from length to width could be achieved as suggested by Day and Wallman (2006a), there could be a greater margin of error with the conversion if the width measures change minutely over time. This is because the degree of disproportion in the change of length and width could mean that the smaller changes in width could be misrepresented by the larger changes in length and this could ultimately lead to an incorrect age estimation. The length could potentially be used as the sole parameter for estimating larval age, but the width should not be used on its own as the incremental differences in the width could reduce the resolution and thus skew the estimation of the larval age.

Pupal casing and third instar larvae preservation

The focus of this study was to determine which preservatives would work best to retain the structural integrity of the lengths and widths of the pupal casings and the third instar larvae. When selecting a preservation technique, it is important to be cognisant of the evidence to be obtained from the samples (Brown et al., 2012). If the morphometric features are to be examined, ethanol would be the recommended preservative. However if the specimens are to undergo DNA or toxicant testing, the preservative selected should cause as little damage to the molecular and biochemical properties of the specimen as possible (Brown et al., 2012). The literature has recommended higher concentrations of ethanol be used for preservation because the internal fluids of the specimen will dilute in proportion to the amount of specimen that is being preserved (Martin, 1977; Amendt et al., 2015). The models showed that the lengths and widths of the larvae decreased with increased preservation time. This indicates that preservation time has more of an effect on the morphological integrity of the specimens than the different concentrations of ethanol used to preserve them.

The data in this chapter seems to suggest that in terms of retaining morphometric features of the pupal casings and third instar larvae, there is no significant difference between preserving in no preservative (for the pupal casings), 100%, 70% and 50% ethanol (for the pupal casings and the third instar larvae). What this means is that the selection of a preservative for these specimens does not need to take into consideration the morphometric

integrity, but rather the DNA and toxicological preservation should take precedence. Bisanti et al. (2009) showed that higher concentrations of ethanol were more effective at preserving DNA integrity, so as per their recommendations, concentrations of >70% should be used for specimen preservation.

In terms of the pupal casings, Amendt et al. (2015) did suggest storing them dry and without any preservatives. This study showed that there was no difference in the proportional length or width changes between the empty pupal casings stored using the different treatments. In this case, it is important to consider the evidence that is being obtained from the empty pupal casings. The recommendation would then be that dry preservation be used for specimens used for toxicological analysis (Amendt et al., 2015) and specimens used for other analyses be stored in >70% ethanol. These preservation techniques should be used in conjunction with a cold storage method for improved preservation and chemical stability of the specimen.

Conclusion

The correctness of the experimental design is a very important factor to consider because as this study has shown, very basic elements of the design can have cascading effects on the results and their interpretation. It is important to standardise the parameter used and the preservation technique as this makes results between studies more comparable and ultimately more admissible in the court of law. The incremental changes in width are much smaller in relation to length and this might lead to inaccurate estimations of the PMI_{min}. The results have also shown that there is no significant difference in the proportional changes in the lengths and widths of empty *Chrysomya chloropyga* pupal casings and *Calliphora croceipalpis* third instar larvae stored in different concentrations of ethanol. However, increased preservation time may cause differences to the dimensions of the specimens. Future research should be aimed towards testing and standardising other parameters within the experimental design. Once all this research has been conducted, a complete experimental design should be composed and tested and ultimately used for all entomotoxicological studies.

Chapter 4: General discussion

The aim of this thesis was to revisit the underutilised field of forensic entomotoxicology, highlight its flaws and then potentially begin the process to troubleshooting these issues. In reviewing the literature, it became apparent that the largest shortfall within the field was the lack of standardisation with regards to the manner in which experimental work is conducted. The publication of Chapter 1 (Da Silva et al., 2017) has now hopefully reached and alerted potential researchers to the gap in the literature and steered research towards standardisation of the field. The rest of the thesis focused on the standardisation of two parameters highlighted in Chapter 1; particularly the feeding substrate and the importance of measurement parameters and storage techniques in further enhancing the accuracy of age determination of insects and thus the PMI_{min} in forensic cases.

From the literature reviewed in Chapters 1 and 2, it became clear that the possibility of artefactual results was quite high because of the inconsistency with regards to the feeding substrates being used for the experimental work. The aim of Chapter 2 was to select an already existing dietary medium and modify it for potential use in entomotoxicological studies. The selected dietary medium was that used by Rabêlo et al. (2011). The differences between the diet used in Chapter 2 and the diet used by Rabêlo et al. (2011) were that different protein sources were used and nipagin (an antifungal agent) was excluded from the dietary medium. The experiments were done to establish a baseline for the growth rates of the larvae without the presence of toxicants and to establish whether the feeding substrate met the criteria set out by Da Silva et al. (2017) (Chapter 1). The results from Chapter 2 showed that the fish based diet was not ideal due to the ammonia that it produced which might have resulted in the death of the larvae. The no meat treatment (NMT) larvae did not reach pupation stage and a possible explanation for this was that they did not have sufficient protein in their diet to successfully complete each growth stage. Future studies should possibly test the contents of these diets to provide conclusive reasons for the observed differences in growth and mortality between diets based on their nutrient content. The dietary media which yielded the largest larvae which also completed all life stages were the larvae reared on the beef and pork based diets.

Numerous authors have explored the possibility of using artificial diets for the purpose of mass rearing insects (Osuji, 1978; Leal et al., 1982; Estrada et al., 2009; Rueda et al., 2010;

Rabêlo et al., 2011; Zheng et al., 2017). None of these diets have been selected as a standard diet for mass rearing, or for specific use in forensic entomotoxicology. Each of these diets outlines the basic nutrients required for growth and development in the respective insects being reared. Future studies should focus on testing the stability of different toxicants in a standardised dietary medium and once the ideal medium is selected, it should be used for all entomotoxicological studies.

The focus of chapter 3 was to address the debate in the literature with regards to which parameter should be used to quantify growth rate, as well as which storage method best preserves the morphometric integrity of pupal casings and third instar larvae. Day and Wallman (2006a) suggested that the width could be used as an alternative measure to the length because some larvae can undergo "head curling" as a result of the killing and preservation methods used and this could affect the accuracy of the measurement taken. The results in chapter 3 showed that the proportional change in width over time was smaller than the proportional change in length. This could result in a lack of resolution which could ultimately result in a greater margin of error when estimating the PMI_{min}.

In terms of the *Chrysomya chloropyga* empty pupal casings and the *Calliphora croceipalis* third instar larvae, there was no significant difference in the proportional length and width changes between treatments, suggesting that the morphometric integrity of these specimens is not affected by the preservative used. The specimens were affected by preservation time, as increased preservation time resulted in decreases in specimen length and widths. Bisanti et al. (2009) showed that in order to preserve DNA integrity, specimens should be stored in higher concentrations of ethanol. This means that when a preservation technique is selected, other analyses should take precedence (i.e. preservation techniques should be selected for the preservation of other characteristics such as DNA and toxicological integrity).

The aim of this thesis was to critically analyse the available literature on the field of forensic entomotoxicology, to select parameters to standardise, and to set baselines for these parameters. The three parameters selected were the dietary medium, measurement parameters and storage techniques. As mundane as these parameters might seem in the larger scheme of an experimental protocol, the literature review in chapter 1 showed that the discrepancies in the literature could be caused by these very simple factors which are often overlooked. Fields such as toxicology have set standard operating procedures (SOP's) in place

which are meant to be adhered to when experiments are conducted (SOFT & AAFS, 2006). Having SOP's in place ensures that any discrepancies in results can easily be identified and troubleshooted. It also means that results become more comparable between experiments because the differences in experimental design do not play a role in the differences in outcomes if the experimental designs are the same. The idea is to steer the direction of the research within entomotoxicology towards standardisation because currently, the lack of standardisation has led to discrepancies in results as well as the inability to make these results comparable (Da Silva et al., 2017).

The results from this thesis have shown the necessity of standardisation. Even within the experiments there were discrepancies between treatments. This issue would be further exacerbated by the introduction of toxicants. It would thus be ideal to ensure that every aspect of the experimental protocol is standardised, as this decreases the potential for artefactual effects in the results. The three parameters selected for standardisation in this thesis were the dietary medium, measurement parameters and storage techniques. Other parameters which should be considered for standardisation include the method of toxicological analysis (although the selected method is dependent on the toxicant), the concentrations of the toxicants that should be used, as well as the experimental design itself. Once SOP's such as those used in toxicology are set for entomotoxicology, results can be more comparable and moreover, results can be more admissible in the court of law which is ultimately the point of the research.

References

Adams, Z.J.O., Hall, M.J.R., 2003. Methods used for the killing and preservation of blowfly larvae, and their effect on post-mortem larval length. Forensic Sci. Int. 138, 50–61. https://doi.org/10.1016/j.forsciint.2003.08.010

Aderjan, R., Mattern, R., 1980. Validity of digoxin concentrations in blood determined post mortem (author's transl). Z. Rechtsmedzin J. Leg. Med. 20, 13–20. https://doi.org/10.1007/BF00200973

Al-Misned, F.A.M., 2001. Biological effects of cadmium on life cycle parameters of *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae). Kuwait J. Sci. Eng. 28, 179–188.

Al-Misned, F.A.M., 2003. Effect of cadmium on the longevity and fecundity of the blowfly *Chrysomya albiceps* (Wiedemann)(Diptera: Calliphoridae). Kuwait J Sci Eng 30, 81–94.

Amendt, J., Campobasso, C.P., Gaudry, E., Reiter, C., LeBlanc, H.N., J. R. Hall, M., 2007. Best practice in forensic entomology—standards and guidelines. Int. J. Legal Med. 121, 90–104. https://doi.org/10.1007/s00414-006-0086-x

Amendt, J., Richards, C.S., Campobasso, C.P., Zehner, R., Hall, M.J.R., 2011. Forensic entomology: applications and limitations. Forensic Sci. Med. Pathol. 7, 379–392. https://doi.org/10.1007/s12024-010-9209-2

Amendt, J., Anderson, G., Campobasso, C.P., Dadour, I., Gaudry, E., Hall, M.J.R., Moretti, T.C., Sukontason, K.L., Villet, M.H., 2015. Standard Practices, in: Tomberlin, J., Benbow, E. (Eds.), Forensic Entomology: International Dimensions and Frontiers. CRC Press, pp. 381–398.

Anderson, G.S., 2010. Factors that influence insect succession on carrion, in: Byrd, J.H., Castner, J.. (Eds.), Forensic Entomology: The Utility of Arthropods in Legal Investigations. CRC Press, Boca Raton, pp. 201–250.

Andretic, R., van Swinderen, B., Greenspan, R.J., 2005. Dopaminergic Modulation of Arousal in Drosophila. Curr. Biol. 15, 1165–1175. https://doi.org/10.1016/j.cub.2005.05.025

Archer, M.S., Elgar, M.A., 2003. Female breeding-site preferences and larval feeding strategies of carrion-breeding Calliphoridae and Sarcophagidae (Diptera): a quantitative analysis. Aust. J. Zool. 51, 165–174.

Azam, I., Afsheen, S., Zia, A., Javed, M., Saeed, R., Sarwar, M.K., Munir, B., 2015. Evaluating insects as bioindicators of heavy metal contamination and accumulation near industrial area of Gujrat , Pakistan. BioMed Res. Int. 2015.

Baia, T.C., Gama, R.A., Silva de Lima, L.A., Lima, K.M.G., 2016. FTIR microspectroscopy coupled with variable selection methods for the identification of flunitrazepam in necrophagous flies. Anal. Methods 8, 968–972. https://doi.org/10.1039/C5AY02342D

Bakr, R., Ramadan, R., El - Sawy, S., Hussien, S., 2012. Ultrastructure of the midgut of the third larval instar of *Chrysomya megacephala* (Diptera: Calliphoridae) fed on malathion treated diet. Egypt. Acad. J. Biol. Sci. 3, 13–26.

Baqué, M., Amendt, J., 2013. Strengthen forensic entomology in court—the need for data exploration and the validation of a generalised additive mixed model. Int. J. Legal Med. 127, 213–223. https://doi.org/10.1007/s00414-012-0675-9

Beuter, L., Mendes, J., 2013. Development of *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) in different pig tissues. Neotrop. Entomol. 42, 426–430. https://doi.org/10.1007/s13744-013-0134-4

Beyer, J., Enos, W., Stalic, M., 1980. Drug identification through analysis of maggots. J. Forensic Sci. 25, 411 – 412.

Bisanti, M., Ganassi, S., Mandrioli, M., 2009. Comparative analysis of various fixative solutions on insect preservation for molecular studies. Entomol. Exp. Appl. 130, 290–296. https://doi.org/10.1111/j.1570-7458.2008.00821.x

Boehme, P., Spahn, P., Amendt, J., Zehner, R., 2013. Differential gene expression during metamorphosis: a promising approach for age estimation of forensically important *Calliphora vicina* pupae (Diptera: Calliphoridae). Int. J. Legal Med. 127, 243–249. https://doi.org/10.1007/s00414-012-0699-1

Bourel, B., Hédouin, V., Martin-Bouyer, L., Bécart, A., Tournel, G., Deveaux, M., Gosset, D., 1999a. Effects of morphine in decomposing bodies on the development of *Lucilia sericata* (Diptera: Calliphoridae). J. Forensic Sci. 44, 354–358.

Bourel, B., Hédouin, V., Martin-Bouyer, L., Bécart, A., Tournel, G., Deveaux, M., Gosset, D., 1999b. Morphine perfused rabbits: a tool for experiments in forensic entomotoxicology. J. Forensic Sci. 44, 347–350.

Bourel, B., Creusy, C., Gosset, D., Cailliez, J.-C., Fleurisse, L., Goff, M.L., Hédouin, V., 2001a. Immunohistochemical contribution to the study of morphine metabolism in Calliphoridae larvae and implications in forensic entomotoxicology. J. Forensic Sci. 46, 596–599.

Bourel, B., Gosset, D., Tournel, G., Goff, M.L., Hédouin, V., 2001b. Determination of drug levels in two species of necrophagous Coleoptera reared on substrates containing morphine. J. Forensic Sci. 46, 600–603.

Bourel, B., Tournel, G., Hedouin, V., Deveaux, M., Goff, M.L., Gosset, D., 2001c. Morphine extraction in necrophagous insects remains for determining ante-mortem opiate intoxication. Forensic Sci. Int. 120, 127–131.

Brown, K., Thorne, A., Harvey, M.L., 2012. Preservation of *Calliphora vicina* (Diptera: Calliphoridae) pupae for use in post-mortem interval estimation. Forensic Sci. Int. 223, 176–183. https://doi.org/http://dx.doi.org/10.1016/j.forsciint.2012.08.029

Byrd, J.H., 1998. Temperature dependent development and computer modelling of insect growth: Its application to forensic entomology. University of Florida, Gainesville.

Byrd, J.H., Castner, J.L., 2010. Insects of forensic importance, in: Byrd, J.H., Castner, J.L. (Eds.), Forensic Entomology: The Utility of Arthropods in Legal Investigations. CRC Press, Boca Raton, pp. 39–126.

Byrd, J.H., Lord, W.D., Wallace, J.R., Tomberlin, J.K., Haskell, N.H., 2010. Collection of entomological evidence during legal investigations, in: Byrd, J.., Castner, J.L. (Eds.), Forensic Entomology: The Utility of Arthropods in Legal Investigations. CRC Press, Boca Raton, pp. 127–175.

Byrd, J.H., Tomberlin, J.K., 2010. Laboratory rearing of forensic insects, in: Forensic Entomology: The Utility of Arthropods in Legal Investigations. CRC Press, Boca Raton, pp. 177–200.

Campobasso, C.P., Di Vella, G., Introna, F., 2001. Factors affecting decomposition and Diptera colonization. Forensic Sci. Int. 120, 18–27.

Campobasso, C.P., Gherardi, M., Caligara, M., Sironi, L., Introna, F., 2004. Drug analysis in blowfly larvae and in human tissues: a comparative study. Int. J. Legal Med. 118, 210–214. https://doi.org/10.1007/s00414-004-0448-1

Carvalho, L.M., Linhares, A.X., Trigo, J.R., 2001. Determination of drug levels and the effect of diazepam on the growth of necrophagous flies of forensic importance in southeastern Brazil. Forensic Sci. Int. 120, 140–144.

Castner, J.L., 2010. General entomology and insect biology, in: Forensic Entomology: The Utility of Arthropods in Legal Investigations. CRC Press, Boca Raton, pp. 17–38.

Child, G., 1939. The effect of increasing time of development at constant temperature on the wing size of vestigial of Drosophila melanogaster. Biol. Bull. 77, 432–442.

Clements, W.H., Cadmus, P., Brinkman, S.F., 2013. Responses of aquatic insects to Cu and Zn in stream microcosms: understanding differences between single species tests and field responses. Environ. Sci. Technol. 130617142015009. https://doi.org/10.1021/es401255h

Cook, D.S., Braithwaite, R.A., Hale, K.A., 2000. Estimating antemortem drug concentrations from postmortem blood samples: the influence of postmortem redistribution. J. Clin. Pathol. 53, 282–285.

Cooper, G.A.A., Paterson, S., Osselton, M.D., 2010. The United Kingdom and Ireland Association of Forensic Toxicologists. Sci. Justice 50, 166–176. https://doi.org/10.1016/j.scijus.2010.09.005

Da Silva, C., Villet, M.H., 2006. Effects of prophylactic progesterone in decomposing tissues on the development of *Chrysomya chloropyga* (Wiedeman)(Diptera: Calliphoridae). Afr. Entomol. 14, 199.

Da Silva, E.I.T., Wilhelmi, B., Villet, M.H., 2017. Forensic entomotoxicology revisited—towards professional standardisation of study designs. Int. J. Legal Med. 131, 1399–1412. https://doi.org/10.1007/s00414-017-1603-9

Davies, K., Harvey, M.L., 2013. Internal morphological analysis for age estimation of blow fly pupae (Diptera: Calliphoridae) in postmortem interval estimation. J. Forensic Sci. 58, 79–84. https://doi.org/10.1111/j.1556-4029.2012.02196.x

Day, D.M., Wallman, J.F., 2006a. Width as an alternative measurement to length for post-mortem interval estimations using *Calliphora augur* (Diptera: Calliphoridae) larvae. Forensic Sci. Int. 159, 158–167. https://doi.org/10.1016/j.forsciint.2005.07.009

Day, D.M., Wallman, J.F., 2006b. Influence of substrate tissue type on larval growth in *Calliphora augur* and *Lucilia cuprina* (Diptera: Calliphoridae)*. J. Forensic Sci. 51, 657–663. https://doi.org/10.1111/j.1556-4029.2006.00127.x

Day, D.M., Wallman, J.F., 2008. Effect of preservative solutions on preservation of *Calliphora augur* and <i>Lucilia cuprina</> larvae (Diptera: Calliphoridae) with implications for post-mortem interval estimates. Forensic Sci. Int. 179, 1–10. https://doi.org/10.1016/j.forsciint.2008.04.006

de Aguiar França, J., Brandão, M., Sodré, F.F., Caldas, E.D., 2014. Simultaneous determination of prescription drugs, cocaine, aldicarb and metabolites in larvae from decomposed corpses by LC–MS–MS after solid–liquid extraction with low temperature partitioning. Forensic Toxicol. 33, 93–103. https://doi.org/10.1007/s11419-014-0255-4

de Carvalho, L.M.L., Linhares, A.X., Badan Palhares, F.A., 2012. The effect of cocaine on the development rate of immatures and adults of *Chrysomya albiceps* and *Chrysomya putoria* (Diptera: Calliphoridae) and its importance to postmortem interval estimate. Forensic Sci. Int. 220, 27–32. https://doi.org/10.1016/j.forsciint.2012.01.023

DeForest, D.K., Brix, K.V., Adams, W.J., 2007. Assessing metal bioaccumulation in aquatic environments: The inverse relationship between bioaccumulation factors, trophic transfer factors and exposure concentration. Aquat. Toxicol. 84, 236–246. https://doi.org/10.1016/j.aquatox.2007.02.022

Denno, R.F., Cothran, W.R., 1976. Competitive interactions and ecological strategies of sarcophagid and calliphorid flies inhabiting rabbit carrion. Ann. Entomol. Soc. Am. 69, 109–113.

Dowell, F.E., Noutcha, A.E.M., Michel, K., 2011. The Effect of Preservation Methods on Predicting Mosquito Age by near infrared spectroscopy. Am. J. Trop. Med. Hyg. 85, 1093–1096. https://doi.org/10.4269/ajtmh.2011.11-0438

Drummer, O.H., Gerostamoulos, J., 2002. Postmortem drug analysis: analytical and toxicological aspects. Ther. Drug Monit. 24, 199–209.

Drummer, O.H., 2008. Postmortem toxicological redistribution, in: Essentials of Autopsy Practice. Springer, pp. 1–21.

El - Samad, L.M., El - Moaty, Z.A., Makemer, H.M., 2011. Effects of tramadol on the development of *Lucilia sericata* (Diptera: Calliphoridae) and detection of the drug concentration in postmortem rabbit tissues and larvae. J. Entomol. 8, 353–364. https://doi.org/10.3923/je.2011.353.364

Estrada, D.A., Grella, M.D., Thyssen, P.J., Linhares, A.X., 2009. Taxa de desenvolvimento de *Chrysomya albiceps* (Wiedemann) (Diptera: Calliphoridae) em dieta artificial acrescida de tecido animal para uso forense. Neotrop. Entomol. 38(2), 203–207.

Fathy, H.M., Attia, R.A.H., Yones, D.A., Eldeek, H.E.M.E., Tolba, M.E.M., Shaheen, M.S.I., 2008. Effect of codeine phosphate on developmental stages of forensically important Calliphoride fly: *Chrysomya albiceps*. Mansoura J. Forensic Med. Clin. Toxicol. XVI, 41–59.

Ferraz, A.C.P., Dallavecchia, D.L., Silva, D.C., Figueiredo, A.L., Proenca, B., Silva-Filho, R.G., Aguiar, V.M., 2014. Effects of the Antibiotics Gentamicin on the Postembryonic Development of *Chrysomya putoria* (Diptera: Calliphoridae). J. Insect Sci. 14, 279–279. https://doi.org/10.1093/jisesa/ieu141

Frampton, M., Droege, S., Conrad, T., Prager, S., Richards, M.H., 2008. Evaluation of specimen preservation for DNA analyses of bees. J. Hymenopt. Res. 17, 195–200.

Gagliano-Candela, R., Aventaggiato, L., 2001. The detection of toxic substances in entomological specimens. Int. J. Legal Med. 114, 197–203.

George, K.A., Archer, M.S., Green, L.M., Conlan, X.A., Toop, T., 2009. Effect of morphine on the growth rate of *Calliphora stygia* (Fabricius) (Diptera: Calliphoridae) and possible implications for forensic entomology. Forensic Sci. Int. 193, 21–25. https://doi.org/10.1016/j.forsciint.2009.08.013

Gerostamoulos, D., Beyer, J., Staikos, V., Tayler, P., Woodford, N., Drummer, O.H., 2012. The effect of the postmortem interval on the redistribution of drugs: a comparison of mortuary admission and autopsy blood specimens. Forensic Sci. Med. Pathol. 8, 373–379. https://doi.org/10.1007/s12024-012-9341-2

Goff, M.L., Omori, A.I., Goodbrod, J.R., 1989. Effect of cocaine in tissues on the development rate *Boettcherisca peregrina* (Diptera: Sarcophagidae). J. Med. Entomol. 26.2, 91–93.

Goff, M.L., Brown, W.A., Hewadikaram, K.A., Omori, A.I., 1991. Effect of heroin in decomposing tissues on the development rate of *Boettcherisca peregrina* (Diptera, Sarcophagidae) and implications of this effect on estimation of postmortem intervals using arthropod development patterns. J. Forensic Sci. 36, 537–542.

Goff, M.L., Brown, W.A., Omori, A.I., 1992. Preliminary observations of the effect of methamphetamine in decomposing tissues on the development rate of *Parasarcophaga ruficornis* (Diptera: Sarcophagidae) and implications of this effect on the estimations of postmortem intervals. J. Forensic Sci. 37, 867–872.

Goff, M.L., 1993. Estimation of postmortem interval using arthropod development and successional patterns. Forensic Sci. Rev. 5, 81–94.

Goff, M.L., Brown, W.A., Omori, A.I., LaPointe, D.A., 1993. Preliminary observations of the effects of amitriptyline in decomposing tissues on the development of *Parasarcophaga ruficornis* (Diptera: Sarcophagidae) and implications of this effect to estimation of postmortem interval. J. Forensic Sci. 38, 316–322.

Goff, M.L., Brown, W.A., Omori, A.I., LaPointe, D.A., 1994. Preliminary observations of the effects of phencyclidine in decomposing tissues on the development of *Parasarcophaga ruficornis* (Diptera: Sarcophagidae). J. Forensic Sci. 39, 123–128.

Goff, M.L., Miller, M.L., Paulson, J.D., Lord, W.D., Richards, E., Omori, A.I., 1997. Effects of 3,4-Methylenedioxymethamphetamine in decomposing tissues on the development of *Parasarcophaga ruficornis* (Diptera: Sarcophagidae) and detection of the drug in postmortem blood, liver tissue, larvae and puparia. J. Forensic Sci. 42, 276–280.

Goff, M.L., Lord, W.D., 2010. Entomotoxicology: Insects as toxicological indicators of drugs and toxins on insect development, in: Byrd, J.H., Castner, J.L. (Eds.), Forensic Entomology: The Utility of Arthropods in Legal Investigations. CRC Press, Boca Raton, pp. 427–436.

Gordon Gibson, G., Skett, P., 2008a. Pathways to drug metabolism, in: Introduction to Drug Metabolism. Nelson Thornes Publishers, United Kingdom, pp. 1–34.

Gordon Gibson, G., Skett, P., 2008b. Factors affecting drug metabolism: Internal factors, in: Introduction to Drug Metabolism. Nelson Thornes Publishers, United Kingdom, pp. 119–145.

Gordon Gibson, G., Skett, P., 2008c. Induction and inhibition of drug metabolism, in: Introduction to Drug Metabolism. Nelson Thornes Publishers, United Kingdom, pp. 87–118.

Gosselin, M., Di Fazio, V., Wille, S.M.R., Ramírez Fernandez, M. del M., Samyn, N., Bourel, B., Rasmont, P., 2011a. Methadone determination in puparia and its effect on the development of *Lucilia*

sericata (Diptera, Calliphoridae). Forensic Sci. Int. 209, 154–159. https://doi.org/10.1016/j.forsciint.2011.01.020

Gosselin, M., Wille, S.M.R., del Mar Ramirez Fernandez, M., Di Fazio, V., Samyn, N., De Boeck, G., Bourel, B., 2011b. Entomotoxicology, experimental set - up and interpretations for forensic toxicologists. Forensic Sci. Int. 208, 1–9.

Grassberger, M., Reiter, C., 2001. Effect of temperature on *Lucilia sericata* (Diptera: Calliphoridae) development with special reference to the isomegalen-and isomorphen-diagram. Forensic Sci. Int. 120, 32–36.

Gunatilake, K., Goff, M.L., 1989. Detection of organophosphate poisoning in a putrefying body by analyzing arthropod larvae. J. Forensic Sci. 34, 714–716.

Gunn, J.A., Shelley, C., Lewis, S.W., Toop, T., Archer, M., 2006. The determination of morphine in the larvae of *Calliphora stygia* using flow injection analysis and HPLC with chemiluminescence detection. J. Anal. Toxicol. 30, 519–523.

Han, E., Kim, E., Hong, H., Jeong, S., Kim, J., In, S., Chung, H., Lee, S., 2012. Evaluation of postmortem redistribution phenomena for commonly encountered drugs. Forensic Sci. Int. 219, 265–271. https://doi.org/10.1016/j.forsciint.2012.01.016

Hédouin, V., Bourel, B., Bécart, A., Tournel, G., Deveaux, M., Goff, M.L., Gosset, D., 2001. Determination of drug levels in larvae of *Protophormia terraenovae* and *Calliphora vicina* (Diptera: Calliphoridae) reared on rabbit carcasses containing morphine. J. Forensic Sci. 46, 12–14.

Hendricks, J.C., Finn, S.M., Panckeri, K.A., Chavkin, J., Williams, J.A., Sehgal, A., Pack, A.I., 2000. Rest in *Drosophila* is a sleep-like state. Neuron 25, 129–138.

Hendricks, J.C., Kirk, D., Panckeri, K., Miller, M.S., Pack, A.I., 2003. Modafinil maintains waking in the fruit fly *Drosophila melanogaster.* Sleep 26, 139–149.

Herskowitz, I.H., 1951. A list of chemical substances studies for the effects on *Drosophila*, with a bibliography. Am. Nat. 85, 181–199.

Holland, M.G., Schwope, D.M., Stoppacher, R., Gillen, S.B., Huestis, M.A., 2011. Postmortem redistribution of Δ9-tetrahydrocannabinol (THC), 11-hydroxy-THC (11-OH-THC), and 11-nor-9-carboxy-THC (THCCOOH). Forensic Sci. Int. 212, 247–251. https://doi.org/10.1016/j.forsciint.2011.06.028

Hörter, D., Dressman, J.B., 2001. Influence of physicochemical properties on dissolution of drugs in the gastrointestinal tract. Adv. Drug Deliv. Rev. 46, 75–87.

House, H.L., 1962. Insect nutrition. Annu. Rev. Biochem. 653–672.

Introna, F., Campobasso, C.P., Goff, M.L., 2001. Entomotoxicology. Forensic Sci. Int. 120, 42–47.

Introna, F., Lo Dico, C., Caplan, Y.H., Smialek, J.E., 1990. Opiate analysis in cadeveric blowfly larvae as an indicator of narcotic intoxication. J. Forensic Sci. 35, 118–122.

Ireland, S., Turner, B., 2006. The effects of larval crowding and food type on the size and development of the blowfly, *Calliphora vomitoria*. Forensic Sci. Int. 159, 175–181. https://doi.org/10.1016/j.forsciint.2005.07.018

Irish, S., Lindsay, T., Wyatt, N., 2014. Key to adults of Afrotropical species of the genus *Chrysomya* Robineau-Desvoidy (Diptera: Calliphoridae). Afr. Entomol. 22, 297–306. https://doi.org/10.4001/003.022.0210

Kaneshrajah, G., Turner, B., 2004. *Calliphora vicina* larvae grow at different rates on different body tissues. Int. J. Legal Med. 118, 242–244. https://doi.org/10.1007/s00414-004-0444-5

Kharbouche, H., Augsburger, M., Cherix, D., Sporkert, F., Giroud, C., Wyss, C., Champod, C., Mangin, P., 2008. Codeine accumulation and elimination in larvae, pupae, and imago of the blowfly *Lucilia sericata* and effects on its development. Int. J. Legal Med. 122, 205–211. https://doi.org/10.1007/s00414-007-0217-z

King, J.R., Porter, S.D., 2004. Recommendations on the use of alcohols for preservation of ant specimens (Hymenoptera, Formicidae). Insectes Sociaux 51, 197–202. https://doi.org/10.1007/s00040-003-0709-x

Kintz, P., Godelar, B., Tracqui, A., Mangin, P., Lugnier, A.A., Chaumont, A.J., 1990. Fly larvae: a new toxicological method of investigation in forensic medicine. J. Forensic Sci. 35, 204–207.

Knapp, M., 2012. Preservative fluid and storage conditions alter body mass estimation in a terrestrial insect: Sampling and storage affect body mass. Entomol. Exp. Appl. 143, 185–190. https://doi.org/10.1111/j.1570-7458.2012.01247.x

Lan, Y.H., Novakofski, J., McCusker, R.H., Brewer, M.S., Carr, T.R., McKeith, F.K., 1995. Thermal gelation of myofibrils from pork, beef, fish, chicken and turkey. J. Food Sci. 60, 941–945.

Langer, R., 1998. Drug delivery and targeting. Nature 392, 5–10.

Lawai, V., Abdul Rahim, N.A., Ngaini, Z., 2015. Blowfly larval tissues as a secondary detector for determining paraquat-related death in rabbit carcass. J. Forensic Sci. 60, 1620–1624. https://doi.org/10.1111/1556-4029.12852

Leal, T.T., Prado, Â.P. do, Antunes, A.J., 1982. Rearing the larvae of the blowfly *Chrysomya chloropyga* (Wiedemann)(Diptera, Calliphoridae) on oligidic diets. Rev. Bras. Zool. 1, 41–44.

Lentini, J.J., 2009. Forensic science standards: where they come from and how they are used. Forensic Sci. Policy Manag. Int. J. 1, 10–16. https://doi.org/10.1080/19409040802596315

Magni, P.A., Pacini, T., Pazzi, M., Vincenti, M., Dadour, I.R., 2014. Development of a GC–MS method for methamphetamine detection in *Calliphora vomitoria L.* (Diptera: Calliphoridae). Forensic Sci. Int. 241, 96–101. https://doi.org/10.1016/j.forsciint.2014.05.004

Martin, J.E.H., 1977. The insects and arachnids of Canada. Part 1: Collecting, preparing and preserving insects, mites and spiders. Reserach Branch, Canada Department of Agriculture, Hull, Quebec, Canada.

McClung, C., Hirsh, J., 1998. Stereotypic behavioral responses to free-base cocaine and the development of behavioral sensitization in *Drosophila*. Curr. Biol. 8, 109–112.

McIntyre, I.M., Escott, C.M., 2012. Postmortem Drug Redistribution. J. Forensic Res. 3. https://doi.org/10.4172/2157-7145.1000e108

Midgley, J.M., Villet, M.H., 2009. Effect of the killing method on post-mortem change in length of larvae of *Thanatophilus micans* (Fabricius 1794) (Coleoptera: Silphidae) stored in 70% ethanol. Int. J. Legal Med. 123, 103–108. https://doi.org/10.1007/s00414-008-0260-4

Miller, M.L., Lord, W.D., Goff, M.L., Donnelly, B., McDonough, E.T., Alexis, J.C., 1994a. Isolation of amitriptyline and nortriptyline from fly puparia (Phoridae) and beetle exuviae (Dermestidae) associated with mummified human remains. J. Forensic Sci. 39, 1305–1313.

Miller, M.L., Lord, W.D., Goff, M.L., Donnelly, B., McDonough, E.T., Alexis, J.C., 1994b. Isolation of amitriptyline and nortriptyline from fly puparia (Phoridae) and beetle exuviae (Dermestidae) associated with mummified human remains. J. Forensic Sci. 39, 1305–1313.

Moe, S.J., Stenseth, N.C., Smith, R.H., 2001. Effects of a toxicant on population growth rates: sublethal and delayed responses in blowfly populations. Funct. Ecol. 15, 712–721.

Moriarty, F., 1988. Ecotoxicology. Hum. Exp. Toxicol. 7, 437–441. https://doi.org/10.1177/096032718800700510

Mullany, C., Keller, P.A., Nugraha, A.S., Wallman, J.F., 2014. Effects of methamphetamine and its primary human metabolite, p-hydroxymethamphetamine, on the development of the Australian blowfly *Calliphora stygia*. Forensic Sci. Int. 241, 102–111. https://doi.org/10.1016/j.forsciint.2014.05.003

Musvasva, E., Williams, K.A., Muller, W.J., Villet, M.H., 2001. Preliminary observations on the effects of hydrocortisone and sodium methohexital on development of *Sarcophaga (Curranea) tibialis* Macquart (Diptera: Sacrophagidae), and implications for estimating post mortem interval. Forensic Sci. Int. 120, 37–41.

Nassar, A.F., Hollenberg, P.F., Scatina, J. (Eds.), 2009. Drug metabolism handbook: concepts and applications. Wiley, Hoboken, N.J.

National Research Council Committee on Identifying the Needs of the Forensic Sciences Community [NRCCINFSC] (Ed.), 2009. Strengthening forensic science in the United States: a path forward. National Academies Press, Washington, D.C.

Niederegger, S., Wartenberg, N., Spiess, R., Mall, G., 2013. Influence of food substrates on the development of the blowflies Calliphora vicina and Calliphora vomitoria (Diptera, Calliphoridae). Parasitol. Res. 112, 2847–2853. https://doi.org/10.1007/s00436-013-3456-6

Nuorteva, P., 1977. Sarcophagous insects as forensic indicators, in: Tedeschi, C.G., Eckert, W.G., Tedeschi, L. (Eds.), Forensic Medicine: A Study in Trauma and Environmental Hazards. W.B. Saunders Co, Philadelphia, pp. 1072–1095.

Nuorteva, P., Nuorteva, S.-L., 1982. The fate of mercury in sarcosaprophagous flies and in insects eating them. Ambio 34–37.

O'Brien, C., Turner, B., 2004. Impact of paracetamol on *Calliphora vicina* larval development. Int. J. Legal Med. 118, 188–189. https://doi.org/10.1007/s00414-004-0440-9

Oliveira, H.G., Gomes, G., Morlin Jr, J.J., Von Zuben, C.J., Linhares, A.X., 2009. The Effect of Buscopan® on the Development of the Blow Fly *Chrysomya megacephala* (F.) (Diptera: Calliphoridae). J. Forensic Sci. 54, 202–206. https://doi.org/10.1111/j.1556-4029.2008.00926.x

Oliveira, J.S., Baia, T.C., Gama, R.A., Lima, K.M.G., 2014. Development of a novel non-destructive method based on spectral fingerprint for determination of abused drug in insects: An alternative entomotoxicology approach. Microchem. J. 115, 39–46. https://doi.org/10.1016/j.microc.2014.02.009

Olson, K.N., Luckenbill, K., Thompson, J., Middleton, O., Geiselhart, R., Mills, K.M., Kloss, J., Apple, F.S., 2010. Postmortem redistribution of Fentanyl in blood. Am. J. Clin. Pathol. 133, 447–453. https://doi.org/10.1309/AJCP4X5VHFSOERFT

O'Malley, K., Crooks, J., Duke, E., Stevenson, I.H., 1971. Effect of age and sex on human drug metabolism. Br Med J 3, 607–609.

Ortloff, A., Peña, P., Riquelme, M., 2012. Preliminary study of the succession pattern of necrobiont insects, colonising species and larvae on pig carcasses in Temuco (Chile) for forensic applications. Forensic Sci. Int. 222, e36–e41. https://doi.org/10.1016/j.forsciint.2012.04.022

Osuji, F.N., 1978. An assessment of the performance of *Dermestes maculatus* DeGeer (Coleoptera, Dermestidae) in some dietary media. Entomol. Exp. Appl. 24, 185–192.

Parry, S., Linton, S.M., Francis, P.S., O'Donnell, M.J., Toop, T., 2011. Accumulation and excretion of morphine by *Calliphora stygia* , an Australian blow fly species of forensic importance. J. Insect Physiol. 57, 62–73. https://doi.org/10.1016/j.jinsphys.2010.09.005

Penders, J., Verstraete, A., 2006. Laboratory guidelines and standards in clinical and forensic toxicology. Accreditation Qual. Assur. 11, 284–290. https://doi.org/10.1007/s00769-006-0131-y

Pien, K., Laloup, M., Pipeleers-Marichal, M., Grootaert, P., De Boeck, G., Samyn, N., Boonen, T., Vits, K., Wood, M., 2004. Toxicological data and growth characteristics of single post-feeding larvae and puparia of *Calliphora vicina* (Diptera: Calliphoridae) obtained from a controlled nordiazepam study. Int. J. Legal Med. 118, 190–193. https://doi.org/10.1007/s00414-004-0441-8

Pounder, D.J., 1991. Forensic entomo-toxicology. J. Forensic Sci. Soc. 31, 469–472.

Prins, A.J., 1982. Morphological and biological notes on six South African blow-flies (Diptera, Calliphoridae) and their immature stages. Ann. South Afr. Mus.

Rabêlo, K.C.N., Thyssen, P.J., Salgado, R.L., Araújo, M.S.C., Vasconcelos, S.D., 2011. Bionomics of two forensically important blowfly species *Chrysomya megacephala* and *Chrysomya putoria* (Diptera: Calliphoridae) reared on four types of diet. Forensic Sci. Int. 210, 257–262. https://doi.org/10.1016/j.forsciint.2011.03.022

R Core Team, 2017. R: A language and environment for statistical computing [Internet]. VIenna, Austria.

Rainbow, P.S., 2007. Trace metal bioaccumulation: Models, metabolic availability and toxicity. Environ. Int. 33, 576–582. https://doi.org/10.1016/j.envint.2006.05.007

Rashid, R.A., Osman, K., Ismail, M.I., Zuha, R.M., Hassan, R.A., others, 2008. Determination of malathion levels and the effect of malathion on the growth of *Chrysomya megacephala* (Fabricius) in malathion-exposed rat carcass. Trop. Biomed. 25, 184–190.

Rezende, F., Alonso, M.A., Souza, C.M., Thyssen, P.J., Linhares, A.X., 2014. Developmental rates of immatures of three *Chrysomya* species (Diptera: Calliphoridae) under the effect of methylphenidate hydrochloride, phenobarbital, and methylphenidate hydrochloride associated with phenobarbital. Parasitol. Res. 113, 1897–1907. https://doi.org/10.1007/s00436-014-3837-5

Richards, C.S., Paterson, I.D., Villet, M.H., 2008. Estimating the age of immature *Chrysomya albiceps* (Diptera: Calliphoridae), correcting for temperature and geographical latitude. Int. J. Legal Med. 122, 271–279. https://doi.org/10.1007/s00414-007-0201-7

Richards, C.S., Rowlinson, C.C., Cuttiford, L., Grimsley, R., Hall, M.J.R., 2013. Decomposed liver has a significantly adverse affect on the development rate of the blowfly Calliphora vicina. Int. J. Legal Med. 127, 259–262. https://doi.org/10.1007/s00414-012-0697-3

Richmond, E.K., Rosi-Marshall, E.J., Lee, S.S., Thompson, R.M., Grace, M.R., 2016. Antidepressants in stream ecosystems: influence of selective serotonin reuptake inhibitors (SSRIs) on algal production and insect emergence. Freshw. Sci. 35, 845–855.

Rivers, D.B., Thompson, C., Brogan, R., 2011. Physiological trade-offs of forming maggot masses by necrophagous flies on vertebrate carrion. Bull. Entomol. Res. 599–611. https://doi.org/10.1017/S0007485311000241

Roeterdink, E.M., Dadour, I.R., Watling, R.J., 2004. Extraction of gunshot residues from the larvae of the forensically important blowfly Calliphora dubia (Macquart) (Diptera: Calliphoridae). Int. J. Legal Med. 118, 63–70. https://doi.org/10.1007/s00414-003-0408-1

Rohlfs, M., 2006. Genetic variation and the role of insect life history traits in the ability of Drosophila larvae to develop in the presence of a competing filamentous fungus. Evol. Ecol. 20, 271–289. https://doi.org/10.1007/s10682-006-0002-3

Rook, E., Hillebrand, M., Rosing, H., Vanree, J., Beijnen, J., 2005. The quantitative analysis of heroin, methadone and their metabolites and the simultaneous detection of cocaine, acetylcodeine and their metabolites in human plasma by high-performance liquid chromatography coupled with tandem mass spectrometry. J. Chromatogr. B 824, 213–221. https://doi.org/10.1016/j.jchromb.2005.05.048

Rueda, L.C., Ortega, L.G., Segura, N.A., Acero, V.M., Bello, F., 2010. *Lucilia sericata* strain from Colombia: Experimental colonization, life tables and evaluation of two artifcial diets of the blowfy *Lucilia sericata* (Meigen)(Diptera: Calliphoridae), Bogotá, Colombia Strain. Biol. Res. 43, 197–203.

Saar, E., Beyer, J., Gerostamoulos, D., Drummer, O.H., 2012. The time-dependant post-mortem redistribution of antipsychotic drugs. Forensic Sci. Int. 222, 223–227. https://doi.org/10.1016/j.forsciint.2012.05.028

Sadler, D.W., Fuke, C., Court, F., Pounder, D.J., 1995. Drug accumulation and elimination in *Calliphora vicina* larvae 71, 191–197.

Sadler, D.W., Robertson, L., Brown, G., Fuke, C., Pounder, D.J., 1997. Barbiturates and analgesics in *Calliphora vicina* larvae. J. Forensic Sci. 42, 481–485.

Shaw, P.J., Cirelli, C., Greenspan, R.J., Tononi, G., 2000. Correlates of sleep and walking in *Drosophila melanogaster*. Science 287, 1834–1837.

Shen, W., St-Onge, M.P., Wang, Z., Heymsfield, S.B., 2005. Study of body composition: An overview, in: Heymsfield, S.B., Lohman, T.G., Wang, Z., Going, S.B. (Eds.), Human Body Composition. Human Kinetics, United States of America, pp. 3–14.

Sherman, R.A., Wyle, F.A., Thrupp, L., 1995. Effects of seven antibiotics on the growth and development of *Phaenicia sericata* (Diptera: Calliphoridae) larvae. J. Med. Entomol. 32, 646–649.

Shi, Y.-W., Liu, X.-S., Wang, H.-Y., Zhang, R.-J., 2009. Seasonality of insect succession on exposed rabbit carrion in Guangzhou, China. Insect Sci. 16, 425–439. https://doi.org/10.1111/j.1744-7917.2009.01277.x

Simkiss, K., Taylor, M.G., 1989. Metal fluxes across membranes of aquatic organisms. Rev. Aquat. Sci. 1, 173–188.

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

SOFT & AAFS, 2006. Forensic Toxicology Laboratory Guidelines.

Souza, C.M., Thyssen, P.J., Linhares, A.X., 2011. Effect of nandrolone decanoate on the development of three species of *Chrysomya* (Diptera: Calliphoridae), flies of forensic importance in Brazil. J. Med. Entomol. 48, 111–117. https://doi.org/10.1603/ME09291

StatSoft Inc, 2011. STATISTICA. Tulsa.

Sukontason, K., Narongchai, P., Kanchai, C., Vichairat, K., Sribanditmongkol, P., Bhoopat, T., Kurahashi, H., Chockjamsai, M., Piangjai, S., Bunchu, N., Vongvivach, S., Samai, W., Chaiwong, T., Methanitikorn, R., Ngern-Klun, R., Sripakdee, D., Boonsriwong, W., Siriwattanarungsee, S., Srimuangwong, C., Hanterdsith, B., Chaiwan, K., Srisuwan, C., Upakut, S., Moopayak, K., Vogtsberger, R.C., Olson, J.K., Sukontason, K.L., 2007. Forensic entomology cases in Thailand: a review of cases from 2000 to 2006. Parasitol. Res. 101, 1417–1423. https://doi.org/10.1007/s00436-007-0659-8

Tabor, K.L., Fell, R.D., Brewster, C.C., Pelzer, K., Behonick, G.S., 2005. Effects of antemortem ingestion of ethanol on insect successional patterns and development of Phormia regina (Diptera: Calliphoridae). J. Med. Entomol. 42, 481–489.

Tracqui, A., Keyser-Tracqui, C., Kintz, P., Ludes, B., 2004. Entomotoxicology for the forensic toxicologist: much ado about nothing? Int. J. Legal Med. 118, 194–196.

Turner, B.D., 1991. Forensic entomology, in: Maehly, A., Williams, R.L. (Eds.), Forensic Science Progress. Springer, Berlin Heidelberg, pp. 129–151.

United Nations Office on Drugs and Crime, 2015. World Drug Report.

Villet, M.H., 2007. An inexpensive geometrical micrometer for measuring small, live insects quickly without harming them. Entomol. Exp. Appl. 122, 279–280.

Villet, M.H., Richards, C.S., Midgley, J.M., 2010. Contemporary precision, bias and accuracy of minimum post - mortem intervals estimated using development of carrion - feeding insects,

in: Amendt, J., Goff, M.L., Campobasso, C.P., Grassberger, M. (Eds.), Current Concepts in Forensic Entomology. Springer Netherlands, Dordrecht, pp. 109–137.

Villet, M.H., 2011. African carrion ecosystems and their insect communities in relation to forensic entomology. Pest Technol. 5, 1–15.

Villet, M.H., Amendt, J., 2011. Advances in Entomological Methods for Death Time Estimation, in: Turk, E.E. (Ed.), Forensic Pathology Reviews. Humana Press, Totowa, NJ, pp. 213–237.

von Hoermann, C., Ruther, J., Ayasse, M., 2012. The attraction of virgin female hide beetles (Dermestes maculatus) to cadavers by a combination of decomposition odour and male sex pheromones. Front. Zool. 9, 1.

Voss, S.C., Spafford, H., Dadour, I.R., 2009. Annual and seasonal patterns of insect succession on decomposing remains at two locations in Western Australia. Forensic Sci. Int. 193, 26–36. https://doi.org/10.1016/j.forsciint.2009.08.014

Voss, S.C., Cook, D.F., Dadour, I.R., 2011. Decomposition and insect succession of clothed and unclothed carcasses in Western Australia. Forensic Sci. Int. 211, 67–75. https://doi.org/10.1016/j.forsciint.2011.04.018

Wallis, D.I., 1962. The sense organs on the ovipositor of the blowfly, *Phormia regina* Meigen. J. Insect Physiol. 8, 453–467.

Wang, J., Li, Z., Chen, Y., Chen, Q., Yin, X., 2008. The succession and development of insects on pig carcasses and their significances in estimating PMI in south China. Forensic Sci. Int. 179, 11–18. https://doi.org/10.1016/j.forsciint.2008.04.014

Wang, W.-X., Rainbow, P.S., 2008. Comparative approaches to understand metal bioaccumulation in aquatic animals. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 148, 315–323. https://doi.org/10.1016/j.cbpc.2008.04.003

Williams, H., 1984. A model for the aging of fly larvae in forensic entomology. Forensic Sci. Int. 25, 191–199.

Williams, K.A., Villet, M.H., 2014. Effects of hydrocortisone and sodium methohexital on growth rate of *Chrysomya chloropyga* Wiedemann (Diptera: Calliphoridae): developmental and behavioural indications of presence of drugs. Durb. Nat. Sci. Mus. Novit. 37, 25–29.

Wilson, Z., Hubbard, S., Pounder, D.J., 1993. Drug analysis in fly larvae. Am. J. Forensic Med. Pathol. 14, 118–120.

Wood, M., Laloup, M., Pien, K., Samyn, N., Morris, M., Maes, R.A.A., De Bruijn, E.A., Maes, V., De Boeck, G., 2003. Development of a rapid and sensitive method for the quantitation of benzodiazepines in *Calliphora vicina* larvae and puparia by LC-MS-MS. J. Anal. Toxicol. 27, 505–512.

Zheng, L., Crippen, T.L., Dabney, A., Gordy, A., Tomberlin, J.K., 2017. Evaluation of sterilized artificial diets for mass rearing the *Lucilia sericata* (Diptera: Calliphoridae). J. Med. Entomol. 54, 1122–1128.

Zou, Y., Huang, M., Huang, R., Wu, X., You, Z., Lin, J., Huang, X., Qiu, X., Zhang, S., 2013. Effect of ketamine on the development of *Lucilia sericata* (Meigen) (Diptera: Calliphoridae) and preliminary pathological observation of larvae. Forensic Sci. Int. 226, 273–281. https://doi.org/10.1016/j.forsciint.2013.01.042