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A comparison of the methods and validating a standardised method for entomotoxicology.

A dissertation submitted as part of the requirement for the BSc Forensic Science.

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12th May 2017

Abstract

Entomotoxicology has become increasingly popular especially since the realisation that different drugs that are accumulated by different species of fly larvae can alter their development rates. This is important from a forensic aspect as many fly species can determine the PMI of a deceased, however with drugs demonstrating the alteration of development of these flies, the PMI can therefore be altered. Throughout the literature there are different rearing methods and analytical methods used to determine the qualitative and/or quantitative concentration of drugs in fly larvae. This IRP will explore the current literature, and focus on interpreting the rearing substrates and analytical methods used to determine a standardised protocol for future researchers wanting to perform experiments with regards to entomotoxicology.

This IRP has partly achieved the aim when regarding the best rearing substrate and the best analytical method to use in relation to entomotoxicology. This IRP also raises the issues to why a standardised protocol cannot be made as there are too many gaps in the current literature to gain a fully reliable interpretation.

Acknowledgements

I would like to express my appreciation to my project supervisor Dr Andrew Whittington for his constant support, guidance and enthusiasm throughout the durations of my IRP. I would also like to give my thanks to Dr Richard Paul, for the help and direction with the sample preparation and analytical methods, despite not being my assigned supervisor.

I would also like to thank my family for their continuous support, not only through my IRP but also my whole time at university.

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Abbreviations

IRP – Independent Research Project

PMI -Post-Mortem Interval

ADD – Accumulated degree days

ADH – Accumulated degree hours

RIA – Radioimmunoassay

FPIA – fluorescence polarization immunoassay

HPLC – High Performance Liquid Chromatography

LC-MS – Liquid Chromatography Mass Spectrometry

LC-MS/MS – Liquid Chromatography Tandem/Triple/Quadrupole Mass Spectrometry

UPLC-MS/MS- Ultra-Performance Liquid Chromatography Tandem/Triple/Quadrupole Mass Spectrometry

GC - Gas Chromatography

GC-MS – Gas Chromatography Mass Spectrometry

LLE – Liquid-liquid extraction

SLE – Supported liquid extraction

SPE – Solid-phase extraction

L1-L3- Larval stages of first, second and third instar

Mg/Kg - milligram/kilogram

Ng/g - nanogram/gram

Ng/mL – nanogram/millilitre

Ng/mg – nanogram/milligram

Mg – milligram

µmol/L – micromole/litre

µg/kg- microgram/kilogram

µg/mg- microgram/milligram

pg/mg -picogram/microgram

SI units:

Gram (g) = 1

Mg = $10^{-}3$g

µg = $10^{-}6$g

ng = $10^{-}9$g

pg = $10^{-}12$g

Chapter 1- Introduction

1.1. Rationale

In forensic science, the post-mortem interval (PMI) can be determined by applying knowledge of insect growth curves to estimate time since hatchings (Goff and Flynn 1991). Some of the first insects to colonise a corpse in the UK include species of *Calliphora, Protophormia* and *Lucilia* (Lane 1975; Ames and Turner 2003). In Northern Europe, *Calliphora* species such as *Calliphora vicina* and *Calliphora vomitoria* are considered the most important as they are found in temperate regions. In conditions that are favourable, within a short time after death, these species will lay their eggs in or around natural orifices or wounds on fresh corpses. These eggs hatch to larvae, which grow in three stages. The most important stage is the third stage, where the larvae stop feeding and migrate from the corpse and burrow down in the ground or surroundings to pupate. The longest stage of development is the pupal stage before emerging into adults (Ames and Turner 2003). Many factors can contribute to the development of these flies, consequently altering the estimation of the PMI. Two examples include; temperature and drug and/or toxins.

The study of the effects of temperature on different fly species has been significantly well researched to confidently provide evidence that the rate of development of flies can be affected dependent on the temperature of the surroundings. Grassberger and Reiter produced an isomorphen-diagram, was founded, that represented all morphological stages with different temperatures and can facilitate a quick and more precise estimate of the PMI (Grassberger and Reiter 2001).

With regards to studies focusing on drugs and/or toxins on the development of different fly species, data is lacking and further studies are necessary to establish an overall picture of the specific drugs and/or toxins that can affect the development of different species of flies.

This is a particularly fascinating field of research proving to be a valuable tool used in criminal investigations. The studies that have already been undertaken have provided significant evidence that different drugs can influence the development of the same and different species of flies. A range of techniques have been reported in the literature, however there is no publication, to date, that has reviewed these techniques to establish which techniques are best for particular circumstances, therefore this IRP will use several publications to interpret and establish the best conditions.

1.2. Temperature findings

In many temperature regions *Calliphora* species are considered the most important. As these insects are poikilothermic, the rate of development in insects is governed by ambient temperature; therefore, the higher the temperature the faster the development occurs and the lower the temperature the slower the development (Grassberger and Reiter 2001; Ames and Turner 2003; Vélez and Wolff 2008). By knowing the ambient temperature and the progress of the blowfly development, as estimation of time since oviposition can be established and therefore the PMI can be determined (Ames and Turner 2003). There are two ways in which the PMI can be calculated; (i) larvae size can be compared with data on larval size related to temperature and time in an isomegalendiagram discovered by Grassberger and Reiter (2001) and (ii) to measure accumulated degree days (ADD) or hours (ADH) needed to reach a particular stage of development. ADD is the product of temperature above a species minimum development threshold and the time spent at that temperature. This is more defined as predicting the insect development by focusing on the range where the relationship between development rate and temperature is constant (Ames and Turner 2003).

Prior to Grassberger and Rieter (2001) establishing the new isomorphen-diagram, Reiter published the isomegalen-diagram in 1984, this diagram being used in their 2001 study.

They discovered that an age range of the larvae can be estimated between the points where the measured larval lengths cuts the graph at the maximum and minimum temperatures recorded when temperature is variable. Equally, their new isomorphen-diagram facilitated a quicker and more precise estimate of PMI. They concluded that if the temperature is roughly constant, the use of both these diagrams could provide a quick and precise estimate for the PMI (Grassberger and Reiter 2001). Later, Grassberger and Reiter (2002) concluded that the development times from oviposition to adult enclosion might possibly differ in various regions of the world. The question raised to whether it is valid to assume that the thermal constant of Holarctic species is the same everywhere. Geographic adaptation could explain a difference in temperature dependent development but continuous data is needed to improve values, and further develop precise values for each stage of the life cycle of flies.

Overall, the effect of temperature on different fly species is well documented worldwide and is always being updated. Models for analysis have been designed to estimate the PMI of flies' dependent on the temperature of the environment.

1.3. Rearing

In one study by Reiter and Grassberger (2002), at a constant temperature of 20 degrees, *L. argyrostoma* from Egypt required more days for total immature development, indicating poor cold adaption. Different experimental conditions, such as the food source, was suggested to influence the development, however this remained unclear. One study found that rearing in field conditions endorsed a precise estimate to the conditions of growth in a real forensic case, however this related back to the climate conditions (Vélez and Wolff 2008). The lack of clarity for the rearing substrates, initiates the need, in my IRP, to discover the reliability of the rearing substrate. Whether this involves the insects being artificially placed on a drug injected foodstuff or insects feeding on tissues isolated from a

deceased animal that has been injected with the drug/toxin or human fatalities consequently by a drug/toxin.

1.4. What is Entomotoxicology?

Entomotoxicology is a combination of entomology and toxicology and is the study of the application of analysis regarding toxicology to carrion-feeding insects and other arthropods, to identify drugs and toxins present within intoxicated tissues (Bourel et al. 1999; Introna et al. 2001). There are two pathways that forensic entomotoxicology has recently been developed. One area involves later stages of decomposition of a body where often no tissues remain, therefore toxicology analysis cannot be undertaken. Due to these circumstances, insects are used as an alternative source for areas of analysis to determine whether or not a drug is present (Goff and Lord 1994; Bourel et al. 1999). The second area considered is how the specific drugs and/or toxins found in decomposing tissues effect the development of the insect larvae that feed on these tissues. This is extremely important as these effects can alter the stages of development and consequently introduce an error in the PMI when using entomological techniques (Bourel et al. 1999; Introna et al. 2001). The most important aspect of entomotoxicology is to answer the following questions; what it the PMI?, what is the cause of death? And finally, where was the site of death? (Gagliano-Candela and Aventaggiato 2001)

1.5. History

The history of entomotoxicology is somewhat short but started with Sohal and Lamb's (1977) first report in the late 1970's. They demonstrated the accumulation of various metals including; copper, iron, zinc and calcium in tissues of adult house flies, *Musca domestica* (Sohal and Lamb 1977; Gagliano-Candela and Aventaggiato 2001). Later, Nuorteva and Nuorteva (1982) reported the presence of mercury from a variety of species

of *Calliphoridae*, including larvae, puparia and adults, all that fed on fish-containing mercury. Since, there have been many cases that have demonstrated the presence of many metals in insects that have helped with criminal cases (Introna et al. 2001).

Entomologists detected drugs in insects from 1980 and this was first reported by Beyer and colleagues regarding prescription drugs (Pounder 1991). Beyer et al (1980) exemplified the suicide with barbiturates of a young woman, aged 22 years, found in skeletonisation, only 14 days after she had been seen alive. This exhibited no organic fluids or tissues, preventing from any toxicological analysis to be undertaken. When using gas chromatography (GC) and thin-layer chromatography (TLC) to analyse *Calliphoride* species, the presence of phenobarbital, was found (Introna et al. 2001). Since, other authors have analysed drugs and narcotics, however some toxicologists are yet cynical of the value of entomotoxicology in forensic cases in relation to the PMI of insects and arthropods (Gosselin et al. 2011b).

1.6. Project definition

Entomotoxicology is an important area within the forensic aspect and is becoming increasingly more popular to research. This IRP compared, discussed and interpreted the best rearing substrate and analytical method to use from all the experiments that have been undertaken. Consequently, a summary to why and how these are the best methods with regards to entomotoxicology has been established. The results ascertained by this exercise have shed light of the difficulties established with regards to entomotoxicology and how these difficulties may be overcome in future studies.

1.7. Methodology

The entirety of this IRP consisted of desk-based research, which although may seem straight forward to produce, encountered some of the issues that, from a forensic aspect,

face from the lack of published data regarding entomotoxicology. The first step was to collate all papers that focus on entomotoxicology research experiments and to locate all the relevant literature regarding entomotoxicology and their views on this topic area, these were obtained using Google Scholar, Bournemouth University Library Catalogue, my-search (the university's online research), Research Gate (including requesting full-texts from Goff) along with other journals and books. The second step following this research, consisted of interpreting the experiments and distinguishing whether each experimental paper was qualitative or quantitative. Interpretation also included what each author used regarding the following: the rearing substrates, type of drug, species of fly, the analytical method used and whether all these aspects contributed to successful and reliable results. The main focus of this IRP consisted of the interpretation of the rearing and analytical methods used in the current literature, to produce a standardised protocol for any researchers wanting to undertake entomotoxicology experiments, regarding the best rearing substrate and analytical method to use. Once complete, an in-depth discussion was undertaken, which includes any possible limitations that arise from the research, along with further research possibilities and how this contributes to a forensic aspect.

1.8. Aims and Objectives

The aim of this IRP was to provide individuals, researching entomotoxicology with an initial protocol on the best method to use, regarding substrate rearing and analytical methods. It has been well established through a series of experimental studies and cases that entomotoxicology is an important area within the forensic aspect. To date, there has not yet been a paper published on the best way to undertake these experiments, regarding the rearing substrate and analytical methods. There are two routes in this IRP analysis; qualitative and quantitative. Qualitative provided a simple yes or no answer to whether the methods used show drug accumulation in maggots. Quantitative provided specific drug

concentrations accumulated by the larvae. As this piece of work focused on the uptake by maggots of a drugs/toxins (spiked foodstuff or animal model), some aspects will be excluded. Different authors have collected their maggots or flies from different sources and cultured their specimens differently before starting the main body of their research. These aspects may be done differently dependent on how the researcher wished to do so, however the collection and culturing of the maggots did not affect the drug accumulation by larvae. Additionally, when interpreting the analytical methods used, different authors validate their methods in accordance to their usual standards. This IRP did not interpret the calibration standards, linearity and sensitivity of each experiment, as these are standards that will not be a significant factor to change the drug accumulation by larvae.

To successfully satisfy the criteria of this aim, a series of objectives was completed and are as follows:

- Collate all entomotoxicology papers that are available
- Evaluate any literary information found on the current findings of entomotoxicology
- Interpret the rearing substrate and analytical methods used in each experiment
- Detail findings of the best rearing substrate and why
- Detail findings of the best analytical method and why
- Discuss the limitations of these
- Suggest any further study that should be conducted in response to this research.

Chapter 2 – Research

2.1. Rearing substrate

In the experiments regarding drugs accumulated in different fly species, relevant as a forensic indicator, the way in which these drugs are incorporated into the substrate can affect the outcome of the drugs detected in the fly. In summary, throughout the papers, with regards to entomotoxicology, there has been three ways in which these drugs have been accumulated by the fly larvae: **(i) using an animal model, most commonly a rabbit, where drugs have been input by an appropriate infusion method**(Goff et al. 1989; Goff et al. 1991; Goff et al. 1992; Goff et al. 1993, 1994; Goff et al. 1997; Bourel et al. 1999; Hédouin et al. 1999a, 1999b; Bourel et al. 2001b; de Carvalho et al. 2001; Hédouin et al. 2001; Tabor et al. 2005; Rashid et al. 2008; El-Samad et al. 2011; Bushby et al. 2012; de Carvalho et al. 2012); **(ii) a food source, such as minced meat, where the drug has been incorporated** (Sadler et al. 1997a; Sadler et al. 1997b; Bourel et al. 2001a; Bourel et al. 2001b; Bourel et al. 2001c; Musvasva et al. 2001; Wood et al. 2003; O'Brien and Turner 2004; Pien et al. 2004; Gunn et al. 2006; Kharbouche et al. 2008; George et al. 2009; Oliveira et al. 2009; Gosselin et al. 2010; Gosselin et al. 2011a; Parry et al. 2011; Bushby et al. 2012; Magni et al. 2014; Mullany et al. 2014; Magni et al. 2016); **(iii) using the body of a deceased human by either; analysing the flies of interest found initially on the body** (Beyer et al. 1980; Gunatilake and Goff 1989; Kintz et al. 1990a; Kintz et al. 1990b; Goff and Flynn 1991; Manhoff et al. 1991; Nolte et al. 1992; Kintz et al. 1994; Miller et al. 1994; Levine et al. 2000; Definis-Gojanović et al. 2007) **or using the tissues obtained at autopsy from known drug abusers, and the flies have been placed artificially onto these tissues** (Introna et al. 1990; Wilson et al. 1993; Sadler et al. 1995; Bourel et al. 2001c; Campobasso et al. 2004). Throughout the papers interpreted, there are additional

differences within each of the three categories, that may also influence the final drug detection accumulated in the flies, these discussed below.

2.1.1. Comparison of animal model

Rabbits are a popular selection for incorporating the drug into tissues for many reasons. Aside from being readily available, catheters are easily placed within the main artery of the ear for a continuous perfusion of the drug of interest. In addition, rabbits are large enough to be a single animal source, that can supply enough tissues needed to rear enough larvae needed for toxicological studies. Around 400 larvae can easily be distributed on a rabbit weighing 3kilograms (Hédouin et al. 1999b). Throughout the 16 animal models, 14 of these were rabbit models, 9 of which larvae were placed on the liver removed from the rabbit and 5 that placed larvae directly onto the carcass. When comparing Bourel at al (1999), where the eggs were deposited in the eyes, nostrils and mouth of the carcass to Goff et al (1991), where the eggs were placed on the livers taken from the deceased rabbits, both experiments detected morphine in liver and larvae samples by radioimmunoassay (RIA). When comparing two animal models; Bushy et al (2012) determined methylphenidate in *Calliphora vicina* when larvae were placed on half the rat brain that the rat had previously received this drug via intravenous injection whereas Hedouin et al (2001) used a rabbit model using *Calliphora vicina* and *Protophormia terraenovae* that had been reared on rabbit carcasses to determine morphine concentrations. Bushby's work determined drug concentrations by liquid chromatography-tandem mass spectrometry (LC-MS/MS) this showed high detection of methylphenidate with around 95% accuracy, and Hedouin determined morphine concentrations by RIA and concluded a good correlation between the concentration of morphine administered and tissue concentrations, particularly in muscle, skin and fat. When using animal models for clinical experiments, Matute-Bello et al (2008) discovered that rabbits were 57% similar to

humans with a specific immune cell gene whereas rats were only 48% similar to humans. This may only be relevant to the cell gene in relation to modelling a lung injury, therefore a summary in relation to toxicology is inconclusive.

2.1.2. Comparison of foodstuff

Throughout the literature a variety of substances can be used as foodstuff, some including: beef liver (Magni et al. 2014); kangaroo meat (Mullany et al. 2014); minced beef (Bourel et al. 2001b; Bourel et al. 2001c) or an artificial food diet, usually made by a combination of muscle, powdered egg and agar (Sadler et al. 1997b). A key factor of using food/meat sources, as the substrate, is whether homogenisation has been undertaken to ensure a uniform spread of the drug (George et al. 2009). 13 experiments out of 20 in the literature have undertaken homogenisation of the substrate to ensure an even spread of the drug. However, Bourel et al (2001a; 2001b; 2001c) did not undertaken homogenisation in any experiments when using minced meat and morphine. The question to consider is whether undertaking homogenisation in Bourel's studies had any effect on the results compared to studies were homogenisation has been undertaken.

In Bourel et al 2001c study, RIA was used to confirm whether the theoretical concentrations of morphine had been incorporated into the minced meat. The results showed that the theoretical concentrations of 1, 2, 5, 100 and 1000 milligram/kilogram (mg/kg) had a higher experimental concentration in the minced meat, whereas 10mg/kg was lower in the experimental concentration than theoretical, but overall morphine concentrations had been incorporated effectively. Focusing on the feeding stages of the larvae (second and third instars) that had been placed on the substrates, theoretical concentrations 1 and 2 mg/kg were not detected in the second instar, but were detected in theoretical concentrations 5, 10, 100, 1000 mg/kg of 0.101, 0.305, 2.309, 3.326, respectively. In the third instar concentrations for theoretical concentrations of 1, 2, 5, 10

and 100mg/kg were found to be of 0.022, 0.003, 0.403, 0.454, 2.365, respectively. 1000mg/kg data was non-available. In the Bourel et al (2001b) study, RIA was used to confirm these concentrations as demonstrated in the 2001c study. The theoretical drug concentrations of 1000, 2500, 5000 and 10,000 nanogram/gram (ng/g) showed similar concentrations, however there were slight variations between the theoretical drug concentration and the experimental concentration in the minced meat, as shown in Table 1. Additionally, larvae analysed in the second and third instars, of these experiments showed smaller differences. Converting the concentrations of the 2001b study, from ng/g to mg/kg to establish an easier comparison, 1000, 2500, 5000 and 10,000ng/g into 1, 2.5, 5 and 10 mg/kg respectively. The theoretical concentrations of 1, 2.5, 5 and 10mg/kg in the second instar larvae were 0.0017, 0.0206, 0.0672 and 0.1023 mg/kg respectively and for the third larvae; 0.0125, 0.0288, 0.1227 and 0.1397, respectively. Table 2 and 3 show these comparisons for each instar larvae and experiment undertaken by Bourel et al (2001b;2001c).

Table 1. Drug concentrations of morphine in the substrate from both experiments performed by Bourel et al (2001b; 2001c)

Mg/kg	1	2 / 2.5 (2001b)	5	10	100	1000
2001c	1.635	2.848	9.219	7.637	120.824	1172.64
2001b	1.3029	2.6405	4.8116	12.0252	Not performed	Not performed

Table 2. Drug concentrations of morphine in the second instar from both experiments performed by Bourel et al (2001b; 2001c)

Mg/kg	1	2 / 2.5 (2001b)	5	10	100	1000
2001c	0	0	0.101	0.305	2.309	3.326
2001b	0.0017	0.0206	0.0672	0.1023	Not performed	Not performed

Table 3. Drug concentrations of morphine in the third instar from both experiments performed by Bourel et al (2001b; 2001c)

Mg/kg	1	2 / 2.5 (2001b)	5	10	100	1000
2001c	0.022	0.003	0.403	0.454	2.365	Non-available
Mg/kg	1	2 / 2.5 (2001b)	5	10	100	1000
2001b	0.0125	0.0288	0.1227	0.1397	Not performed	Not performed

As shown in the tables there are only slight variations, with exception to the anomaly highlighted in table 3, to the amount of morphine detected in the second and third instars. These slight variations may be due to the species of the fly used in the two different experiments, as in 2001c experiment *Lucilia sericata* was used and in 2001b *Thanatopilus sinuatus* was used. As reported in most of the literature, the metabolism of different species of flies may affect the amount of drug detected in the larvae (de Carvalho et al. 2012), this discussed later in the report.

On the contrary, Gunn et al (2006) investigated morphine concentrations in *Calliphora stygia* that have fed on **homogenised** minced beef injected with morphine. These concentrations of morphine in the drug substrate was confirmed by High Performance Liquid Chromatography (HPLC). Gunn et al study, added theoretical morphine concentrations of 0.5, 1, 2.5, 5 and 10 mg/kg to the minced beef. The measured concentrations and concentrations incorporated in the flies are shown in table 4.

Table 4. Shows the theoretical, measured and larvae concentrations of morphine (mg/kg) in Gunn et al (2006) study.

Theoretical concentrations (mg/kg)	Measured concentrations (mg/kg)	Larvae concentrations (mg/kg)
0.5	0.812	0
1	1.330	0
2.5	2.220	0.765
5	5.370	2.720
10	8.970	3.010

The table demonstrates that the theoretical morphine concentrations correlate well to the measured concentrations in the substrate as expected, showing that incorporation of the drug has been established.

Sadler et al (1997a) investigated barbiturates and analgesics in *Calliphora vicina* that had fed on homogenised artificial foodstuff containing lower-limb muscle, powdered egg, agar and desired amount of drug concentrations. These concentrations for each drug in the substrate was analysed by HPLC. As there was two series of experiments undertaken, the concentrations of the target concentration, actual concentrations and concentrations of these drugs analysed in the larvae on harvested day 8 (series 1 experiment) or harvested at day 6 (series 2 experiment) are show in table 5.

Table 5. Target and actual concentrations of drug concentrations in foodstuff and the concentrations of drug incorporated in *Calliphora vicina* at harvested day 8 (series 1) or day 6 (series 2) (mg/kg) in Sadler et al (1997a) study.

Drug Conc. In Foodstuff (mg/kg)				Mean Drug concentration in Larvae (mg/kg) (Range) (n=3)
Drug	Target	Actual Mean (n=5)	Range	
Series 1				
Phenobarbitone	100	58.4	51.8-62.9	7.2 (4.1-12.4)
Amylobarbitone	100	106.8	102-113	n.d.
Paracetamol	250	241	22-258	n.d.
Acetylsalicylic acid	1000	800	726-982	n.d
Series 2				
Sodium salicylate	2000	1870	1620-2200	160 (40-285)
Sodium salicylate	4000	3100	2450-3430	331 (61.8-285)
Paracetamol	2000	2290	2000-2720	5.6 (4.9-6.4)
Aminohippurate	2000	1520	1390-1620	147 (71.6-236)
Amphetamine	50	44.8	43.3-45.9	18.5 (11.3-22.5)

n.d. = not detected.

The table clearly shows that the drugs have been incorporated well within the substrate. It also shows the importance of the analyses of the substrate as well as the larvae, this demonstrated by the range of the actual mean drug concentration differing from the target drug concentration, this may influence the amount of drug accumulated in the flies once they have fed on the foodstuff.

When comparing the morphine concentrations in both Bourel studies (non-homogenised) in comparison to Gunn's study (homogenised) there is not a great distinction between the studies, as shown in table 6.

Table 6. Comparison of drug concentrations of morphine in the substrate from Bourel et al (2001b;2001c) studies (non-homogenised) and Gunn et al (2006) study (homogenised).

Mg/kg	0.5	1	2 / 2.5 (2001b)	5	10	100	1000
Bourel et al 2001c	Not performed	1.635	2.848	9.219	7.637	120.824	1172.64
Bourel et al 2001b	Not performed	1.3029	2.6405 (using 2.5)	4.8116	12.0252	Not performed	Not performed
Gunn et al 2006	0.812	1.330	2.220	5.370	9.970	Not performed	Not performed

There are some relevant distinctions demonstrating that homogenisation of the substrate can slightly affect the substrate concentration, this demonstrated by the differences throughout the substrate concentrations. In Gunn's study the differences between the actual and theoretical concentrations differ from 0.3-0.9mg/kg whereas Bourel (2001c) study differed from 0.6-4.2mg/kg and Bourel (2001b) study differed from 0.1-2.0mg/kg, thus showing that homogenisation provides closer concentrations to actual and theoretical concentrations. Additionally, both Bourel studies did not mention any calculated mean of the drug concentration in the substrates thus assuming only one portion of the substrate was used to sample each drug concentration. Gunn's study was performed to gain mean

concentrations in the substrate, as did Sadler's study, therefore both of Bourel studies may be considered unreliable. This unreliability considered in relation to the morphine concentrations distributed throughout the substrate as these have only been performed once for each drug concentration, therefore the drug concentration may have ranged throughout each substrate. Sadler's study demonstrates that the drug concentration in food substrates varies throughout the substrate and therefore can influence how much of this drug is accumulated by the larvae, when they feed on different parts across the substrate area.

In relation to the amount of drug incorporated into the larvae; in both of Bourel studies majority of the life stages of the fly have been measured, most importantly the feedings stages; second and third instars. Gunn and Sadler experiments neither specify in their results which larvae stage they have used to evaluate their drug concentration findings. For example, in Gunn, they analyse larvae up to the prepupal stage, therefore the second and third instar are included in the findings, however they do not specify whether they have combined the results of all the larvae up to the prepupal stage to obtain their average drug concentration in the 5 larvae they have used. In Sadler's study, no specific larvae stage was analysed, only whether the larvae were harvested at day 6 or 8 and were performed in triplicate readings. Table 2 displays that the theoretical concentrations of morphine differ in the second and third instars of the larvae demonstrating the importance of analysing the larvae at different stages throughout entomotoxicology experiments.

When comparing the results of the morphine concentrations measured in both Bourel study and Gunn study, there is a clear distinction that both Bourel studies show lower doses of morphine in the larvae stages compared to those displayed in Gunn's study. The larvae in Gunn's study show a much higher morphine concentration than those in Bourel 2001b study, for example theoretical values of 10mg/kg demonstrated a larvae concentration six times more in Gunn's study compared to that of Bourel 2001b study. When comparing

these results there are two factors that need to be considered; (i) homogenisation of the substrate allowed for an even spread of the drug and a fair chance of all the flies accumulating the same amount of drug and (ii) the species of the fly, therefore the metabolism, this discussed later in the report. The results obtained from Sadler's study also showed a relatively high concentration of a variety of drugs incorporated into the larvae, also using a different species of fly than the fly species used in Gunn's study. Factoring in the homogenisation of the substrate, from the results obtained from Gunn and Sadler's studies it can be concluded that homogenisation of the substrate is more likely to offer reliable results when undertaking experiments in relation to entomotoxicology.

2.1.3. Comparison of food and animal model

Importantly in both rearing substrates, foodstuff or animal, the concentrations of each drug was selected as reported overdoses from the body tissues of human fatalities, therefore reflecting those of real-life scenarios. When considering what rearing substrate is best to consider, there are factors to consider, some which include; metabolism of the rabbit, metabolism of the fly and whether the metabolite of the parent drug has been considered. Comparison of Goff et al (1992) and Magni et al (2014) studies have been considered to distinguish whether any differences can be established, due to the different rearing substrates used. Goff's study researched the effects of methamphetamine on the development rate of *Parasarcophaga ruficornis,* that had been reared on tissues from rabbits, that had been administered different doses of this drug, using RIA as the analytical method. The concentration of methamphetamine and the metabolite, amphetamine, are shown in Table 7.

Table 7. Concentrations of methamphetamine and amphetamine detected in rabbit's liver tissue samples, after administration of three theoretical concentrations (Goff et al 1992).

Theoretical concentration of methamphetamine administered (mg/Kg)	Concentration of drugs in rabbit's liver sample (nanogram/millilitre)	
	Methamphetamine	Amphetamine
0	0	0
37.5	3026	2157
71.4	7594	5249
142.9	55,698	7607

The table demonstrates that the rabbit receiving the methamphetamine concentrations, metabolise this drug to form a smaller concentration of its metabolite, amphetamine. As noted in the table, the concentrations of the drugs detected in the liver samples are in ng/mL, whereas the concentration of the methamphetamine administered is mg/Kg. The analyse made of the ten larvae from each colony, was determined using RIA. A cut off value of 50ng/mL was established as positive findings, and these samples, including the control, showed to have a weak positive reaction. This uniform weak reaction of the control was possibly a cause of the analytical method used when analysing the larvae, as the control liver samples was negative, therefore the preferred choice of analytical method is discussed later. When compared to Magni et al (2014) study, this study determines concentrations of 5 and 10 nanogram/milligram (ng/mg) of methamphetamine in larvae of *Calliphora vomitoria* that fed on homogenised methamphetamine spiked liver, detected by GC-MS. The concentrations of methamphetamine in the liver substrate and the larvae are shown in table 8.

Table 8. Quantification of methamphetamine in the liver and larvae samples (Magni et al 2014).

Sample	Quantification of methamphetamine (ng/mg)		
	Control (0)	5	10
Liver	<0.10	5.01 +/- 0.04	10.07 +/- 0.12
Larvae (second instar)	<0.10	0.45 +/- 0.01	0.67 +/- 0.02
Larvae (third instar)	<0.10	0.78 +/- 0.01	6.70 +/- 0.06

Focusing on both studies, Goff established the methamphetamine concentrations of 37.5, 71.4 and 142 mg by the weight of the rabbits, therefore the units of mg/kg are the same as those of Magni's study, when using the lethal dose known to cause human fatalities in ng/mg. Once establishing that the ratio of mg/kg to ng/mg is 1:1, it clearly demonstrated that Goff used a much higher dose given to the rabbits than those used in Magni's study. When comparing the amount of the methamphetamine found in both substrates, excluding the 142.9mg/kg (2 x median lethal dose for rabbits) quantification of the actual methamphetamine concentration in the liver substrate from Magni study, displayed a closer correlation to the theoretical concentrations compared to those of demonstrated in Goff's study. Goff's study showed a greater difference of methamphetamine in the theoretical concentration and the concentration of the methamphetamine measured in the liver tissue, which can be due to the metabolism of the rabbit. Additionally, Goff's study evaluated the concentration of the metabolite amphetamine, therefore considering the metabolism of the rabbit, as in the case of a human metabolism if any human were to ingest methamphetamine, whereas Magni's study did not considered this factor. Mullany et al (2014) considered the metabolite p-hydroxymethamphetamine as well as methamphetamine in their study when using homogenised kangaroo meat as the foodstuff that *Calliphora stygia* fed on. Their findings showed that the parent drug and metabolite were detected both in meat and *C. stygia* samples by HPLC analysis. This showed that both substances were consequently ingested by the larvae, however methamphetamine could not be detected in the first and second comparison intervals in the larvae, these results differing from Wilson et al (1993) study. Wilson's study reared *Calliphora vicina* on skeletal muscles from a suicidal overdose of co-proxamol and amitriptyline, and consequently amitriptyline and nortriptyline, it's metabolite, were both detected in the larvae. The difference could be due to many factors, some which include; the species of fly

therefore its metabolism and the analytical method used. Focusing on Mullany's study, it demonstrated that the use of the metabolite, injected in the foodstuff, will be consequently ingested and detected in the fly larvae which can mirror the metabolite produced in a human fatality.

2.1.4. Comparison with a deceased person

There are two parts to which a deceased person can be used when regarding analyses of fly larvae for drug analysis; (i) analysing the flies of interest found initially on the body (Beyer et al. 1980; Gunatilake and Goff 1989; Kintz et al. 1990a; Kintz et al. 1990b; Goff and Flynn 1991; Manhoff et al. 1991; Nolte et al. 1992; Kintz et al. 1994; Miller et al. 1994; Levine et al. 2000; Definis-Gojanović et al. 2007) or (ii) using the tissues obtained at autopsy from known drug abusers and fly larvae have been artificially placed onto these tissues (Introna et al. 1990; Wilson et al. 1993; Sadler et al. 1995; Bourel et al. 2001c; Campobasso et al. 2004).

When comparing foodstuff and muscle obtained from a deceased known to have consumed drugs, Bourel et al (2001c) compared both in one study. In the foodstuff, theoretical concentrations of 1-1000 mg/kg of morphine were used, whereas in the 200g of human psoas taken from nine cadavers of nine heroin abusers, the morphine concentration ranged from 0 to 0.418 mg/kg, thus differing immensely to the foodstuff experiment. When comparing the results obtained from the 1mg/kg theoretical concentration in the foodstuff to the results from two of the deceased, the amount of morphine detected in the larvae showed similarity. These are shown in Table 9.

Table 9. Actual concentrations of morphine found second and third instar larvae when comparing two cases (A and E) against theoretical concentration (1mg/kg) (Bourel et al. 2001c)

Case/Theoretical concentration (mg/kg)	Actual Concentration (mg/kg)	Second instar (mg/kg)	Third instar (mg/kg)
A	0.418	0.027	0.005

Case/Theoretical concentration (mg/kg)	Actual Concentration (mg/kg)	Second instar (mg/kg)	Third instar (mg/kg)
E	0.329	0.018	0
1	1.635	0	0.022

The table demonstrates that the concentrations in the second and third instars from both experiments were almost proportional to the concentrations in both substrates. The use of either the foodstuff or the muscle obtained from known heroin abusers did not differ hugely in this experiment, however foodstuff concentrations of morphine would be easier to control, therefore, foodstuff can be deemed a good alternative for larvae to feed on when regarding entomotoxicology experiments.

Additionaly, Notle et al (1992) used *Calliphora vicina* larvae taken from a deceased as well as skeletal muscle to determine what drugs the deceased had taken, this analysed by RIA and GC/MS. The results of cocaine (C) and its metabolite benzoylecgonine (BE) are shown in table 10.

Table 10. Analytical results on muscle and insect larvae (Notle et al 1992).

	RIA (C & BE)	GC (C)	GC/MS (C)	GC/MS (BE)
Muscle	Positive	N/A	Positive	0.33 mg/kg
Larvae	Positive	0.49 mg/kg	Negative (14 months after specimen obtained)	0.03 mg/kg

As displayed in the table, both the muscle and larvae showed presence of cocaine and its metabolite at small but notifiable concentrations to indicate that cocaine was used only a few hours before death, as cocaine has a relatively short half-life (Baselt and Cravey 2011). Goff et al (1989) determined the effect of cocaine on fly larvae *Boettcherisca peregrina* that fed on the liver samples obtained from deceased rabbits that had been given cocaine doses of 35, 69 and 137mg. The concentration of cocaine in the liver and larvae was

determined by RIA. The results of this study showed that the analysed liver sample showed the presence of cocaine and its metabolite benzoylecgonine from all the rabbits that had received the drug, however no definite number was given in this experiment. The larvae reared on the livers of these rabbits, receiving all doses, were positive for cocaine, benzoylecgonine, or both at levels >0.30mg/kg. Comparison of Notle and Goff studies, when focusing on cocaine alone, both studies predominantly show the same concentration of cocaine found in the larvae when feeding on tissue samples taken from a deceased.

Table 11 demonstrates that not all species of flies have been reared on the three substrates discussed above. Due to the lack of data on entomotoxicology a conclusive answer to provide knowledge of the best rearing substrate cannot be determined however a comparison of preferred ways may be distinguished. Not only due to the lack of data but also due to a variety of factors that must be considered, a final conclusion cannot be made at this point when researching entomotoxicology.

Table 11. Demonstrates the extent of the research that has been undertaken with regards to the three different substrates used in comparison to the different species used.

		Substrate		
		Animal	Food Source	Deceased
Species	*Lucilia sericata*	✓	✓	✓
	Calliphora vomitoria	*X*	✓	*X*
	Calliphora vicina	✓	✓	✓
	Chrysomya putoria	✓	✓	*X*
	Chrysomya albiceps	✓	✓	*X*
	Parasarcophaga ruficornis	✓	*X*	*X*
	Boettcherisca peregrina	✓	*X*	*X*
	Chrysomya megacephala	✓	*X*	✓
	Chrysomya rufifacies	*X*	*X*	✓
	Calliphora stygia	*X*	✓	*X*
	Cochlomyia maccellaria	*X*	*X*	✓

	Animal	Food Source	Deceased
Sarcophaga tibialis	*X*	✓	*X*
Phormia regina	✓	*X*	*X*
Calliphoridae	*X*	*X*	✓
Sarcophagidae	*X*	*X*	✓
Phoridae	*X*	*X*	✓

2.2. Drug vs Species

Forensic entomology's objective is determining the time elapsed since death of a human or animal. The insects involved, depending on the life cycle, have become a key standard to estimate PMI (Marks et al. 2009). It has been demonstrated by many authors that the lifecycle of different flies can be affected by the presence of different drugs, therefore possible altering the PMI (Goff et al. 1991; Goff et al. 1992; Goff et al. 1993; Goff and Lord 1994; Bourel et al. 1999; de Carvalho et al. 2001; Introna et al. 2001; O'Brien and Turner 2004; Gosselin et al. 2011b). As previously noted by many authors, the same drug can exert different effects on different species regarding the whole fly development (i.e. maggot, larval, pupal stages). Table 12 demonstrates the effects of different drugs on the development rate on the same species of fly and *vice versa*, at larvae stages only, that have been noted by the current literature. The table also displays many gaps, demonstrating that experiments have not been undertaken for all the different species of flies listed against all the drugs listed, therefore this section of this report focuses on how different drugs affect the same species of fly. Additionally, not all the literature regarding entomotoxicology establish the effect of the drug on the species of fly. Instead, they use analytical methods to detect the presence of a specific drug accumulated by the fly species, and whether the analytical method and fly species used in the authors experiment is reliable, this is discussed later in the report.

Table 12. Demonstrates the effects of different drugs on the development of different species of flies that have been researched in the current literature.

		Species											
		L. sericata	*C.vomitoria*	*C. vicina*	*C. putoria*	*C. albiceps*	*P. ruficornis*	*B. peregrina*	*C. megacephala*	*C. rufifacies*	*C. stygia*	*S. tibialis*	*P. regina*
		ALS	ALS	ALS	ALS	ALS	ALS	ALS	ALS	ALS	ALS	ALS	L3
Drug	Morphine	-									0		
	PA			+									
	Diazepam				+	+							
	Cocaine				+	+		+					
	AM						-						
	MA		0				+				+		
	PC						+						
	MDMA						+						
	Heroin							+					
	Buscopan								-				
	Methadone	-											
	Codeine	+											
	Nicotine		0										
	HCS											-	
	SMH											-	
	NOR			0									
	Malathion								-	-			
	Ethanol												-

Key	
+	Increase normal growth
-	Retard normal growth
0	No change
ALS	All larval stages
HCS	Hydrocortisone
SMH	Sodium methohexital
MA	Methamphetamine
PC	Phencyclidine
AM	Amitriptyline
NOR	Nordiazepam
PA	Paracetamol

Focusing on the species *Lucilia sericata,* only opiates/opioids have been used to determine these drugs on the development rates. When considering the effects of these drugs different results were obtained. Bourel et al (1999) discovered that the presence of morphine appeared to retard the growth of *L. sericata* during the larval stages, indicating that an underestimation of PMI of up to 24 hours is

possible. Similarly, Gosselin et al (2011a) discovered that high methadone concentrations slightly deaccelerated the development stages of *L. sericata* too. On the contrary, Kharbouche et al (2008) discovered that the presence of codeine stimulates the growth of *L. sericata* during the larval period, but no significant difference of codeine effecting the pupal stage, this differing from Bourel and Gosselin studies. In the study of El-Samed et al (2011), it was discovered that Tramadol, a prescribed opioid (Shipton 2000) accelerated the growth of *L.sericata* in the larval stages only, but retarded the total development. These studies show the importance of how the same species of fly react differently towards two drugs belonging to the same family (Kharbouche et al. 2008). Additionally, these studies show the importance of knowing what stage of their lifecycle these flies are collected at when determining PMI.

When focusing on the fly *Calliphora vicina* and the drugs that have an effect on the development growth, only two studies have been undertaken (O'Brien and Turner 2004; Pien et al. 2004). In O'Brien and Turner study they determined the effects of paracetamol on the development of this species of fly. Their results showed that paracetamol and/or its metabolites slightly accelerate larval growth rates during 2-4 days, which may generate a difference of approximately 12 hours in PMI, however Pien's study demonstrated that the development stages of *C. vicina* were not affected by their controlled nordiazepam study. Pien's study did not determine concentrations to establish an overdose, therefore more studies are needed to determine a definite conclusion to compare nordiazepam with *C. vicina* and future studies are needed to monitor different drugs to determine whether they have any effect on the development of this fly.

Chrysomya albiceps and *Chrysomya putoria* development rates have been monitored with two different drugs but have been undertaken by the same authors (de Carvalho et al. 2001; de Carvalho et al. 2012). Both species of fly that had been exposed to diazepam, showed

accelerated growth in the larval stages and weighed significantly more than those of the controls (de Carvalho et al. 2001). Similarly, when focusing on the effects of cocaine on the development rate of both species of flies, the overall larval stage and pupal stage were significantly shorter, therefore cocaine accelerated the development rate of *C. albiceps* and *C. putoria*, however throughout the experiment the development stages of these species of fly differed. At both the 6th and 18th hour observation *C. albiceps* had developed faster than those of the control, whereas this was the opposite to *C. putoria*. At 24 hours, *C. albiceps* larvae were less developed than those of their controls, whereas no change was noted in *C. putoria*. At 30 and 42 hours, no differences were made to *C. albiceps*, however *C. putoria* had developed significantly faster than those of the control. At 54h post exposure, both species had developed more than those of the controls and overall exposure to cocaine accelerated development rates of both species(de Carvalho et al. 2012). This shows the importance of the time in which the species of flies are exposed to cocaine, as this differs the development throughout. Additionally, this demonstrates the need for more research to be undertaken for these flies when exposed to a variety of drugs.

Goff et al first used *Boettcherisca peregrina* when investigating the effects of heroin (Goff et al. 1991) and cocaine (Goff et al. 1989) on their development rates. The presence of heroin, as morphine, shortened the duration of the larval stages in *B. peregrina,* however this was the opposite for the pupal stage. If the PMI was based on the larval stage the estimate would be greater than the actual interval because of accelerated rate of development, however, if based on adult emergence or pupal duration, the estimate would be shorter than the actual interval because of the retardation of development. Cocaine and its metabolites increased the development stages of both the larval and pupal stages of *B. peregrine* by up to 12-18h.

As shown in Table 12, *Parasarcophaga ruficornis* has been used most frequently when determining the effects of different drugs on the development rate of this fly (Goff et al. 1992; Goff et al. 1993, 1994; Goff et al. 1997). Goff first monitored the effects of methamphetamine on *P. ruficornis* and demonstrated that overall methamphetamine increased the rate of development of the larvae reaching maximum length quicker but were smaller in size at the maximum length (Goff et al. 1992). Similarly, phencyclidine increased the rate of development of this species as the duration of the larval stages of the treated colonies took less time. This was the opposite in the puparial stage, therefore overall there was no significant differences in the rate of growth in *P. ruficornis* (Goff et al. 1994*).* Amitriptyline affected the larval stages and pupal stages of *P. ruficornis,* by decreasing the larval growth, effectively resulting in an error of up to 30 hours of the PMI if based on larval stage. Additionally, the pupal stage also took longer to complete, therefore amitriptyline decreasing the growth of this life cycle, resulting in an error of up to 77 hours of PMI when combining both life cycles (Goff et al. 1993). MDMA had the opposite effect on *P. ruficornis* development when compared to those seen by amitriptyline but similar to the effects demonstrated by methamphetamine and phencyclidine. MDMA at median lethal dose of 67mg increased the rate of development of *P. ruficornis,* primarily based on the increased development observed in the larval stages (Goff et al. 1997). Studies performed by Goff have provided only a snapshot of how different drugs can affect the development of the same species of fly, and the importance of knowing these in a forensic context. These findings initiates the need for more studies to be undertaken to form a data collection of what drug exert what effects on different species of fly, if any, and how does this effect the PMI of a deceased.

Chrysomya megacephala have been used by two different authors when determining the effects of two products that can be bought legally; Buscopan, a medication (Oliveira et al.

2009) and malathion an organothiophosphate used as an insecticide (Rashid et al. 2008). When *C. megacephala* fed on Buscopan (butylscopolamine bromide) the development time was decreased and mortality rates increased. Similarly, malathion delayed the development of *C. megacephala* larvae to almost 3 days, therefore the PMI may be incorrect if malathion in the larvae is not considered in the first 6 to 36 hours. This is supported by the case of malathion poisoning by Gunatilake and Goff (1989) where the development stages of *C. megacephala* and *C. rufifacies* had indicated that the minimum PMI was that of 5 days, however the victim had been seen alive 8 days prior to the discovery of the body. The findings of Rashid's study magnify the important of these studies as they can reflect to real life scenarios where larvae can be analysed.

Calliphora stygia species have been used by two different authors determining the effect of morphine (George et al. 2009) and methamphetamine (Mullany et al. 2014) on the development rates. George's study determined that morphine with concentrations of 2, 10 and 20ng/g had no effect on *C. stygia* in any of the life stages. Mullany's study determined that the larvae development stages had increased when these larvae fed on tissues containing methamphetamine. Additionally, puparation was complete in all methamphetamine treated pupae before the control, consequently the PMI could be overestimated by up to 44h when based on the larval stage and by up to 78h when based on the pupal stages. These results show the need for future research to be undertaken on *C. stygia* and until future research is obtained this fly cannot be assumed to be a reliable model for aging corpses when methamphetamine is present.

Magni et al studies (2014; 2016) investigated the effects of methamphetamine and nicotine, respectively, on the development rate of *Calliphora vomitoria.* Magni's first study in 2014, focused on the effects of methamphetamine on this species of fly. Their findings demonstrated that methamphetamine had no effect on the development rate of the

larval stages (L1-L3), however the overall time for the development rate for *C. vomitoria* from egg to enclosion was found to be greater for those that fed on tissues with methamphetamine compared to those of the control. In the 2016 study nicotine and its metabolite, cotinine, can affect the length and survival of *C. vomitoria* however the growth rate is not affected by the presence of nicotine. Their findings suggest the need for more studies to be undertaken on this species of fly and more studies to investigate the effects of nicotine on different fly species, as this is a popular legal substance used throughout the UK and Europe (West and Shiffman 2007).

Sarcophaga tibialis development rates have only been studied by Musvasva et al (2001) with legal medications, hydrocortisone and sodium methohexital. Sodium methohexital is an ultra-short-acting barbiturate used for as an anaesthetic (Wyant and Chang 1959) and hydrocortisone is the treatment used widely for rheumatoid arthritis, ulcerative colitis, and asthma but most specifically for acute adrenal insufficiency (Hart 1955). The presence of this drug and/or its breakdown products significantly retarded the development of *S. tibialis*, especially at lower doses, however, pupae showed the opposing trend, resulting in the total development time not significant between the barbiturate concentrations. Larval development was significantly delayed with the presence of hydrocortisone and/or its breakdown products. Pupae showed a significant result and although the overall trend seemed opposite to that presented in the larvae stage, results showed that development at half the lethal concentration was significantly different from the other treatment groups. The total development time showed significant differences, this being entirely due to slower development rates at the lethal dose. Overall, development rates of *S. tibialis* were affected by both drugs, but the effects dependent on the stage of the fly and the drug dosage. In both cases, the opposing trends found in larvae and pupae resulted in total development time not being significantly different, therefore, the total immature lifespan is

largely unaffected by these drugs and PMI may not be affected (Musvasva et al. 2001). If *S. tibialis* are collected at the larval stages, then the PMI may be altered therefore caution must be considered when these drugs are detected at death.

Overall many illegal and legal substances can affect the development rates of different fly species that feed on decomposing tissues. As clearly demonstrated throughout this report, the lack of literature provides an inconclusive answer when comparing the effects of different drugs on the same species of fly and *vice versa*. Additionally, results differ greatly dependent on species and type of drug. For example, morphine has no effect on *C. stygia* (George et al. 2009) but methamphetamine can accelerate the growth of the same species (Mullany et al. 2014). Furthermore, the effects of morphine on *L. sericata* retarded the development rate (Bourel et al. 1999), but on the contrary codeine, which can be metabolised into morphine, accelerated the growth of *L. sericata* indicating that the same family of drug can differ the development stages of the same species of fly (Kharbouche et al. 2008). Future studies are therefore needed to provide a database of this information. Additionally, Tabor et al (2005) investigated the effects of antermortem ingestion of ethanol on the successional patterns of *Phorma regina* when feeding on ethanol treated pigs. The results showed that antemortem ingestion by pigs did not seem to alter the successional patterns of the insect, however the time required for maggots feeding on tissue from ethanol-treated required approximately 11.9 hours more to reach the pupal stage, this mainly due to the third-instars taking significantly longer to reach the pupal stage. When *P. regina* feed on ethanol-treated pigs given 3x more the legal human limit in the US, this can retard the growth. This study shows the importance of how legal, common substances like ethanol can influence the development rate of *P. regina*. Additionally, the question to consider is whether using pigs as the rearing substrate may be a better animal model to use over rabbits and rats. The animal of choice for the study was the domestic

pig, *Sus scrofa* L., because this species was found to be the most suitable for use as a human model in successional studies (Payne 1965; Catts and Goff 1992).

2.3. Drug vs Technique

Several studies have demonstrated that the toxicological analyses of insects are able to provide more reliable and sensitive results than from highly decomposed tissues and remains (Kintz et al. 1990a; Kintz et al. 1990b; Nolte et al. 1992), however the quantification of some experiments lack relevant and reliable data due to the analytical method used. The aim of this section focuses on the same analytical methods used with different drugs. Table 13 shows the extent at which the same technique has been used for different drugs.

Table 13. Summary of the techniques and different drugs that have been used when regarding entomotoxicology.

1	Detected	Technique								
0	No detection	RIA	FPIA	LC-MS/MS	LC-MS	GC	GC-MS	HPLC	UPLC-MS/MS	TOTAL
Drug	Morphine	1					1	1		3
	MPH			1						1
	Diazepam						1			1
	Nordiazepam			1						1
	Amitriptyline						1	1		2
	MA	0					1	1		3
	Phencyclidine						1			1
	MDMA				1					1
	Heroin	1								1
	Malathion					1				1
	Methadone			1					1	2
	Codeine				1		1			2
	Nicotine						1			1
	Cocaine	1	1				1			3
	Amphetamine						1			1
	Triazolam							1		1
	Oxazepam							1		1

		RIA	FPIA	LC-MS/MS	LC-MS	GC	GC-MS	HPLC	UPLC-MS/MS	
	Alimemazine							1		1
	Clomipramine							1		1
	Phenobarbital						1	1		2
	Trazodone							1		1
	Trimipramine							1		1
	Propoxyphene					1				1
	Temazepam							1		1
	Acetaminophen							0		1
	Secobarbital						1			1
	Tramadol							1		1
	Ethanol					1				1
	Analgesics							1		1
	Barbiturates							1		1
	TOTAL	4	1	3	2	3	11	15	1	

The table demonstrates that the most frequent drug used in the current literature is morphine and that most drugs have only been used once. Additionally, HPLC has been used most frequently and most of the analytical methods used have been performed on more than one drug. The gaps in the table show the extent as to how much research is missing with regards to entomotoxicology and therefore the importance to why more experiments are needed. The two displayed in red demonstrate that these drugs have not been detected by the analytical method used.

2.3.1. Radioimmunoassay (RIA)

When focusing on RIA a total of 4 different drugs have been used to see whether these drugs could be detected in larvae. In total, 11 of the current literature have used RIA for the detection; 6 experiments have been undertaken with morphine (Introna et al. 1990; Hédouin et al. 1999a, 1999b; Bourel et al. 2001b; Bourel et al. 2001c; Hédouin et al. 2001), 3 experiments have been undertaken with cocaine (Goff et al. 1989; Manhoff et al. 1991; Nolte et al. 1992), 1 experiment has been undertaken with heroin (Goff et al. 1991)

and 1 experiment has been undertaken with methamphetamine (Goff et al. 1992). When focusing on the results obtained from experiments using RIA, a variety of results were obtained. Methamphetamine exhibited a weak positive result from both the control and treated larvae, this being unreliable. The authors explained the false positive was most likely be the result of a non-specific reaction to a substance other than methamphetamine and amphetamine. Additionally, when reading this literature there was no reference to any sample preparation, which could have affected the RIA method (Goff et al. 1992). When interpreting the rest of the literature that has used RIA, focusing on detecting morphine concentrations, Bourel et al (2001c) established that the morphine concentrations, detected in the larvae stages of *L. sericata* were almost proportional to those concentrations injected into to the minced. Hedouin et al (2001) study, found that concentrations of morphine in the development stages of *P. terraenovae* and *C. vicina* were significantly lower than those in tissues, similar to the results obtained for *L. sericata* in a previous study (Hédouin et al. 1999a). Hedouin compared these results to those of Introna et al (1990), and found that in Introna's study the concentration detected in the larvae were very similar to those detected in the liver tissues, as was established in both Bourel (2001c). Hedouin noted that whilst their experiment and Introna's experiment both used the same analytical method (RIA), sample preparation and fly larvae of *C. vicina*. that only one difference did exist. This difference included the rearing substrate; Introna's study used human liver whereas Hedouin used an entire rabbit carcass. Additionally, Bourel (2001c) study used *L. sericata* as did Hedouin (1999a) study, but the results differed as stated above, where Bourel found that the concentrations of morphine were proportional to those of the larvae, whereas Hedouin found the opposite. The difference in this experiment was again the rearing substrate whereby Bourel used minced meat whereas Hedouin used rabbit carcasses. Using rabbits as a model can provide an inconclusive analysis as rabbits are particularly resistant

to morphine intoxication whereas this is not the case in human intoxications (Hédouin et al. 1999b). Concentrating on the analytical method used, RIA assay process used in these studies measured only free morphine and the metabolism of morphine in insects is unknown at present, therefore a better analytical method should be used to detect the presence of unknown metabolites.

Goff et al (1989) used RIA and fluorescence polarization immunoassay (FPIA), to detect cocaine in *B. peregrine.* The results demonstrated that the larvae were positive for cocaine and its metabolite with RIA, however with FPIA the larvae tested for morphine and phenobarbital, establishing a false FPIA response, making FPIA an unreliable method of detection for drugs in larvae.

When regarding both studies that used RIA for the detection of cocaine in larvae (Manhoff et al. 1991; Nolte et al. 1992), RIA was only used as initial screening for cocaine and its metabolite before using a further analytical method to obtain quantitative drug concentrations. Additionally, Kintz et al (1990a) also used RIA for initial screening to indicate presence of more than one family of drug, before undertaking HPLC for further analysis.

In summary, RIA only establishes a limited amount of data, providing only a detection limit of a specific drug. In Goff et al (1992) study methamphetamine was found to be positive in the control, resulting in a false positive. As noted above, some authors use RIA as a preliminary observation before undertaking a more specific and reliable analytical method.

2.3.2. High Performance Liquid Chromatography (HPLC)

Throughout the literature of entomotoxicology, HPLC has been used most frequent to detect drugs in larvae and/or substrate. Throughout the whole literature of using HPLC,

only two detection methods have been used; (i) spectrophotometric detectors (Wilson et al. 1993; Sadler et al. 1995; Sadler et al. 1997a; El-Samad et al. 2011; Mullany et al. 2014), whereby this detector is based on the absorbance of UV radiation (Kealey and Haines 2002) or (ii) chemiluminescence detector (Gunn et al. 2006; George et al. 2009; Parry et al. 2011), this categorised as a electrochemical detector, which provides high sensitivity and low background detections (Griffin et al. 2014; Kricka and Park 2014). The results obtained using these two different detection methods significantly differ, this clearly demonstrated between studies undertaken by Parry et al (2011) and Mullany et al (2014). In Parry's study the use of chemiluminescence exhibited three neat peaks excluding any background peaks; two peaks representing morphine detected at 2.8 micromole/litre (µmol/L) and the other representing the assumption of an unidentified metabolite, structurally similar to morphine and detected at a concentration of 8.7µmol/L. This unidentified metabolite was highly likely to have been detected because of the metabolism of *C. stygia* larvae.

In Mullany's study analytes were detected by UV. Chromatograms displayed many peaks, representing background peaks had been detected. In addition, detection of methamphetamine by UV could not be detected in larvae in the first and second comparison intervals, however the sample preparation method for Mullany's study displayed a crude method of homogenisation which may have affected the results, nonetheless, the use of chemiluminescence detectors may yield more conclusive results.

2.3.3. Liquid Chromatography Mass Spectrometry (LC-MS)

When looking through the literature only two experiments have been undertaken using LC-MS with liquid-liquid as both of their extraction methods (Goff et al. 1997; Kharbouche et al. 2008). Comparing both experiments, the detected drug concentrations in the larvae relating directed to those of the dose administered, demonstrating an increase of larval drug

concentration with increasing drug concentration in the substrate. Focusing on the MS detection methods used, Goff did not determine the Limit of Quantification (LOQ), however Khabourche had and found that the LOQ was 5ng/500mg, this being a relatively good LOQ compared to another study where the LOQ for codeine was 0.5 microgram/kilogram (µg/kg) this being equivalent to 5ng/500mg (Badawi et al. 2009).

2.3.4. Liquid Chromatography Tandem/Triple/Quadrupole Mass Spectrometry (LC-MS/MS)

Throughout the literature of entomotoxicology LC-MS/MS has been used as the analytical method to detect drug concentrations in substrate and/or larvae (Wood et al. 2003; Pien et al. 2004; Gosselin et al. 2010; Gosselin et al. 2011a; Bushby et al. 2012). Using LC-MS/MS instead of LC-MS enhances the sensitivity of toxin determination in samples by measuring characteristic fragmentation patterns of molecules (Pietsch et al. 2001). Pien et al (2004) performed the same analysis as described in Wood et al (2003) and both studies found that the drug concentration detected in a single larva were detected in picogram/milligram, this demonstrating that the analytical method was very sensitive to detect such small quantities of a drug concentration. In Wood's study the LOQ for the different benzodiazepines detected ranged from 7.63 to 20.63 picogram/milligram (pg/mg) and the chromatographs produced single distinct peaks with no background peaks, thus existing as a sensitive and precise method. When this study was compared to that undertaken by Gosselin et al (2010) the lowest quality control (QC) level found in the third instar larvae was 71.3 and 79.2 pg/mg of EDDP and methadone, respectively, these concentrations to be detected at higher concentrations than those of Wood study. The only difference between these studies regarding the analyses was the sample preparation; Gosselin used LLE whereas Wood determined the best sample preparation between SPE, LLE and simple acetronitrile precipitation test. Wood determined that extraction recoveries

of diazepam, nordiazepam and prazepam obtained by SPE were very low (<8%) and LLE were also low compared to the drugs recovered by the simple acetronitrile precipitation which was >90% for all of the benzodiazepines investigated. Gosselin used this analytical method to analyse drug concentration of larvae from human remains and found that the methadone LOQ was 6.08-6.10 and EDDP LOQ was 6.42-6.44 pg/mg, demonstrating to be a sensitive method, however Wood did not use this method in practice with human remains. Additionally, Gosselin et al (2011) progressed to establish the methadone and EDDP concentrations in larvae, using Ultra-Performance LC-MS/MS (UPLC-MS/MS) using LLE as the extraction method, with >86% recovery for all compounds. The results of this study showed that the LOQ was 4.13-4.14 and 3.81-3.82 pg/mg of methadone and EDDP respectively. UPLC compared to LC improves sensitivity and resolution, as well as significantly reducing analysis time and the mobile phase solvent consumption (de Villiers et al. 2006; Ortelli et al. 2009; Guillarme et al. 2010; Vidal et al. 2010).

2.3.5. Gas Chromatography (GC)

Gas chromatography has been used 4 times throughout the literature of entomotoxicology (Gunatilake and Goff 1989; Wilson et al. 1993; Tabor et al. 2005; Rashid et al. 2008) but with only three different drugs; malathion, propoxyphene and ethanol, all these substances that can be bought legally. Results obtained from the two papers that determine malathion concentrations (Gunatilake and Goff 1989; Rashid et al. 2008), differ quite significantly, these are shown in Table 14. The units have been made to microgram/milligram (µg/mg) to exhibit an easier comparison.

Table 14. Malathion drug concentrations in fat sample and larvae from Guntilake and Goff (1989) study and malathion drug concentrations in liver sample and larvae from Rashid et al (2008) study.

	Malathion drug concentrations (µg/mg)	
	Fat sample/Liver sample	Larvae
Guntilake and Goff (1989)	0.017	2.05
Rashid et al (2008)	1.892 (+- 0.93)	0.157 (+-0.092)

As clearly demonstrated in the table malathion drug concentration in fat sample from Guntilake and Goff's study is lower than those detected in the larvae, though this is the opposite in Rashid's study. When comparing the detector used in GC, Guntilake and Goff used a nitrogen-phosphorus detector whereas Rashid used an electron capture detector. As noted in some studies, these two detection methods can be used simultaneously to gain reliable results (Gaillard et al. 1993; Jiménez et al. 2001), however dependent on the sample preparation method this can defect the results (Jiménez et al. 2001). The difference is sample preparation is the likely problem in Guntilake and Goff study as they used LLE described in a previous experiment (Chaturvedi et al. 1983), whereas Rashid's study used SPE, this said to be a cleaner extraction method (Scheurer and Moore 1992). On the contrary, Wilson et al (1993) used SLE for propoxyphene, and the results obtained displayed that the muscle to larvae ratio was significantly proportional, nevertheless it is noted in other literature that LLE and SLE can give rise to many interfering peaks on other analytical techniques, as well as not providing a putrefied sample extract (Scheurer and Moore 1992).

2.3.6. Gas Chromatography Mass Spectrometry (GC-MS)

Throughout the literature of entomotoxicology, GC-MS has been the second most frequent analytical method used (Introna et al. 1990; Nolte et al. 1992; Goff et al. 1994; Kintz et al. 1994; Miller et al. 1994; Sadler et al. 1995; Levine et al. 2000; de Carvalho et al. 2001; Definis-Gojanović et al. 2007; Magni et al. 2014; Magni et al. 2016). In one study, GC-MS analysis using an ion-trap mass selective detector, quantitative analysis of amphetamine was less clear in maggots, providing only a positive result that amphetamine was present in the larvae (Definis-Gojanović et al. 2007). Nolte (1992) used GC-MS to determine the concentration of benzoylecgonine in muscle and larvae 14 months after the first analysis

undertaken by GC, and found that 0.03mg/kg of this metabolite was detected in the larvae. There were no specific details of the specific MS mode, this would allow for a better comparison of the analytical method to be undertaken. Most recent experiments undertaken using GC-MS in electron impact ionization (EI) mode operated in the selected ion-monitoring (SIM) mode by Magni et al (2014; 2016) have shown to be a useful technique with regards to determining drug concentrations in fly larvae, using LLE prior to analysis. Magni et al (2014) study established a LOQ of 0.33ng/mg and determined concentrations as low as 0.67ng/mg with only +/- 0.01-0.02, thus demonstrating that this analytical method is useful to detect low concentrations of methamphetamine in the second and third instars of the *C. vomitoria* larvae.

Chapter 3 - Discussion

The following discussion will be drawn upon information collected regarding the experiments undertaken in relation to entomotoxicology to make comparisons between findings and interpretations regarding the best rearing substrate and analytical method to use. The experiments that have been undertaken range in date starting from as 1980 to 2016, therefore the analytical methods used in the earlier experiments have progressed immensely, thus making the comparison of some results difficult.

When regarding the rearing substrate, the factors including; metabolism of the rabbit, metabolism of the fly and whether the metabolite of the parent drug has been considered are important and can differ the final results. Initially, Payne (1965) established that the use of mice and rats amongst other smaller animals, ascertained difficulty when observing the arthropods presence due to the fast rate of decay and the small size of the study animal, however the use of decaying pigs attracted different species of flies demonstrating slower rates of decay. The use of rat as an animal model cannot be deemed as a reliable animal

source for entomotoxicology as seen in experiments performed by Rashid et al (2008) and Bushby et al (2012). A major problem that must be considered when using rabbit models and other animal models to determine the effects of morphine on fly species to reflect PMI is the metabolism of this drug. The use of rabbits as a model can provide inadequate analysis since rabbits are particularly resistant to morphine intoxication, although this is not the case in human intoxications (Hédouin et al. 1999b). Additionally, the rates of metabolites formation and excretion differ between human and other animals (Milne et al. 1996). With regards to researching morphine in entomotoxicology experiments, the preferred rearing substrate is foodstuff that has been homogenised with known concentrations of morphine as successfully achieved in previous studies (Bourel et al. 2001b; Bourel et al. 2001c). When using foodstuff as substrates, the metabolites must be considered, as metabolites would be present in any human intoxication and therefore accumulated by larvae when feeding on the deceased. Another factor that must be considered is the analytical method used, for both the detection of the drug in the foodstuff substrate and drug accumulated by the species of fly. Some studies that used foodstuff as the substrate did not test whether the drug had been incorporated within the substrate (Musvasva et al. 2001; Wood et al. 2003; O'Brien and Turner 2004; Pien et al. 2004; Oliveira et al. 2009; Magni et al. 2014; Magni et al. 2016), however many studies did (Sadler et al. 1997a; Sadler et al. 1997b; Bourel et al. 2001a; Bourel et al. 2001b; Gunn et al. 2006; Kharbouche et al. 2008; George et al. 2009; Gosselin et al. 2011a; Mullany et al. 2014). The findings of the literature that analysed their foodstuff showed the importance as to why foodstuff should be analysed as well as the larvae, as seen in Table 6, demonstrating the difference between theoretical drug concentrations and actual concentrations within the substrate.

Obviously in any case it would be best to use a deceased person, as the factors can be reduced, however this can be difficult to establish the effects of larger drug concentrations on the development of flies. When experiments are undertaken not using a deceased, a big factor is not considered, this known as the post-mortem drug redistribution. A study was undertaken and the results demonstrated that for many drugs, post mortem concentrations in the blood are dramatically site dependent (Pounder and Jones 1990). This can be a real danger for individuals that have taken therapeutic doses of a drug an artefactually elevated post mortem drug level may be misinterpreted as a lethal overdose (Jones 1988), therefore using foodstuff as the rearing substrate may provide results that do not mirror this problem, especially when focusing on the effects of these drugs on different fly species for the need of data.

As established throughout the literature, different drugs can affect different species of flies and at different stages of these fly life-cycles. Wilson et al (1993) found that amitriptyline, nortriptyline and propoxyphene were all detected in the third-instar larvae but puparia and adults were negative. Similarly, Sadler et al (1995) observed that larvae metabolise and eliminate drugs with varying levels of efficiency, this demonstrated by the reduction of drug concentrations throughout larval development. Sadler et al (1997a; 1997b) studies both demonstrated that different drugs show unpredictable larval drug accumulation and excretion suggesting that literature of different fly species metabolisms is lacking (Definis-Gojanović et al. 2007). Additionally, George et al (2009) found that pure morphine concentrations had no effect on the blow fly *C. stygia*, these findings differing from Bourel et al (1999) where morphine concentrations where lower than those used in George's study, but morphine retarded the growth of *L. sericata* during the larval stages. These findings may be due to the difference in morphine metabolism between the study species used (Goff et al. 1991; Bourel et al. 1999; Bourel et al. 2001c; O'Brien and Turner 2004).

The study undertaken by Kharbouche et al (2008) focused on the development rates of *L. sericata*, the same species of fly used by Bourel et al (1999) study, however the results in Kharbouche study demonstrated that codeine, metabolised to morphine, increased the development rates in the larval stages of the same species of fly, demonstrating that the same family of drugs, can have a different effect on the same species of fly, however the knowledge of different fly metabolisms is missing (George et al. 2009).

When regarding the analytical method used to detect drugs in larvae, the results throughout the literature show a great importance. Firstly, the extraction method is considered an important factor prior to performing analytical analyses. Studies that do not provide information on the extraction method, cannot form reliable conclusions as this can affect the results, as seen with Goff et al (1992; 1993) where both studies did not demonstrate any description of the extraction method and both results displayed false positives in the control samples. Studies that have defined their extraction methods to LLE, SLE and/or SPE have been successful, however it is said that SPE methods provide a cleaner method of extraction, yet this is a more expensive method of extraction, compared to LLE (Scheurer and Moore 1992). In one experiment it was found that both extraction methods (LLE and SPE) are reliable extraction methods in clinical samples (Saar et al. 2009), however when regarding post-mortem blood from decomposed bodies, the extraction efficiency and LC-MS matrix efficiency showed considerable differences. It was found that blood from decomposed bodies can be viscous with an oily consistency sometimes blocking SPE cartridges, demonstrating lower extractions of a drug when using SPE, thus further research must be undertaken to consider this problem in post-mortem blood samples (Saar et al. 2009) and possibly larvae samples.

As established in the literature, larvae are useful as qualitative toxicological specimens, however they are of limited quantitative analysis, though as the years have progressed so

have the analytical method making quantitative analysis more achievable (Introna et al. 2001; Campobasso et al. 2004). Immediately it is clear that using RIA allows for preliminary drug screening and limited results are achieved compared to the results obtained from other analytical methods using GC-MS and HPLC, as demonstrated in Goff et al (1992) study, where the detection of false positives in the control samples occurred, therefore RIA should not be used as the preferred choice of analytical method when undertaking entomotoxicology. When regarding GC, in most instances only one drug or parent drug can be detected, requiring time-consuming multiple extractions. Due to the polarity of morphine; poor peak shapes, low sensitivities and long retention times are established as most of this molecule is absorbed onto the column material (Kintz et al. 1989), consequently a suitable derivative must be prepared (Holder 1986). When comparing, the difference between GC and GC-MS, the most recent papers using GC-MS have detected smaller drug concentrations in ng/mg (Magni et al. 2014; Magni et al. 2016), whereas GC alone, has detected larger concentrations in µg/mg thus GC is established as a less sensitive method (Rashid et al. 2008). Additionally, GC detectors such as electron capture (EC), nitrogen-phosphorus (NP) and flame ionisation (FI) have been used less frequently since GC-MS has been introduced as MS can be easily adjusted, allowing for the selection of particular molecular and fragments ions, thus avoiding interferences (Alder et al. 2006). HPLC is complementary to GC, however HPLC has better efficiency, resolution and is inherently more versatile. It is not limited to volatile and thermally stable samples and additionally a much wider range of stationary and mobile phases can be used in comparison to GC, facilitating in the selectivity of the separation process (Fifield and Kealey 2000). A disadvantage of HPLC is that this method separates mixtures into micrograms to grams as noted in the literature (Fifield and Kealey 2000), therefore the use of a combination of analytical techniques such as GC-MS and LC-MS/MS detects smaller

quantifications of drug concentrations, consequently being preferred methods. When regarding the comparison of GC-MS and LC-MS/MS, the limit of quantification (LOQ) must be considered, more precisely the lower and upper classifications. The lower LOQ (LLOQ) is the lowest concentration of the standard curve which can be measured with accuracy and precision, therefore the lower the LOQ the better the method. Additionally, the mean value should be within +/- 15% and not deviate more than or less than 20% (Shah et al. 2000). When focusing on the LC-MS compared with LC-MS/MS, the use of MS/MS enhances sensitivity and therefore better results (Lee and Kerns 1999). Furthermore, Gosselin et al (2011a) used UPLC-MS/MS, this analytical method establishing a better LOQ in pg/mg with a 0.01 pg/mg variation between each mean concentration compared to the LOQ found in LC-MS/MS (Wood et al. 2003; Gosselin et al. 2010; Bushby et al. 2012). When comparing the methods between LC-MS/MS and GC-MS, Definis-Gojanovic et al (2007) found that the quantitative analysis of GC-MS was less clear, therefore further analysis would be needed to form a reliable conclusion. Additionally, both recent methods undertaken by Magni et al (2014;2016) established the LOQ in ng/mg, thus being less specific than the detection of drugs found by Gosselin (2011a) using UPLC-MS/MS. Nonetheless, GC-MS is still a useful technique as it has the ability for the separation of many samples and is predominantly the most popular analytical technique for the identification of biological samples in comparison to LC techniques. The difficulty with using LC techniques is the transfer of the separated components of the analytes to the MS without interference of the solvent, however, LC-MS demonstrates less peaks so less fragmentations, excluding background interferences allowing for the detection of impurities in synthesised drugs which can be a great advantage in toxicology (Kealey and Haines 2002).

When regarding entomotoxicology, there are some authors that have published papers and consequently their research does not bring any help or clarity when comparing the methods used or the reliability of their findings. Throughout the findings of entomotoxicology papers, Verma (2013) published a paper insinuating, by the title, to establish the effects of codeine and sodium pentothal on the growth rate of *C. rufifacies*. The context of the paper, did not mention these drugs, instead alcohol and cannabis was mentioned, unrelated to the title of the paper, thus showing the extent of the unreliability of this paper. Additionally, the results from this paper cannot be deemed valid nor reliable towards entomotoxicology research.

Chapter 4

4.1. Conclusion

The driving aim of this project was to discuss and interpret the current methods used in entomotoxicology regarding the rearing substrate and analytical method used to establish a standard protocol for any researcher wishing to undertake any future experiments in this area of research. Using the aforementioned set of objects one could argue that this IRP has achieved some of the objectives, however, some objectives have not.

With regards to the best rearing substrate, this is dependent on the drug. One conclusion that can be made from the current literature is that rabbits and different animals produce different or more metabolites when given morphine compared to those in humans, therefore using a homogenised foodstuff substrate should be the preferred rearing substrate regarding entomotoxicology experiments. Additionally, studies that have used homogenised foodstuff, as the rearing substrate, have established that the amount of drug accumulated in the larvae demonstrated higher drug concentrations, since an even spread of the drug was determined in the substrate, therefore an even chance of the flies

accumulating these drugs occurred. Animal models and foodstuff rearing substrates results were compared against those from human fatalities, although the concentrations in the substrates were much higher for the set-up experiments, the concentrations in the larvae from all experiments were almost proportional to the correlating concentrations detected in the substrates the larvae had fed on, thus demonstrating that both rearing methods can be used, however, drug concentrations in homogenised foodstuff is easier to control.

When regarding the best analytical method to use, again this is dependent on the drug and costing of the experiment. Preparation of samples using SPE and LLE have shown to work throughout the literature however, SPE provides a cleaner but more expensive sample preparation, therefore SPE should be priority, though if there is limited budget LLE can provide reliable results. On the contrary, it was found that blood from decomposed bodies can be viscous with an oily consistency sometimes blocking SPE cartridges, demonstrating lower extractions of a drug when using SPE, therefore further research is required to form a preferred sample extraction method.

Extracted from the current literature, the best analytical method to use regarding entomotoxicology is a coupled method, this being either GC-MS or LC-MS/MS as these provide a better yield of the quantification of drugs and establises the LOQ which demonstrates the precision of the method. Additionally these methods can analyse complex samples and less time is needed to undertake analysis (Kealey and Haines 2002). LC-MS/MS in the wider scope with increased sensitivity and better selectivity provides a better method to use when compared to GC-MS. Additionally, performance without derivatisation, makes LC-MS/MS the preferred method for the determination of pesticides such as malathion, an organophosphate (Alder et al. 2006) used twice in the literature of entomotoxicology (Gunatilake and Goff 1989; Rashid et al. 2008). These two pieces of literature used GC as the analytical method, but future studies should be directed to use

LC-MS/MS as the preferred method. GC-MS can achieve better performances when detecting organochlorides than those demonstrated by LC-MS/MS (Alder et al. 2006), therefore the preferred choice of the analytical method is drug dependent.

When analysing fly larvae samples, it is very important to monitor all larval stages as the metabolism throughout the lifecycle can differ the drug concentration. If this factor is not taken into consideration, inconclusive results may be obtained, especially if the mean concentration has been calculated from all larval stages.

4.2. Further Study

Due to the lack of publication regarding entomotoxicology further research is not only necessarily in forensic aspect but it is inevitable. From the findings already available for analysis, a clear correlation has been made between the effects of different drugs on different species of fly thus altering the development stages, regardless there is much more to research to form a solid conclusion regarding the best method to use for different types of drugs. Most of the current literature regarding entomotoxicology has not been repeated with the advances of new analytical methods, thus providing an insufficient correlation between different drugs on the same species of fly and *vice versa*. Additionally, there is more research required regarding the metabolism of different species of fly and how different drugs are metabolised, to understand the levels to which different drugs and metabolite concentrations are found in the larvae and consequently affecting the development rates, if any. Ideally, once further experiments have been undertaken with the most recent analytical methods regarding different drugs and different species of flies then the main aims of this IRP would be completed. For now, future work lies on; firstly, the effects of different drugs on the development rate of the most common species of flies found on a deceased to help with the PMI; secondly, in the continuation and replications of the current literature findings with the most recent analytical methods to provide more

quantitative analyses than qualitative; and finally, determining the best rearing method that mirrors a human overdose, including whether the use of larger animals, such as pigs, may act as a better animal model compared to those such as rabbits.

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Appendices

Appendix 1: Evaluative supplement

This Independent Research Project having been the largest and complex piece of literature I have produced to date; this section will aim to look back over at the project undertaken upon completion. Therefore, this will include clarifying, understanding and reinforcing the knowledge I have gained, whilst identifying the projects strengths and weaknesses. I have always had a keen interest in forensic toxicology, and it was enjoyable to learn a new area of forensic toxicology, this being entomotoxicology, especially as this has such an impact from a forensic view point. Regardless of originally wanting to undertake a laboratory project, but unfortunately the university not being able to facilitate an undergraduate project regarding entomotoxicology, the challenge of undertaking an entire desk-based IRP has helped me in so many other ways especially in my academic writing, something that will be greatly beneficial in any research career. Despite being challenging at times I am somewhat satisfied with the outcome and conclusions that I have drawn from the current literature of entomotoxicology. The objectives of this dissertation were; to determine the best rearing substrate and the best analytical method to form a standardised protocol for future researchers wanting to undertake experiments regarding entomotoxicology. I feel as though I have achieved parts of these objects however due to the lack of literature, further research must be undertaken to form a more definite standardised protocol.

The strengths of this dissertation had been the ability to be able to develop and refine my knowledge of entomotoxicology and provide an in-depth comparison of studies undertaken as well as interpreting the results from analytical techniques, something that I have not completed in depth whilst my time at Bournemouth University, to provide a valid comparison of the current literature. As an area I have not previously explored in depth throughout my modules over the three years at university, I discovered my interest for entomotoxicology through toxicology modules and entomology lectures. By combining my known scientific knowledge with literature from current entomotoxicology papers, and

having Dr Andrew Whittington, as my assigned supervisor, to guide me down the correct path and understand more of the basics of entomotoxicology, I feel I have provided future researchers with knowledge regarding the preferred options of the rearing substrate, and the preferred choices of the analytical methods, this being dependent on the drug. Additionally, I feel like this IRP has identified the rearing substrates and analytical methods that should also be avoided in relation to this area of forensic toxicology, as the results obtained were less reliable and provided less benefits towards entomotoxicology research. Due to the current gaps in the literature, this was a big problem when trying to achieve my aims and objectives as there was not enough data to form a valid conclusion. Additionally, as the papers ranged from 1980 to 2016, some results obtained from the current literature was hard to compare against one another, as the analytical methods have advanced, and therefore the sensitivity of the analytical methods have increased. Another problem I faced throughout this project is the lack of literature regarding the metabolism of different fly species, which is a big factor to consider when undertaking entomotoxicology. Without this knowledge, it is hard to form a solid conclusion, and unfortunately drugs in the same family cannot be grouped together when regarding the effects of these drugs, on the same species of fly, as drugs within the same family can exert different effects on the same species of fly, as noted in the current literature. The need for more studies is essential, especially in a forensic aspect, when the PMI of a deceased relies on the identification of different species of flies. When regarding the rearing substrate, it has been founded in papers within the literature that the use of pigs, as the rearing substrate, may provide results mirroring those found in a human fatality, therefore more studies are needed to validate this conclusion.

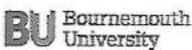
Comparing the results obtained from the current literature, it was easy to identify that the analytical methods RIA should only be used as a preliminary test, as this provides more of

a qualitative analysis than quantitative when compared to the results obtained using more sensitive analytical methods. Overall, the combination of two analytical methods should be used as this provides a more sensitive and quantitative analysis. The preferred methods of choice, dependent on the drug used, when undertaken experiments in entomotoxicology should be either GC-MS and/or a type of LC-MS/MS (including UPLC-MS/MS). I therefore think this information is something that will be useful for future researchers, as they can identify straight away from this IRP that these analytical methods have obtained the most reliable results.

As a result of completing this project my research skills have advanced immensely, especially when using tools such as Google Scholar, Bournemouth University Library Catalogue, my-search (the university's online research), Research Gate (including requesting full-texts from Goff) along with other journals and books. These tools formed the creation of my entire project, and developed my understanding of entomotoxicology allowing myself to undertake the comparison of studies and consequently find gaps within the current literature to obtain my findings. Additionally, developing my academic, critical and evaluative writing throughout this report, including planning and creating Gantt charts to stay on top my work and consequently meet the required deadlines, this has allowed myself to produce a final dissertation that I am proud, which have provided me with a set of skills I will need for any future career in research. From this report, I feel like I am confident to undertake another literature review as I am fully aware of the organisation and time needed to produce a high standard report. This research has expanded on my entomotoxicology knowledge, an area I have grown to enjoy. Additionally, this project would potentially work towards a baseline for many masters or even a PhD project to be undertaken which may include; producing a standardised protocol (once more literature

has been published) or undertaking experiments to provide more data for entomotoxicology, something I wish to pursue.

Appendix 2: Learning Contract



LEARNING CONTRACT: INDEPENDENT RESEARCH PROJECT

Student Name:	Bethany Roome
Degree Programme:	BSc (Hons) Forensic Science
Proposed Project Title:	Literature review: Comparison between the current methods used to analyse entomotoxicology in relation to Forensic Science.
Supervisor:	Andrew Whittington

Research Proposal Attached ☒ YES ☐ NO and includes:

YES	NO	
☐	☒	Risk Assessment for fieldwork and evidence of COSSH assessment for all laboratory procedures (online risk assessment completed)
☐	☒	Completed booking forms for all field equipment
☐	☒	Letters of permission where appropriate providing evidence of access to such things as field sites and/or museum archives
☒	☐	Completed Ethics Checklist

Copies of all relevant forms may be found on myBU - SciTech tab - Projects - Project Forms

INTERIM INTERVIEW – Progress evaluation

Good progress is being made & I recommend that Bethany proceed with the IRP and aims to complete the thesis within time limits

Assessment Due: [signature] 08 December 2016

FINAL ASSESSMENT – RESEARCH PAPER/REPORT

PTO

As the student undertaking the above project I agree to:

- E-mail my supervisor on a fortnightly basis with a progress report
- Meet with my supervisor at least once a month to discuss progress and I understand that it is my responsibility to organise these meetings
- Comply with the terms of this learning contract and the guidance set out in the Guide to Independent Research Projects
- I understand that this is an *independent* project and that I am solely responsible for its completion
- I agree to comply with all laboratory and fieldwork protocols established by the Faculty.

As the supervisor of this project I agree to:

- Meet with the student undertaking this project on at least a monthly basis and to respond to the progress e-mails as appropriate
- To meet formally with the student during the first week in November to undertake the interim interview
- To provide guidance and support to the student undertaking this project bearing in mind that it is an *independent* research project. This is inclusive of commenting on drafts of the final report in a timely fashion.

Both of the undersigned parties agree to be bound by this learning contract:	
Student Signature:	B Roome
PRINT NAME:	Bethany Violet Roome
Date:	05/10/16.

Supervisor Signature:	[signature]
PRINT NAME:	A. E. WHITTINGTON
Date:	6/10/2016

When completed, this form should be handed in to SciTech Admin (C114) and a copy retained by the student to be included in an appendix to the final IRP document.

Appendix 3: Interim Interview Comments Form

Independent Research Project Interim Interview : Agreed Comments Form

Student Name: Bethany Roome	Programme: BSc (Hons) Forensic Science
Date: 8th December 2016	IRP Title: Literature Review: A comparison of the methods and validating a standardised method for entomotoxicology
Supervisor Name: Andrew Whittington	

- All the relevant papers have been collated for entomotoxicology.
- For the introduction, papers regarding rearing of flies for medical reasons and temperature findings must be included.
- Targets were set to interpret the papers by creating tables to separate the rearing substrate and analytical methods used. These tables would help visualise any of the gaps missing from the current literature.
- New timetable for deadlines were set.
- Next meeting with Andrew to be arranged in January/February to ensure the interpretation of data was being completed at the correct standard.

Two copies of this form are needed – student to retain one copy the other is to be handed in to the student admin office C114.

Student Signature:	Supervisor Signature:
B Roome	