Int J Legal Med
DOI 10.1007/s00414-017-1603-9

REVIEW

Forensic entomotoxicology revisited—towards professional standardisation of study designs

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Received: 19 October 2016 / Accepted: 3 May 2017

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

Keywords Forensic · Entomotoxicology · Ecotoxicology · Standardisation

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Introduction

The term *forensic entomotoxicology* was coined by Derrick Pounder [1] in 1991. Forensic entomotoxicology is concerned with the use of insect specimens as an indirect source of toxicological evidence in the absence of direct forensic matrices, such as blood, urine, soil or water, in determining the presence of a toxicant in those insects' environments, which may be a dead body, a river or even an entire landscape.

Environmental forensic entomotoxicology has emphasised the use of organisms (specifically insects) as bioindicators of environmental toxicants like pollutants [2–5], while medicolegal forensic entomology has tended to focus on using insects as surrogate or proxy samples when bodies are too decomposed to provide toxicological samples [6, 7]. Secondary applications of forensic entomotoxicology are best developed in medicolegal forensic entomology, where knowledge about toxicants in bodies may have implications for the estimation of postmortem intervals [6, 7].

The first publications in environmental entomotoxicology appeared about 40 years ago [4], and were soon followed by work in medicolegal entomotoxicology [6]. Over 60 primary studies are now published. The literature of medicolegal entomotoxicology has been reviewed intermittently [7–10] and its goals critiqued [11]. Like the rest of forensic science [12], forensic entomotoxicology faces both academic and practical challenges to its validity as a source of forensic evidence and these have not been evaluated rigorously. Toxicology laboratories are required to have well-documented and consistently applied standard operating procedures (SOPs) [13–15], and there is a clear disciplinary understanding of the need for standards in forensic entomology [16–18], but few standard experimental protocols and procedures have been transferred from the general field of toxicology to ensure that the results of academic entomotoxicological studies are fit for purpose in

Published online: 31 May 2017

forensic settings. Because of the lack of standard experimental protocols, research results in forensic entomotoxicology are often hard to compare or generalise [12], let alone theorise.

This paper reviews practically all of the primary research publications (Table 1), written in various languages (see References), that are currently available on forensic entomotoxicology. From this basis, we evaluate potential challenges to the validity of this source of evidence and outline components of a standardised methodology for future studies in forensic entomotoxicology.

The scope of entomotoxicology

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

The primary literature on entomotoxicology currently (1977–2016) includes over 63 papers (Table 1), mostly focused on the detection of toxicants. Although the findings of medicolegal entomotoxicology have been reviewed [7, 8, 10], they have not been put into the broader context of medical or environmental toxicology. In the following discussion, we examine entomotoxicological questions in the light of pharmacological and physiological mechanisms that affect toxicants in bodies, because this can serve as a model for the dynamics of toxicants in environments in general.

Qualitative toxicant detection

Insects can provide indirect samples to establish the presence of certain toxicants in their environment when processes like putrefaction or drainage have affected the media that are usually sampled (i.e. blood, urine, water, soil or air) [9]. Insects may also bioaccumulate certain toxicants (especially through food chains) to levels where they can be more readily detected [2, 4, 52]. The improving sensitivity of analytical instrumentation is eroding the significance of bioaccumulation for toxicological detection, and even putrefactive attrition in primary samples is a diminishing technical concern [11]. One exception where entomotoxicology remains relevant is that in the extreme case of totally skeletonised bodies, the only remaining traces of toxicants may be in the associated insect faeces (termed *frass*) and cast exoskeletons of larvae that have fed on the corpse [32, 54]. Similarly, insects may retain traces of toxicants even if the toxicant in the environment has been broken down, washed downstream or blown away.

It is important to understand the metabolism of toxicants by insects because this could influence whether a toxicant is detected. Knowing how toxicants are metabolised allows one to use appropriate extraction techniques to target diagnostic metabolic products. For example, the prior presence of codeine is indicated by the current presence of its metabolites, norcodeine and morphine [61]. The detection of a toxicant in an insect sample is highly dependent on the extraction and detection efficiencies of an analytical method and if more is known about relevant metabolites, then appropriate protocols could be deployed. The literature comparing the metabolism of toxicants across species has focused on mammals and little research has included insects [7], although some species have been tested fairly intensively (e.g. *Drosophila* [72]; *Calliphora* [28, 29]).

Quantitative toxicant detection

Can insects provide samples to establish the quantity of a toxicant present in their environment? The quantity of toxicant in the environment to which an insect is exposed depends on processes of transportation, catalysis and sequestration of the toxicant (Fig. 1) and on the insect's ecological function within that environment. Once the insect encounters it, the toxicant undergoes tropism (preferential movement to certain tissues) through pharmacokinetic processes such as absorption, distribution and excretion, metabolism and sequestration (Fig. 1), which is why the insect is considered an indirect sample. The degree to which an indirect sample represents a primary matrix depends on the magnitude of these complex processes.

Absorption, distribution and excretion may occur at different rates so that even when no other processes affect the quantity of toxicant in an insect, it may be at a different concentration from that in the environment. The environmental concentrations to which insects are exposed are affected by similar processes (Fig. 1). In corpses, for example, the manner in which the toxicant is ingested by a human being can affect the rate at which it is metabolised and distributed in the body [73]. A toxicant administered intravenously bypasses the absorption phase (unlike orally administered toxicants), reducing the lag time between administration and the appearance of detectable toxicant concentrations in blood [74]; analogously, toxicants may diffuse into an environment or be dumped.

The distribution of toxicants through particular tissues is also affected by blood flow within the tissues, ionisation characteristics of the toxicant, degree of protein binding and the affinity of the toxicant for each tissue (for example, THC has a

Table 1 List of literature examined for this review, with the toxicants, insect species and research goal of each study

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case study	
Calliphora dubia	1	Gunshot residue		X			[19]
Calliphora stygia	1	Methamphetamine		X	X		[20]
Calliphora stygia	1	Morphine		X			[21]
Calliphora stygia	1	Morphine		X			[22]
Calliphora stygia	1	Morphine			X		[23]
Calliphora vicina	1	Alprazolam	X	X			[24]
Calliphora vicina	1	Clonazepam	X	X			[24]
Calliphora vicina	1	Diazepam	X	X			[24]
Calliphora vicina	1	Flunitrazepam	X	X			[24]
Calliphora vicina	1	Lorazepam	X	X			[24]
Calliphora vicina	1	Nordiazepam	X	X			[24]
Calliphora vicina	1	Oxazepam	X	X			[24]
Calliphora vicina	1	Prazepam	X	X			[24]
Calliphora vicina	1	Temazepam	X	X			[24]
Calliphora vicina	1	Triazolam	X	X			[24]
Calliphora vicina	1	Nordiazepam		X	X		[25]
Calliphora vicina	1	Morphine hydrochloride		X			[26]
Calliphora vicina	1	Morphine		X		X	[27]
Calliphora vicina	1	Amitriptyline		X		X	[28]
Calliphora vicina	1	Temazepam		X		X	[28]
Calliphora vicina	1	Trazodone		X		X	[28]
Calliphora vicina	1	Trimipramine		X		X	[28]
Calliphora vicina	1	Acetylsalicyclic acid		X			[29]
Calliphora vicina	1	Sodium salicylate		X			[29]
Calliphora vicina	1	Paracetamol		X			[29]
Calliphora vicina	1	Aminohippuric acid		X			[29]
Calliphora vicina	1	Amphetamine sulphate		X			[29]
Calliphora vicina	1	Sodium amylobarbitone		X			[29]
Calliphora vicina	1	Sodium phenobarbitone		X			[29]
Calliphora vicina	1	Sodium thiopentone		X			[29]
Calliphora vicina	1	Sodium barbitone		X			[29]
Calliphora vicina	1	Sodium brallobarbitone		X			[29]
Calliphora vicina	1	Paracetamol			X		[30]
Calliphora vicina	1	Co-proxamol		X			[31]
Calliphora vicina	1	Acetaminophen		X			[31]
Calliphora vicina	1	Amitriptyline		X			[31]
Calliphora vicina	1	Nortriptyline		X			[31]
Calliphora vicina	1	Morphine hydrochloride		X		X	[32]
Calliphora vomitoria	1	Methamphetamine		X	X		[33]
Calliphora vomitoria	1	Morphine hydrochloride		X		X	[32]
Calliphora vomitoria	1	Morphine hydrochloride		X			[34]
Calliphoridae larvae (species unidentified)	1	Triazolam		X		X	[35]
Calliphoridae larvae (species unidentified)	1	Phenobarbital		X		X	[35]
Calliphoridae larvae (species unidentified)	1	Alimemazine		X		X	[35]

Table 1 (continued)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case study	
Calliphoridae larvae (species unidentified)	1	Clomipramine		X		X	[35]
Calliphoridae larvae (species unidentified)	1	Oxazepam		X		X	[35]
Chrysomya albiceps	1	Cadmium			X		[36]
Chrysomya albiceps	1	Cadmium			X		[37]
Chrysomya albiceps	1	Diazepam			X		[38]
Chrysomya albiceps	1	Cocaine			X		[39]
Chrysomya albiceps	1	Codeine			X		[40]
Chrysomya albiceps	1	Methylaphenidate chloride			X		[41]
Chrysomya albiceps	1	Methylphenidate hydrochloride			X		[41]
Chrysomya albiceps	1	Phenobarbital			X		[41]
Chrysomya albiceps	1	Nandrolone decanoate			X		[42]
Chrysomya chloropyga	1	Medroxyprogesterone acetate			X		[43]
Chrysomya chloropyga	1	Norethisterone enanthate			X		[43]
Chrysomya chloropyga	1	Hydrocortisone			X		[44]
Chrysomya chloropyga	1	Sodium methohexital (hydrocortisone sodium succinate)			X		[44]
Chrysomya megacephala	1	Buscopan® (Butylscopolamine bromide)		X	X		[45]
Chrysomya megacephala	1	Malathion	X	X	X		[46]
Chrysomya megacephala	1	Methylphenidate chloride			X		[41]
Chrysomya megacephala	1	Methylphenidate hydrochloride			X		[41]
Chrysomya megacephala	1	Phenobarbital			X		[41]
Chrysomya megacephala	1	Nandrolone decanoate			X		[42]
Chrysomya megacephala	1	Malathion			X		[47]
Chrysomya megacephala	1	Flunitrazepam	X	X			[48]
Chrysomya megacephala	1	Flunitrazepam	X	X			[49]
Chrysomya megacephala	1	Malathion		X		X	[50]
Chrysomya putoria	1	Gentamicin			X		[51]
Chrysomya putoria	1	Gentamicin sulphate (Hyatamicina)			X		[51]
Chrysomya putoria	1	Methylphenidate chloride			X		[41]
Chrysomya putoria	1	Methylphenidate hydrochloride			X		[41]
Chrysomya putoria	1	Phenobarbital			X		[41]
Chrysomya putoria	1	Nandrolone decanoate			X		[42]
Chrysomya rufifacies	1	Malathion		X		X	[50]
Chrysomya putoria	1	Cocaine			X		[39]
Cochliomyia macellaria	1	Flunitrazepam	X	X			[49]
Cochliomyia macellaria	1	Phenobarbital		X		X	[6]
Creophilus maxillosus	6	Methyl mercury		X			[52]
Crocothemis servilia	9	Cadmium		X			[2]
Crocothemis servilia	9	Chrome		X			[2]
Crocothemis servilia	9	Nickel		X			[2]
Crocothemis servilia	9	Zinc		X			[2]
Crocothemis servilia	9	Copper		X			[2]
Danaus chrysippus	8	Cadmium		X			[2]
Danaus chrysippus	8	Chrome		X			[2]
Danaus chrysippus	8	Nickel		X			[2]
Danaus chrysippus	8	Zinc		X			[2]

Table 1 (continued)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case study	
Danaus chrysippus	8	Copper		X			[2]
Dermestes frischi	4	Morphine hydrochloride		X			[53]
Dermestes frischi	4	Morphine hydrochloride		X		X	[32]
Dermestes maculatus	4	Amitriptyline		X		X	[54]
Dermestes maculatus	4	Nortriptyline		X		X	[54]
Lucilia sericata	1	Morphine hydrochloride		X		X	[32]
Lucilia sericata	1	Tramadol		X	X		[55]
Lucilia sericata	1	Methadone		X	X		[56]
Lucilia sericata	1	2-ethylidene-15-dimethyl-33-dipheylpyrrolidine (EDDP).		X	X		[56]
Lucilia sericata	1	Cadmium		X	X		[57]
Lucilia sericata	1	Morphine hydrochloride			X		[58]
Lucilia sericata	1	Ampicillin			X		[59]
Lucilia sericata	1	Mezlocillin			X		[59]
Lucilia sericata	1	Cefazolin			X		[59]
Lucilia sericata	1	Ceftizoxime			X		[59]
Lucilia sericata	1	Gentamicin			X		[59]
Lucilia sericata	1	Clindamycin			X		[59]
Lucilia sericata	1	Vancomycin			X		[59]
Lucilia sericata	1	Opiates		X		X	[60]
Lucilia sericata	1	Cocaine		X		X	[60]
Lucilia sericata	1	Barbiturates		X		X	[60]
Lucilia sericata	1	Clomipramine		X		X	[60]
Lucilia sericata	1	Amitriptyline		X		X	[60]
Lucilia sericata	1	Nortriptyline		X		X	[60]
Lucilia sericata	1	Levomepromezine		X		X	[60]
Lucilia sericata	1	Thioridazine		X		X	[60]
Lucilia sericata	1	Phenobarbital		X		X	[60]
Lucilia sericata	1	Norcodeine		X	X		[61]
Lucilia sericata	1	Codeine		X	X		[61]
Lucilia sericata	1	Morphine		X	X		[61]
Lucilia sericata	1	Ketamine		X	X		[62]
Lucilia sericata	1	Morphine hydrochloride		X		X	[32]
Megaselia scalaris	2	Amitriptyline		X		X	[54]
Megaselia scalaris	2	Nortriptyline		X		X	[54]
Oxya hyla hyla	10	Cadmium		X			[2]
Oxya hyla hyla	10	Chrome		X			[2]
Oxya hyla hyla	10	Nickel		X			[2]
Oxya hyla hyla	10	Zinc		X			[2]
Oxya hyla hyla	10	Copper		X			[2]
Phormia regina	1	Ethanol			X		[63]
Protophormia terraenovae	1	Morphine hydrochloride		X		X	[32]
Protophormia terraenovae	1	Morphine hydrochloride		X			[26]
Sarcophaga peregrina	3	Cocaine		X	X		[64]
Sarcophaga peregrina	3	Heroin		X	X		[65]
Sarcophaga ruficornis	3	MDMA		X	X		[66]
Sarcophaga ruficornis	3	Methamphetamine		X	X		[67]

Table 1 (continued)

Insect species	Family	Toxicant	Research goal				Reference
			Method	Detection	Effect	Case study	
Sarcophaga ruficornis	3	Amitriptyline			X		[68]
Sarcophaga ruficornis	3	Nortriptyline			X		[68]
Sarcophaga tibialis	3	Hydrocortisone			X		[69]
Sarcophaga tibialis	3	Sodium methohexital (hydrocortisone sodium succinate)			X		[69]
Tenebrio molitor	7	Methyl mercury		X			[52]
Thanatophilus sinuatus	5	Morphine hydrochloride		X			[53]
Unknown		Paraquat		X			[70]
Unknown		Amitriptyline	X	X			[71]
Unknown		Carbamazepine	X	X			[71]
Unknown		Bromazepam	X	X			[71]
Unknown		Clonazepam	X	X			[71]
Unknown		Diazepam	X	X			[71]
Unknown		Flunitrazepam	X	X			[71]
Unknown		Cocaine	X	X			[71]
Unknown		Benzoylecgonine	X	X			[71]
Unknown		Aldicarb sulfone and sulfoxide metabolites	X	X			[71]

'Method' refers to a focus on method development; 'Detection' refers to a focus on the detection of toxicants; 'Effect' refers to a focus on the effects of toxicants on insects; and 'Case study' refers to a focus on a forensic case as an example in which forensic entomotoxicology was used

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

higher affinity to muscle and adipose tissue whereas alcohol is evenly distributed throughout the body [74]). The processes may also not reach an equilibrium that can be related back to the original dose, particularly if the duration of exposure is not known. For instance, when absorption exceeds excretion, toxicants will bioaccumulate [75, 76].

Catalysis of toxicants in the environment or their metabolism within a body will generally give them a characteristic half-life, which affects their recovery from forensic samples. Various metabolic processes modify toxicants within an animal's body, particularly in organs such as the vertebrate liver [77], which enhances tropism [78]. The metabolism of toxicants depends primarily on their physiochemical properties, including their physical state (solid, liquid or gaseous), lipophilicity and solubility [79, 80]. For instance, the metabolism of mercury by larvae of *Calliphora vicina* is affected by whether the mercury is methylated [52]. The pathways and rates of metabolism are affected by intrinsic factors like species, genetics, sex, age, hormone activity, pregnancy and disease and extrinsic factors like diet and environment [78, 81, 82]. Postmortem changes to toxicant concentrations also occur in bodies [74, 83]. This phenomenon is known as postmortem drug redistribution (PMR) [84]. PMR occurs as a result of the rupturing on cell membranes, causing changes to the concentrations of toxicants as a result of diffusion through different tissues [85]. This process may cause toxicant concentrations to increase post mortem in some tissues, e.g. antipsychotics (such as amisulpride, paliperidone, chlorpromazine, clozapine, haloperidol, olanzapine, promethazine, quetiapine, risperidone and zuclopenthixol) [86], THC and its metabolites [87], digoxin [74, 88] and fentanyl [89].

Sequestration in insects' environments may involve binding of toxicants to sediments or ligands that can make them unavailable; in bodies, this is a component of toxicant tropism [75, 76]. Each body tissue has its own unique chemical and physical properties [90], and these can have an effect on how a toxicant distributes and deposits in the body. For instance, hydrophobic molecules may deposit in lipid-rich tissues, while hydrophilic molecules accumulate in more aqueous tissues [80]. Patterns of tropism may vary with the age and sex of the body [82]. Similar processes affect the distribution of toxicants in other environments (Fig. 1), but tropism in the insect itself is usually not a forensic concern unless frass or exoskeletons are being used for toxicological analysis. Even so, blowfly larvae may accumulate toxicants that are then deposited into the intestine of the pupa and excreted with the meconium by the adult [52], leaving almost no trace of the toxicant in the adult insect.

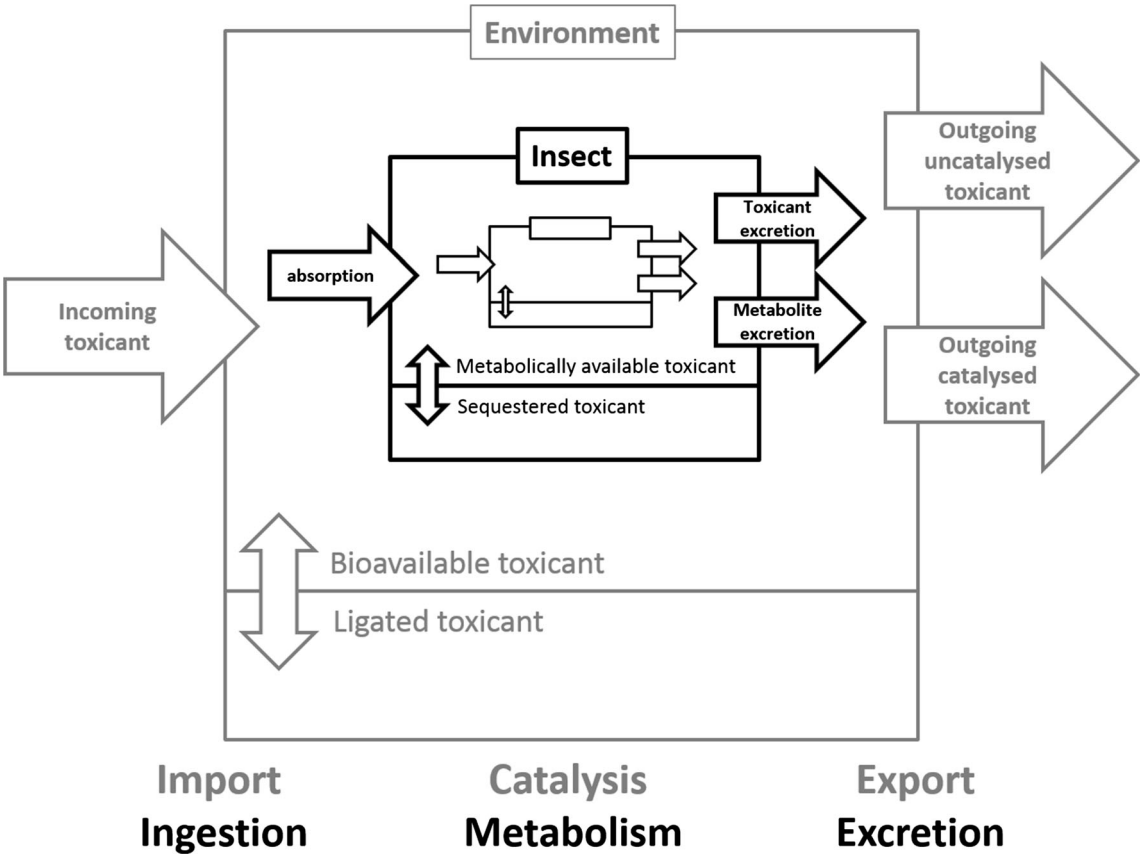

Fig. 1 Conceptual model of the nested processes of transport, storage and modification that affect the distribution of toxicants in the environment (*grey*), insects (*bold black*) and tissues (*feint black*)

In principle, it may be possible to estimate the quantity of toxicant in a source by correcting for these metabolic processes empirically (e.g. using an empirical ensemble rate for the whole organism) provided that the duration of exposure is known, but in medicolegal cases, this duration (i.e. the postmortem interval) is commonly the issue in question. Toxicants show unusual empirical patterns of transfer to insect samples (e.g. heavy metals), and these are typically specific to both the toxicant and the insect species [91]. In addition, insects feeding in particular parts of an environment or body may eat quantities of a toxicant that are uncharacteristic of the environment or body as a whole, thus providing quantitatively unrepresentative toxicological samples. In particular, fly larvae may wander about a body and feed from different sites on it, and if there is significant toxicant tropism in the body, it may require many replicated samples of insects to represent the general quantity of toxicant in the body accurately.

The implication of these processes (Fig. 1) for medicolegal forensic entomotoxicology is that the concentrations of toxicants that are detected in insect specimens are affected by (a) toxicant tropism in the body, (b) pre- and postmortem changes and (c) the extraction and detection efficiencies of the analytical techniques. This means that the quantities of toxicant detected in insects are best interpreted qualitatively or by using empirical experimental surrogates such as dead pigs. Environmental toxicology has made progress in refining the interpretation of indirect samples (e.g. [76, 91]), and approaches such as comparative modelling [76], biokinetic modelling [76] and saturable uptake kinetics modelling [92] can be adopted and adapted by medicolegal entomotoxicology.

Postmortem interval estimation

Thirty-three studies have addressed the effects of different toxicants on the growth rates of different insect species of forensic interest (Table 1). However, with the lack of standardisation and replication in methods and conflicting outcomes of these studies, it is difficult to determine whether the results are drug effects or artefacts. There are indications that neuroactive toxicants have similar effects on insects and mammals. For instance, cocaine [93], caffeine [94, 95], modafinil [96] and methamphetamine [97] are pharmacological stimulants for *Drosophila*, while antihistamines cause them to sleep [95]. It is therefore understandable that cocaine should cause accelerated feeding and growth in blow flies [39, 64]. Vertebrate hormones like progesterone can be expected to have little effect on insects, and this appears to be the case (e.g. [43]). The effects of steroid hormones are less predictable [29].

Toxicants that affect insect development and behaviour may have dose-dependent effects on growth, but because of our currently incipient understanding of insect pharmacokinetics (Fig. 1), it is difficult to model these unless one knows the duration of exposure, which is the unknown post-mortem interval that many medicolegal entomotoxicological investigations need to estimate [69]. Furthermore, the dose-dependence is also not necessarily linear, and may show hormesis [69], where initial dose-dependent trends reverse at even higher concentrations [97].

Toxicant-induced variation in development may be confounded with variation in development caused by feeding on different tissues, particularly vertebrate liver [98–102]. This effect can be problematic for medicolegal investigations if larvae have migrated around a body to any degree while feeding, and also needs to be taken into account in the selection of toxicant matrices for laboratory experiments.

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

Toxicant source localisation

Toxicants in the environment may originate from point sources, such as a dumping site, or diffuse sources such as agricultural run-off. Point sources are typically traced by mapping the concentrations of the toxicant in samples and identifying gradients emanating from the source. In rivers, it can also be shown that toxicants are absent upstream of a point source. Diffuse sources have shallow gradients. Pioneering work in this direction was done on mercury in flies, fish and birds in Finland [4].

Analytical review of the available literature

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

Insect taxa

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

In total, ten families of carrion-feeding insects have been studied (Table 1), although the list of potential research targets is much larger (e.g. [103–108]). The most studied family was the blow flies (Diptera, Calliphoridae) which are featured in 70% (43 papers) of the literature, followed by the flesh flies (Diptera, Sarcophagidae) (10%, 6 papers) and the hide beetles (Coleoptera, Dermestidae) (5%, 3 papers).

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

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

The early stages (fresh, bloated and active decay) of decomposition are colonised the blow flies *L. sericata*, *C. vicina* and *C. megacephala*, which fulfil the above-mentioned criteria and would therefore be ideal taxa in those stages. The hide beetle *Dermestes maculatus* (Coleoptera, Dermestidae) would be ideal as an indicator of toxicants later in the decomposition process (during the advanced decay and skeletonised stages), and this species also fulfils these criteria [103, 109, 110].

Feeding substrates

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

(1) The toxicant should be stable and homogenously distributed throughout the matrix. Stability and homogeneity of the toxicant in the matrix allow the concentration of the

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

(2) The matrix should not react significantly with the toxicant (i.e. there should be no metabolites present). Unreactive matrices help to ensure that the experimental insects are exposed to consistent doses of toxicants, and avoid the confounding effects of metabolites. Liver has been identified as a particularly poor matrix in this regard [61, 102].

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

It has been suggested that the matrix should closely represent matrices in forensic investigations to ensure that experimental results can be applied to field investigations without criticism [111]. Considering that the ultimate goal of the matrix is to deliver controlled amounts of nutrients and toxicants to the insect, the importance of its similarity to matrices found in forensic investigations is debateable. If the insects are provided with all of the necessary nutrients to reach the landmarks of its life (e.g. growth, ecdysis, reproduction) and there are no observable confounding effects as a result of the feeding substrate (control), then it does not necessarily have to mimic more complex matrices.

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

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

We classified the feeding substrates used in the literature by donor organism and the donor organ used in each study. Rabbit was used most often (25%, 15 papers), followed by cow (20%, 12 papers) and human (15%, 9 papers). Two studies used kangaroo meat, which is not easily available in most countries. Ideally, one would use a tissue type that is easily and widely accessible. Another factor which should be taken into consideration is the religious constraints on using certain animals in different countries (e.g. the use of beef in a Hindu country or pork in a Muslim country). When considering which donor organism to use for a study, it is important to consider the statistical analyses required because there needs to be a suitable number of replicates. More research needs to be done to determine the number of treatments, controls and replicates per treatment and control that would be required. Due to ethical constraints on using animals such as rabbits, it is seldom possible to get sufficient numbers of animals.

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

Taking all of these factors into consideration, the ideal donor organisms would be chicken and fish. These could be incorporated into standardised artificial diets.

In terms of the organs used, 31% (20 studies) used liver, followed by whole carcasses (17%, 10 studies) and muscle and mince (12%, 8 studies). We classified muscle and mince as being different because the fat content of mince is most likely to be variable across different studies. The liver is catalytically active [61], which violates an important criterion for matrix selection. Most published studies did not consider the stability of the toxicant within the feeding substrate and so it is not known whether the concentration of the toxicant remained consistent throughout the duration of these studies. Kharbouche et al. [61] noted that metabolism of codeine (into norcodeine and morphine) occurred in their study as a result of enzymes that remained active after autolysis of the liver tissue

that they used. This shows that liver is not an inert tissue type, which violates our first criterion for matrix selection.

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

Toxicants

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

Toxicants most often reported in the literature were morphine (8%, 10 studies), amitriptyline (6%, 7 studies) and cocaine, cadmium, flunitrazepam, nortriptyline and phenobarbital (3%, 4 studies each). Morphine is a metabolite of heroin [112] which is an abused drug in many regions of the world [113]. Amitriptyline is an antidepressant associated with overdose and suicide cases. Cocaine is a drug of abuse and, like heroin, is a problem in many countries [113].

Other toxicants examined such as heavy metals (cadmium, copper, nickel, zinc and chrome), insecticides and accelerants are relevant to the fields of ballistics, environmental ecotoxicology and arson. In these cases, the insects were successfully used as bioindicators for potential toxicants in the environment [2, 36, 37, 52, 57] and gunshot residues (Table 1).

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

Toxicological analysis

The preparation of a forensic sample for toxicant detection is highly dependent on the type of detection method that will be used. When developing an extraction technique, it is important that the extraction efficiency is known and that this known value will remain constant for all extractions. The extraction efficiency on its own needs to be tested prior to analysis to ensure that the toxicant will be extracted from the insects [114].

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

After extraction, the matrices should be tested to confirm the presence and concentration of the toxicants; otherwise, it is impossible to confirm that the observed effects are a direct result of the presence and amount of the toxicant. Of the 55 papers examined, 21% (12 studies) did not use any analytical methods to detect or quantify the toxicant in the insects. When analytical methods had been used, 18% (10 studies) used HPLC or GC/MS and 11% (6 studies) used RIA. Recently, Oliveira et al. [48] and Baia et al. [49] developed quantification methods which are not invasive or destructive to insect evidence. From an entomological perspective, this could be highly beneficial, especially if the insects are also needed to estimate a PMI or for DNA analysis.

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

Statistical analysis

There was a high level of inconsistency in the numbers of controls, treatments and replicates amongst the studies. More studies should be conducted to determine what the appropriate number of the above-mentioned parameters should be for the results to be statistically robust. As things stand, it is possible that there are many Type II errors of inference (false positives) in published studies because they used as few as three replicates (ten would be better and nearer 30 would be ideal). Toxicant concentrations in dose-response regressions should be geometrically-spaced rather than simple multiples of the lowest concentration; there should be at least six concentrations in a regression analysis; and standard toxicological

analyses (e.g. logistic regression) should be deployed where appropriate.

Experimental vs. applied: the application of entomotoxicology

Most studies in medicolegal forensic entomotoxicology have been experiments done in the controlled settings of a laboratory, while ecotoxicological research usually draws samples from the environment. Although laboratory results are indicative of what could possibly happen in an actual case, there are confounding factors (such as weather conditions) that cannot be controlled accurately in the field. When using results from laboratory studies, it is crucial to determine how applicable they would be to an actual case by taking into account the processes summarised in Fig. 1.

Another factor to consider is the feasibility of conducting these tests in a mortuary or forensic laboratory. Unfortunately, due to the equipment and expertise required, and the challenge of meeting the SOPs for toxicological testing (e.g. routine calibration, logging protocols, interinstitutional benchmarking) [114], there is a high chance that many mortuaries and forensic laboratories would not be able to conduct these tests. The focus should also lean towards getting forensic laboratories ISO 17000 accredited as this would further validate the results being produced by these laboratories. In particular, ISO/IEC 17025 provides the general standard for competence of testing and calibration laboratories, and most laboratories must hold such accreditation to be deemed technically competent by many suppliers and regulatory authorities.

The future: a basic protocol for future entomotoxicological studies

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

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

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

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

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

Acknowledgements We thank Dr. Claudia Tocco (Rhodes University) for editorial advice and our reviewers for their constructive comments. Funding was provided by the National Research Foundation (NRF) of South Africa. Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the NRF.

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