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CONTINUING MEDICAL EDUCATION REVIEW

# Forensic entomology: applications and limitations

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**Abstract** Forensic entomology is the science of collecting and analysing insect evidence to aid in forensic investigations. Its main application is in the determination of the minimum time since death in cases of suspicious death, either by estimating the age of the oldest necrophagous insects that developed on the corpse, or by analysing the insect species composition on the corpse. In addition, toxicological and molecular examinations of these insects may help reveal the cause of death or even the identity of a victim, by associating a larva with its last meal, for example, in cases where insect evidence is left at a scene after human remains have been deliberately removed. Some fly species can develop not only on corpses but on living bodies too, causing myiasis. Analysis of larvae in such cases can demonstrate the period of neglect of humans or animals. Without the appropriate professional collection of insect evidence, an accurate and convincing presentation of such evidence in court will be hampered or even impossible. The present paper describes the principles and methods of forensic entomology and the optimal techniques for collecting insect evidence.

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## Definition and application

Forensic entomology is the analysis of insect evidence for forensic and legal purposes [1]. The most important and most frequently requested task is the estimation of the minimum time since death [2]. Techniques devised recently allow experts in the field to collect robust entomological evidence that can provide vital information in a death investigation, to answer questions concerning movement or storage of the remains following death, submersion interval, time of decapitation and/or dismemberment, identification of specific sites of trauma, post-mortem artefacts on the body, use of drugs (entomotoxicology), linking a suspect to the scene of a crime, sexual molestations and the identification of suspects [3]. It is also possible to demonstrate the period of neglect of living humans and animals by examining the insects recovered from infested wounds.

## Estimating the minimum post-mortem interval

Post-mortem interval (PMI) refers to the time between the death and discovery of a corpse [2]. There are several natural processes associated with decomposition, such as rigor mortis or livor mortis, that can be used to estimate the PMI [4], but many of these are reciprocal functions and become inaccurate in application very quickly [5]. Furthermore, they are limited to the first 72 h after death [4]. However, during that 72 h and well beyond, insects can be a very powerful tool for estimating the minimum time since

death. Depending on the level of accessibility and environmental conditions, necrophagous insects will promptly colonize a fresh corpse. Usually the first taxa to arrive on a body are flies (Diptera), mainly blowflies (Calliphoridae), which can locate an odor source with great spatial precision and deposit their eggs on a corpse within minutes–hours of death. Larvae (often called "maggots") hatch from the eggs and feed on the underlying tissues. As they grow they shed their cuticle twice, a process termed "ecdysis," to enable further growth. After each ecdysis (moult) a new larval instar (stage) is formed. When third instar larvae finish feeding they enter the post-feeding stage, most species migrate away from the body to find shelter, either within soil or underneath objects e.g. stones or leaves (outdoor crime scenes), or furniture (indoor crime scenes). Here, they form pupae within a protective outer case, the puparium (the hardened cuticle of the third instar larva), from which adult flies emerge at the completion of metamorphosis.

Decomposition as a result of insect activity in and on the corpse is a continuous process that can be measured, allowing accurate minimum PMI estimates to be made up to several months after death depending on the circumstances [4]. The assumption behind these estimates is that by calculating the age of developing insects on a body, it is possible to calculate the time of colonization, which infers a minimum PMI ($PMI_{min}$) [2], i.e., the time when insects first colonized the body, rather than the actual time of death [1]. Because blowflies are usually the first group to colonize a body, the focus of $PMI_{min}$ estimates is often on them when using entomological evidence [2].

The rate of development of an insect is mainly governed by temperature and can differ between even closely related species [6]. Hence, a three-step process of, (1) accurately identifying the species found on a corpse, (2) reconstructing crime scene temperatures, and (3) modelling the rate of development of the immature insects found on a corpse, is absolutely essential to enable a forensic entomologist to calculate the age of a sampled insect.

## Identifying entomological evidence

The identification of insects is a highly skilled procedure and should always be conducted by an expert in insect taxonomy. Museums and universities are generally the best equipped organizations to process insect identifications and should always be a first point of contact. For detailed identification keys on forensically important insects see e.g. Zumpt [7], Smith [8] and Szpila [9].

## Modelling of crime scene temperatures

To obtain the temperatures of a crime scene while the body was in situ, a record of ambient temperatures is gathered, usually from the weather station nearest to the body discovery site [1, 8, 10]. Unfortunately, there can be a significant difference between the ambient temperatures of the weather station and the crime scene, e.g., because they might be at different altitudes to one another, or experience different exposure to identical weather conditions [10]. For this reason, ambient temperature is recorded on site for several days after the body is discovered, and a regression relationship is then derived between these temperature and simultaneously recorded weather station temperatures [1]. This derived equation is then used to correct the weather station temperature records to ambient site temperature for the duration that the body was thought to be in situ [10].

Once ambient environmental temperature has been estimated for the duration that the body was in situ, an estimate of $PMI_{min}$ can be made using this information together with reference development data for the particular species, usually obtained experimentally in the laboratory.

## Modelling insect development

Developmental data are simply data pertaining to the duration of development of immature stages recorded at different temperatures (usually constant but sometimes fluctuating), which are then summarized in one or more of three developmental models, namely isomorphen diagrams [11], isomegalen diagrams [12] and thermal summation models [13]. Size (e.g. length or weight) and developmental event (e.g. 1st larval moult/ecdysis or pupariation), are the only two available measures to calculate the age of immature insects, and developmental models use one of these two measures to estimate $PMI_{min}$.

### *Isomorphen diagram*

The most basic of these models is the isomorphen diagram which is a simple scatterplot that models the duration of developmental events (egg hatching, 1st ecdysis, 2nd ecdysis, onset of wandering, onset of pupariation, adult eclosion; *X*-axis) against temperature (*Y*-axis). Each contour in this model represents one of these developmental events (Fig. 1). By knowing the stage of development of the oldest immature insects on the body, and the average environmental temperature of the crime scene while the body was in situ, it is possible to calculate an accurate $PMI_{min}$ from this model, using the min/max error bars to estimate the confidence window for $PMI_{min}$.

The advantage of this model is that it is simple to use, with concepts that are easily explained in court. However, this simplicity compromises the accuracy of the output i.e. in cases where environmental temperatures fluctuate, e.g. outdoor scenarios, the average environmental temperature

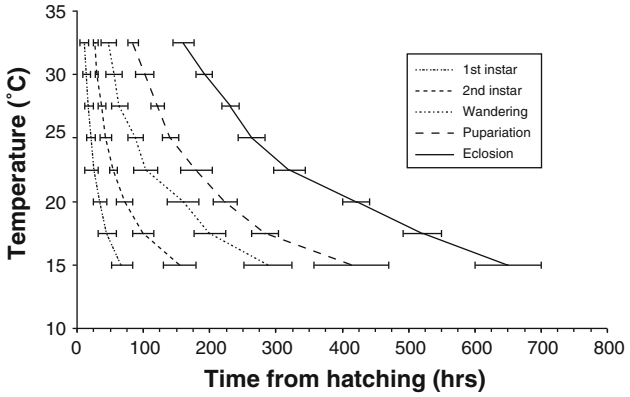


**Fig. 1** Isomorphen diagram of hypothetical development data. The contours of the graph represent the time taken for developing immature blowflies to reach a particular developmental event (refer to key on graph), at a range of constant temperatures. *Error bars* represent 95% confidence intervals for each developmental event. (*Note*: "egg hatching" is not included in this graph as it is measured in hours that are an order of magnitude smaller than those of "wandering," "puparation" and "eclosion.")

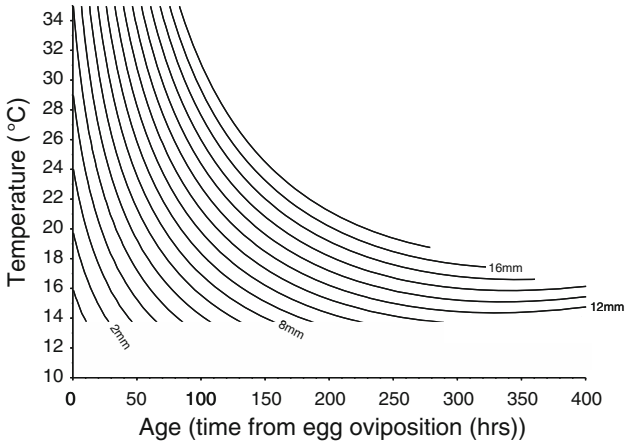


**Fig. 2** Isomegalen diagram of hypothetical development data. Each contour represents larval length (*Z*-axis) in relation to their age (*X*-axis) and the temperature experienced during development (*Y*-axis). The dimension of the *Z* axis (length) is not needed visually to calculate a $PMI_{min}$, and is therefore, not illustrated. (*Note*: *no error bars* are presented in this diagram and the window for $PMI_{min}$ is calculated from the standard deviation or min/max data of the entomological evidence.)

of the crime scene used to calculate $PMI_{min}$ compromises the accuracy of the estimate.

As the isomorphen diagram only models the timing of developmental events, and shows no gradation between events, the estimate is only accurate if derived from live entomological evidence which is reared through to the next developmental event at a known, constant temperature.

However, entomological evidence is not always collected alive, and in some cases, live specimens may be killed and preserved before being analyzed. In these cases the estimated age of insect specimens modelled from an isomorphen diagram will always be less than their actual age, simply because it has to be based on the last recorded developmental stage (e.g. the moult from 2nd to 3rd instar larvae for all 3rd instars, no matter how long ago that moult was), resulting in an underestimate of $PMI_{min}$.

### *Isomegalen diagram*

The isomegalen diagram is a more sophisticated model that can estimate $PMI_{min}$ using dead larvae, and has the capability of accounting for and analyzing fluctuating environmental temperature data. The isomegalen diagram is a 3D contour plot that models larval size (length, weight or width) (*Z*-axis) as a measure of age, against temperature (*Y*-axis), and time (*X*-axis) (Fig. 2). Larval size is a measure of age with a higher resolution than developmental event and shows detail of growth between two developmental events.

This higher resolution input provides a more accurate estimate of $PMI_{min}$. The diagram details the full range of larval sizes (from egg hatching to the onset of puparation) where each contour on the graph represents a particular larval size (Fig. 2). The largest larvae from the body and crime scene are collected, identified and measured, and these data are then summarized and modelled, along with related environmental temperatures, to estimate $PMI_{min}$. The process of estimating $PMI_{min}$ from fluctuating environmental temperatures, using an isomegalen diagram, is illustrated in Villet et al. [14].

Despite the fact that this model provides more accurate $PMI_{min}$ estimates than the isomorphen diagram, it has several criticisms. Firstly, it is well documented that size is not always an accurate measure of age, particularly for blowfly larvae [15]. Larvae are known to shrink in size before entering the next developmental event i.e. 1st ecdysis, 2nd ecdysis, and particularly before puparation when larvae migrate from the food source [11]. Therefore, it is possible for larvae of a small size to be older than larvae of a larger size. Additionally, stunted larval or puparial forms, smaller than normal, can be commonly observed in experimental cultures, possibly as a result of the scarcity of the available food source, competition between larvae at high densities [4] and slow developing "laggards" [16]. Secondly, this model is limited to larvae only, which only accounts for approximately half the total duration of development from egg to adult. The other half being egg and puparation. Thirdly, larval size can change significantly during preservation, as different preservatives and the duration of preservation causes shrinkage or expansion of larvae [17]. This change in size directly influences the calculation of age and subsequent estimate of $PMI_{min}$. Additional models are required to correct for this change in

size, which adds another source of error to the $PMI_{min}$ calculation and reduces the precision of the estimate. Lastly, it is labor intensive to collect the experimental data for an isomegalen diagram and the model is not as simple to build as the isomorphen diagram. This is evident by the dearth of published studies reporting isomegalen diagrams of forensically important blowfly species [6, 11, 12, 15].

*Thermal summation models*

Thermal summation models are the most sophisticated of the three available models. They have the capability of processing both size and developmental event data, as well as fluctuating environmental temperatures. Because this model can process more complex data sets, it is less affected by the limitations imposed on the two iso-diagrams, and is therefore, the preferred model to use when estimating $PMI_{min}$.

There is a recognized linear regression model for analyzing developmental data [13, 18]. Traditionally, it has been used to calculate development thresholds for application in biological control [19] and has been modified for forensic entomology. This method is commonly referred to as the thermal summation model, or accumulated degree–hours (ADH) model, where the x-intercept and the inverse of the slope of the linear regression are used to calculate $PMI_{min}$. These coefficients are termed thermal summation constants and form the basic assumptions of linear models of development. These assumptions are that there is a positive linear relationship between development rate and increasing temperature (slope), and that development ceases below a minimum developmental threshold (intercept) [20]. In these models, development is measured as thermal time (temperature multiplied by time), hence the phrase 'degree–hours', and each developmental stage, e.g. egg, 2nd instar, or pupariation, requires a specific number of accumulated degree–hours to complete development, hence the name 'ADH' [13]. The principle behind estimating $PMI_{min}$ is to calculate the time taken to reach the ADH required for the species identified on or around the body to have developed to the oldest recorded stage collected at the environmental temperatures experienced while the body was in situ.

A criticism of this model is the high variance in the upper and lower developmental temperature extremes. Because these values are the end points of the regression, and are thus heavily weighted values, they significantly influence the confidence of the regression coefficients, which compromises the precision of a $PMI_{min}$ estimate. Recently, a revised regression model [21] has been used to model blowfly development [6]. It identifies the points nearest the extremes of the linear approximation that deviate significantly from it, which results in the calculation of more precise thermal summation parameters.

Insect succession

Numerous insect species and other arthropods will occur on or around a cadaver during the process of decomposition. However, their occurrence on the body does not necessarily indicate that they have oviposited. Depending on their ecological and biological preferences they will be attracted by specific olfactory cues and colonize a body during different stages of decomposition. Some groups do not feed on dead tissue at all, but are attracted to a corpse to feed as predators on necrophagous insects. Smith [8] proposed four different ecological categories of insects which can be found on corpses:

- Necrophagous species—which feed on the dead tissue.
- Predators and parasites—of insects and other arthropods (note that some species start their development as necrophages, before becoming predacious in their later developmental stages).
- Omnivorous species—for example wasps, ants and some beetles, which are not obligate necrophages, but will use the resource, the cadaver, as a food source if available.
- Adventive species—which are specific to the habitat of the scene of crime and merely use the body as an extension of that habitat, e.g., for cover as they would use a fallen tree trunk, e.g., spiders, springtails or caterpillars.

It is important to recognise that many insects are attracted to certain stages of decomposition and they will not all occur simultaneously on a cadaver, but in a more or less predictable chronological sequence, known as insect succession, first proposed by Megnin [22]; see also for a recent update Anderson [23]. For estimating the $PMI_{min}$ from succession patterns forensic entomologists mainly use the first two groups of insects to arrive at a corpse, i.e. flies and beetles. Blowflies prefer fresh cadavers and dominate during the first days and weeks of decomposition, while other fly groups, for example the cheese skippers (Diptera: Piophilidae) occur in the later stages of decomposition. Several Coleoptera, such as the larder beetles (Dermestidae), are adapted to utilise dry foods like skin, and even hairs and bones: they tend, therefore, to be attracted to the very last stages of decomposition.

The manner of deposition or storage of a corpse will also influence the species composition. Thus, while blowflies will not normally colonize buried cadavers, even if attracted to their general vicinity by decomposition odours, scuttle flies (Diptera: Phoridae) are found regularly colonizing such bodies, with *Muscina* species additionally colonizing shallow burials [24]. So, while burial usually restricts the access of many carrion insects to a body,

others are able to take advantage, such as *Megaselia scalaris* (Loew) (Dipt., Phoridae) [25].

Several other parameters like, habitat, or time of year, influence the composition of the species community of a corpse. This may help to identify the season of the year when death occurred, even months and years post-mortem, or demonstrate the post-mortem transport of a corpse from, for example, the city into a forest after first colonization. Hence, succession patterns can provide important insight into an investigation. However, as the speed of decomposition is influenced by many internal and external parameters, the estimated $PMI_{min}$ window can be so large as to be of limited value, lacking in statistical validation. Nevertheless, succession may be the only tool available to give some idea of a $PMI_{min}$, in cases where PMI is longer than several months or even years. Anderson [23] gives an excellent overview of the factors which influence insect succession on carrion. As these factors are diverse (e.g. wrapping, scavenging, burying, effects of season and sun exposure) and because each scene of crime is unique, there is a great need for further research to develop a better understanding of these processes. To date, most studies have simply demonstrated that patterns of insect assembly do exist, and that these patterns change under different conditions; however, according to VanLaerhoven [26], "*asking if patterns exist in nature is akin to asking if bears shit in the woods.*" What we need is a better understanding of the mechanisms that influence and modify community assembly [26], otherwise a prediction of $PMI_{min}$ from insect succession on carrion remains on shaky ground. Recent studies [e.g. 27–29] have made significant advances towards improving the forensic evaluation of insect succession on carrion.

## Myiasis

Myiasis has been defined as, "the infestation of live human and vertebrate animals with dipterous larvae, which, at least for a certain period, feed on the host's dead or living tissue, liquid body-substances, or ingested food" [7]. It can be a cause of significant economic loses and a major animal welfare issue for the livestock industry, affect wildlife and also be a problem in human health, causing significant pain and suffering and, in extreme cases, death [30, 31] (Fig. 3). Myiasis can be considered in three main categories, according to the degree of parasitism of the host:

- Obligate parasites—these can only develop on the living tissues of living hosts and do not develop on carrion.
- Facultative parasites—these usually develop on carrion but they can also develop on living hosts, in which cases they usually require some predisposing condition, such as neglected wounds with necrotic tissues, or bacterial growth in soiled fur or fleece.
- Accidental parasites—these can cause minor health problems but are not normally parasitic, being found in host tissues by accident, e.g., after ingestion or inhalation.

**Fig. 3** Collecting larvae from cases of interdigital myiasis in a Greek sheep flock, predisposed to myiasis because of bacterial foot-rot. Close-ups of two cases illustrate the "head-down" feeding behaviour of larvae. Most larvae are of the fleshfly *Wohlfahrtia magnifica* (Diptera: Sarcophagidae), but *arrowed* in the *lower-left* close-up is a secondary infesting larva of the blowfly, *Chrysomya albiceps* (Diptera: Calliphoridae). (Image Martin Hall, copyright Natural History Museum, London)

The most significant group of myiasis-causing flies for forensic entomology are the facultative parasites, which can be associated with cases of extreme neglect, both of humans and animals. The species involved are typically blowflies (Calliphoridae) or fleshflies (Sarcophagidae), usually the same species that are involved as the primary indicators of $PMI_{min}$. However, other fly families can also be involved [32, 33].

In veterinary cases, the role of the forensic entomologist is to determine the period of infestation of livestock or pet animals in cases of cruelty or neglect. In some countries farmers can be prosecuted on animal welfare grounds for having livestock with cases of clearly neglected myiasis. It can be difficult to produce conclusive evidence of neglect based solely on clinical signs, but forensic entomology can provide powerful, scientifically validated evidence of the period of neglect or abuse to strengthen a prosecution. The role of forensic entomology in the prosecution of neglect of domestic animals is illustrated by a case from the UK (M. Hall, 2007, unpublished). The stage of development of *Lucilia* larvae removed by a veterinarian from a severe wound on the left hind foot of a dog indicated that it had

been injured a minimum of 30–35 h beforehand, rather than on the day that it was taken to the vets as the defendant initially claimed. Faced with this and other evidence of neglect, the defendant changed his plea from not guilty to guilty of cruelty and was sentenced accordingly. Anderson and Huitson [34] discuss in more detail the strong potential for forensic entomology techniques to assist in determining the period of neglect or abuse of animals.

In a forensic context, human infestations are most common in the young, the very elderly or debilitated, or others who are unable to respond to infestations or maintain a high level of personal hygiene, especially at sites of wounding such as ulcers and bed sores [35, 36]. Cases of personal neglect and a setting of high fly density can also give rise to human myiasis, with homelessness, alcoholism and peripheral vascular disease reported as other cofactors in US cases [36]. Similar to alcoholism, the influence of drugs can also make humans self neglect and become unaware of flies settling and ovipositing on uncovered wounds [37]. Infected wounds or necrotic tissues are a good food source for the growth of fly larvae.

A case from the UK illustrates the potential for myiasis to accompany neglect in a domestic situation. On admission to hospital, a male infant aged 13 months was found to be passing large numbers of live third instar larvae of the common housefly, *Musca domestica*, from his bowels (K. Ugonna, J. Wales, M. Hall and N. Wright, 2004, unpublished). It is highly likely that the larvae, aged 3–4 days, entered the rectum to feed on faeces after hatching from eggs laid in his soiled underwear and around his anus. This infestation was just the tip of the iceberg of neglect for this child and his three siblings, but it was a part of the evidence submitted to court that led to his parents being convicted of cruelty and sentenced to 7 years in jail.

While neglect can occur in a domestic situation, it can also occur in a hospital and be indicative of poor standards of health care, standards that might lead to litigation (see [18], pp 168–169). In cases of hospital acquired infestations, termed nosocomial, the primary objective of the forensic entomologist is to establish the minimum period of infestation. An example is given by Hira et al. [32] of the discovery of a nasal infestation of a young, comatose boy in the intensive care unit of a hospital in Kuwait with 2–3 days-old larvae of *Lucilia sericata* (Diptera: Calliphoridae): the boy had been admitted 4 days earlier, therefore, the infestation must have been acquired within the hospital. Smith and Clevenger [38] reviewed six similar cases of nosocomial nasal myiasis in the USA, in which *L. sericata* was involved in five. Mielke [39] reviewed 23 cases of general nosocomial myiasis and listed the major predisposing conditions as: (1) access by flies (e.g. due to unscreened windows); (2) poor wound dressings; (3) patient's mental/physical functions impaired.

Sometimes the neglect might simply be due to ignorance of the potential for myiasis, rather than to a deliberate act of negligence. For example, cases of myiasis of elderly nursing home residents in Hong Kong caused by Old World screwworm fly, *Chrysomya bezziana* [40], appeared to be due to the relatively recent increase in the population size or distribution of that species [41], bringing the flies into contact with susceptible human hosts for the first time. Seven of the eight cases reported were in bedridden patients with advanced dementia and five had oral infestations as a result of poor oral hygiene associated with feeding tubes. The authors conclude that these cases highlight the need for education of nursing staff as to the importance of wound and dental care of the institutionalized. Clearly, in a litigious society this is crucial.

Establishing the period of infestation in cases of myiasis uses exactly the same techniques as are used in estimating the $PMI_{min}$ of dead bodies. Determining the temperatures to which the larvae were exposed in their development is crucial. In living humans and animals these temperatures clearly tend to be higher than are found on a corpse, other than where there is a large larval mass. Normal human body temperature is in the range 36.1–37.8°C [42], but nostril temperatures tend to be lower, about 30°C [43].

## Entomotoxicology

### Detection of drugs in insects

The potential use of insects as alternative samples for detecting drugs and toxins has been well documented in the literature [44–49]. Fly larvae and pupae are often found on decomposing bodies and in their surroundings long after tissues traditionally sampled for toxicological analyses, such as blood, urine or solid organs, have disappeared. In such badly decomposed bodies, these immature stages and their remnants are not only useful for estimating the $PMI_{min,}$ but they can also be used as a reliable substrate for toxicological analysis, and can sometimes provide a more suitable biosample without any decomposition interference [47]. Most of the substances involved in drug-related deaths are detectable through analyses of maggots: opiates such as morphine and codeine, cocaine and benzoylecognine, amphetamines, tricyclic antidepressants, phenotiazines and benzodiazepines, steroids, barbiturates and several salicylates such as paracetamol [46, 48, 49]. Drugs and toxins have also been detected through analyses of empty puparial cases [50] and even beetle exuviae and fecal material [51]. Some studies have demonstrated that drugs could be detected in larvae but not in associated soft tissues [52, 53]. However, the absence of a drug from feeding larvae does not necessarily imply that it is not present in the food source.

A drug or toxin can be detected in larvae when its rate of absorption exceeds the rate of metabolism, but it is not yet known exactly how larvae accumulate or metabolize drugs, and how these affect larval development. Bourel et al. [54] demonstrated morphine accumulation inside the cuticle of *Calliphora vicina* maggots. In the same area, lying between the endocuticle and exocuticle of *Chrysomya albiceps* larvae, Alves et al. [55] found an intense immunoreaction positive for cocaine. Based on such findings, Diptera larvae are certainly useful as qualitative toxicological specimens, but they are still of limited quantitative value. Several earlier studies suggested that a correlation between concentrations of drugs and toxins in larvae and the tissues on which the specimens had fed might exist, particularly for opiates and cocaine [56], while other authors showed no relevant correlation [47]. In all reports, the concentration of xenobiotics was significantly lower in the larvae analysed than in the tissues they used as a food source. Such a toxicological finding was confirmed by a comparative study [57], where concentrations in post-feeding larvae were significantly lower than those in actively feeding maggots. Several authors [56, 58, 59] have noted that insects metabolize and eliminate the substances ingested during their development. However, the wide variations observed show that the accumulation of a drug by larvae is unpredictable and quantitative extrapolations are unreliable [58]. This may result from internal redistribution of the drug through the body after death, also explaining why drug concentration varies depending on the tissue and site-to-site variability in the same organ [53]. Potentially, this can have major implications on $PMI_{min,}$ even on non-intoxicated tissues, e.g., Kaneshrajah and Turner [60] found that the development rate of *C. vicina* maggots on pork liver is faster than the rate on other tissues such as lung, kidney, heart or brain.

Drug induced changes in the development rate of necrophagous insects can be large enough to significantly alter PMI estimates, leading to significant errors if overlooked and not taken into account during a death investigation. Some drugs can delay the colonization by insects for several days as observed with malathion by Gunatilake and Goff [61] and with flunitrazepam-ethanol-mix by Fremdt et al. [62]. Drugs may also influence the development of the necrophagous insects reared on contaminated tissues and, therefore, any modification of growth rate would bias the $PMI_{min}$ estimates [63]. For example, larvae of the sarcophagid fleshfly, *Boettcherisca peregrina*, developed more rapidly on tissues contaminated with cocaine and heroin than on uncontaminated tissues [64, 65]. A significantly accelerated growth rate was also observed by Bourel et al. [66] for *Lucilia sericata* larvae feeding on tissues contaminated with morphine and by O'Brien and Turner [67] for *Calliphora vicina* larvae feeding on tissues contaminated with paracetamol. Diazepam also appeared to affect the size and shape of *Chrysomya albiceps* puparium [49]. Such studies demonstrate the risk of calculating an incorrect $PMI_{min}$ due to the drug modified rate of development of the immature stages [63] which can vary from a minimum of 18 h up to 96 h depending on the drug, the fly species, and the stage of development. Therefore, all reasonable steps must be undertaken to perform as comprehensive a drug screen as possible in bodies where there is a suspicion that the death may be drug-related [52].

It is noteworthy that remnants of insect evidence, such as fly puparia, may remain at the scene for several years; these can retain drug residues, detection of which could help to resolve the history and identity of the dead body which was the source of food for the larvae that gave rise to the remnants.

## Molecular analysis of insects

### Species determination

Crucial for any evidence in forensic entomology is the association of development data to a certain species [6]. However, species identification on a morphological basis may be hampered by the lack of taxonomic experts who are able to differentiate species using morphological characters, especially in the early larval stages. Within many genera (e.g. *Sarcophaga*), discrimination of individual species is not possible from larval morphology alone. Rearing to adults would facilitate determination (at least of male *Sarcophaga*), but this can be time consuming and might not be possible due to rearing difficulties or because the larval evidence is presented dead. Therefore, molecular techniques as alternative tools for identification of forensically important species have been established within the last decade [68, 69], based on species specific nucleotide sequences of certain genes. The gene for subunit I of the mitochondrial encoded cytochrome oxidase (CO I) is well established for this purpose and reference sequences are available for a large variety of species, including blowflies and other groups of forensic interest via Genebank [http://www.ncbi.nlm.nih.gov/]. In addition to aiding with the identification of whole organisms, an analysis of insect fragments or empty puparia is possible using sensitive molecular techniques [70].

The common way to analyze the nucleotide sequence is a three step procedure: (1) extraction of DNA, (2) amplification of the gene of interest (in most cases CO I) and (3) determining the nucleotide sequence of the generated PCR product. Usually sequences of 350–650 bp are sufficient for analysis.

The quality of the extracted DNA is a critical parameter, heavily affected by the storage conditions of the insect specimen. Good results can be expected using ethanol (70–100%) preserved specimens, or completely dried material, such as pinned adults. Storage conditions are crucial because DNA may be degraded by the influence of microorganisms that grow in humid conditions. The DNA can be extracted after years of storage, as described using classical methods like phenol–chloroform extraction or commercially available extraction kits. However, ethylacetate or formaldehyde must be avoided for killing and preservation because these substances may damage DNA.

After PCR amplification of the target gene, analysis of the nucleotide sequence can be easily achieved using commercially available kits, followed by capillary electrophoresis and subsequent data analysis. If the analyzed nucleotide sequence of the specimen of interest matches a reference sequence, usually this leads to identification of the species in question. However, it cannot be excluded that closely related species may exhibit a very similar nucleotide sequence, which may hamper an unequivocal species determination. Therefore, extensive knowledge of all relevant species is mandatory. Examples for this are CO I sequence data from *Lucilia caesar/L. illustris* or *Chrysomya megacephala/C. saffranea* [71, 72]. In these cases extended sequence analysis may be helpful.

Within this context the occurrence of intraspecific variation of the nucleotide sequence must also be taken into consideration, often associated with geographical isolation. Variability of more than 1% can be observed in certain species (e.g. *L. illustris* [71], and Zehner, unpublished data). By assuming a reliable species differentiation in case of more than 3% sequence differences even differences of 1–3% between the sequence of a specimen in question and a reference sequence does not necessarily mean that two different species are present.

Nevertheless, this technique leads to a maximum amount of genetic information, while alternative techniques like RFLP analysis presents only data concerning a small area of the nucleotide sequence i.e. the restriction site. Single nucleotide changes affecting these restriction sites lead to altered restriction patterns and may end in a false exclusion. Therefore, sequence analysis is highly recommended to achieve a reliable species determination, which is essential in a forensic context [71].

In addition, due to biological complexity unanticipated observations can be made. Two examples are presented below:

1. Blowflies originating from Hawaii which were morphologically identified as *Lucilia cuprina* showed a CO I sequence which assigned them to *L. sericata*. However, the sequences of nuclear encoded 28 S rRNA did show a *L. cuprina* type. This result may be due to a hybridization event. This is not solely a problem for island populations, as similar observations have been made in South Africa [73, 74].
2. It has been demonstrated that cytochrome oxidase sequences based classification of flies of the genus *Protocalliphora* cannot be performed reliably in *Wolbachia* infected individuals. Although this has not been described as a phenomenon in forensically relevant insects, it has to be kept in mind that a CO I sequence has to be interpreted with caution [75].

Despite potential problems, sequence analysis as an identification tool has become indispensable. Projects like the international DNA barcoding project where a unique CO I sequence, the so-called "barcode," is assigned to every species, emphasizes the general importance of this method (http://www.boldsystems.org).

## Detection and typing of human DNA within insect larvae

Examination of human DNA from the gastrointestinal tract of maggots is a further molecular approach in applying forensic entomology to rare cases [76]. This kind of analysis is recommended if the food source of the larvae sampled at the scene is in doubt, e.g. there may be an alternative food source near the body which the larvae may have fed on. Another scenario is that in which larvae are found but no corpse is present. The detection of human DNA in larval guts will indicate that a decomposing body was previously in that location but has subsequently been relocated, e.g., where larvae are found in the boot of a car known to have been used to transport a body, but the suspect claims they were just escaped fishing bait. If necessary, the identity of the person can be determined by forensic STR typing, by comparing it with existing genetic profiles [77, 78]. This kind of analysis can also be undertaken on non-feeding stages, e.g., sheep specific DNA were detected within 2 days old pupae of *Calliphora dubia* [79].

## Gene expression studies

In addition to DNA sequence analysis, a potential future tool is indicated by gene expression studies, which can help determine the age of developing individuals. Because the gene expression pattern is dependent on the age of the maturing insect, the monitoring of an increase or decrease of certain gene products, which are essential in specific development stages, can give a time scale for age estimation. This is performed by determining the amount of mRNA of specific genes through RNA extraction, reverse

transcription into cDNA and quantification of this cDNA, e.g., using a real time PCR system.

Age estimation of premature individuals is of special importance for pupae because an age dependent change in size and weight cannot be used for age estimation—pupae do not change in size during development, and furthermore, the pupal period lasts approximately 50% of the total time span of development. However, puparia can be opened to observe the physical changes associated with development of the pupae within, which correlate with age.

After extensive and pioneering development studies on *Drosophila*, necrophagous flies became a subject of this research. Tarone et al. [80] investigated the expression pattern of three genes by analyzing eggs of *L. sericata* and could make predictions for their age due to altered expression rates. Age dependency of gene expression had also been demonstrated in pupae for several gene products [81].

## Sampling and evaluation of insect evidence

For a professional, high quality entomological examination and analysis, sampling should follow strict standards or guidelines [1, 82].

### Where to collect

Colonization of different body regions can occur in a different sequence. The first areas of a body to be colonized are the natural orifices (eyes, nose, mouth, anal or genital area). Any other mass occurrence of larvae in the early stages of decomposition may indicate a wound site. Different orifices and body regions of a single corpse could be randomly infested by different species, which therefore, should all be examined and sampled. If the corpse was wrapped or enclosed (e.g. in a carpet or bag), these wrapping materials, as well as the clothes of the victim, should be checked for insects.

As immature insects generally disperse away from a body after feeding (e.g. for pupation), it is necessary to search the area around the body intensively. In the field, the leaf litter and soil underneath and surrounding the body should be searched for insects to a radius of between 2 and 10 m, depending on the soil type. In indoor scenarios, migrating larvae or pupae may be found under and/or in carpets, mats or rugs, and furniture e.g. couches, cupboards, chests and possibly under baseboards or floorboards. Any crime scene should be checked carefully for nutrient sources other than the corpse (e.g. animal cadavers, organic waste) as these may give rise to contaminating insect evidence.

### What to collect and how to store

Several soil samples should be taken to a depth of at least 10 cm. Storage at cool temperatures (e.g. in a fridge at 4°C) is recommended until they are examined, to prevent further development of most species [1].

Insects of every type, size and shape (e.g. eggs, small and large larvae, pupae and adults) should be collected, using different vials for each type of insect and sampling site. The sampling should not be restricted to just the largest larvae or putative oldest stages: small larvae of one species could be older then large larvae of another species. It is also important to collect empty puparia, as they clearly indicate the completed development of at least the first "wave" of colonization.

According to Amendt et al. [1], sample size will vary depending on the number of larvae found, but as a rough guide, it should range from all of the larvae, where fewer than 100 are available, to 1–10% of the larvae, where thousands are available. Adult or immature specimens that are already dead on collection should be stored in 70–95% ethanol.

If there is the possibility to collect specimens alive, storage should take place in vials kept at cool temperatures (e.g. in a fridge at 4°C). The vials should allow entry of air but prevent the escape of maggots. Coarse sawdust or tissue paper will help to absorb fluids produced by the maggots. All living samples should be transferred to an expert for rearing within 24 h.

Remaining specimens should be killed as soon as possible. It is recommended to kill fly larvae in very hot (>80°C) water [17] and beetle larvae at extremely cold temperatures, i.e. in a freezer at −20°C [83]. The use of 70–95% ethanol for preserving dead specimens is recommended. Formalin/formaldehyde should be avoided for health and safety reasons and because storage of samples in them will increase the chances of degraded DNA.

### Additional data required

A general description (ideally photographic documentation) of the condition of the corpse as well as the ecology of the scene will be helpful. It is essential to record the ambient temperatures at the scene of crime and to obtain weather data for the area of discovery of the body from the nearest meteorological station for the period from last sighting of the deceased up to discovery of the body. If possible, the hourly temperature for the 5–10 days after discovery should be collected at the position of the corpse [1]. This will enable an estimation to be made of the temperatures at the scene of crime prior to discovery of the body, a necessary step for establishing the period of

development of the insects at the scene (see above *Modelling of crime scene temperatures*).

## Limits and pitfalls

Forensic entomology is still the most reliable method for establishing the minimum time since death in the first weeks post-mortem. Longer periods may be estimated up to the season or even the month of the year, i.e. not as precise as in the early weeks.

Several parameters can lead to a delay in colonization (e.g. the wrapping of the corpse, low temperature, rain, burial, or the inactivity of most flies at night) which can lead to an underestimate of $PMI_{min}$. Forensic entomologists should be aware of the possibility that the scene of discovery of the body might not be the scene of death, nor even where the body was first placed after death. So the thermal history of any corpse, and of the insects developing on it, can be extremely complex.

Larval-generated heat, produced by tens of thousands of larvae feeding gregariously on a cadaver, must be considered as a potential factor influencing larval development. Values for larval masses of up to about 25°C above ambient temperature can be measured in extreme scenarios [14]. It is still under debate how to handle this fact. As the development of ectothermic organisms like insects are driven by the microclimate temperatures they experience, there might be the need to reconsider larval-generated heat when calculating the $PMI_{min}$. There are several ways in which larvae could regulate this thermal stress, e.g. by migration or evaporative cooling; and it is almost impossible to account for all these factors in determining a $PMI_{min}$. For reasons of clarity, Villet et al. [14] emphasize the need to measure the temperature of the larval mass on site, recording the location and temperature of each sample, also sampling larvae from smaller aggregations that generate less heat. The simplest tools for non invasive surface temperature measurements are hand-held digital infrared thermometers. Forensic entomologists and pathologists should also be aware that heat generated from larval aggregations can reduce the cooling effect of mortuary refrigeration units. Therefore, even in such mortuary coolers, larval masses could continue to consume body tissues and destroy other physical evidence. Therefore, corpses heavily colonized by larval masses should be considered a matter for urgent forensic pathology examination, and the autopsy should be performed as soon as possible [3].

The effect of drugs and toxins on the rate of Diptera development should also be considered when using maggots for $PMI_{min}$ determination (see entomotoxicology section).

As previously stated, the period of insect activity does not always correspond to PMI [1]. In this respect, myiasis can be a significant point of confusion, because the period of insect activity could be far longer than the actual PMI [84]. The possibility of a pre-mortem myiasis infestation must always be borne in mind by forensic entomologists, as well as by forensic pathologists faced with insect evidence collected from a dead body. If the infestation on a body was initiated before death, then assuming incorrectly that it started after death would clearly lead to an inaccurate estimate of $PMI_{min}$ (see [84] for examples). The difficulty with determining if infestations are unequivocally pre- or post-mortem is highlighted by three cases discussed by Benecke et al. [85].

Intravital and post-mortem insect activity may modify the corpse in ways that can be frequently misinterpreted as ante-mortem inflicted injuries. Ants, for example, are opportunistic feeders on fly eggs and maggots, but may feed on the cadaver itself. Drying of the post-mortem ant bites can give the impression of friction, cigarette or chemical burns [86].

Another potential source of error in determining $PMI_{min}$ is the fact that laboratory derived data might just reflect the developmental biology of the local population from where the culture of flies was initially established. An important question is "*how well do those developmental data relate to populations of the same species, but living hundreds or thousands of miles away?.*" Potential variation in developmental time for geographically distinct populations is now receiving attention [15]. Reference data from different studies present different developmental rates for the same species [87, 88]. Differences in ADH/ADD calculations for the most commonly encountered blowflies in forensic entomology have been observed [1] as great within publications as between publications mainly due to geographical population but also experimental methods.

Furthermore, Catts [2] and Greenberg [89] warned about the false perception of accuracy each model can give, when calculating $PMI_{min}$, due to the complexity of integrating all factors and parameters (diet, diapause, competition, maggot-generated heat, etc.) affecting insect development into a single algorithm. According to Villet et al. [14], "*assessing the interactions amongst their varying precisions is even more of a Gordian knot.*" These examples highlight the need for a careful interpretation of the evidence, keeping in mind that every case has its own, very specific history. Determining the PMI is extremely difficult, and precision is impossible, even by using the entomological method, due to a wide biological variability.

## Outlook

The majority of published data dealing with the time of development for different forensically relevant species

were produced in the laboratory. It might be questioned whether these data sets always mirror the reality in the field. Competition, stress and other, so far, neglected parameters (e.g. humidity), could explain some of the observed variations or deviations in development patterns and should be considered when designing further studies. There is still a need for new tools in forensic entomology [81, 90], and several taxa remain largely unexplored [91, 92].

Using development and succession studies in the court room needs a serious statistical background; unfortunately, forensic entomology still suffers from a lack of statistical confidence. However, this is now slowly changing [14, 93, 94]. Accompanying efforts in quality assurance and accreditation [26, 63] will lead to a higher level of acceptance of forensic entomology in forensic science and the courtroom.

## Key points

1. A Forensic Entomology analysis gives an estimate of the period of insect activity on the body. This equates to an estimate of minimum time since death or minimum post-mortem interval.
2. A quantitative collection of insect evidence and climatic data is mandatory for an accurate and reliable report.
3. Using insect development and succession studies in court can provide a reliable estimate of minimum time since death but needs rigorous statistical support.
4. DNA sequence analysis (e.g. by barcoding) is a very powerful tool, especially for the youngest developmental stages of insects which can be difficult to identify to species level by morphological techniques.
5. Toxicological and molecular examinations of insects found on a corpse may help reveal the cause of death, the identity of a victim, or even link a suspect to a crime.
6. Some fly species can infest living humans and other vertebrates, causing the disease myiasis. Analysis of larvae in such cases can demonstrate the period of neglect of humans or animals; this possibility of a pre-mortem infestation by flies must always be borne in mind when stating a minimum time since death in a forensic report.

## Appendix

CME questionnaire

1. The term “myiasis” means:
   - ☐ maggot therapy
   - ☐ period of insect activity
   - ☐ post-mortem interval
   - ☐ Dipteran parasitism of living vertebrates
   - ☐ investigation of homicide
2. Forensic toxicologists can detect and identify all of the following substances in immature insect specimens:
   - ☐ barbiturates
   - ☐ cocaine
   - ☐ opiates
   - ☐ benzodiazepines
   - ☐ all of the above
3. Which development model/s is/are regarded as the most sophisticated model when estimating $PMI_{min}$?
   - ☐ Thermal Summation Models
   - ☐ Isomorphen diagram
   - ☐ Isomegalen diagram
   - ☐ Isomorphen diagram and Isomegalen diagram
   - ☐ none of the above
4. Which development model/s is/are used to estimate PMImin from the size (length or weight) of blowfly larvae?
   - ☐ Thermal Summation Models
   - ☐ Isomorphen diagram
   - ☐ Isomegalen diagram
   - ☐ Thermal Summation Models and Isomegalen diagram
   - ☐ none of the above
5. Which factors may delay or prevent the insect colonisation of a cadaver?
   - ☐ pollen circulating in the air
   - ☐ heavy rain
   - ☐ cold temperatures
   - ☐ bleeding wounds
   - ☐ noise
6. What additional data (beside the insects itself) are NOT required for an appropriate entomological report?
   - ☐ temperatures from the scene of death
   - ☐ temperatures from the nearest weatherstation
   - ☐ stomach content of the deceased
   - ☐ soil samples
   - ☐ pictures/description of the scene of death
7. An old man, in need of care, died approx. 24 h before his discovery. Which of the following findings could indicate negligence?
   - ☐ insect infestation of the anogenital-area solely, maggots 10–15 mm in size
   - ☐ insect infestation of the face (eyes, nose) solely, just eggs
   - ☐ no insect infestation

- ☐ several dead flies on the windowsill
- ☐ many living flies on the walls, windows and the ceiling lamp

8. On the unused area of an old waste yard the skeletal remains of an unknown man were discovered. In the soil beneath the corpse numerous empty puparіae of the blowfly *Calliphora vicina* were found. Which of the following examinations are reasonable?

   - ☐ a DNA-analysis of the empty puparia will help to reveal the identity of the unknown man
   - ☐ a toxicological analysis may help to answer the question of a possible intoxication or drug overdose
   - ☐ as we know the species of the blowfly the estimation of the time since death right to the day is possible
   - ☐ as we know the species of the blowfly it is possible to answer the question of a post mortal relocation of the body
   - ☐ the distribution pattern of the puparia in the soil clearly indicates a high loss of blood

9. During road works, roadmen discover one male and one female corpse at a depth of approximately 1.5 m. While the female body shows a heavy infestation by blowfly larvae, the male cadaver is colonized just by some scuttle flies and a few soil organisms like mites. Which of the following assumptions is reliable from an entomological point of view?

   - ☐ as the insect colonisation of a corpse is sex-related these differences are quite normal
   - ☐ the female body was buried in spring, while the male corpse was buried in late summer
   - ☐ the female body was infested by maggots prior to her burial
   - ☐ the bodies were buried during night time
   - ☐ the victims were still alive when burial startet

10. How long is the maximum storage time of insects after collection to make successful DNA analysis?

    - ☐ weeks to months if conserved in formaldehyde
    - ☐ not longer than 3–4 weeks in general
    - ☐ at least years if properly conserved
    - ☐ at least years but only if killed with ethylacetate
    - ☐ DNA is a very easily damaged and has to be analyzed immediately

CME questionnaire answers

1. Dipteran parasitism of living vertebrates
2. all of the above
3. Thermal Summation Models
4. Thermal Summation Models and Isomegalen-diagram
5. heavy rain, cold temperatures
6. stomach content of the deceased
7. insect infestation of the anogenital-area solely, maggots 10–15 mm in size
8. a toxicological analysis may help to answer the question of a possible intoxication or drug overdose
9. the female body was infested by maggots prior to her burial
10. at least years if properly conserved

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