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REVIEW

Entomology-based methods for estimation of postmortem interval

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Abstract: Forensic entomology involves the use of insects and other arthropods to estimate the minimum time elapsed since death, referred to as minimum postmortem interval ($_{min}$PMI). This is based on the assemblage of insects found in association with remains, and most often, the time required for development of the first colonizing insects to develop to their size/life stage at time of collection. This process involves the accumulation of appropriate data for the development of the species of insect at a variety of relevant temperatures and consideration of the other biotic and abiotic factors that may affect developmental rate. This review considers the approaches to the estimation of $_{min}$PMI, focusing largely on the age estimation of specimens collected from remains and the limitations that accompany entomology-based PMI estimations. Recent advances and newly developed techniques in the field are reviewed in regard to future potential.

Keywords: forensic entomology, PMI, blowfly, decomposition, death investigation

Introduction

Forensic entomology, the use of arthropods as tools in legal investigations, primarily focuses on the estimation of the time length between death and the discovery of decomposing remains in cases of homicide, suicide, or accidental death.[1–3] Termed "minimum postmortem interval" ($_{min}$PMI), the entomological estimation of this time period is based on the assumption that insects, commonly found in association with decomposing remains, arrive at a carcass shortly after death.[4] Decomposing remains present a transient habitat and food resource opportunity for numerous insect species.[5,6] Within hours of death, insect groups such as blowflies (Diptera: Calliphoridae) are olfactorily attracted to decomposing remains which are both a source of protein for egg development and a site for oviposition.[7,8] The colonization time, development time, and departure time of the different insect species inhabiting remains are closely linked to the progression of carcass decomposition.[9] As such, the age of the oldest immature insect specimen collected from remains, in the context of expected arrival time of adult females, provides an indication of the minimum time that the decomposing remains were available for insect colonization and thus $_{min}$PMI.[10–12]

As insect development is primarily governed by temperature, where this relationship has been quantified for a species, the age of a specimen can be determined based on the level of development and the thermal history at which that specimen developed.[13] Where immature insect specimens are not present or have already completed development, as is often the case in advanced stages of decomposition, $_{min}$PMI is estimated based on the assemblage of insects associated with the remains, referred to as the predictable

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process of insect succession.[14] The insect species present are compared to known patterns of insect colonization and the time frames associated with each phase of colonization. Such estimates are not as precise as age-based estimates, but they do provide a broad time frame within which death occurred.[15] Forensic entomology is most commonly used to provide $_{min}$PMI estimates where early colonizers are still in association with remains, and thus, the fundamental issue in such cases is to estimate the age of a specimen collected from remains. This review concentrates on the methods used to achieve this, considering the strengths and weaknesses of such approaches and scope for further research and technique development.

Entomological approaches for age determination

Current approaches to age estimation are based around the species-specific time required for an immature insect to progress through developmental landmarks such as length, weight, and life cycle stage in relation to temperature. While measures of developmental duration based on length and weight are valuable, life cycle stage is a preferred landmark for age estimation due to the confounding issues of diet, competition, and application of different preservation methods for forensic specimens (shrinkage) on weight and length.[16,17] Thus, determination of specimen age is predominantly based on predetermined development data detailing the predictable relationship between temperature and insect growth for the onset and completion of each life stage of insect development.[18–20] Applicable reference data details the duration of development of specific life stages of immature insects encompassing egg, larval instars, pupation, and eclosion under a range of constant or fluctuating temperatures.[21] The application of data detailing the relationship between insect development and temperature is then used in several modeling approaches to predict insect specimen age based on the thermal history of the collected forensic specimens.

Isomorphen and isomegalen diagrams

The simplest approach is termed an "isomorphen diagram" which is essentially a scatter plot of the time from eggs hatching until eclosion plotted against constant temperature.[1,22] Associated error bars provide a 95% confidence interval for each developmental event. A slight variant, termed "isomegalen diagram", plots larval size since hatching (length, weight, or width) rather than life stages against temperature.[22] Use of size as a component within the isomegalen diagram has the advantage of greater time point resolution compared to life stage event landmarks for age estimation; however, size measures have been reported as poor indicators of age.[23,24]

Estimation of specimen age is achieved with considerable accuracy where the thermal history of the specimens examined is consistent with the constant temperatures used to generate the reference data of the diagram. Considerable error occurs, however, in the derived age estimation using this approach when the ambient temperatures under which specimens are developing on decomposing remains fluctuate over time.[22] As the majority of crime scenes within which decomposing remains are found experience fluctuating temperature conditions, a series of mathematical models have been developed which are generally more applicable and widely used.

Thermal summation model

The most commonly applied method for modeling insect development rates in a forensic context is the thermal summation model which applies a linear regression analysis to the positive relationship between temperature and development.[1] Insect development can be measured at close intervals over a range of temperatures, and where the rate of development (measured as reciprocals of development time, $1/D$) is plotted against temperature, a sigmoid-shaped curve results.[25] At temperature extremes, insect development is either slowed or completely halted corresponding to an upper and lower developmental threshold.[26] A large proportion of the relationship between temperature and development is linear between the upper and lower developmental threshold (species specific). Linear regression can thus be used to determine an x-intercept (lower developmental threshold, T_L) and inverse of the slope of the linear regression (thermal summation constant, K) which allow prediction of development time from the thermal history of a specimen.[1] Under this linear regression model, development is measured as physiological time with units of "degree days" or "degree hours", where one degree day is equal to one degree above the lower developmental threshold over either 24 hours or 1 hour, respectively. Each life stage (egg, first instar, or pupation) requires a certain amount of accumulated degree hours to develop to the next life stage and complete development equating to K.[26] Standard practice, under this method, would be to rear insects collected from a scene at a constant, controlled temperature, record time elapsed at point of eclosion, and subtract the physiological time required for laboratory development from the total physiological time required for development in this species. When used in conjunction with crime scene temperatures, the period of time elapsed between oviposition and insect collection at the scene may then be calculated.

Problematically, while linear models have the advantage of simplicity and allow estimation of lower developmental thresholds and thermal summation constants, they do not incorporate the nonlinearity observed in insect development at low and high temperatures.[27] Several alternative models have been proposed including a revised linear model[28] that calculates an improved fit for T_L and K by accounting for the high variability at extremes of the linear range and multiple nonlinear approaches[29–31] encompassing a recent "new simulation model" termed "ExLAC".[32,33]

Curvilinear models/ExLAC

Nonlinear or curvilinear models can more accurately describe the relationship between development and temperature for insect populations by incorporating the curvilinearity observed at the upper and lower temperature extremes of the plotted relationship between development rate and temperature.[27,34] While offering improved parameters for estimation, the complexity of such models, however, reduces the practicality of application to forensic estimation of $_{min}$PMI. Additionally, no one curvilinear model, above others, has been identified that consistently outperforms linear models across relevant species data.[35]

A recently proposed curvilinear model known as ExLAC has been demonstrated to offer an alternative to linear thermal summation modeling.[32,33] While the ExLAC model shows minimal performance improvement over linear modeling, it has the advantage of generating error rates associated with the age estimate derived by the model. Again, the development model is based on the duration of each life stage during development as a function of temperature.[33] An individual exponential function is, however, applied to each life stage, and additional parameters are included in the model that account for variation in measurement of input values such as thermal history data and the strength of the relationship between life stage duration and temperature.[32,33] The included measurement of error for the inputted temperature data offers a distinct advantage over the currently applied thermal summation model. Standard practice involves the use of temperature data gathered at the weather station nearest to the crime scene as a measure of the insect specimen's thermal history prior to collection.[36] This data is corrected for potential variation between locations using regression modeling incorporating data acquired at the crime scene following the discovery of decomposing remains. While this aspect of the proposed model offers a measure of potential error in estimates of $_{min}$PMI, the performance of the model is only marginally better than the linear thermal summation method for establishing specimen age.[32] To date, the ExLAC model has yet to be thoroughly evaluated and assessed prior to implementation in forensic practice, and the linear thermal summation model is still the preferred method of estimating specimen age and thus $_{min}$PMI.

Current issues

Regardless of the developmental modeling approach taken to determine age, several problems arise in regard to the available development data used in such models. Available reference data for use in forensic practice is typically focused on the development of early colonizing dipteran species. A considerable body of reference literature exists documenting the development of forensically relevant fly species under varied constant temperatures within the laboratory.[11,37–41] Problematically, reference data is not available for all species reported in association with crime scenes, particularly in the case of alterative indicators of $_{min}$PMI such as beetles and parasitic wasps. Aspects of life history and development in relation to temperature are often unknown for specimens collected off remains or of limited scope for application in development models.

Additionally, research has indicated that populations of the same species can differ physiologically depending on their geographic origin.[2,42,43] For instance, there are often considerable discrepancies in the reported development time of blowfly populations from different geographic origins when reared at the same developmental temperature.[44,45] Such differences have been attributed to regional genetic variation between populations, although the effect of differing environmental factors between regions cannot be discounted. The possibility that inherent biogeographical variation exists between populations of species in relation to development time is of considerable consequence in respect to the accuracy of $_{min}$PMI estimation. At present, development data for a species from a single source location are applied to the same species from different geographic locations, even though there is little evidence supporting the validity of such procedures and considerable evidence to the contrary.[17,44–46] At present, the applicability of extrapolating development data to a population from a different geographic origin to that of the source data is likely to be called into question under cross examination during court proceedings. Thus, further work is urgently needed to address and quantify this issue.

Further error in the accuracy of $_{min}$PMI can arise in regard to a species physiological response to fluctuating temperature regimes, a common occurrence at crime scenes. Thermal summation assumes that a species development rate at a given

constant temperature is independent of the overall thermal regime.[47] A number of studies, however, have indicated that development rate under fluctuating temperatures does not correspond to development under the resulting mean constant temperature.[24,48,49] As reference data detailing the relationship between temperature and insect development is typically generated using constant temperatures, the use of this data to model specimen age for forensic estimation of $_{min}$PMI can lead to erroneous estimates where daily ambient temperatures at crime scenes fluctuate. As such, there exists a need for expanded research of species-specific development under both variable and constant temperatures to provide comprehensive reference data for use in forensic case work.

Moreover, temperature is considered the primary factor influencing insect development; however, a variety of abiotic and biotic factors additionally influence the rate of development including, humidity,[50] photoperiod,[51] and diet (nutrition).[52–54] These factors are essentially ignored in the majority of studies documenting developmental timeframes for use in forensic practice, yet are likely to have an impact on development rate and hence the accuracy of any associated $_{min}$PMI estimation. Other limitations in the currently available reference data include the lack of a standardized rearing methodology between research studies. Where development data is generated using different food substrates, photoperiods, sampling protocols (ie, sampling interval, larval density), and rearing conditions unrelated to temperature, reported development rates for a particular species often vary.[55,56] Thus, inconsistency in the methodology behind the generation of development data for use in estimating forensic specimen age can contribute to inaccuracies in the associated $_{min}$PMI estimate.

Estimates of $_{min}$PMI based on modeling of the relationship between insect development and temperature are widely accepted in criminal courts throughout the world; however, there are substantial issues regarding the appropriateness of the reference data available in the literature and used to formulate such estimates. As such, further research on aspects of forensically relevant insect growth and life history are still needed to establish a comprehensive, global, reference database of applicable developmental data for use in both linear and nonlinear models.

Recent advances in age estimation methodology

Current approaches to age estimation are generally based on measurement of the timeframes associated with the start and end of the life stage collected, such as the onset of an instar or pupation. The duration of a life stage such as the pupal stage can be considerably long, and thus, the use of life stage landmarks alone (ie, onset of pupation and eclosion) as indicators of specimen age can introduce considerable error into associated $_{min}$PMI estimates.[57–59]

Several approaches have been proposed to address this issue, and provide refined approaches to limiting the possible age range of a specimen. These range from observations of insect morphological features as an indication of age to quantitative approaches based on gene expression or hormone levels. These approaches vary in their utility, objectivity, and reliability.

Morphology

Calliphorids are the primary colonizers of carrion, and as such generally represent the oldest insects present on a corpse and perceived best indicators of $_{min}$PMI. They exhibit holometabolous development, meaning a complete metamorphosis through egg, larval, pupal, and adult stages occurs. These life stages are all morphologically distinct from each other. Within each life stage, relative degrees of development based on size or the relative level of development of certain features may be useful in characterizing the insects as having experienced a certain amount of physiological time. In the egg stage, which is relatively short, physical markers refining age estimates are less important than in the longer persisting larval or pupal stages. The pupal phase alone may comprise >50% of the total life cycle of the insect;[57–59] thus, the ability to refine an age estimate from a 2-week window, down to perhaps a 24- to 48-hour window, is highly desirable. Current approaches to pupal aging rely frequently on rearing to adult stage,[60] at times a lengthy process and sometimes compromised by insect death due to the presence of hymenopteran parasitoids or other factors.

External morphology

Size measures are often proffered as useful methods to refine insect age within the larval stage, utilizing width, length, or weight of larvae as an indicator of relative period of development.[61] These measures are subject to numerous influences, summarized suitably by Villet and Amendt,[60] including drugs, maggot mass-generated heat, competition for food, substrate fed on, preservation, and measurement errors. These factors make the use of size measures, as used in isomegalen- and isomorphen-based methods, prone to significant error. An alternative is to concentrate on discernible developmental changes within a life stage.

Eggs, pupae, and adults display no obvious measurable size-related changes that may be utilized for age refinement.

Eggs are generally reared to hatching, and then age is derived via back-calculation of physiological time required for development. Discernible morphological features are generally lacking unless the specimen can be observed to be in the process of emerging from the chorion via the plastron. In larvae, non-size-related changes are largely confined to the posterior spiracle morphology, with instar determined based on the number of slit-like openings to the trachea in each posterior spiracle.[62] These instars are clear intra-life stage events that may be used to further refine larval age.

Adult age estimation is generally confined to the period immediately following eclosion, where wings are gradually unfurled as hemolymph moves into veins, and general body coloration of the fly develops.[62] There is little call for determining age of adult flies, given that their mobility makes connecting them to development on a specific source of carrion problematic. The puparium left behind following eclosion is likely to provide as much information as the adult fly itself.

It is the pupal phase that necessitates the greatest age estimation refinement, given the extended duration of the stage and thus large timeframes provided for $_{min}$PMI based on the landmarks of life stage commencement and end. In the pupal stage, there are few obvious changes on the exterior of the puparium itself, with the exception of the initial tanning and sclerotization process.[62] Pupae are sedentary, and their extended close association with remains makes them useful targets for provision of temporal information regarding remains. It is for this reason that most age refinement methods concentrate on this extended stage.

The calliphorid pupa is coarctate, with the puparium formed following "loosening" of the third instar cuticle. The casing darkens with time,[63] but coloration is obviously altered with soil type/moisture level and/or preservation. Removal of the puparium, however, reveals significant morphological remodeling as the larva metamorphoses to adult form, with the development of key features such as tagmosis, appendages, setae, and coloration.[57] Examination of these features, in consultation with species-specific morphological timelines,[57] may be utilized to refine an age estimate for a specimen. Brown et al[57] utilized 23 morphological features of *Calliphora vicina* pupae to refine an age estimate to within 5% of a pupa's actual age with 95% reliability. This illustrates the utility of the technique for intra-pupal stage aging but is heavily reliant on appropriate datasets for numerous features in all species of relevance, and confirmation that between population variation is minimal, before data may be applied in other localities.

Internal morphology

Internal changes have been studied in the pupal phase of *C. vicina* for their use in age estimation of specimens.[64] Internal metamorphosis during this phase involves histolysis and histogenesis of tissues and organs during the transition to adult form, with changes such as the utilization of larval fat bodies and glycogen stores. Thoracic flight muscles become recognizable, and digestive, reproductive, and nervous systems are modified and developed.[64] These changes can be used to create a chronology of development relative to physiological time, and utilized, either alone or in conjunction with external morphological data, to provide a $_{min}$PMI estimate. Other life stages have not been considered, and future studies are needed to further develop this approach across relevant life stages.

Optical tomography

Optical coherence tomography utilizes benchtop-sized instrumentation to provide high-resolution images of samples without the need for destructive analysis. Specimens may be analyzed while still alive, remaining in the puparium and successfully continuing development and emerging in expected time frames, allowing rearing-based confirmation of age, and species identification from adult morphology. Morphological features such as brain, mouthpart, and leg development have been successfully visualized in calliphorid pupae;[65] however, the puparium limits resolution due to absorption of light and thus limits penetration of light into the insect itself. The method holds promise but requires refinement for extensive use in casework.

Hyperspectral imaging

The ability to analyze specimens nondestructively is also provided using hyperspectral imaging, a technique used in other areas of forensics[66–68] and in agriculture.[69] This method allows specimens to be analyzed either live or preserved in a noninvasive manner, utilizing the technique to provide spatial and spectral information regarding a specimen. Preliminary work assessing the validity of this approach to the analysis of entomological specimens has been conducted using pupae of *Chrysomya rufifacies* and *Calliphora dubia*. Specimens reared at 24°C and 30°C were imaged daily to provide data for predicting age, in conjunction with morphological changes. Hyperspectral imaging allowed determination of pupal age with >82% accuracy and also reliably distinguished between the two species based only on spectral data.[70] The technique has distinct advantages including the portability of the equipment required which, once developed, will allow

analysis of specimens at the crime scene. Additionally, the nondestructive and noninvasive advantages of the technique allow alternative and/or later reanalysis of evidence. Such advantages place hyperspectral imaging at the forefront of future advances in the forensic field, and there is considerable potential for the approach to be developed as a regular tool in forensic case work.[68] Problematically, the technique requires the development of a reference database for relevant species prior to implementation in case analysis. It is anticipated, however, that upon further development, hyperspectral imaging will provide a valuable approach to age estimation of all life stages of insect development.

Molecular methods

Molecular methods form a useful basis for estimation of insect age, as they are based on objective, quantitative data, not subject to the interobserver error associated with morphological methods. In theory, these should be more reliable indicators of insect age. As with morphological methods, the focus is largely on the long-lasting pupal phase.

Steroidogenesis

Steroidogenesis examines levels of ecdysteroids (polyhydroxylated steroid hormones) produced throughout insect development. Moulting and metamorphosis are triggered by such hormones, and therefore, developmental change can be measured relative to quantitative levels of specific steroids. Ecdysteroid levels were examined in pupae of *Protophormia terraenovae* based on enzyme immunoassay, and it was shown that with suitable preservation, ecdysteroid peaks could be determined between 36 and 96 hours after pupariation.[71] This may provide valuable information regarding time of pupariation, to correlate with morphological development observations; however, utility may be limited by state of preservation, for example, when samples have been frozen following collection. Furthermore, conclusions may only be narrowed to quite wide time frames. A more precise method of estimation would be useful, given that the pupal stage may be considerably long.

Gene expression

During insect development, genes are switched on and off at various times, triggering the synthesis of products including proteins. For a gene to be switched on, the DNA within a cell must first be transcribed as mRNA, called a transcript. Gene expression studies measure the level of transcription at a given point in time.[72] Measurement of the level of expression of a particular gene over time can provide a useful indication of age of a specimen, as specimens of known age can be examined to build reference data regarding levels of expression expected at various times in development.

Age estimation of *Lucilia sericata* eggs has been shown to be possible,[73] successfully aging within 2 hours based on transcript levels of three genes. The use of three genes, rather than one gene alone, allows three expression levels to be compared, providing corroboration and refining age estimations to smaller windows. Other studies[59,72] have further extended the approach to larval and pupal stages of the same species with success, providing good support for traditional morphology-based methods of age estimation. Pupae, in particular, are useful targets for gene expression-based aging, given the extensive tissue remodeling and cellular proliferation that take place during this stage.

Cuticular hydrocarbons

On the surface of the insect cuticle is a lipid wax layer of hydrocarbons that function to protect the insect from drying out, provide defense against attack by microorganisms, and may play important roles in behavioral events such as mate selection by acting as pheromones or kairomones.[74] These hydrocarbons may be either saturated or unsaturated compounds, and have been suggested to be useful in forensic entomology for a number of applications, including determining species identity, determining geographical origin of specimens, and estimating age of individual insects for the purpose of refining $_{min}$PMI estimates.[75–77]

Hydrocarbon analysis has been proven useful with forensically significant calliphorids, separating adults of *Phormia regina* according to population origin and sex,[75] and identifying empty puparia to species level.[77] The utility of cuticular hydrocarbon composition to pinpoint larval age in *C. rufifacies* determined that the method is particularly useful for post-feeding larval age estimation,[77] mirrored in a study of *L. sericata* showing distinct difference between young and post-feeding larvae.[78] This is usually limited with respect to morphological changes, so it represents a useful application for the technique. Cuticular hydrocarbons could also be used to help estimate PMI based on weathering of puparia, but this is obviously complicated by the numerous factors that may affect rate and degree of weathering.[79] Other studies have suggested that insect cuticular hydrocarbons can be used for PMI estimation for egg to 8-day-old adults;[80] however, large databases of expected hydrocarbon compositions are required for species of different ages at different locations, given that conspecific populations have been shown to be distinguishable based on hydrocarbon composition.

The possibility of extending analyses to additionally measure volatile organic compounds released by insects to estimate their age has been proposed.[81] Volatile organic compounds were collected daily throughout larval and pupal phase and analyzed using headspace solid-phase microextraction and gas chromatography–mass spectrometry, and profiles were shown to carry in quantity and composition. While promising initial results were reported for *C. vicina*, the effect of age and genetic factors, as well as environmental factors (temperature, diet, geoclimate), requires further investigation. In addition, some compounds are produced by the insects, some by the corpse itself, and some by bacteria, so the source of emission needs to be clarified.[81]

The future

Forensic entomology has become an integral technique for the forensic sciences, providing important information for the investigator regarding time of death. This is dependent initially on examination of the assemblage of insects present, followed by accurate identification, and then refinement of minimum age of insects based on knowledge of developmental rates and ambient temperatures to calculate physiological time. This entire process is limited greatly by lack of data regarding developmental rates in the plethora of decomposition environments that may be encountered, including burials, wrapping, enclosure in vehicles, and aquatic submersion, plus the numerous other complicating variables including drug presence in remains, clothing, rainfall, and humidity. Of all the variables requiring consideration in $_{min}$PMI estimation, temperature is fundamental and is the best studied factor, but there remains a need for significant study into the effect of other variables.

The current methods utilized for $_{min}$PMI estimation such as thermal summation and isomegalen/isomorphen diagrams suffer limitations, and as such, several new approaches have been suggested and preliminary work undertaken. But ultimately, entomology is a locality-specific science, and techniques must be thoroughly examined for reliability and utility in all geographic regions of use with all potentially encountered species. Currently, work has focused largely on calliphorids as the first colonizers, but even data gathered for these species requires consideration of numerous biotic and abiotic factors to ensure it is reliable and repeatable and thus suitable for use in legal proceedings. Ultimately, the most reliable methods for estimation will likely be corroborated using multidisciplinary approaches, for example, the use of gene expression, and external and internal morphology to estimate the age of a pupa in conjunction with ExLAC or thermal summation.

In recent times, a number of approaches to age estimation of insect evidence have been proposed and assessed in preliminary validation studies that appear to offer considerable advances in the accuracy of age determination. However, all require substantial development prior to application in standard investigative practice of forensic cases. Further research and establishment of relevant reference data for application is required and warranted in the case of techniques such as hyperspectral imaging, gene expression, and ExLAC.

There are numerous factors that will induce error into $_{min}$PMI calculations, and fundamental to all conclusions is an awareness, and acknowledgment, that biological data, and organisms themselves, are subject to variation. It is unlikely that a one-size, easy-answer approach will be applicable, as each life stage has different features, and each case introduces a new range of challenges, whether they are factors affecting the development of the insect at the crime scene, altered succession patterns, or human-induced challenges such as preservation errors or damage of specimens. A flexible, but scientifically sound and well-tested approach, that encompasses variables as much as possible, but acknowledges shortfalls, is the model that forensic entomologists are ultimately working toward in the provision of $_{min}$PMI estimates for forensic purposes.

Disclosure

The authors report no conflicts of interest in this work.

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