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Various methods for the estimation of the post mortem interval from Calliphoridae: A review

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Abstract Insects play a fundamental ecological role in the decomposition of organic matter. It is the natural tendency of sarcosaprophagous flies to find and colonize on a food source such as a cadaver as a natural means of survival. Sarcosaprohagous fly larvae are frequently encountered by forensic entomologists during post mortem investigations. The most relevant colonizers are the oldest individuals derived from the first eggs deposited on the body. The age of the oldest maggots provides the precise estimate of the post mortem interval. With advancement in technology, various new methods have been developed by scientists that allow the data to be used with confidence while estimating the time since death.

Forensic entomology is recognized in many countries as an important tool for legal investigations. Unfortunately, it has not received much attention in India as an important investigative tool. The maggots of the flies crawling on the dead bodies are widely considered to be just another disgusting element of decay and are not collected at the time of autopsy. They can aid in death investigations (time since death, manner of death, etc.). This paper reviews the various methods of post mortem interval estimation using Calliphoridae to make the investigators, law personnel and researchers aware of the importance of entomology in criminal investigations. The various problems confronted by forensic entomologists in estimating the time since death have also been discussed and there is a need for further research in the field as well as the laborator. Correct estimation of the post mortem interval is one of the most important aspects of legal medicine.

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Contents

1. Introduction

Entomology is the study of insects and the related arthropods (crustaceans, spiders and so on). When this science is used for aiding in legal investigations, it is called forensic entomology. The most visible type of forensic entomology is used in the investigation of death, abuse and neglect cases.[1] The most important contribution of this science is the estimation of the post mortem interval or time since death. Many types of information can be extracted from the study of arthropods at crime scenes. Suspects have been linked to a scene by the presence of arthropods. The role of a forensic entomologist in a crime investigation can be a major one. His/her role is to collect and identify arthropod specimens and then interpret findings in relation to environmental variables.

Forensic entomologists can provide an objective estimate of time since death as well as other valuable information concerning the circumstances surrounding the victim's demise, including the season of death, location of death, movement or storage of remains after death, specific sites of injury on the body, post mortem artefacts on the body, use of drugs, and can even provide information for linking a suspect to the scene of a crime, to a child neglect or sexual molestation case, as well as in the identification of suspects.[2] Calliphorids have been known to cause myiasis and may be involved in the mechanical transmission of diseases. Myiasis is generally produced by the deposition of eggs or larvae by the female flies on the bodies of persons or animals, usually in body openings or in sores or wounds.[3]

Medico-legal entomology deals with the use of insects or other arthropods associated with a corpse at a murder scene in a legal investigation to provide data not available by using the normal methods of classic pathology.[4] Determination of the post mortem interval is a crucial and fundamental step in any death scene investigation when the death has not been witnessed. Estimation of the post mortem interval is defined as the length of time between death and corpse discovery. At the onset of death, the medical parameters to establish the cause, manner, and time since death begin to degrade. With the progression of time and soft tissue decomposition, a post mortem determination by a pathologist or medical examiner becomes more difficult and less accurate.[5]

Forensic entomology is now an integral part of a death investigation when estimating the time since death beyond 72 h. Forensic entomology is considered the most accurate method for estimating the elapsed time since death, particularly when more than 3 days have elapsed.[6]

Forensic entomology was successfully utilized in the most famous Buck Ruxton case in 1935 in UK.[7] Since then, the importance of forensic entomology has increased dramatically. The credit for the first entomology case goes to the French doctor Bergeret, who used forensic entomology to estimate time since death in 1855.[8] Probably the best study on insects and their relationships with decay rates was reported in 1958 by Reed.[9]

The use of insects to investigate cases of wrongful deaths has increased dramatically in the recent years. Blow flies are among the first insects to discover and colonize on human remains. The larvae of blow flies are also used extensively in forensic entomology, predominantly to establish the minimum time elapsed since death.[10]

In medico-legal death investigations one of the most critical question is, "When did the death take place?" An accurate estimation of the post mortem interval has special relevance in a homicide case because this information can narrow the field of possible suspects in a crime.[11]

The application of the entomological method requires an extensive knowledge of the factors, which interfere with the processes of colonization, the development time and the decomposition of the corpses by insects. Knowledge of the distribution, biology, ecology and behaviour of insects found at a crime scene can provide information on when, where and how the crime was committed.

To take advantage of the potential forensic value of arthropods, evidence must be systematically collected and processed. In 1983, the first paper on proper collection and preservation of forensically important entomological materials was published.[12] A basic understanding of insect biology and anatomy, especially with regard to flies and beetles, shall facilitate search, recognition and collection of insect specimens for evidence. The collected eggs or larvae should be placed directly into a preservative solution. The suggested preservative fluid is KAA (95% ethanol, 80–100 ml; glacial acetic acid, 20 ml; and kerosene, 10 ml), but several different methods exist. An excellent technique for preservation is to blanch the larvae in hot (nearly boiling) water for 60–120 s, and then place the blanched larvae in 80% ethyl alcohol. It is important to understand that with soft-bodied insect larvae, a simple placement of the insect directly in 80% ethyl alcohol is not an adequate method of preservation.[13]

Forensic entomology is recognized in many countries as an important tool in legal investigations.

With advancement in forensic entomology, investigators have also considered the effects of complicating factors which have been discussed in this paper. Various methods to estimate the post mortem interval from flies (Diptera: Calliphoridae) and problems associated with it have been reviewed in this study.

2. Measures for estimation of the post mortem interval

2.1. Stages of succession

This approach takes the advantage of the succession of the arthropod species commonly observed on carrion. The succession of insect communities on carrion varies according to the geographical location. Successional analysis may be used to estimate both minimum and maximum post mortem intervals. The species typically occur in succession and respond to progressive changes of the carcass decomposition stages. The post mortem interval estimation is applicable to the remains in the more advanced stages of decomposition.[9]

Limitations: In order to apply entomological evidence to the estimation of the post mortem interval based on the stages of succession, it is essential to precisely identify the species of insects attracted to the remains. Secondly, the succession patterns are typical for seasonal periods.

2.2. Age-dependent changes in the intestinal contents

Insect life cycles act as precise clocks, which begin within minutes of the death. These six legged insects are attracted by the odour of the decaying corpse. The calliphorids arrive within few minutes and the egg hatches to form the first instar. It undergoes moulting to form the third instar, which then enters the wandering stage. The radiological examinations of the feeding behaviour of the maggots reveal that maggots stop eating immediately upon attaining their maximum length. During the course of their subsequent development, the anterior intestine always remains empty. It is reported that by studying the intestinal filling in blow fly maggots the post mor-tem interval can be estimated efficiently.[14]

Limitations: Determination of changes in the intestinal contents of maggots using radiological techniques is difficult. Such a technique for the aging blow fly larvae has not been reported for most of the species of Calliphoridae.

2.3. On stage of invasion

The entomological method is statistically more reliable and superior when compared to other prevalent methods. The time since death can also be calculated from the stage of the insect present on the cadaver by using the formula:

$$T = A + B \times C$$

where ‘A’ is the stage of invasion, ‘B’ is the stage of the life cycle and ‘C’ represents the climatic factor correction.[15]

Limitations: Many factors affect fly larvae’s growth on a corpse, such as climate, ambient air conditions, the surrounding environment, and the wound situation on the body, which may affect the oviposition and invasion of flies on a corpse.

2.4. Development pattern

This method involves applying blow fly larval development times to investigations. During early decomposition, entomological estimates of the post mortem interval depend upon the time period required for the insect species represented to develop to the growth stage encountered at the death scene. Most often the larvae of the flies belonging to the families Calliphoridae and Sarcophagidae predominate. These flies currently provide the most accurate estimate of time since death. The age of specimens collected from the victim may be estimated to provide a minimum time period since death. This approach requires the detailed knowledge of the fly species used and the conditions at the crime scene, but is relatively conservative. It is mentioned that because of their relatively short developmental cycles, blow flies and flesh flies are typically useful only during the first three to four weeks following death.[16] The growth rate of larvae can be studied by rearing them in the laboratory and this can give a reliable definition of time since death. Temperature and humidity heavily influence insect activities, such as the rate of oviposition and maturity.[17]

Limitations: Development information is necessary with regard to different geographical areas, as the more similar climatic and micro environmental conditions between the experiment and the real case, the more accurate can be the post mortem interval obtained. Maggots appear to lengthen in a continuous manner during growth, developing at a predictable, species specific temperature mediated growth rate. Thus within limits, their age may be estimated from their length, thereby providing a minimum estimate of time since death. The fluctuating temperatures also affect the development rate of blow flies.[18]

2.5. Weights of the larva

The maggots of the blow flies are responsible for the maximum corpse destruction. These maggots quickly invade the areas of the corpse and grow in size and weight. The weight of maggots can also serve as an important factor for age estimation. In this method the weight of larvae is studied under controlled laboratory conditions. Egg hatching to post feeding stage is considered and a statistical model relating to distributions of weights to age is formulated. This statistical model can be used to fit the experimental data and this model considers the weight and age of the larvae. This method is called calibration or inverse prediction. The weight of larva of unknown age can be com-pared to the model via inverse prediction, and a confidence interval on the age of larva can be constructed. In constructing the confidence intervals on age, it is assumed that the weight in question is the weight of one larva that can be reasonably regarded as having been sampled at random from a population of larvae of the same age and under same conditions. With a change in the location there is change in insect species so for each species a separate model should be constructed.[19] Based on this, investigators have developed their own baseline data for the conditions relevant to any particular location, insect spe-cies and environment likely to be encountered in the field.

Limitations: When fully grown the larvae stop feeding (post feeding larval stage), migrate away from carcass and settle under turf.[20] Hence the weight of larvae cannot be used for post mortem interval estimation in the post feeding stage. Scarcity of food or competition between species may result in larval mortality and reduction in size and weight of the larvae. The weight of larvae varies according to the rearing conditions, density of larvae and among individuals. Satisfactory techniques have not been available to quantify the precision of such a post mortem interval estimate.

2.6. Isomegalen/isomorphen diagrams

Blow flies are attracted to dead bodies and often arrive within minutes of death of an animal. They have a complete life cycle that consists of egg, larva, pupa and adult stages. Larvae continue to grow and moult (shed exoskeletons) as they pass through the various stages. Development behaviour of life stages can be studied at different temperatures. In the isomegalen diagram the time from egg hatching (x axis) is plotted against temperature (y axis). The lines in the graph represent the length of larvae from which age estimation can be done. In the isomorphen diagram all morphological stages from oviposition to eclosion are considered and the area in between the lines in the graph shows the morphological stages of the fly. The age of post feeding larvae or pupae is estimated from the isomorphen diagram.[21]

Limitations: The development times from oviposition to eclosion might differ in different geographical regions of the world. If the temperature is roughly constant (corpses found indoors) the use of isomegalen and isomorphen diagrams could provide a precise estimate of the post mortem interval. However in the field the conditions are variable, hence greatest care must be taken while interpreting the results.

2.7. Fly eggs

Forensic entomologists use their knowledge of insects and their life cycles and behaviours to give clues about a crime. Egg laying of a fly may give clues to the investigator about the time since death. Calliphorid flies feed on a fresh corpse and may arrive within few minutes. By studying the time of hatching of blow fly eggs in the laboratory at constant temperatures the data obtained can be used in actual cases. The estimate resulting from the experiment and laboratory developmental data shows the time of egg laying within a period of about 2 h, which determines the time since death, as the Calliphorid species lays eggs immediately after death under favourable conditions. Laboratory rearing of eggs of flies found on a corpse and studying the time of egg hatching or the emergence of the first instar can contribute to the determination of a short post mortem interval.[22] However, immediate oviposition may not occur even with those species which prefer carrion in the early fresh phase but may undergo a preovipositional pause. They may also feed on pooled blood as a protein supplement and then wait for digestion and assimilation of the meal. The gravid female flies can retain eggs for 1–2 weeks in the absence of a suitable oviposition medium.[23] Life table studies may be very time consuming and blow fly studies under laboratory conditions may be of dubious value in evaluating their potential in the field because many biotic and abiotic factors influence their survival, developmental rates, and fecundity.[24] However, while this is in part true, life history studies made under laboratory conditions allow researchers to collect the data needed for life table analysis to estimate their biotic potential under specific conditions, and these data can be used as a basis for simulation models for field use that include other factors.[25]

Limitations: The arrival time of flies depends on many environmental factors such as sun exposed/shaded carcass, cloud, rain and other unfavourable conditions. The time of hatching of eggs in the laboratory at a constant temperature must be used carefully while studying the hatching of fly eggs under variable conditions in the field.

2.8. From insect's gut contents

The age of insects can be considered as a minimum estimate of time since death. Eggs laid by an adult fly quickly develop into the first larva. They feed voraciously and increase in size. However, sometimes due to inadequate supply of a food source they may migrate away in search of food or onto the other nearby corpse. In such cases developing an insect-corpse association is very important in forensic entomology. DNA typing of gut contents of insects provides evidence that a carrion fly maggot developed on a particular victim. This shows that the maggot which is used for estimation of time since death actually fed on the corpse. Proving larvae-corpse association in a homicide case is crucial evidence. DNA typing of the gut contents would distinguish the corpse as the food source and it will ensure that the larvae are useful indicators for determining the post mortem interval.[26]

Limitations: Carrion larvae collected during a forensic investigation may be preserved using a wide variety of fluids. The effect that these preservatives have on subsequent DNA extraction and PCR needs to be evaluated. Analysis presented here can identify a maggot's "last meal", but it is not known how long a maggot can cease feeding before gut content DNA can be recovered.

2.9. From cuticular hydrocarbons

Biochemical changes or shifts in ratios of cuticular hydrocarbons can be used for post mortem interval estimations. This method is useful when the fly puparia are recovered from the crime scene. The colour change of puparium is not a reliable method for age estimation. The weathering of hydrocarbons present in the cuticle of the puparium shows a direct relation-ship with time. These biochemical changes or the gradually changing ratio of cuticular hydrocarbons can be studied through gas chromatography mass spectrometry. Considering the advanced analytical technology, the study of these changes can be helpful in post mortem interval estimations more precisely. The cuticular hydrocarbons of the puparial cases contain a mixture of n-alkanes, methyl branched alkanes and dimethyl branched alkanes. The length of the carbon chain is from C21 to C35 and the hydrocarbon composition shows significant regular changes with the weathering time. For the even numbered n alkanes with low molecular weight, such as n C22, n C24 and n C26, the abundance increases significantly with the weathering time. For n C26, in particular, a linear in-crease in abundance with the weathering time is observed. In addition, for most of the other low molecular weight hydrocarbons (n C26 or below), the abundance decreases significantly with the weathering time.[27]

Limitations: Cuticular hydrocarbon composition may vary for several reasons. Diet and age are the two variables that must be considered in cuticular hydrocarbon analysis.

2.10. Width

The fly larvae during the growth stage increase in length and width. They feed actively on the corpse and when the third in-stars cease feeding they shrink in size and this 'head curling' or shrinkage leads to difficulty in post mortem interval estimation from the length of the larvae. The body width of larvae can be used as an alternative to length for age estimation. The body width of larvae, at the junction of the fifth and sixth abdominal segment, is comparable with the body length for age prediction of maggots. It is mentioned that the conversion of width to length can be done with 95% accuracy from a single linear model. Body width as an alternative to body length for post mortem interval estimation was used for *Calliphora augur*. The initial reduction in width was seen from 0 to 6 h at 20 °C. It possibly relates to elastic properties of the cuticle of the young larva.[28]

Limitations: Such analyses are performed only on one species of flies. More research is required to determine the accuracy of the above method for the aging of Calliphorid larvae.

2.11. From accumulated degree days/hours (ADD/ADH)

Another method of calculating the post mortem interval involves estimation of the accumulated degree days or hours (ADD or ADH). ADH values represent a certain number of "energy hours" that are necessary for the development of insect larvae. The degree day or hour concept assumes that the developmental rate is proportional to the temperature within a certain species-specific temperature range. However, the relationship between temperature and the development rate (reciprocal of development time) is typically curvilinear at high and low temperatures and linear only in between.

The formula for calculating ADH is given by

$$\mathrm{ADH} = T.(\theta - \theta^{\circ})$$

where T is the development time, θ is the ambient temperature, and the minimum developmental threshold temperature is $\theta°$, a species specific value, the so-called development zero, which is the x intercept, and is calculated by the linear approximation method.[29]

Limitations: The ADH method seems to give good results only when the larvae of interest have been exposed to temperatures similar to those used for generating the reference value applied in the post mortem interval calculation. Use of ADH in post mortem interval estimations has shortcomings, particularly during the winter period where low temperatures are involved or where there are sudden summer cold spells during the development period.

2.12. Aging blow fly eggs through gene expression

The blow flies lay their eggs at predictable times in the decay cycle of the corpse. The immatures are difficult to identify up to the species level and they have to be reared up to the adult stage for correct identification and for correct post mortem interval estimations. This causes delay of several weeks before time since death can be estimated. In criminal cases there is a need for quick investigations, hence the reliable method of age estimation could be studying of the expression of three genes (bcd, sll, cs) present in the blow fly eggs as a means of predicting age. These models show that estimating egg age through gene expression made predictions within 2 h of the true age when all expression data are available, while the presence/absence of cs transcripts identified two age classes, together indicating that gene expression can be used to more precisely predict blow fly age.[30]

Limitations: Specimen collection, preservation, the time of storage and DNA extraction strategies need to be refined.

2.13. Effect of body length and crawling speed

Calliphorids set the time clock very soon after death and therefore provide the minimum post mortem interval. Maggot size and maturity are the fundamental elements used for indicating when adult blow flies first encounter a corpse. When larvae complete their development they disperse to find an adequate site for pupation. The crawling speed of larvae is expressed by the following equation:

$$\text{speed(cm/min)} = 5.45 \times \log[\text{length(mm)}] + 0.66 \times \text{temperature(degrees C)} - 12.8$$

This study shows that the larvae crawled at a faster speed with increase in temperature. Furthermore, speed increases as a function of body length. These results are useful for estimating the time that elapsed following the larva's departure from the corpse. This information can provide more accuracy for post mortem interval estimations.[31]

Limitations: It is not always easy to obtain the range of speeds. More comparative data are required to make strong inferences about the relationship between crawling speeds and ecological or morphological characteristics. Data are also required for other major groups of arthropods. In particular, insufficient data exist to calculate the allometric relationships.

2.14. Ontogenetic study

Hydrocarbons are the compounds which contain carbon and hydrogen atoms. Hydrocarbons are also present in the insect's cuticle. These hydrocarbons are present as long chains with carbon atom varying from 21 to 35 carbons. Through gas chromatography the ontogeny of these hydrocarbons can be studied in different growth stages of a fly. The gas chromatography reveals that these hydrocarbon profiles vary in different life stages such as larvae, post feeding larvae, pupae and adults and they also vary with the age of individuals. Even eggs or larvae from different species can be distinguished, provided their profiles are different. This study concludes that the post mortem interval can be determined from the composition of cuticular waxes in Calliphoridae in forensic situations.[32]

Limitations: Use of cuticular compounds in a phylogenetic approach has to take into account the fact that during ontogeny, the composition of these compounds may vary quantitatively and also qualitatively. Some compounds represent basic and general constituents of cuticular wax. Their specificity is not noticeable at the species level but may be useful to separate and characterize different sub families of Diptera. Some controversies exist concerning the possible use of cuticular compounds as phylogenetic markers.

2.15. New simulation model

A new stimulation model has been proposed based on the duration of each development stage which is measured as a function of temperature. The following equation is used

$$T\alpha(\theta) = a\alpha.\exp(-T\alpha.\theta) + T0, \alpha$$

where $T\alpha$ is the duration of one developmental stage and α is a function of temperature θ. The parameter $T\alpha$ defines how strongly the time interval depends on temperature; the higher the parameter the steeper is the gradient of the fitted curve. $T0, \alpha$ represents the minimum time interval required for completing a certain developmental stage and $a\alpha$ provides the absolute normalization.

The time tF is the point at which the maggots are collected, $\theta(t)$ is the time temperature profile and the post mortem interval is calculated by backward calculations as is done in the ADH method. The larval age is calculated successively during certain time steps using the fitted functions corresponding to the current developmental stage. In each developmental stage a the relative developmental progress is $P\alpha$ (values 0–1) where 0 is the beginning and 1 is the finishing point of each developmental stage; e.g., a maggot in the middle of the post feeding stage is $P4 = 0{:}5$, and at the end of the post-feeding stage it is $P4 = 0{:}9$ and so forth. The developmental duration $t_{0,\alpha}$ spent in each individual stage is calculated by the following equation:

$$P\alpha = \int_{t\alpha+1,0}^{t\alpha,0} dt/T\alpha(\theta(t_F - t))$$

where $\mathrm{d}t/T(\theta(t))$ is the infinitesimal relative development. The calculation starts with the developmental stage of the maggot at the time of collection, the development at each stage is summed backwards until the beginning of the egg stage is reached. The calculation for each collection stage uses $t\alpha + 1{,}0 = tF$. The total development time $t0$ or the post mortem interval is then given by[33]

$$t_0 = \sum_{\alpha} t\alpha, 0$$

Limitations: Growth data of flies with known error values are required to refine the inclusion of uncertainties.

2.16. Larval dispersal

The blow fly life cycle begins with the oviposition of eggs into eyes, mouth and nasal passages where moisture and protection from the sun is optimal. Within hours (dependent on species and ambient temperatures) the eggs hatch and produce the first instars. These moult into second and third instars. Feeding ends when larvae acquire the fat needed for pupariation. These larvae migrate to find a suitable site for pupariation. The lighter larvae tend to move longer distances for pupariation. This dispersal process can be important while estimating the mini-mum post mortem interval. The investigator should be careful while drawing conclusions about the minimum post mortem interval on the basis of size of those larvae that have dispersed to considerably long distances. Some of them may also migrate in search of a new food source. The time taken for such dispersal should also be taken into consideration if pupae found away from the corpse are used to determine the minimum time since death. The larvae may also migrate on the decaying corpse from the other nearby corpse. Hence the larval dispersal is also an important factor and it should not be underestimated while estimating the post mortem interval.[34]

Limitations: The larvae may be at a risk of predation, parasitization and desiccation during the post feeding stage. If one wave of blow fly infestation has occurred and the post feeding larvae have dispersed and are overlooked, the post mortem interval will be incorrectly determined.

2.17. Length of larvae

The post mortem interval is directly correlated with the length of the larva. The post mortem interval can be determined from the larvae of blow flies using the growth parameter and larval length as a 'biological clock'. The insect larvae found on a corpse need to be collected and transferred to the laboratory in living conditions for the purpose of identification and estimation of the post mortem interval. The growth parameter of the egg, first, second and third larval instars, total larval period, pupal period and egg-to-adult period can be studied in the laboratory. It is believed that older the maggots the more time has elapsed since death and this growth depends on the temperature. Hence the age of the oldest (longest) larvae provides the minimum post mortem interval.[35]

Limitations: Larval specimens placed alive in most preservatives such as 70%, 75%, 80%, 90% and 100% ethyl alcohol, Kahle's solution and 10% formalin show colour changes, desiccation, sunkenness and agglomeration and head curling. Reaction to the preservative type might be species specific and that different instars of some species might also react differently.[36]

2.18. Pupae

In certain indoor cases, the corpse may be discovered only after the maggots have finished feeding and left it to pupate. In these cases it is not possible to estimate the time of death by examination of larvae, but structural and morphological changes in pupae or pupal cases should, in principle, enable a useful estimate to be reached. During the growth stage several species of blow flies spend 50% of their juvenile development in the pupal stage; therefore, methods which are suitable for age estimation within this period would be valuable in entomological post mortem interval estimates. During pupal development or metamorphosis, changes occur in gene expression also. The gene expression patterns of transcripts, which are differently expressed during pupal development, show an age-dependent differential gene expression.[37]

Limitations: Preservation methods for the pupal stage are poorly defined and inappropriate methods may result in nucleic acid degradation. These studies have used preservation at −80 °C or fresh pupae only, followed by analysis of differentially expressed genes to ascertain age. This application imposes the greatest limitations on the choice of the preservative method, as RNA is an inherently unstable molecule. Although newer molecular techniques are quite powerful, the technology is still expensive and DNA sequences are available for only some carrion breeding species at the present time. Furthermore, specific gene sequences are not appropriate for all species.

2.19. Internal morphological analysis of pupae

This is the recent technique for estimation of the post mortem interval based on changes in pupae during metamorphosis. This technique illustrates that time since death can be estimated from pupae collected at the time of autopsy. The age of maggots and pupae found on corpses indicates the time since death. Estimation of time since death from pupae is very difficult and time consuming. The larvae crawl away from the corpse to find a suitable site for pupariation. The colour change in puparium is not a reliable method for estimation of the post mortem interval. During metamorphosis some changes also occur internally in the puparium. For the estimation of age these change are reliable factors and they can be studied using histological techniques. To study the internal features the pupae should be killed in hot water after piercing through three tagma and then preserved in 80% alcohol. Pupal sections are then stained with hematoxylin and eosin stains. The section of pupae shows that the thoracic and brain muscle development is different throughout the pupal development. This difference in muscle development can be used as an indicator of age, and hence an estimation of the post mortem interval can be made.[38]

Limitations: It is the standard practice to chemically fix tissues for histological examination. Internal morphological analysis requires reliable cutting of complete pupal sections and is dependent on preservative uptake and wax impregnation. Improper choice of preservatives may lead to discolouration and alteration of weight of pupae. Discolouration can be more pronounced without a prior hot water kill and subsequent preservation/storage in ethanol.

2.20. Differential gene expression during metamorphosis

Usually larvae are used for aging purposes because their growth such as increase in length and weight is visible with the naked eye, but the changes which occur in pupae at the time of metamorphosis is not visible with the naked eye, hence it is mainly ignored while estimating the post mortem interval. The differential expression of two arbitrarily-named genes (15_2, 2014192) and two other genes (actin, arylphorin receptor) during fly metamorphosis is studied and quantified through real time PCR. This new technique is useful in estimating the age of pupa. The regulation of these transcripts depends on temperature and is age specific.[39]

Limitations: PCR-RFLP produces a particular set of DNA fragment sizes that can be measured on an agarose or polyacrylamide gel. This method requires an exact match between the fragment profile of an unknown specimen and the profile of a known reference specimen. However, reference profiles are not available for many species of sarcosaprophagous flies.

2.21. Estimation of age with 3D micro computed tomography

The internal and external changes that occur in the puparium at the time of metamorphosis can be studied by a most recent technique called micro computed tomography. A high spatial resolution of 17.2 μm shows the external and internal morphological features of a blow fly puparium. The age of blow fly pupae can be estimated with a higher degree of accuracy and precision with both external and internal features. This recent advancement in forensic entomology can help in estimation of the post mortem interval in cases where only fly puparia are recovered.[40]

2.22. Volatile organic compounds released by larvae and pupae

In forensic entomology these volatile compounds have an important role in estimating the age of blow fly larvae and pupae. This is estimated by a recent technique in which the volatile profiles released by larvae and pupae of blow fly are studied through headspace solid phase microextraction, followed by gas chromatography mass spectrometry. This study shows that there are branched and unbranched hydrocarbons, alcohols, esters and acids present in the volatile organic compounds. There is a variation in volatile profiles with the age of the larva and pupa and they vary in composition and quantity. This recent advancement may increase the accuracy of post mortem interval estimation.[41]

Use of microscopic computed tomography (micro CT) scanning continues to grow in biomedical research. The estimation of age with 3D microcomputed tomography and from volatile organic compounds released by larvae and pupae is the new perspective in forensic entomology. Hence, many limitations have not been explored yet. After the decay stage (31 + days), when most of the flesh is gone, changes in the corpse and the insect fauna occur at a much slower rate, and it becomes difficult to determine the post mortem interval accurately. In the dry stage when only bones and dry skin are left the post mortem interval can be estimated from the successional stages of dermestid beetles, ants, cheese skipper flies, soldier flies and ground beetles.[42] Succession data have been used to very accurately calculate post mortem interval as large as 52 days[43] and could be applied to a much greater interval. Approaches for estimation of the post mortem interval based on insect succession and development are complementary. Measuring insect development is a powerful method for providing estimates of the post mortem interval, but there are many crucial considerations and limitations in making such estimates. The determination of insect development is an estimation process and more research is required to decrease the variation in such estimates and to provide precise data for determination of the post mortem interval.[44]

3. Problems in estimation of the post mortem interval

3.1. Myiasis

Myiasis is the feeding by maggots on living tissues or dead tissues associated with a wound. In forensic context, myiasis is most frequently associated with facultative parasites in the families Calliphoridae, Sarcophagidae and Miscidae. Myiasis occurs primarily in indoor cases because sufferers are generally helpless infants or elderly people and may set in hours to weeks before death.[45] If not fully appreciated, myiasis can be a significant point of confusion for the forensic entomologist, appearing to give an estimate of post mortem far longer than the actual period of time since death.[46]

3.2. Premortem condition of the deceased

The maggots found on the corpse can give the clue as to what actually happened. The study of these insects can reveal the ante mortem condition of the deceased or the manner of death. The presence of drugs and toxins taken internally prior to death can influence the estimation of time since death. Presence of drugs in tissues can lead to under- or overestimation of the post mortem interval. There is necessity of considering the possible effects of drugs in tissues on insect growth rates when estimating the post mortem interval using entomological techniques.[47]

3.3. Maggot mass effect

There can be a variation in the corpse temperature and ambient temperature. This is due to the activity of gregarious fly maggots present on the corpse. The maggot activity can lead to an increase in temperature approximately 1–3 °C above the ambient temperature. This increase in temperature can lead to an increase in development of maggots; therefore, it can have detrimental effects on the accuracy of post mortem interval estimates.[48]

3.4. Fly pupae and puparia as contaminants

In forensic investigations, immature stages of the fly (egg, larva or puparia) are used as entomological evidence at death scenes. The puparia represent the longest developmental time, which makes them useful indicators of time since death. However, post feeding larvae, which have originated from other remains, may migrate and pupate on forensic entomology samples at death scenes. These contaminants may erroneously lengthen post mortem interval estimates if no pupae or puparia are genuinely associated with the body.[49]

3.5. Nocturnal oviposition

It was believed earlier that Calliphorid flies do not oviposit at night. But the research of some workers revealed that the flies crawl towards the food source and the oviposition occurs at a reduced rate. This can lead to an error of around 12 h in estimation of time since death.[50]

3.6. Maggot development during morgue storage

The effects of refrigeration on insect development must also be considered. The insects present on the corpse are collected, refrigerated and transferred to a laboratory for further studies. Even when the body infested with maggots is stored at 4 °C, before autopsy, it is seen that the low temperatures disrupt the insect's development. Low temperatures can also be indicated by the presence of dead larvae on the corpse.[51] A debate exists as to whether or not refrigeration has an effect on maggot development. As long as temperatures are sufficient for maggot development (above minimum developmental thresholds), movement of the body and changes in ambient temperature do not seem to retard or alter maggot feeding. If the assumption is made that no insect development takes place during preautopsy refrigeration (−1 to +4 °C), potential error rates in the PMI estimation of 8.6–12.8% occur.[52]

3.7. Effects of the photoperiod

The photoperiod that larvae and pupae experience can also influence the development time, which in turn could affect post mortem interval estimates. Temperature appears to have a greater effect on development than the photoperiod; however, under constant light there is an increase in variation in the overall adult developmental time and it significantly delays the development. The knowledge that light triggered stimuli cause developmental rates to slow under constant light can help in correct estimates of time since death.[53]

3.8. Fluctuating temperatures

In some insects the rate of development is constant at fluctuating temperatures but in some insects the fluctuating temperatures have a greater impact on development. Some species develop more rapidly under fluctuating temperatures and some insects take a longer time to develop under fluctuating temperatures. Negligence of fluctuating temperatures in legal cases can lead to distinctly wrong estimates of the post mortem interval.[54]

3.9. Indoors forensic entomology

The bodies of socially isolated people may remain undiscovered in their own houses for prolonged periods. Medico-legal investigations of these cases are extremely difficult. Entomological examinations of the insect remains may estimate the time since death. But the oviposition of flies in bodies found inside the buildings shows a decreased rate of egg batches and there is a delayed oviposition of about 24 h. The delayed oviposition must be considered before post mortem interval estimation is done.[55]

3.10. Under sized larvae

Due to the intra specific and inter specific competition for food there is increased mortality, increased developmental rates and the production of under sized larvae and adults. Due to scarcity of food, the larvae may also migrate away from the corpse in search of food. This may result in wrong estimations of the post mortem interval. Hence, the factors that have effect on larval development should be considered.[56]

3.11. Weather conditions

The insect fauna vary with the geographical location. The first colonizers on the corpse are the blow flies and their life cycle is important for estimation of time since death. It is dependent on temperature and environmental conditions of the scene. The crime scene temperature and meteorological station temperature are also considered. Although the gross weather conditions may be quite similar, microhabitat conditions may differ considerably and may not be comparable. Vegetational cover, air drainage and slope exposure all influence microhabitat conditions greatly and this may also affect the development of the forensically important flies.[23]

3.12. Dispersal time

The larvae of flies feed on the corpse and moult into three instars. There is rapid loss in the corpse weight and the post feeding maggots begin to leave the corpse. In order to find a suitable site for pupariation they migrate some distance away from the corpse. This period is known as the wandering stage. For pupariation the larvae prefer an environment protected from light and predators and may have a longer dispersal time in order to reach an appropriate pupation site. Hence, the dispersal time can vary and may influence the total time of development which may lead to an erroneous calculation of the post mortem interval.[57]

3.13. Precocious egg development

The presence of fertilized eggs and larvae in the genital tract of female blow flies shows that it can lead to wrong estimations of the post mortem interval. The samples developed from precocious eggs could lead to variation of about 24 h in time since death estimates.[58]

4. Discussion

Diptera are the insects of greatest forensic interest. Flies are considered to be nothing more than ecological scavengers and a nuisance to man. However, they are the important indicators of the post mortem interval usually in the first few weeks after death.[59] The examination of insects at the crime scene can provide other useful information, such as linking a suspect to a crime scene, providing analysis of badly decomposed bodies, indicating movement of body, period of neglect, and time of day and weather conditions at the time of insect colonization.[60]

Various problems encountered in post mortem interval estimations should be taken into account. These areas are in need of immediate research. The erroneous estimations could impede justice. Due to ethical reasons, animal models are used for the experimental studies. Care must be taken when apply-ing data from one carcass type (non human/animal) to estimate the post mortem interval to another type (human). Forensic entomologists often rely on specimens of maggots collected and preserved using a variety of techniques by police or medical examiners. The kind of solution in which the maggots are killed or preserved has significant effects on their length.[61] Hence, standard techniques should be used for collecting maggots at the crime scene and at the time of autopsy.

Some alternative approaches of post mortem interval estimation have been presented. With advancement in technology and science these new methods have become more reliable. Nowadays, DNA technology is a useful tool that can help in species identification as well as in time since death estimations. Within the past few years, the use of flies (Order: Diptera) has became a convenient tool in estimating the post mortem interval in criminal investigations but in India this field is yet to gain popularity. The most dominant species present on a corpse are typically the blow flies. The analysis of differentially expressed genes during blow fly metamorphosis can lead to an understanding of the process of formation of the adult fly and may also represent a new approach to predict the pupal age in death investigations. Such research work is required for the Indian species as well.

Blow flies (Diptera: Calliphoridae) are the first organisms to arrive on a dead body. Their offspring can give a good estimate of the time a body has been exposed to insects. The age of the maggots can be calculated based on measurements of these morphological characters. Following the feeding stage, larvae disperse from the food source in order to pupate. The pupal stage can last up to 50% of the whole juvenile development. During this stage, age estimation is increasingly difficult because length, for instance, does not significantly change during metamorphosis. The colour change in the puparium is also not a reliable method for post mortem interval estimation. Several different measures have evolved to estimate age from pupae such as internal morphological analysis of pupae through histological techniques, hydrocarbon profiles and through the study of gene expressions. The gene expressions, like human DNA fingerprints, are unique and this information allows the forensic entomologists to correctly identify the insect species and hence the post mortem interval.

Dead body progresses through a recognized sequence of decomposing stages, from fresh to skeletal, over a period of time. The arthropods occur in predictable sequence depending on the stage of decay. In the past few years time since death was estimated from the stage of succession of insects, but the most recent methods such as age estimation from 3D micro computed tomography and from volatile organic compounds released by blow fly larvae and pupae may further increase the accuracy of post mortem interval estimates.

The entomoforensic science is nowadays used mostly for estimating the post mortem interval.[62] There has been a renewed interest on the part of scientists in the techniques for estimation of post mortem intervals using entomological evidence. The amount of research work has increased on this subject. This renewed interest has resulted in publication of a number of baseline studies detailing the roles of insects in the decomposition process.[63] These published studies have detailed methodologies for estimation of the post mortem interval for remains which have been readily available for insect colonization either inside of dwellings or outdoors.

Numerous case studies have demonstrated that forensic entomology is a valuable adjunct to death investigations.[64] Extensive knowledge of the factors that interfere with the process of colonization is required. Lack of information can result in various difficulties in post mortem interval estimation. The problems discussed in this paper relate to estimation of the post mortem interval, a key element in any death investigation.

Precise developmental data for forensic indicator blow fly species are essential for accuracy in the estimation of the post mortem interval. Collecting living organisms from the corpse and studying their development under laboratory conditions can lead to much better results.[65] But the determination of the post mortem interval is sometimes conflicting. To use larval development in estimating the post mortem interval, accurate information on the development of individual species is essential. Currently, much of the available information comes from relatively few studies, often with limited data.[66] Studies should include experimental field as well as laboratory research.

Another important contribution that anthropods can make in determining the cause of death is in the area of toxicology (entomotoxicology), i.e. where the body has decomposed to the point where it is in the dry stage, containing only skin and bones or has become skeletonized with only bones remaining, it is still possible to determine if the victim died as a result of a drug overdose or poisoning. In the cases where sufficient hair is not present, then anthropods may contain some of the drug or poi-son and it can be extracted from the body of the insect and identified by modern instrumental techniques.

The preference of Calliphorids for a fresh corpse makes them a high priority at crime scenes whenever they are encountered. Studies are necessary within each region since differences in community composition and assumptions could lead to erroneous conclusions pertaining to the estimation of time since death. Constant surveys of blow fly populations must be conducted for regions. This study is done to gather information on necrophagous flies for post mortem interval estimations.

India needs to develop its own internal database for future reference. Apart from the above mentioned references, more publications are needed, particularly with reference to Indian species so that the insect evidence can be used in medico-legal investigations.

The application of forensic entomology to criminal cases has also increased but this science has not gained the required popularity in India. To make pathologists, police officers and law personnel aware of the importance of entomology, a standard protocol is required for collection and preservation of entomological specimens containing taxonomic keys for identification of Indian species. Each geographic region is com-prised of unique factors that affect carrion decomposition rates and arthropod succession patterns. Arthropod succession has been studied in India in the state of Punjab.[67] Such studies are also required for other geographic regions of India. Accurate developmental data are required for all carrion species.

Due to the recent advancement in the field of forensic entomology, there is a need to understand the importance of the rate of development of flies in relation to temperature, detailed development data are needed for Indian species to allow more precise post mortem interval estimates.

5. Conclusion

The primary objective of this study is to gather baseline data and to stimulate further study of the forensically important flies in India, especially those species that have established themselves as the primary indicators of time since death. The study presented here is limited and can shed light on the importance of blow flies (Diptera: Calliphoridae) as post mor-tem interval indicators. The new approaches for estimating time since death discussed above seem more reliable and can be used with confidence in medico-legal cases given the inherent difficulties in generating a precise post mortem interval estimate are considered.

The study detailed herein is designed to make investigators and law personnel aware of the importance of forensic entomology in criminal cases. Investigators should not be completely dependent on the few baseline studies. Estimation of post mortem interval is based on few valid assumptions and errors in any of these assumptions can lead to wrong estimates of time since death. The study will make the officials and the criminal investigation team aware of and familiar with forensic entomology, a step which may initiate future studies and interest in the application of insect evidence in legal investigations in India.

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Conflict of interest

None declared.

Ethical approval

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