Forensic Genetic Analysis of Insect Gut Contents

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Abstract: Entomological evidence is most often used for estimating the postmortem interval, but fly larvae can also be a source of vertebrate DNA. Forensic analysis of DNA recovered from a larva's gut can be used to identify what the larva had been feeding on. During our previous research studies, we used the same DNA extraction for the dual purpose of identifying the insect species and associating a maggot with its last meal. In our experience, we have encountered several situations where this method for associating a maggot with a corpse would have been useful, such as removal of remains from a suspected crime scene, an alternative food source is nearby the scene or the body, and a chain-of-evidence dispute. However, since maggot gut content analysis is a quite brand-new area of study, many of the limitations of the technique have not yet been explored. The results of our most recent research studies suggest that third-instar larvae actively feeding on the corpse can be considered the best source of human DNA, better than postfeeding or starved larvae. In this paper, the state of the art of forensic genetic analysis of maggot gut contents is reviewed.

Key Words: forensic science, forensic entomology, DNA analysis, maggot crop

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Maggots are commonly used during a death investigation to estimate the period of insect activity based on aging the oldest insect specimen associated with the corpse.[1,2] In estimating the postmortem interval, it is assumed that all of the maggot's development and feeding period occurred on the corpse since usually the larvae are collected directly from or near the corpse. However, such a direct assumption cannot always be made. In our experience, we have encountered several situations where an alternative method for associating a maggot with a corpse would have been useful, such as removal of remains from a suspected crime scene, an alternative food source is nearby the scene or the body, and a chain-of-evidence dispute.[3] We successfully recovered both human and animal host DNA from the crops of necrophagous fly larvae. The same DNA extraction served the dual purpose of identifying the insect species and associating a maggot with its last meal.[4] Most animal molecular-systematic techniques rely on the mitochondrial DNA (mtDNA) molecule.[5,6] Due to the high copy number per cell and maternal inheritance, mitochondrial DNA analyses provide a very useful tool for the identification of human tissue that is degraded or that requires comparison to a maternal relative.[7–15]

When detecting host DNA in the insect alimentary canal, primer specificity is essential.[16] Identification of humans usually relies on 2 particularly hypervariable regions (HVI and HVII) within the noncoding control region or D-loop.[17–22] Vertebrate animal species determination may also be based on the D-loop; however, for most animals the locus of choice is a protein-coding gene (eg, cytochrome B, Cyt B) for vertebrates[23–25] and cytochrome oxydase subunits 1 and 2 (COI+II) for insects.[26–31]

Vertebrate DNA has been successfully amplified and analyzed from several insect sources. DNA recovered from insect blood meals has been used to identify the species or individual identity of the host.[32,33] Gokool et al[34] detected human DNA from mosquito blood meals using microsatellites referred as variable numbers of tandem repeats (VNTR). Minisatellites were used to detect human DNA in the mosquito blood meal up to 26 hours after ingestion,[35] demonstrating that DNA isolated from mosquitoes is qualitatively and quantitatively sufficient for DNA typing. In a forensic context, human DNA has been isolated and amplified from crab-louse blood meals[36] and crab-louse excreta.[37] This may be useful in a forensic investigation to identify individuals involved in certain cases of body violence because a louse can be transferred from assailant to victim during a sexual assault. MtDNA has been also typed from beetle larvae recovered from human bones subjected to environmental exposure for several months,[38] thus demonstrating that mtDNA may survive late-stage decompositional events and

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be subsequently recovered even from late-succession insects. Genetic analyses of insect gut contents may prove to be crucial evidence to link a suspect to the victim or the scene, reconstruct the circumstances of the crime, or establish the credibility of the statements made by witnesses.

Maggot Gut Content Analysis and Wash Methods

As previously demonstrated,[3,4] *Diptera* larvae (also called maggots) can be a suitable source of human mtDNA. Such analyses have focused on the crop, an anterior portion of the gut that forms a food-storage organ. It must be certain that the extracted DNA comes from the gut and not from possible contamination on the maggot's exterior. In fact, exterior contamination would interfere with making correct inferences about whether a maggot had been feeding on alternative or multiple food sources (human body, animals, etc) and even on the identity of the missing person. For this reason, it is strongly advisable to provide DNA extraction of the crop rather than the entire maggot, also because removal of an internal structure preserves taxonomically important cuticular surface structures and because the crop contents are relatively undigested compared with those of the stomach or intestine.

The method usually followed for maggot dissection has been illustrated in detail by Linville and Wells.[39] It includes that the 2 or 3 most posterior segments of the maggot are usually cut off with iris scissors followed with a ventral incision made from the posterior to the anterior end of the maggot. During this incision, the scissors must be kept just under the cuticle to prevent damaging the crop. Following the incision, the cuticle can be folded back exposing the viscera, and the crop can be easily removed with forceps.

In their research study, the above authors[39] demonstrated that before dissection of the maggot and removal of its crop, washing maggots with 20% bleach solution is an effective method for sterilizing the external surface of a blow fly larva, thus reducing significantly any kind of external contamination without any interference with crop mtDNA analysis. Some maggots were intentionally contaminated by soaking them in cow blood for 3 hours and then washed by using 3 different methods (DNase enzyme, distilled water, and 20% bleach solution). Based on the PCR and sequencing results, only washing the maggots in 20% bleach reduced significantly the amount of vertebrate DNA amplified from the maggot's exteriors.

From these experiences, several more questions arose about the preservation method's effect on genetic analysis, the persistence of recoverable vertebrate DNA in blowfly larvae throughout their development, and the digestion time of DNA in maggots after their removal from the food source.

Preservative Conditions and Genetic Analysis of Host DNA

Since maggot gut content analysis is a new area of study, many of the limitations of this technique have not yet been explored and we tried to investigate several of them. For example, the method of maggot preservation may affect the investigator's ability to successfully extract vertebrate DNA from the maggot's crop. In fact, the type of preservation fluid can change the physical characteristics of the maggot, which may inhibit the investigator's ability to dissect the maggot and remove the crop intact. This assumption is consistent with the observations made by Tantawi and Greenberg,[40] who demonstrated a severe shrinkage effect of several killing and preservative solutions in maggots. Also, the chemical properties of the preservation fluid may inhibit the extraction of DNA from the crop. Formalin-containing solutions are commonly used to preserve the external identifiable features of entomological evidence, but formalin has been known to degrade high-molecular-weight DNA[41] and inhibit the extraction of DNA from formalin-fixed tissues.[42]

Regarding the ability of storage temperature and type of preservation fluid to alter the stability of human or other vertebrate DNA within the maggot crop, preliminary results suggest that maggots should be stored and preserved as soon as possible in ethanol or at a low temperature.[43] Using 8 preservative conditions on maggots dissected after different time intervals (2 weeks, 8 weeks, and 6 months) it was demonstrated that ethanol should be considered a better preservative solution at refrigeration or room temperatures since other kind of solutions such as formaldehyde or Kahle's solution can seriously prevent host DNA analysis over time.[43] In maggots preserved in 95% ethanol at room temperature, although the dehydrated crop became attached to other internal organs and often broke during dissection, this did not prevent the recovery of host DNA. In maggots preserved in formaldehyde, although the crop was easily removed, quantitation results suggest a reduced amount of DNA had been extracted, in most cases preventing the amplification of the HVII region. Maggots stored without any preservation fluid degraded over time at both refrigeration and room temperatures. However, the best results were observed in maggots stored at −70°C without any preservation fluid.[43]

Maggot's Development and Digestion Time of Host DNA

Another factor the investigator should consider during analysis is the maggot's stage of development, as the relative size and condition of the entire maggot and its crop may affect the strategy of dissection and extraction. The size of the maggot and its crop may render different strategies for extracting vertebrate DNA from the gut contents. In fact, young maggots may be too small for dissection and crop removal. In older postfeeding maggots, the larvae stop feed-

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ing and the crop contents are emptied into the remainder of the maggot gut. Alternative methods of analysis, such as extraction of the entire maggot, may provide better results for maggots that are too small for dissection or for postfeeding maggots when the crop is no longer visible. In this matter, we investigated the degradation of host DNA during the maggot development and after a maggot is removed from the food source. The purpose was to answer the 2 main questions dealing with genotyping host DNA: when can human DNA be recovered throughout a maggot's development? How long can a maggot cease feeding before gut content DNA cannot be recovered?

In the attempt to answer to the above questions, maggots of *Calliphora vicina* were collected at half-day intervals for 6 days: one group was immediately preserved, and some other maggots were kept alive off the food source for 24 hours and for 48 hours. Preliminary results suggest that mtDNA was successfully amplified from most of the groups of maggots collected in different stages of development that had been immediately preserved and some kept 24 hours off the food source.[44] DNA was not recovered from any maggots that had been removed from the tissue and kept alive for 48 hours.[45] DNA sequencing failed in most maggots less than 2 days old. Maggots 2–2.5 days old were too small for dissection, but extraction of the entire maggot did allow for the recovery of human DNA. In older postfeeding maggots with near-empty crops, extraction of the intestines failed to recover human DNA. These data are consistent with those illustrated by Dadour et al,[46] who were able to detect ingested host DNA throughout all stages of the life cycle of *Calliphora dubia* (*Diptera*: Calliphoridae), including first-stage larvae at day 1 and pupae at day 2, that is, approximately 24 hours after the pupal case has formed and the larva has stopped feeding and, therefore, comparable to 24 hours postfeeding period of *Calliphora vicina*. In fact, no DNA was amplified in pupae of *C dubia* at day 3, consistent with 48-hour postfeeding period of *C vicina* maggots or kept alive for so long.

However, in our results STR analysis was successful only for maggots 2.5–4.5 days (fully third-instar larvae) immediately preserved. Young and postfeeding individuals, as well the group of maggots kept alive for 24 hours and 48 hours, failed to produce a STR genotype at any locus as shown in Table 1. Quantiblot results revealed that the total human DNA recovered from this latter group of maggots was very low, falling below the detection limit of 0.06 ng/μL (Linville et al, unpublished data). We believe that the cause of the sporadic mtDNA success and failed STR attempts is essentially due to the amount of food stored inside the gut content, as clearly expressed by the crop size. In *C vicina* third-instar larvae, the crop is commonly 7 mm long (one third of the total maggot length) at the peak feeding, but just a day later (24 hours), after the crop is emptied into the gut, it is reduced to 3 mm long.[47] In our experience, all maggots whose crops were approximately 1 mm or less did not produce consistent results. In fact, in postfeeding maggots 48 hours after peak feeding, the size remains about the same, approximately 1 mm long. Therefore, the smaller crops of postfeeding maggots, as those of young maggots, reduce the chance of successfully amplifying DNA and obtaining a genetic profile. Another factor affecting DNA analysis to be considered is the lipid deposition that usually occurs in maggots towards the end of the third instar since postfeeding larvae show high lipid concentrations. In this respect, removal of the maggot crop should be always preferred over extraction of the entire maggot for genetic analysis of insect gut contents.

TABLE 1. Host mtDNA and STR Results of Maggots Immediately Preserved and Kept Off the Food Source for 24 and 48 Hours

Age	Alive, Hours	Crop/ Intestine	mtDNA	STR
Less than 2 days	0	No/no	No	No
Young third instar (2–2.5 days)	0	Yes/yes	Yes	No
Fully third instar (2.5–4.5 days)	0	Yes/yes	Yes	Yes
Postfeeding (4.5–6.5 days)	0	Yes/yes	Yes	No
Less than 2 days	24	No/no	No	No
Young third instar (2–2.5 days)	24	No/no	No	No
Fully third instar (2.5–4.5 days)	24	Yes/yes	Yes	No
Postfeeding (4.5–6.5 days)	24	No/no	No	No
Less than 2 days	48	No/no	No	No
Young third instar (2–2.5 days)	48	No/no	No	No
Fully third instar (2.5–4.5 days)	48	No/no	No	No
Postfeeding (4.5–6.5 days)	48	No/no	No	No

Regarding biochemical alterations of DNA during food digestion, there does not appear to be any severe enzymatic breakdown of host DNA in the crop over time. Primarily digestion does not occur within the crop, because proteolytic enzymes are not secreted into this area of the foregut, which mainly acts as a food container. However, enzymes are present in the saliva of the maggot for preoral digestion and are reincorporated with the food into the crop.[48,49] Thus, some degradation of DNA certainly occurs within the crop, but it seems to be not the main reason to a failure of DNA typing from the crop material. Zehner et al[50] have recently demonstrated that also the time of storage of the maggots and

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the length of the postmortem interval up to 16 weeks appeared to have no particular influence on the quality of DNA results.

CONCLUSION

Based on the above preliminary results, we do not imply that amplification of DNA from small crops is impossible since DNA laboratories better equipped for low-level DNA analysis could get better results from young and post-feeding maggots or even from pupae, as demonstrated by Dadour et al.[46]

It seems for sure that some other laboratories can certainly get better STR results very useful for investigators; for example, to determine the identity or the sex of the individual on which the maggots feed. This is consistent with the results reported by Clery,[51] who detected prostate-specific antigen (PSA) and obtained a male Y-STR type from maggots colonizing a cadaver in a simulated postmortem sexual assault; PSA was recorded from sonication of whole post-feeding larvae after 145 hours while correct Y-STR profiles were recorded from the crops of actively feeding second-instar larvae after 48 hours of initial semen deposition. Also Zehner et al[50] performed STR typing and HVR amplifications using crop contents of maggots collected from corpses after various postmortem intervals.

Our results suggest that host DNA analysis is possible in maggots fully developed and actively feeding on the corpse but is not as likely in older postfeeding or starved maggots with empty crops approximately 1 mm long or less (see maggots kept off the food source for 24–48 hours). Since crop contents greatly decrease within 24 hours off the food source, it is crucial to preserve maggots immediately at the crime scene in proper preservative conditions (ethanol or at low temperatures). A morphologic study of the crop (color and length) before gut-content analysis could provide useful information for genotyping host DNA, as well for aging the maggots, as suggested by Greenberg.[47] It must be taken into consideration that crop length can vary, depending on larval development, as well as on the species; for example, the crop of *P sericata* empties rapidly during the first day after peak feeding, while the crop of *C rufifacies* empties gradually. Therefore, since degradation of DNA within the crop, time of storage of the maggots, and length of the postmortem interval do not seem to influence DNA typing, based on the results illustrated above and relevant literature examined, we believe that the crop size is the main factor affecting the ability to analyze host DNA in maggots and the success of the genetic analysis.

Actually, we are trying to expand our experience, and future work is scheduled to analyze vertebrate DNA from maggots that have been collected on different food sources, as well as from different species of *Diptera*.

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