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## MOSQUITO BLOOD FEEDING AS AN INDICATOR FOR ESTIMATING THE TIME OF A CRIME USING PCR ANALYSIS

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### ABSTRACT

Blood-fed female mosquitoes can carry human DNA away from a crime scene, but that DNA does not last. This study measured how long human DNA stays amplifiable inside the blood meal of *Culex* sp. Eighteen fully engorged females were fed on a human host under controlled laboratory conditions and were held in three post-feeding groups of six (24, 48 and 72 h). A further 20 extracts were prepared, 10 from human-fed and 10 from rat-fed mosquitoes. Each abdomen was dissected, homogenised in phosphate-buffered saline and processed on a silica-membrane spin column. DNA yield and purity were measured by UV spectrophotometry, and the first hypervariable region (HVR1) of the human mitochondrial control region was amplified by PCR. Products were resolved on ethidium bromide-stained agarose gels. Mean DNA yield was  $86.00 \pm 22.71$  ng  $\mu\text{L}^{-1}$  with a mean  $A_{260}/A_{280}$  ratio of  $1.90 \pm 0.04$  for the time-course extracts, and  $47.60 \pm 12.38$  ng  $\mu\text{L}^{-1}$  with a ratio of  $1.90 \pm 0.10$  for the human- and rat-fed set. A clear 343 bp HVR1 product was obtained from every sample at 24 h. At 48 h the product was faint and was seen in only part of the samples. At 72 h no product was obtained. Human DNA in a mosquito blood meal is therefore recoverable within a short and definable window, and the chance of a result falls sharply after 48 h. The finding sets a practical collection deadline for investigators who plan to use blood-fed mosquitoes as biological evidence, and it supports the use of forensic entomology as a complement to conventional trace evidence in death-scene and identification casework.

**Keywords:** *Culex* sp.; forensic entomology; blood meal; human mitochondrial DNA; HVR1; post-feeding interval; PCR; DNA degradation.

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### INTRODUCTION

Recovering human DNA from non-standard biological evidence has become an important part of modern forensic practice. It offers a route to identification when conventional traces are degraded, diluted or deliberately removed from a scene. Blood-feeding insects have been proposed as short-term reservoirs of host nucleic acids, because the blood they ingest stays in the gut for a measurable period. Mosquitoes fit this model well. They are common in human settlements, the females must take blood, and they rest close to the feeding site while the meal is digested and the eggs mature. These traits make the mosquito blood meal a possible source of human DNA for identification and for reconstructing where and when a person was present [1].

Mosquitoes of the family Culicidae pierce vertebrate skin and draw blood into the midgut. Digestion then proceeds through proteolysis followed by nucleolytic breakdown. At a crime scene a mosquito may feed on the victim, on the suspect, or on both, and it often remains trapped in the same room or enclosure [2]. Nucleated host cells and free nucleic acids are broken down during digestion, yet fragments of host DNA can persist in the gut lumen for some time before they are fully catabolised. The forensic value of such a sample therefore depends on how much time has passed between feeding and collection. Knowing the length of that window is essential before blood-fed mosquitoes can be used as evidence, and it is needed to set realistic collection schedules for investigators [4].

Molecular genetics has enormously improved the sensitivity for detecting tiny and degraded templates. Central to this is the polymerase chain reaction (PCR). Mitochondrial DNA is a particularly useful target for forensic work because it is present in thousands of copies per cell and is more resistant to degradation than single-copy nuclear DNA. The hypervariable sections of the mitochondrial regulatory region are short enough to be amplified from partially digested material, so even a little residual of host DNA in a mosquito stomach may be sufficient for detection and, in favourable instances, for identification [3].

From an investigative point of view, a gravid mosquito caught at or near a scene within hours of feeding can be highly informative. Engorged females tend to rest in sheltered places close to the host, so capturing one places an individual at a location within a limited time frame. This matters most in enclosed scenes, in bodies wrapped in fabric, and on surfaces where ordinary biological traces are easily disturbed. Linking human DNA in a blood meal to a named person can connect a victim or a suspect to a place, and can support scene reconstruction and the testing of statements. All of this rests on how long host DNA survives inside the insect, which is governed by gut physiology, ambient conditions and the kinetics of nucleic acid degradation [5].

Published data on the persistence of human DNA in mosquito blood meals remain limited, especially under controlled conditions that resemble real casework. This study was designed to close part of that gap. Its aim was to determine, under standardised laboratory conditions, the longest post-feeding interval at which human mitochondrial DNA can still be extracted and amplified from the blood meal of *Culex* sp., and to compare extracts from human-fed and rat-fed mosquitoes. The specific objectives were (i) to measure DNA yield and purity from dissected blood meals, (ii) to test HVRI amplification at 24, 48 and 72 h after feeding, and (iii) to define the practical collection window for forensic use.

## 2. MATERIALS AND METHODS

### 2.1 Study design

This was an experimental laboratory study with a cross-sectional structure at three fixed post-feeding intervals. Blood-fed female mosquitoes were held for 24, 48 or 72 h after the meal and were then processed in the same way, so that detection of host DNA could be compared across time. All runs used the same reagents, the same instruments and the same operator, which removes between-batch variation as an explanation for the results. The full sequence of steps is summarised in Figure 1.



Figure 01: Workflow of the study, from blood feeding through post-feeding holding, abdominal dissection, silica-column DNA extraction and spectrophotometric quantification to PCR amplification of the human mitochondrial HVRI region and agarose gel electrophoresis.

### 2.2 Setting and duration

All laboratory work was carried out in the Biotechnology and Genetic Engineering Laboratory, Department of Biology, College of Science, University of Babylon, from normal for molecular biology work. Instruments were calibrated and control reagents were run with every batch. October 2024 to March 2025. Temperature and humidity in the laboratory were kept within the range

### 2.3 Mosquito collection, feeding and grouping

Female *Culex* sp. mosquitoes were allowed to feed on a human host under laboratory conditions. Only females that were clearly fully engorged were kept; partially fed females were discarded, because the volume of blood in the midgut cannot be assumed in those specimens. Eighteen engorged females were used for the time-course experiment and were divided into three groups of six: Group I (24 h), Group II (48 h) and Group III (72 h). The engorged females were held at  $26 \pm 2^\circ\text{C}$  and  $70 \pm 5\%$  relative humidity with access to 10 % sucrose solution. At the end of each interval the mosquitoes were killed by freezing, which stops enzymatic digestion in the gut and prevents further loss of host DNA before analysis. A second, separate set of 20 females was used to compare host species: 10 were fed on human blood and 10 on the blood of laboratory rats.

### 2.4 Ethical approval

The protocol was approved by the Scientific Research Ethics Committee, College of Science, University of Babylon, under approval number 2023/78 dated 15 October 2024. The human volunteer who acted as the blood-meal host gave written informed consent. Handling of laboratory rats followed the institutional guidelines for the care and use of laboratory animals, College of Science, University of Babylon.

### 2.5 Dissection and preparation of blood meal samples

The mosquitoes were dissected under aseptic circumstances at the end of each holding interval. The blood-filled abdomen was cut off using a sterile blade to avoid surface contamination. Each abdomen was

transferred to a sterile microcentrifuge tube containing 200  $\mu$ L of phosphate-buffered saline (PBS) and physically disrupted to give a homogenous solution of insect tissue and blood. Homogenisation was mild but complete to liberate cellular and extracellular nucleic acids from the blood meal matrix. The homogenates were processed immediately for extraction to prevent additional deterioration.

## 2.6 Genomic DNA extraction

Genomic DNA was isolated from the homogenates by a silica-membrane spin column kit (FAVORGEN) based on the manufacturer's instruction. The homogenate (200  $\mu$ L) was combined with proteinase K (30  $\mu$ L, 10 mg mL<sup>-1</sup>) and incubated at 60 °C for 15 min to breakdown protein and lyse cells. Lysis buffer (GB buffer, 200  $\mu$ L) was then added and the tubes were incubated at 70 °C for 15 min with occasional inversion to release nucleoprotein. Absolute ethanol (200  $\mu$ L) was added immediately to promote binding to the silica membrane, and the mixture was passed through the spin column at 14,000  $\times$  g. The column was washed with WI buffer and then with the ethanol-based wash buffer to remove residual protein, salts and PCR inhibitors. DNA was eluted in 100  $\mu$ L of pre-warmed elution buffer and stored at -24 °C until use [6].

## 2.7 DNA concentration and purity

Concentration and purity were measured by UV absorbance at 260 and 280 nm on a NanoDrop spectrophotometer. Concentration was read at 260 nm and is reported here in ng  $\mu$ L<sup>-1</sup>, and purity was taken as the  $A_{260}/A_{280}$  ratio, which reflects carry-over of protein and organic contaminants. Ratios between 1.8 and 2.0 were accepted as suitable for PCR. Each sample was read in duplicate and the mean of the two readings was used [7].

## 2.8 PCR amplification

Human mitochondrial DNA was detected by amplifying the first hypervariable region (HVR1) of the control region with the primer pair listed in Table 1; the expected product is 343 bp. Each reaction had a final volume of 20  $\mu$ L and contained 12.5  $\mu$ L of pre-mixed PCR master mix, 1.5  $\mu$ L of forward primer (10 pmol  $\mu$ L<sup>-1</sup>), 1.5  $\mu$ L of reverse primer (10 pmol  $\mu$ L<sup>-1</sup>), 3  $\mu$ L of template DNA (about 50 ng) and nuclease-free water to volume. A no-template control and a positive control of known human genomic DNA (positive control) were included in every run. Cycling conditions are given in Table 2 [8]. The same reaction set-up was used for the rat-fed samples, in which a rat-specific caspase-3 fragment was amplified with the primers and product size given in Table 01.

Table 01: Primers used for amplification of the human mitochondrial HVR1 region and of the rat caspase-3 fragment.

| Target            | Forward primer (5' $\rightarrow$ 3') | Reverse primer (5' $\rightarrow$ 3') | Product size |
|-------------------|--------------------------------------|--------------------------------------|--------------|
| Human mt DNA HVR1 | 5'-CTCCACCATTAG CACCCAAAGC-3'        | 5'-TGATTTCACGG AGGATGGTG-3'          | 343 bp       |
| Rat caspase-3     | 5'-GGTATTGAGAC AGACAGTGG-3'          | 5'-CATGGGATCT GTTCTTTGC-3'           | 280 bp       |

Table 02: Thermal cycling programme used for PCR amplification.

| Step                 | Temperature | Time  | Cycles |
|----------------------|-------------|-------|--------|
| Initial denaturation | 95 °C       | 2 min | 1      |
| Denaturation         | 95 °C       | 30 s  | 30     |
| Annealing            | 55 °C       | 1 min | 30     |
| Extension            | 72 °C       | 30 s  | 30     |
| Final extension      | 72 °C       | 5 min | 1      |

## 2.9 Agarose gel electrophoresis

PCR products were separated on 2 % agarose gels prepared in Tris-borate-EDTA (TBE) buffer and stained with ethidium bromide. A 100 bp DNA ladder was run alongside the samples to estimate fragment size. Electrophoresis was carried out at 70 V for 90 min. Gels were visualised under ultraviolet light in a gel documentation system, and images were recorded to show the presence or absence of a product of the expected size at each post-feeding interval.

## 2.10 Image processing

Gel photographs were adjusted only by uniform, linear changes of brightness and contrast applied to the whole image, followed by a light unsharp mask. No part of any image was altered selectively, and no band was added, removed or moved. The original unprocessed images are available from the corresponding author.

## 2.11 Statistical analysis

Data were analysed with SPSS version 16. DNA concentration and purity are reported as mean  $\pm$  standard error (SE). PCR detection was scored as a binary outcome (product present or absent) at each post-feeding interval, so the detection data are described as counts and proportions rather than tested with a parametric method. An independent-samples t-test was used to compare mean DNA concentrations between the two post-feeding interval groups

(significance set at  $P \leq 0.05$ ). A value of  $P \leq 0.05$  was taken as significant.

### 3. RESULTS

#### 3.1 DNA yield and purity

Genomic DNA was recovered from every blood meal processed. Yields varied between samples, which is expected given differences in the volume of blood taken, the stage of digestion reached and the efficiency of extraction from abdominal homogenates. Even so, the amount recovered was sufficient for standard PCR with human mitochondrial primers.

Mean DNA concentration was  $86.00 \pm 22.71 \text{ ng } \mu\text{L}^{-1}$  in the time-course set and  $47.60 \pm 12.38 \text{ ng } \mu\text{L}^{-1}$  in the human- and rat-fed set. Mean  $A_{260}/A_{280}$  ratios were  $1.90 \pm 0.04$  and  $1.90 \pm 0.10$ , respectively (Table 3, Figure 2). Both ratios fall inside the 1.8–2.0 range accepted for downstream enzymatic work, which indicates that the silica-column protocol removed most protein and most PCR inhibitors from a difficult matrix.

The pattern of concentrations across samples is consistent with the increasing enzymatic breakdown of host nucleic acids in the midgut, a process that decreases the amount of recoverable DNA at later post-feeding intervals. The yield and purity results together suggest that the extraction process produced templates that were suitable for PCR and for electrophoretic analysis.

Table 03: DNA concentration and purity of blood-meal extracts (mean  $\pm$  SE).

| Sample set                                   | Concentration ( $\text{ng } \mu\text{L}^{-1}$ ) | Purity ( $A_{260}/A_{280}$ ) |
|----------------------------------------------|-------------------------------------------------|------------------------------|
| Set I — post-feeding time course (human-fed) | $86.00 \pm 22.71$                               | $1.90 \pm 0.04$              |
| Set II — human- and rat-fed mosquitoes       | $47.60 \pm 12.38$                               | $1.90 \pm 0.10$              |

SE: standard error. In the original data table the mean and the standard error were transposed; the values are shown here as mean  $\pm$  SE. Confirm the number of samples behind each mean and the unit of concentration.

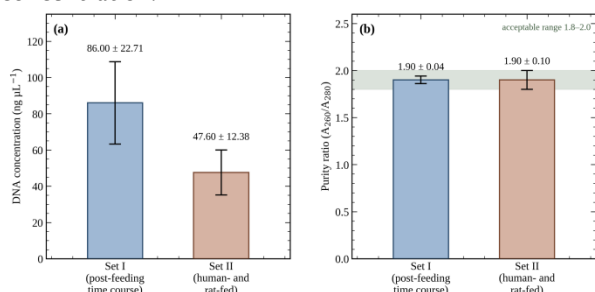


Figure 02: DNA recovered from mosquito blood meals. (a) Mean DNA concentration and (b) mean  $A_{260}/A_{280}$  purity ratio for the two sample sets. Error

bars show the standard error. The shaded band in (b) marks the 1.8–2.0 range accepted for PCR-grade DNA.

#### 3.2 PCR detection of human mitochondrial DNA across post-feeding intervals

The HVRI target was selected because mitochondrial DNA is present in high copy number and is more resistant to digestion than nuclear DNA, making it the more realistic target in a partially digested blood meal. All extracts were amplified using the same optimised procedure.

At 24 h, all samples had a crisp band of the predicted size, indicating that host mitochondrial DNA was generally intact at this time (Figure 3a). At 48 h the picture changed, a faint product was seen in just a part of the samples and the remaining lanes did not show any discernible band (Figure 3b). No amplification product was detected in any of the samples at 72 h (Figure 3c). The pattern is summarised in Table 4.

The loss of signal over time is a direct manifestation of nucleic acid degradation during digestion. It also contains a practical message: evidentiary samples of this kind must be taken early if a molecular result is to be expected.

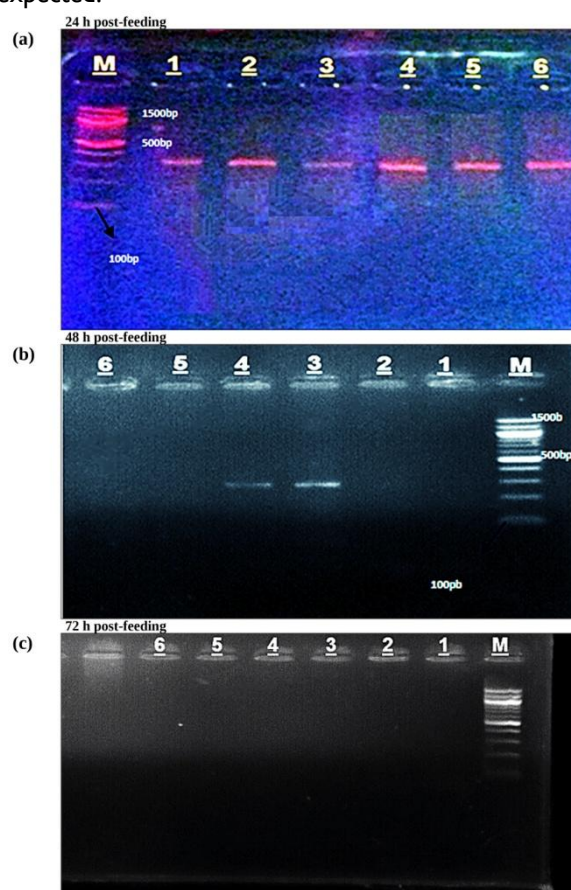


Figure 03: Agarose gel electrophoresis of HVRI PCR products from mosquito blood meals at (a) 24 h, (b) 48 h and (c) 72 h after feeding. M, 100 bp DNA ladder; lanes 1–6, individual blood-meal extracts. Images were adjusted only by uniform brightness and contrast changes applied to the whole image.

Table 04: PCR detection of human mitochondrial DNA in mosquito blood meals at each post-feeding interval.

| Post-feeding interval | Samples tested | Samples with a product | PCR result                                 | Detectability of human DNA |
|-----------------------|----------------|------------------------|--------------------------------------------|----------------------------|
| 24 h                  | 6              | 6                      | Clear amplification                        | Detectable                 |
| 48 h                  | 6              | 4                      | Faint amplification in part of the samples | Partly detectable          |
| 72 h                  | 6              | 0                      | No amplification                           | Not detectable             |

### 3.3 Extracts from human-fed and rat-fed mosquitoes

A second set of 20 extracts was prepared to check that the assay behaved as expected with two different host species. DNA was recovered from both groups at the concentration and purity given in Table 3. Amplification of the human HVRI target gave a product of about 343 bp in the human-fed samples (Figure 4a), while the rat-fed samples were amplified with the rat caspase-3 primers (Figure 4b). The result confirms that the extraction and amplification steps recover amplifiable host DNA from the blood meal, and that the human-specific target does not carry over from a non-human host.

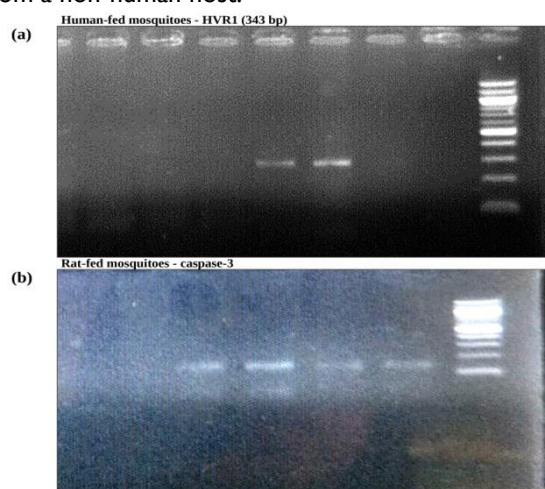


Figure 04: Agarose gel electrophoresis of PCR products on 2 % agarose at 70 V for 90 min. (a) Human mitochondrial HVRI product (343 bp) from human-fed mosquitoes. (b) Rat caspase-3 product from rat-fed mosquitoes. Lane order and ladder positions to be confirmed by the authors: M, 100 bp DNA ladder (Bioneer, Korea); lanes 1–6, individual mosquito extracts.

## 4. DISCUSSION

This work provides a controlled measurement of how long human DNA remains amplifiable inside a mosquito blood meal. Standardised extraction, spectrophotometric quantification, PCR of the human mitochondrial HVRI region and gel detection were combined in a single protocol. The results show a clear time dependence. Amplification was reliable at 24 h, partial at 48 h and absent at 72 h. In other words, the blood meal of a *Culex* female is a real but short-lived pool of human genetic material. Figure 5 summarises how the state of the blood meal, the state of the host DNA and the PCR outcome change together across the three intervals.

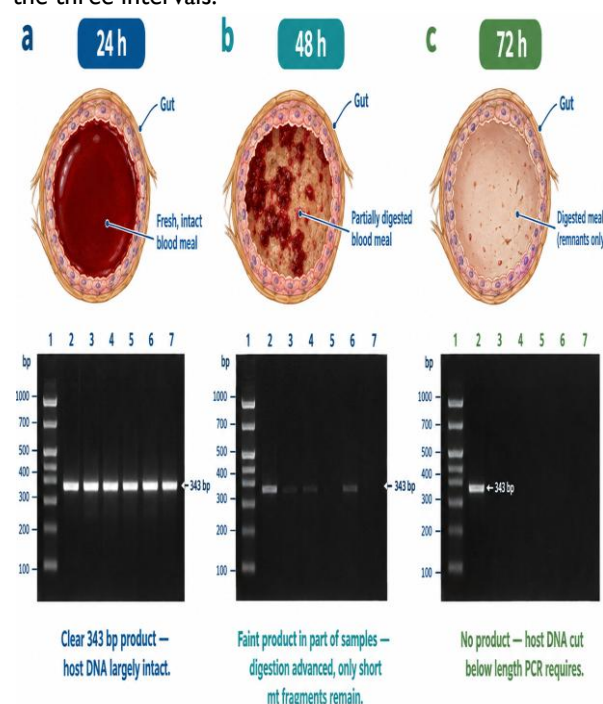


Figure 05: Persistence and breakdown of host DNA in the mosquito midgut. At 24 h the blood meal is fresh and host DNA is largely intact, giving a clear 343 bp product. At 48 h digestion is advanced and only short mitochondrial fragments remain, giving a faint product in part of the samples. At 72 h the meal is digested and host DNA is cut below the length that PCR requires, so no product is obtained.

The strong signal in the first day after feeding follows from the biology of blood digestion. Immediately after ingestion the meal is still close to whole blood, with nucleated cells and free nucleic acids that lyse readily during extraction. Proteolysis and nucleolysis have not yet run to completion, so intact templates survive in the lumen [9]. Hiroshige et al. [10], reported that a mosquito blood meal is fully digested about three days after feeding, which matches the limit found here. Vieira et al. [11], recovered human DNA from *Aedes aegypti* at 1 h and at 24 h after feeding, and Curic et al. [12], showed that a blood-fed mosquito can link a suspect or a victim to a scene even when the body has been moved.

The weak but meaningful signal at 48 h indicates that small mitochondrial fragments are still present after digestion is well advanced. With mitochondrial DNA, there are thousands of copies per cell, therefore some copies of a short target will last longer than any single-copy nuclear locus would. Mitochondrial markers thus constitute the logical initial choice for degraded or low-yield insect material. This pattern is consistent with the most directly comparable study available. Ahmed et al. [13] followed *Culex pipiens* at two-hourly intervals, and obtained full human STR profiles up to 24 h, partial profiles up to 36 h, only weak detection at 48 h and nothing at 72 h, including in mosquitoes fed on mixed human and rodent blood. The agreement of two separate trials employing different markers bolsters the case for a 48 h working restriction.

The complete loss of amplification after 72 h shows that the host DNA has been degraded by nucleases in the midgut to fragments that are too short or too damaged to function as templates. Also, mosquito metabolism gradually takes up blood components, thus removing available host nucleic acid from the lumen. The 72 h marker thus represents a biological ceiling for this type of evidence under the conditions investigated [14].

The implication for casework is straightforward. Amplifiable human DNA may persist for as long as ~48 h suggesting that blood fed mosquitoes collected within this time frame may reveal genetic information that can place a person to a location. Trapping engorged females, which stay at the feeding site while the food is digested, can provide both spatial and temporal information on recent human activity. This is mainly beneficial in indoor and confined settings where mosquitoes gather shortly after eating [12].

The decreasing of detectability over time also advocates for rapid collection and adequate preservation. Freezing blood-fed mosquitoes and stopping digestion allows for rapid recovery and reduces further nucleic acid loss, improving the chances of a useful result. Delayed collection or poor storage permits enzymatic and microbiological degradation to proceed, and can destroy the evidential value of the item altogether. Therefore, protocols for insect-derived DNA evidence should stipulate quick collection, preservation and processing [15].

Methodologically, the silica-column kit was found to be suitable for a mixture of insect tissue and partially digested blood. Purity ratios were within acceptable limits and quantities were measurable in all samples, indicating that protein and inhibitors were adequately lowered for amplification. This justifies the use of commercial extraction kits in forensic entomology since the starting material is rarely pristine [16].

Recovering host DNA from blood-feeding arthropods is not unique to mosquitoes. Human mitochondrial DNA has been isolated and sequenced from the blood meals of the human crab louse, *Phthirus pubis* [17];

human STR profiles have been obtained from the gut contents of fly larvae feeding on decomposing bodies [18]; and human mitochondrial DNA has been sequenced from nitidulid beetle larvae recovered from human bone [19]. Bed bugs have been studied in the same way: Szalanski et al. [20] isolated human DNA of typeable quality from *Cimex lectularius* blood meals, and Silverman et al. [21] detected hepatitis B virus DNA in bed bugs and their excreta for up to six weeks after an infectious meal. Schal et al. [22] later established a time course for human DNA typing from *C. lectularius*, so the earlier statement that no human typing had been achieved from bed bugs no longer holds. The persistence window differs widely between arthropods, which is why each vector has to be characterised separately rather than by analogy.

Some constraints should be mentioned. The quantity of mosquitoes each interval was low, and hence the 48 h result should be interpreted as a point of partial detection rather than an exact detection rate. Only three intervals were sampled therefore the true cut-off remains unresolved, but is between 48 and 72 h. The holding circumstances were constant, whereas real situations fluctuate in temperature and humidity, both known to alter digesting rate. Identification of the donor was not attempted, as detection was based on the existence of a band rather than on sequencing or STR typing. Lastly, only *Culex* sp. was tested. Future work should employ shorter sampling intervals between 48 and 72 h, larger groups, multiple temperature regimes, more than one mosquito genus and quantitative PCR or sequencing to directly evaluate template deterioration.

## 5. CONCLUSION

We effectively recovered and amplified human mitochondrial DNA from blood meals in *Culex* mosquitoes. Amplification was reproducible at 24 h post-feeding, partial at 48 h and absent at 72 h. The 72 h point represents the highest biological limit for molecular detection under the conditions used here. Our findings provide a brief forensic window to use blood-fed mosquitoes as transient repositories for human genetic material. They also show why timing matters at a scene: once more than about 48 h has passed, the chance of amplifying human DNA from a mosquito falls sharply. By fixing that boundary experimentally, the study gives forensic entomologists a practical rule for collection and supports the inclusion of mosquito blood meals among the biological traces considered in human identification and scene investigation.

## 6. AUTHOR CONTRIBUTIONS

G.R.A. designed the study. J.M.U. performed the mosquito rearing, dissection and DNA extraction. J.M.U. carried out the PCR and electrophoresis. J.M.U. and G.R.A. analysed the data and drafted the

manuscript. All authors read and approved the final version.]

## 7. CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

## 8. FUNDING

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## 9. DATA AVAILABILITY

The datasets generated and analysed during this study, including the unprocessed gel images, are available from the corresponding author on reasonable request.

## 10. ACKNOWLEDGEMENTS

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