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Case report

Entomological evidence: Lessons to be learnt from a cold case review

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ABSTRACT

Insects are known to be useful in estimating time since death, but this is only possible if samples are collected and preserved correctly according to best practices. This report describes a case where an 18-year old female was found dead and during the first medico-legal investigation which determined it was a homicide, entomological samples were collected but not considered. The case was then closed with no suspect. However, 9 years after the first investigation the courts decided that the case needed to be re-examined. In doing so the new review team decided that although the remaining entomological evidence was poorly preserved some extra information may be gained from its analyses. On inspection of the remaining samples of larvae no normal morphological analyses could be conducted. Molecular analyses were combined with an unorthodox morphological analysis to provide an estimate of the post-mortem interval based on insect evidence, indicating the value of multidisciplinary approaches to both cold and contemporary cases.

1. Introduction

Medico-legal entomology is the use of insects and other arthropods in forensic investigations involving a dead body (corpses and/or carcasses) and has been used and accepted in many countries [1,2]. The knowledge of the ecology and biology of insects when compared with the crime scene environment may assist forensic investigations especially where medical parameters are no longer of value [3]. The two main issues confronting a Forensic Entomologist in case work following the collection of specimens from a corpse are the identification and the age of the samples [4]. Armed with this knowledge, forensic entomology is a crucial tool in providing a chronological "gold standard" in the estimation of time elapsed since death [5]. To achieve such a standard several publications have highlighted the importance of systematic and quality controlled approaches for the collection and preservation of entomological samples, but so often this does not happen [6].

This case study illustrates the importance of sample collection, and difficulties that may result in analysis of specimens where standard guidelines are not followed. This is also a discussion of a forensic "cold case" [7–9] which is defined as a case where the police investigation has been scaled down and the case is effectively no longer active and still sub judiciary [10]. After a 9 year period this case was re-examined and it was noted that entomological evidence was collected, but it was not considered for examination in the original investigation. On close examination the insect material was desiccated, however it was decided that an attempt should be made to determine firstly the identity of the material, and if successful using an experimental approach then an estimate of the age of the larvae could be calculated.

This case, one of only a few cold cases involving forensic entomology [11–13], has a successful conclusion entomologically, whereby added information was able to be submitted and accepted by a court of law. Critically, this case illustrates the value of combining unorthodox morphological techniques with newer approaches such as molecular analysis to gain valuable information where entomological evidence would otherwise have been neglected.

1.1. Case history

At the beginning of June 2001 an 18-year old girl disappeared from a small city in the Lazio region of central Italy. Three days following her disappearance her body was found in woodland not far from that city in an area usually used by people to discard rubbish. No insect evidence concerning this case was collected at

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the scene. The pathology and police reports stated that the girl was murdered and that the crime scene in the woodland appeared to be the place where she was executed.

The autopsy noted many contradictory observations about the cause of death. Photographs revealed that the girl sustained a bloody wound on her head, and a small mass of fly larvae was present in the eyes of the girl. At the time, the entomological evidence was sampled by the Pathologist, poorly preserved (allowed to dry out) and not used. Instead the Pathologist gave a qualitative determination of the level of moisture on the girl's clothes compared with the amount of rain given in testimony over the previous days to determine her time of death.

Over the ensuing 24 months several medico-legal professionals were consulted and each person presented a contradictory post-mortem interval and a different conclusion about the cause of death. In particular, one report stated clearly that if insect larvae were taken at the time of the crime scene the identity of the species may have been useful in determining a PMI. Two years later the investigation was closed, after a person was found guilty of the crime and imprisoned. However, the evidence used in his conviction was found to be erroneous, and after 2 years of imprisonment the person was exonerated and released.

The case was reopened 5 years later following the exoneration and the prosecutors decided to use a new team of investigators to reconsider the evidence. They decided that an analysis of the insect material found on the body might be useful to determine the time of death.

2. Methods

2.1. Identification

The original evidence was re-examined in collaboration with a new team of investigators. All the entomological samples collected during the autopsy were destroyed prior to 2003. The evidence that had been retained included reports, pictures, a crime scene and autopsy video and the girls' clothes. The clothes were of particular interest because following removal from the refrigerator and the autopsy the clothes were immediately dried in a drying room (65 °C) for 3 h and then packaged in paper boxes. This procedure was well documented using the correct procedures for handling forensic evidence as well as detailing the chain of custody. As a consequence the clothes remained in boxes in cool, dry constant conditions inside a temperature controlled evidence room (20 °C) for the next 9 years.

Fortunately, on examination of the girl's clothing, desiccated insect material was collected from the neck area on the long-sleeved garment. Three egg clusters of approximately 20 eggs and 3 groups of approximately 10 dry fly larvae were removed from the neck region of the long sleeved garment. The eggs and larvae found were completely desiccated and were unable to be identified using a morphological key even after attempts to rehydrate the specimens in water, glycerol and detergent based solutions [14].

Mitochondrial DNA (mtDNA) analysis was therefore used to identify the insect samples. DNA was extracted from batches of approximately 20 eggs, and pooled samples of 10–15 larval fragments, using a phenol chloroform isoamyl alcohol extraction technique. Samples were homogenised in liquid nitrogen using micro-pestles, then 500 μl of Lifton buffer (0.2 M sucrose, 0.1 mM EDTA, 20 mM Tris–HCl at pH 7.5, 0.1% SDS) was added. Samples were left for 15 min at room temperature, then 2 μl of 50 mg/ml Proteinase K added with incubation at 56 °C for 1 h. A further 2 μl Proteinase K was added before overnight incubation at 37 °C. Samples were centrifuged at 14,000 rpm for 30 s and supernatant removed, to which an equal volume of 37 °C Tris–equilibrated phenol was added and vortexed for 1 min. Following centrifugation at 14,000 rpm for 15 min, the aqueous phase was added to an equal volume of chloroform and vortexed for 1 min then centrifuged for 7 min at 14,000 rpm. The aqueous phase was again added to an equal volume of chloroform, vortexed for 1 min then centrifuged for 5 min, before addition of the aqueous phase to 1/10 volume of 3.5 mM NaAc and equal volume of isopropanol, inverted and precipitated at −20 °C overnight. Samples were then centrifuged for 15 min at 14,000 rpm at 4 °C, supernatant discarded and 1 ml of −20 °C, 80% ethanol added. A 5 min centrifugation at 4 °C was followed by removal of supernatant, air-drying of the pellet, and finally re-suspension in 50 μl of sterile water.

DNA was quantified using a NanoDrop 2000c Thermo Scientific spectrophotometer. Amplification of three mitochondrial fragments was attempted, using primers encoding the cytochrome oxidase I (COI) encoding region (Table 1). All PCR reactions were conducted in 50 μl reaction volumes, containing 0.5 unit of Biotherm Taq Polymerase (Genecraft), 1× PCR buffer (Genecraft), 1.5 mM Mg^{2+}, 0.5 mM each primer, 0.2 mM dNTPs and 50 ng of template DNA. Bovine serum albumin (BSA) was added to enhance amplification, at a concentration of 0.05 mg/ml PCR, but 0.1 mg/ml for the primer pair TY-J-1460/C1-N-1687. Thermocycling parameters were set as follows on a Veriti 96-well Thermal Cycler (Applied Biosystems): 94 °C for an initial 1 min denaturation step, followed by 36 cycles of 22 s at 92 °C, 80 s at 72 °C, and finally 60 s at 72 °C. The primer pair TY-J-140/C1-N-1687 was cycled as 36 cycles of 1 min at 92 °C, 1 min at 46 °C and 2 min at 72 °C. PCR products were visualised using 1.5% agarose gel electrophoresis with ethidium bromide staining.

Table 1
Primers used to amplify DNA from eggs and larvae.

Primer	Sequence (5′–3′)	Source
TY-J-1460	TACAATTTATCGCCTAAACTTCAGCC	[23]
C1-N-1687	CAATTTCCAAATCCTCCAATTAT	
C1-J-2495	CAGCTACTTTATGAGCTTTAGG	[23]
C1-N-2800	CATTTCAAGCTGTGTAAGCATC	
UEA7	TACAGTTGGAATAGACGTTGATAC	[27]
TL-2-N-3014	TCCATTGCACTAATCTGCCATATTA	[28]

Successfully amplified products were purified using a glycogen precipitation method with 20 μg of glycogen added to the PCR product along with 3 volumes of absolute ethanol. Following mixing, the reaction was frozen at −20 °C for 1–3 h, then centrifuged at >13,000 rpm for 5 min. The supernatant was removed, and the purified pellet air-dried for 10–15 min before re-suspension in 25 μl of sterile water. PCR products were commercially sequenced (GATC Biotech). DNA sequences were examined using BioEdit (v7.1.3), and similarity to reference sequences computed using blast and tree functions on NCBI to compare with GenBank sequences.

2.2. Larval age

Due to the deteriorated condition of the larval samples and with only remnants of the posterior spiracles evident an experiment was derived to determine the age of the larvae. The larvae on the clothing were completely desiccated. To achieve a similar effect a total of 60 fly larvae were extracted from existing colonies of *Lucilia illustris* (Meigen). The first group consisted of 20 late 1st instar larvae. Ten larvae from each instar (control) were then sacrificed by placing in hot water for 30 s and preserved in 70% ethanol [6,15] and 10 larvae from each instar were placed onto a moist cloth (simulate clothing) into a drying oven until completely desiccated. This process took 3 h at 65 °C. This technique was repeated for 20 late 2nd instar and 20 late 3rd instar fly larvae. Measurements were compared between the length of the experimental samples and the desiccated fly larvae extracted from the girl's clothes.

In addition, meteorological data from the area closest to the crime scene were also available for the time period of the original investigation. No rainfall was recorded only an observation that it had been raining some hours prior to discovery. The average temperature over the period that the girl was missing was approximately 22 °C.

3. Results

The insect samples were poorly preserved (desiccated), and therefore were pooled for extraction. The egg samples yielded an average of 77.1 ng/μl and the larvae 193.93 ng/μl of DNA. Amplification of 831 and 1270 bp fragments was attempted but failed due to presumed degradation of the templates. Amplification of 650, 305 and 220 bp fragments was successful. Sequences were blasted against mitochondrial data from GenBank, the eggs and larvae were determined to be most similar to *L. illustris* with similarity of 98–99% for the samples.

The desiccation experiment demonstrated that the length of the dry samples recovered from the clothes were similar to the 2nd instar samples used for the experiment rather than the 1st and 3rd instar samples. A paired *t*-test between the length of the larvae found on the clothing (mean 1.70 ± 0.07 mm) and instar 1 and instar 3 were highly significantly different (instar 1, $t_9 = 2.26$, $p < 0.01$, mean 1.25 ± 0.08 mm and instar 3 $t_9 = 2.28$, $p < 0.01$, mean 2.25 ± 0.06 mm), whereas with instar 2 there was no significant difference in length ($t_9 = 2.26$, $p > 0.05$, mean 1.72 ± 0.06 mm).

When this case study was initially submitted the case had been closed completely by the prosecutor. This case is now being investigated again but the court has accepted the above entomological evidence. As a consequence a precise