The use of blowfly larvae for offender identification during death investigations with non-consensual sexual contact

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Abstract: Forensic entomology facilitates death investigations by providing evidence into the estimation of time since death and victim identity. However, the application of entomology to identify an offender remains an under-researched area. This study replicated the circumstances of a death with non-consensual sexual contact to explore whether spermatozoa can be visualized from the external surface and crop contents of forensically relevant blowfly eggs and larvae. A sample of each immature stage of blowflies, from three piglet cadavers with a 6 mL deposit of boar semen on their surface, was recovered. Each sample was either submerged in distilled water (dH_2O) and frozen at -18°C or allowed to develop to the third instar before undergoing a crop extraction technique. The samples were all subjected to a differential extraction process, isolating any present spermatozoa from the cadaver's epithelial cells, and stained with hematoxylin and eosin. The results demonstrated that fully intact spermatozoa can be recovered from the external surface of blowfly egg masses and first instar larvae following oviposition upon a location in which semen was directly deposited. Additionally, sperm heads (with no attached flagellum) were recovered from within the crop content of laboratory-reared and feeding third instar larvae from the same cadaver. These findings demonstrate the potential use of forensically relevant blowflies within the investigation of deaths with non-consensual sexual contact.

Keywords: Forensic entomology, crop extraction, larvae, sexual assault, spermatozoa.

Introduction

The number of sexual offences recorded by the police in England and Wales has continuously increased over the last decade. In the year ending March 2022 the Office for National Statistics (ONS) reported 193,566 cases of recorded rapes and sexual assaults by penetration (including attempts) (1). This is a 31% increase of police recorded offences compared to the previous year, and a 265% increase from the 72,977 recorded offences in 2014 (2). However, due to the number of cases that are not reported by victims, it is believed that the true figure is significantly higher (1).

It is reported by the ONS that the majority of these types of offences are committed by biological males (3). Therefore, where a sexual offence has been committed it is likely that there will be semen (containing DNA within the head of the spermatozoa) present if it has been ejaculated by the offender upon sexual arousal. Whilst there are many circumstances in which

semen or sperm cells may not be present including: wearing a condom, not ejaculating, having had a vasectomy, having azoospermia, penetration using an object other than a penis or the offender being a biological female, recovering semen evidence can result in offender identification through DNA analysis and therefore has high evidential value in a sexual assault or rape investigation (4, 5).

Whilst not every sexual assault or rape case ends in the death of the victim, recent UK media has highlighted an increase in cases, such as Sarah Everard (6), Sabina Nessa (7), and Amber Gibson (8) in which the sexual violence resulted in death. Additionally, recent media reports demonstrate cases of this nature are occurring worldwide including in the US (9), Pakistan (10), Paraguay (11), and Argentina (12). In these instances, bodies may be recovered days after the offence was committed and in an advanced stage of decomposition in which no skin is present and thus is unable to be directly swabbed for biological evidence relating to the offender. In this instance, other methods of evidence recovery from the victim would be required.

Forensic Entomology

It is widely reported throughout literature that insects are utilised within death investigations due to their attraction to the products of decomposition and the subsequent colonisation and consumption of a cadaver (13, 14). Most commonly, blowflies and their larvae are used for estimating the minimum post-mortem interval of a cadaver (15, 16).

However, recently several studies have contributed to an advanced use of entomological evidence within forensic research relating to the analysis of the crop contents of blowflies having fed on a victim's cadaver (17). Whilst literature has demonstrated the ability of using gut content analysis for victim identification using a crop extraction process (18, 19), there remains limited research surrounding the success of this technique for the identification of an offender, specifically in a sexual assault or rape case where semen in present. Therefore, the aim of this study was to visualise any present sperm cells recovered from the gut content of larvae in a simulated fatal rape or sexual scene to assess the potential of identifying an offender from any present spermatozoa.

Methods

This study took place in the grounds of Ravelin House at the University of Portsmouth, throughout March, April and May 2021 where the monthly temperatures ranged from 7.6°C to 10.2°C, 6.6°C to 10.8°C and 10.0°C to 14.8°C respectively. During each month, one still-born, frozen-thawed piglet (*Sus scrofa domesticus*) cadaver was placed in a metal crate and left to naturally decompose. On day one of each study, each cadaver had 6 mL of thawed boar semen placed in 2 mL aliquots in three locations: behind the ear, in-between the rear legs and across the surface of the trunk (Figure 1).

FIGURE 1 Three piglet cadavers on day one of the studies in March (a), April (b) and May (c), all with a total of 6 ml of boar semen placed between the rear legs, behind the ear and across the trunk.

On average, each human ejaculation is 2-6 mL in volume (20, 21), therefore 6 mL was opted for to increase the chance of recovering larvae which had consumed semen whilst remaining within the average range. The locations to deposit the semen (specifically between the legs and along the surface) were chosen as they represent areas where seminal fluid is likely to be present upon a cadaver after non-consensual sexual contact has taken place (22). Additionally, these locations (specifically behind the ear and between the rear legs) provide a desirable oviposition site for blowflies due to the warm, dark, and moist environment they provide (23), as well as being areas in which the researcher would have sufficient access to for sample recovery. A sticky trap and an EasyLog EL-USB-2 data logger were placed alongside each cadaver to collect adult insects and measure the hourly ambient temperature.

Sample Collection and Storage

When present on each cadaver, one small mass of eggs was recovered from each of the locations where the semen was originally placed. Each mass was transferred to an Eppendorf tube containing 20 µL of distilled water (dH_2O) and stored at -18°C for subsequent surface sperm recovery.

Once first instar larvae were present, one small mass was recovered from the three locations and preserved as the eggs were with the aim to wash off any sperm cells present on the external surface.

Subsequently, ten individual second instar larvae were recovered from each cadaver. Larvae were not recovered from a specific location on the cadaver as in each instance the mass of larvae colonising the cadaver at this stage were too large to know where they had originally been oviposited. These larvae were reared under specific laboratory conditions (contained at 22°C in a bug dorm with access to sheep liver as a food source and sand for burrowing pre-pupation) and left for approximately 48 hours to develop into the third instar stage as this is where the crop is the largest (24). Once this developmental stage had been reached, all larvae were hot water killed (HWK) and preserved in 80% ethanol (25).

When third instar larvae were present on the cadavers, ten were recovered from each before being HWK and preserved in 80% ethanol. All third instar larval samples that were preserved in ethanol were left at room temperature until required for downstream laboratory processes.

Sample Preparation

All frozen samples in dH_2O were thawed at room temperature and vortexed to release any present spermatozoa from the surfaces of the eggs and larvae. The liquid was removed from each Eppendorf tube with care given not to transfer any entomological material.

A sterile scalpel was used to slice the length of the exoskeleton of all third instars (those reared in the laboratory and recovered at third from the cadaver) being careful not to cut through the whole depth and destroy the crop. The entire crop of each larva was removed, transferred to an individual Eppendorf tube, and submerged in 20 µL of dH_2O. Each sample was agitated with a clean pipette tip and gently vortexed for 30 seconds to release any present spermatozoa from within.

Grade	*Presence of Spermatozoa*
Negative	Spermatozoa not present
1+ Heads	Spermatozoa are hard to find
2+ Heads	Some spermatozoa in some microscopic fields easy to find
3+ Heads	Many or some spermatozoa in most fields
4+ Heads	Many spermatozoa in every field
T	Complete spermatozoa with tails present

Sample Processing

Each sample was subjected to a standard differential extraction process with the aim to denature and remove any excess epithelial cells and therefore isolate any present spermatozoa which are robust enough to withstand the process (26, 27). Each sample was added to 996 µL of lysis buffer and 4 µL of Proteinase K prior to being incubated at 56°C for a minimum of 30 minutes. The samples were centrifuged for five minutes at 8000 x rpm. All supernatant was removed, and 1 mL of phosphate buffered saline (PBS) solution was added. The samples were centrifuged once more for five minutes at 8000 x rpm. Following this, 950 µL of the solution was removed and the pellet was resuspended in the remaining 50 µL by agitation with a pipette tip.

Subsequently, 10 µL of each sample was placed in the centre of a sterile microscope slide and dried on a heat plate at 56°C. Sufficient haematoxylin was pipetted onto each slide and left for two minutes. The haematoxylin was washed off under slow running tap water before two drops of eosin was pipetted onto each slide. After 30 seconds the eosin was washed off and the slides were returned to dry on the heat plate. The presence of spermatozoa on each slide was assessed using Allard's grading system (Table 1) (28) at 40x magnification under a compound microscope. A slide of neat boar semen and four slides from buccal/semen swabs that had undergone the differential extraction processes were also stained and analysed for the presence of spermatozoa. These swabs were an addition to this study as control samples used to assess the success of differential extraction by representing a victim/offender mixed sample which is typically found within sexual assault casework (29).

TABLE 1 —*Allard's grading scale (28) to determine the presence of spermatozoa.*

Results

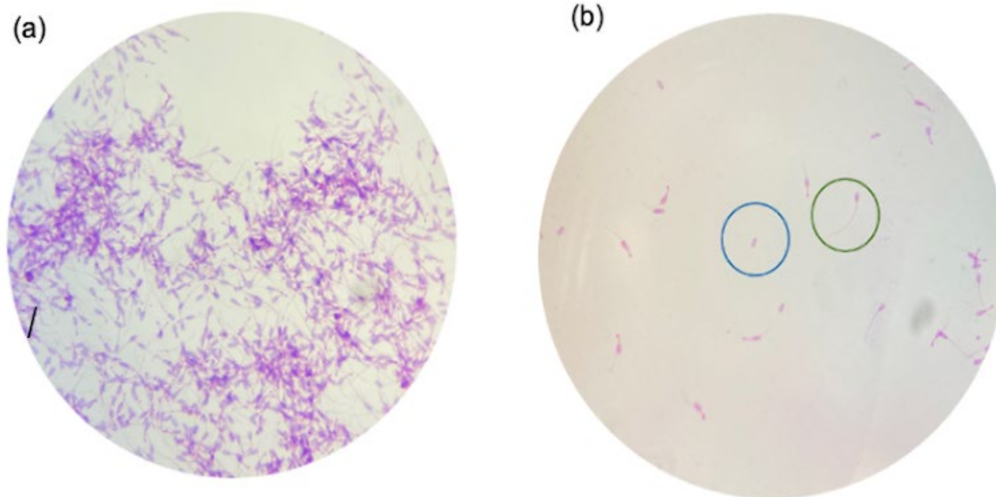
Throughout this study, 38 slides were prepared and assessed for the presence of spermatozoa. Of these 38 slides, one slide contained neat boar semen and was the only slide graded *T* due to the abundance of intact spermatozoa in all fields. All four of the buccal/semen mixed slides were graded *4+ as* the spermatozoa in these slides were mostly intact and easily viewed. Additionally, there was an absence of epithelial cells in each of these slides, therefore demonstrating the success of the differential extraction process (Figure 2a). Of the ten slides prepared from the egg masses, 40% were graded *Negative* and 60% were graded *1+.*

Of the five slides prepared for the first instar larval masses, 20% were graded *Negative,* 60% were graded *1+*, and 20% were graded *2+* (Figure 2b).

Of the ten slides prepared from the third instar larvae that had been reared in the laboratory, 50% were graded *Negative* and 50% were graded *1+.*

Of the eight slides prepared from the third instar larvae recovered from the cadaver, 50% were graded *Negative* and 50% were graded *1+.*

FIGURE 2 (a) A microscope image showing a *4+* grade given to a neat semen sample. (b) A microscope image showing a *2+* grading from a sample of first instar larval mass where both sperm heads (circled, blue) and intact spermatozoa (circled, green) are present.



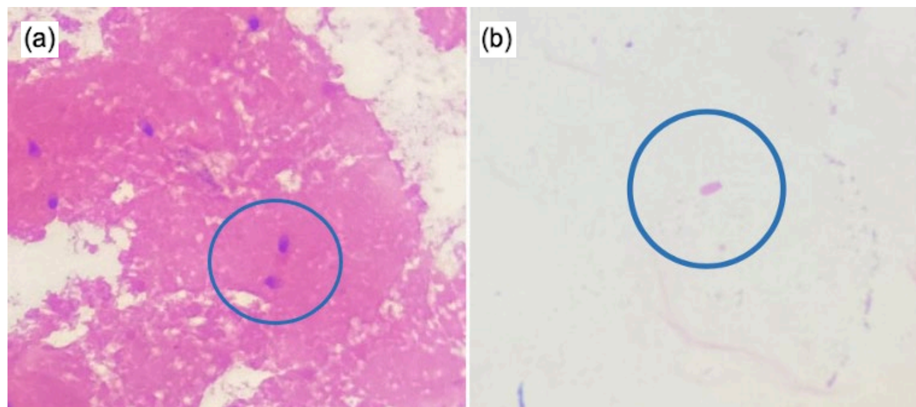
Discussion and Conclusion

These results demonstrate that intact spermatozoa and/or sperm heads were present in at least 50% of all assessed entomological samples. It was identified that the slides prepared from egg masses and first instar larval

masses were graded highest for the presence of spermatozoa and sperm heads. It was additionally noted that these samples had the most intact spermatozoa compared to the reared and feeding third instar samples which had a higher percentage of sperm heads with no attached flagellum. It was expected that a higher number of intact spermatozoa would be seen within the eggs and first instar sample as there was a shorter time period between when semen was deposited on the cadaver and when the potential semen evidence was recovered. Literature surrounding the degradation of sperm cells has highlighted that the separation of the tail from the midpiece and the head is indicative of the start of seminal degradation which typically occurs 12-24 hours post ejaculation (30, 31); a finding which is corroborated by this study.

Whilst the tails are not required for offender identification (due to the DNA being contained within the nucleus in the sperm head), having fully intact cells is beneficial as the presence of the flagellum provides the cell with its uniquely identifiable structure. When visualising sperm heads from mixed samples (containing at least the epithelial cells of the victim and the sperm cells of an offender) it can be difficult to distinguish what is a sperm head and what is unwanted cell debris where the tails are absent (Figure 3). The addition of the differential extraction process in this study allowed the researcher to state with confidence that the only remaining biological material in the sample were sperm cells. Differential extraction is an essential step in current sexual assault and rape investigation casework as the removal of all non-sperm cells from a sample reduces the chances of obtaining a mixed DNA profile (32). Whilst DNA analysis was not performed during this pilot study, completing the differential extraction process at this stage will ensure that samples contain as little non-suspect DNA as possible when DNA analysis is carried as an extension of the current project.

FIGURE 3 (a) An example of a sperm head (circled) from a swab of the trunk of a piglet cadaver with semen present on the surface *before* the differential extraction process (stained with H&E and viewed at 10x magnification). (b) an example of a sperm head (isolated from any unwanted epithelial cells) from the same sample as (a) *after* the differential extraction process was carried out.



It was expected that not all samples would contain spermatozoa due to the large mass of larvae colonizing the cadaver and only 6ml of

boar semen being present. Whilst an effort was made to recover eggs and larvae from the locations where semen was originally deposited on the cadaver, this was not guaranteed due to the movement of the larvae and the advanced stage of decay that was observed by the time third instars were present. This must be considered for industry practice as it is not definite that larvae which will have consumed semen will be recovered during the sample collection stages, especially if the cadaver surface area to sperm volume ratio is lower than what was used in this study. To overcome this limitation, the number of entomological samples that are recovered from a cadaver should be as high as possible, as suggested in standard practice (33, 34). Whilst this would also increase the time in which it takes to prepare the samples for analysis, a methodology in which samples can be batch processed is being developed by the authors.

Furthermore, this research is being expanded to assess the DNA potential of the recovered samples. Thus far, this study has successfully demonstrated the ability to recover spermatozoa/sperm heads from all stages of entomological evidence. If DNA is isolated from the samples, a profile may be obtained with the potential to identify the offender.

Conclusion

The results of this study have demonstrated that both fully intact cells and isolated sperm heads are recoverable from the gut content of blowfly eggs and larvae. This demonstrates the successful application of entomological evidence for offender identification during the investigation of a death with suspected non-consensual sexual contact.

The results also demonstrate the success of the differential extraction process by concentrating spermatozoa from samples which also contain an abundance of epithelial cells. However, further research (currently being conducted by the researcher) with the aim of assessing the potential to obtain a DNA profile from the recovered spermatozoa is necessary. With an advancement in the DNA aspect of this project, this technique can be used within forensic investigations to aid the identification of an offender of a fatal rape or sexual assault.

Author Contributions

All authors conceived and designed the experiments and analyses; AC collected the data and performed the analysis; AC, HM and KB contributed towards writing the paper.

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