Molecular Forensic Investigations into Animal Sexual Abuse

by

KHILONA NATHA
NTHKHI001

SUBMITTED TO THE UNIVERSITY OF CAPE TOWN
In partial fulfilment of the requirements for the degree

MPhil Biomedical Forensic Science

Division of Forensic Medicine and Toxicology
Department of Pathology
Faculty of Health Sciences
UNIVERSITY OF CAPE TOWN

Date of Submission: **27 March 2020**

Primary supervisor: **Dr L.J. Heathfield**
Co-supervisor: **Mr. C.G. Mole**

Division of Forensic Medicine and Toxicology
Department of Pathology
Faculty of Health Sciences
University of Cape Town

DECLARATION

I, Khilona Natha, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I authorise the university to reproduce for the purpose of research either the whole or any portion of the content.

This dissertation has been submitted to the Turnitin module and I confirm that my supervisor has seen my report and any concerns revealed by such have been resolved with my supervisors in any manner whatsoever.

Name: Khilona Natha

Student number: NTHKHI001

Signature: Signed by candidate

Date: 27 March 2020

Abstract

Animal sexual abuse (ASA) involves the sexual molestation of animals by humans. The identification of semen provides a legally-accepted indicator that sexual activity occurred, while forensic DNA analysis provides a lead to a potential suspect. After conducting a systematic literature review, no previous research investigating semen and/or DNA recovery from animals *over time* was found. Therefore, this pilot study aimed to assess the recovery of human semen and DNA from animal fur over a two-week period to establish baseline data pertaining to evidence retention in the ASA context. This pioneer study also attempted to contribute towards the development of a suitable animal fur model on which to perform experiments. Daily swabbing and testing of semen from three fur models (unpreserved baboon fur, preserved nyala hides and faux fur) showed that semen could still be detected at 14 days using standard presumptive and confirmatory tests. Although DNA degradation showed a statistically significant increase over time, forensically usable DNA profiles (≥ 12 fully typed short tandem repeat loci) were consistently obtained. There was significantly higher DNA degradation in samples from the baboon fur compared to the others, while DNA concentrations were significantly different between each fur model. These differences highlight that future research must consider the choice of fur model to best represent the animal of interest; *e.g.* dissected fur from a recently deceased animal would best mimic a fatal ASA case. The insight regarding the choice of animal model hopes to be of benefit for future research, which should focus on the influence of more realistic variables (*e.g.* movement and body heat) on semen and DNA retention on animal fur. Overall, this study successfully generated baseline data, and provides a foundation for additional research, which hopes to eventually assist in the interpretation of forensic evidence in the global burden of ASA.

Acknowledgements

I would first and foremost like to thank the Almighty for blessing me with good health, strength and His guiding light throughout this year.

To my supervisor, Dr Laura Heathfield, for her vital expertise, intuition and guidance throughout this project.

To my co-supervisor, Mr. Calvin Mole, for his knowledgeable input, assistance and willingness to always help.

To Ms Jo Millington for her contribution towards the conceptualisation of this project.

To Mr Lyle Curry for his assistance in the recruitment of sample donors.

To Cape Nature for the provision of the baboon specimens.

To Mrs Carol Royle and eZulu Game Reserve for the donation of the nyala fur.

To Dr Andrea Gibbon, Ms Tracy Bennett and Dr Gloudi Agenbag for providing their guidance and training regarding the molecular techniques used in the project.

To Mrs Yolanda Davies and Ms Michelle Perrins for their assistance and provision of resources for histology.

To the University of Cape Town for providing me with a scholarship award to financially support my studies.

To the Division of Forensic Medicine and Toxicology for funding the project.

To my friends and colleagues who have provided support and assistance when needed.

To my parents and brother. I thank you for your blessings, support and consistent motivation.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AEC	Animal Ethics Committee
ALS	Alternative light source
AP	Acid phosphatase
ASA	Animal sexual abuse
BFB	Brentamine Fast Blue
BFI	Body fluid identification
bp	Base pairs
cm	Centimetre
C_t	Cycle threshold
CT	Confirmatory testing
DI	Degradation index
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
FISH	Fluorescence *in situ* hybridisation
g	Gram
H&E	Haematoxylin and eosin
HREC	Human Research Ethics Committee
h	Hours
IC	Internal control
kV	Kilovolts
LCM	Laser capture microdissection
MeSH	Medical subject headings

mL	Millilitre
µL	Microlitre
µM	Micrometre
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
ng	Nanogram
nm	Nanometre
pg	Page
%	Percent
POP7	Performance optimised polymer 7
PCR	Polymerase chain reaction
PT	Presumptive testing
PSA/p30	Prostate specific antigen
qPCR	Quantitative real time polymerase chain reaction
RFU	Relative fluorescent units
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Seconds
SEM	Scanning electron microscopy
SPCA	Society for the Prevention of Cruelty to Animals
STR	Short tandem repeat
SVSA	Seminal vesicle-specific antigen
TSI	Time since intercourse
Vic	Victim

Vol	Volunteers
Y-STR	Y-chromosomal short tandem repeat

Chapter 1: Literature Review

1.1 Introduction

Human-animal sexual interactions have been known since Biblical times and have been expressed in art, folklore and pop culture.[1–4] Animal sexual abuse (ASA) involves harm inflicted on animals for human sexual pleasure.[5] This can involve fondling of genitalia; vaginal, anal or oral penetration; oral-genital contact; penetration with an object and the injuring or killing of an animal for sexual gratification.[5] Men more commonly partake in the act, with dogs and horses more frequently abused.[2,5–9] Other reports of sexually abused animals include goats, sheep, chickens and cows.[5,10–17]

Determining the true prevalence of ASA is challenging due to under-reporting, which in turn, is due to the inability of animals to talk, dishonesty of perpetrator(s) and social stigmatisation surrounding human-animal sexual interactions.[18,19] In a study on non-accidental injury in animals, Munro and Thrusfield reported an ASA prevalence of 6.3 % based on information provided by selected veterinarians in the United Kingdom.[8] The occurrence of ASA in other cohort-based studies are either outdated or reported as a secondary behaviour to a primary concern, such as criminal behaviour or psychological disorders.[5,18,20–32]

1.1.1 Definitions and classifications of ASA

Various terminology is used to describe ASA, with the colloquial term "bestiality" and the clinical term "zoophilia" commonly used. "Bestiality" describes sexual interactions between human and animal without the human emotionally bonding with the animal.[33] However, there may be a clinical aspect to ASA where a person is unable to control their sexual desire rather than it being malicious in nature. In these instances, when there are *emotional* and sexual *affections* for an animal, the term "zoophilia" is used.[33] Zoophilia is listed as a paraphilia in the Diagnostic and Statistical Manual of Mental Disorders, Fifth edition as well as the International Classification of Diseases, 10th Revision, Clinical Modification.[34,35] Self-identified zoophile populations exist who, despite knowing their actions may be illegal, prefer to have sexual intercourse with animals rather than humans.[2,6,7,9]

When considering malicious human-animal sexual interactions, Beirne used the term "interspecies sexual assault".[36] This term implies that animals of different species may sexually abuse one another, which is highly unlikely in nature. Veterinarians, Munro and Thrusfield,

then suggested the use of the term "animal sexual abuse" to describe any form of human-animal sexual activity or harm brought upon an animal for sexual gratification by a human.[8] In light of this definition, "animal sexual abuse" (ASA) is the terminology that will be used throughout this dissertation.

1.1.2 The use of molecular forensic techniques

Maintaining voluntariness in sexual interactions between humans is legally, ethically and socially important, however, sexual willingness is not a sentiment that an animal can express to a human.[3] Although there have been arguments that an animal's non-retaliating behaviour and assent serves as a form of consent, coercion is always a factor.[18,36,37] The animal does not voluntarily choose to partake in the activity, informed consent cannot be given and the interaction poses the risk of severe pain, injury or even death.[36] Thus, animals which are subjected to sexual relationships with humans are at risk of suffering, and this type of interaction should not be excluded from being punishable by law.[16] ASA is illegal in a majority of countries and warrants criminal and forensic investigation.[5,21,38–43]

During criminal acts, biological evidence deposited at the crime scene or on the victim, such as body fluids, not only aid in crime reconstruction, but also provides a means of identification of the fluid donor. Molecular forensic techniques are usually used for body fluid identification (BFI) and subsequent human identification and thus forms an integral part of forensic investigations. Semen corroborates an act of sexual intercourse and by detecting human semen on or in animals, an instance of ASA can reasonably be supported.

Human semen is made up of seminal fluid and spermatozoa. Seminal fluid contains organic and inorganic components such as fructose, amino acids, acid phosphatase (AP) and prostate specific antigen (PSA or p30).[44] The average volume of semen present in an ejaculate from a healthy male ranges from two to six (2 - 6) ml with approximately 60 million spermatozoa/ml.[45] In an ejaculate from a healthy male, there is approximately 200 – 500 million spermatozoa.[45]

Various techniques are available for BFI which are either presumptive or confirmatory in nature. Techniques used to screen for the possible presence of semen are usually performed through enzymatic or chemical assays that target the various seminal fluid components,[46] while microscopic identification of spermatozoa confirms the presence of semen.[47] Guidelines on how to address the medico-legal examination of ASA are provided by Stern and Smith-Blackmore.[5]

The recovery of semen in ASA can become challenging as an animal cannot report the crime and evidence can be lost over time due to the animal's natural physiological processes, uncontrolled daily activities and environmental exposures. Although case reports of ASA exist, it appears that there is a paucity of literature exploring the extent of human semen persistence and recovery from animals over time. Therefore, this systematic literature review aimed to document the retention and recovery of human semen and resultant DNA over time in a medico-legal context, with particular attention to the recovery thereof from animals.

1.2 Objectives of the literature review

The objectives of this literature review were to:

- Quantify the number of studies which documented human semen recovery in relation to time in the context of ASA, and portray this against those in the context of sexual offences.
- From the included studies, calculate the frequency of the different molecular forensic methods used to analyse semen.
- Assess retention and recovery rates of human semen and resulting DNA over time, from animals and other substrates, when common detection methods were used; and using this data, describe factors which may affect semen retention.

1.3 Methods

1.3.1 Search strategy

Three literature databases; PubMed, Scopus, and the meta-database, Web of Science™, were searched using search queries that incorporated several themes surrounding the research aim (Appendix A, Table A1). An additional specific search for ASA case reports was also done in PubMed using terminology specific to ASA (Appendix A, Table A1). All terminology and relevant MeSH terms (medical subject headings used in PubMed) are noted in Table A2 and Table A3 (Appendix A) respectively.

The resultant literature was then evaluated according to the inclusion and exclusion criteria (section 1.3.2). Every reference from the included articles was then "hand-searched" and evaluated against the same inclusion and exclusion criteria. For review articles, hand-searching

was limited to relevant articles pertaining to semen detection and DNA recovery from semen. Data not referenced in review articles could not be hand-searched. One round of hand-searching was performed.

1.3.2 Inclusion and exclusion criteria

Articles were only included if it adhered to the criteria listed below. Literature obtained from the database searches were read in full to determine if the article was to be included. Hand-searched articles were evaluated based on the title, abstract and/or scanning through the article.

Article context and content:

- Studies investigating the recovery of human semen and/or subsequent DNA in the context of sexual offences were included.
- The persistence of semen and/or DNA from semen **must** have been made in reference to time and/or post-exposure to water.
- DNA must have been obtained from human semen for the purposes of *human identification*. Articles where DNA or RNA was used for the purposes of *BFI* were excluded.
- An article or data was included regardless of the exhibit or body region that semen was recovered from, except if samples were obtained from the penis.
- Articles pertaining to reproductive health or storage effects on samples were excluded.
- Articles pertaining to method development, optimisation or validation of BFI techniques as well as age-estimation of stains, were excluded.
- Studies aimed at assessing skills and methodology between different forensic analysts and laboratories were excluded.

Article data:

- Articles were included if it contained some data that met the requirements above. Thus, sometimes only subsets of data could be extracted and included in this review.
- Data presented in a format that could not be interpreted quantitatively were excluded from the analyses.

- Data where secondary transfer of semen was explicit were excluded.
- DNA profiling data were only included if achieved by short tandem repeat (STR) analyses and not by obsolete methods (*e.g*. restriction digestion, blood typing).
- DNA profiling data were only included if it was explicit that DNA was extracted from semen (as opposed to epithelial cells, reference samples or unknown).
- To avoid bias, data pertaining to recovery *rates* of human semen with regards to presumptive and/or confirmatory testing were excluded if they did not represent the full data set (*e.g*. if presumptive testing recovery rates were only reported for the positive confirmatory tests, this would not reflect a true rate).
- Data in instances where semen deposition was not possible (*i.e*. it was reported explicitly that penetration and/or ejaculation did not occur, or condoms were used) and spermatozoa recovery from explicitly classified azoospermia cases were excluded.

Article type and quality:

- Only peer-reviewed, original articles from accredited journals were included. Review articles, editorials and letters to the editor were not included in the article count, but were included for hand-searching purposes only.
- Articles had to be available in English.
- Articles of poor scientific quality or where representation of data was unclear, were excluded.

1.3.3 Data collection and analysis

Variables pertaining to the cohort, sample site/substrate, analytical method(s) used, time since intercourse (TSI) and, successful recovery of semen and/or DNA over time were extracted from the included literature and collated for analyses. For the purposes of this literature review, presumptive testing (PT) comprised all techniques that screened for the presence of semen except those that were based on spermatozoa identification. Confirmatory testing (CT) was based on the detection of spermatozoa and DNA analyses was based on the performance of STR profiling only.

To assess semen recovery, the detection of spermatozoa was considered positive regardless of quantity. PT was considered positive according to the respective study's cut-off reaction times

or quantitative thresholds. DNA analyses were deemed successful regardless of the completeness of the profile. The success rate of DNA profiling, as defined by the authors, was captured or calculated.

The recovery rates of AP and spermatozoa were calculated as a percentage of the total number of samples tested for that category (*i.e.* time interval or number of washes). The retention of semen over time was assessed by Spearman's Rank Correlation and regression analyses at a significance level of $\alpha = 0.05$. This was done using recovery rates from all available studies, irrespective of sample site, size or cohort. The recovery rates of AP and spermatozoa from laundered semen stains were calculated and represented separately. Graphical representations and statistical analyses were performed using R and R studio Version 3.4.

1.4 Results

1.4.1 Literature search and included articles

The database searches resulted in a total of 216 non-redundant articles of which **24** met the inclusion criteria (Figure 1.1). These, along with a total of 21 review articles, were hand-searched, where a further **52** articles met the inclusion criteria for analysis. Of the review articles, **one** article also contained new data for a case report. These data were included, bringing the number of included articles to **77**. Twenty-one articles in total were unavailable and could not be assessed.

Additional searching in PubMed specifically for ASA case reports yielded 29 case reports (Appendix B, Table B1). However, only **one** of these case reports contained data with respect to time and could be included for analyses. One article was unavailable and could not be assessed.

Of all literature obtained, three articles (a review, an ASA case report and a sexual offences case series) described instances where molecular forensic techniques were used to investigate ASA, but no reference to the TSI was given, and thus, these articles were excluded.[5,12,14] Thus, a total of **78** articles were included in this review.

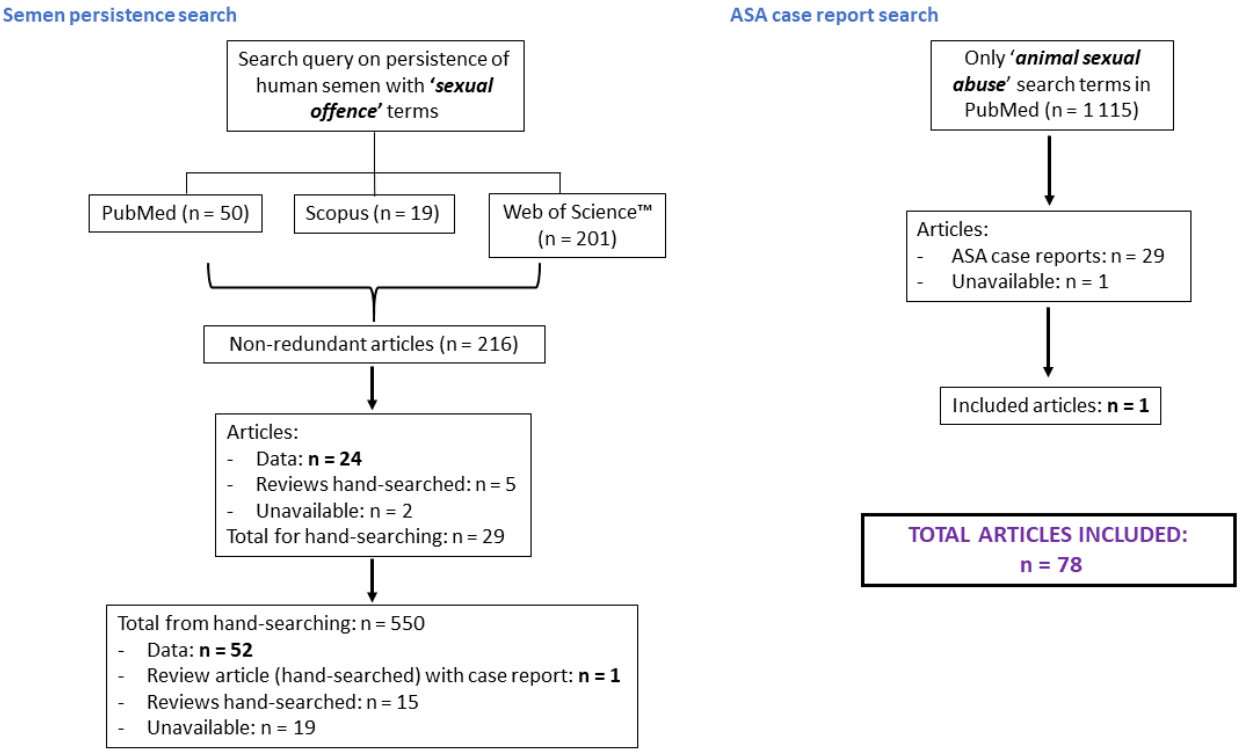

Figure 1.1: Flow diagram depicting results from the literature search and animal sexual abuse (ASA) case report search. Data was extracted from a total of 78 articles, one of which was a review article which also contained a case report.

1.4.2 Study design and cohort characteristics

Three main "study cohorts" were identified in the literature; namely (i) humans (living and deceased sexual offence victims, volunteers and human models), (ii) animals (ASA victims) and (iii) inanimate surfaces and objects. The frequency of these, along with the different study designs used, are represented in Figure 1.2. Ten articles addressed two different cohort types. Living sexual offence victims was the most commonly studied cohort (n = 32/88; 36.4 %), followed by inanimate surfaces and objects (n = 23/88; 26.1 %).

Of the ten articles that addressed two different cohorts, seven articles used the same study design for both cohorts. Prospective study designs were the most common as it was utilised 42 times in the 78 included studies (n = 42/78; 53.8 %). All three study designs were found in the "inanimate surfaces and objects" cohort. Only one ASA case report was obtained,[11] and no prospective or retrospective studies had been conducted with regards to the longitudinal detection of human semen in ASA over time.

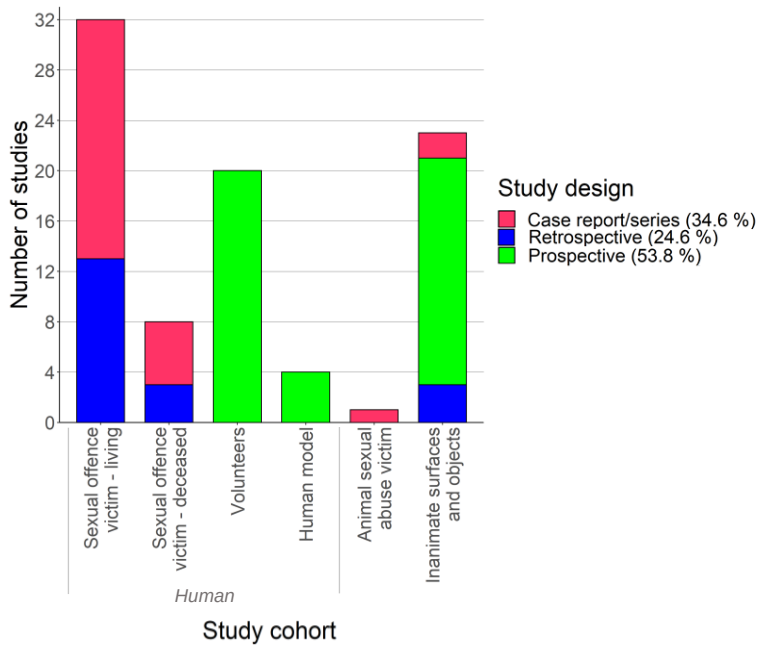

Figure 1.2: The frequency of case report/series (pink), retrospective (blue) and prospective (green) studies grouped according to sample cohort type. Living sexual offence victims were the most frequently studied cohort (36.4 %). Ten studies addressed more than one cohort type and seven of these used the same study design for both cohorts. Only one animal sexual abuse case report which provided the time since the assault was found.

Various sample sites were assessed in the different cohorts, with the human vagina (internal as well as the labia, vulva, and perineum) being the most frequent (n = 58/130; 44.6 %, Figure 1.3 A). Vaginal, anal/rectal (including the perianal region), oral (including the lips) and "other" samples were analysed in both living and deceased sexual offence victims. "Other" sample sites comprised of human skin, hair, urine and gastric fluid. This sample site was studied in all four human-based study cohorts. "Human models" consisted of two studies that addressed the retention of human semen on human skin models,[48,49] and two in simulated gastric fluid.[50,51]

The only ASA case report is represented as "animal site" (Figure 1.3 A) and comprised of vaginal and anal samples from a sheep.[11] "Inanimate surfaces and objects" comprised of a range of textiles and surfaces containing semen stains (Figure 1.3 B) with stained cotton fabric being more frequently analysed (n = 14/40; 35.0 %). "Undefined" textiles comprised clothing, linen and synthetic cloths.

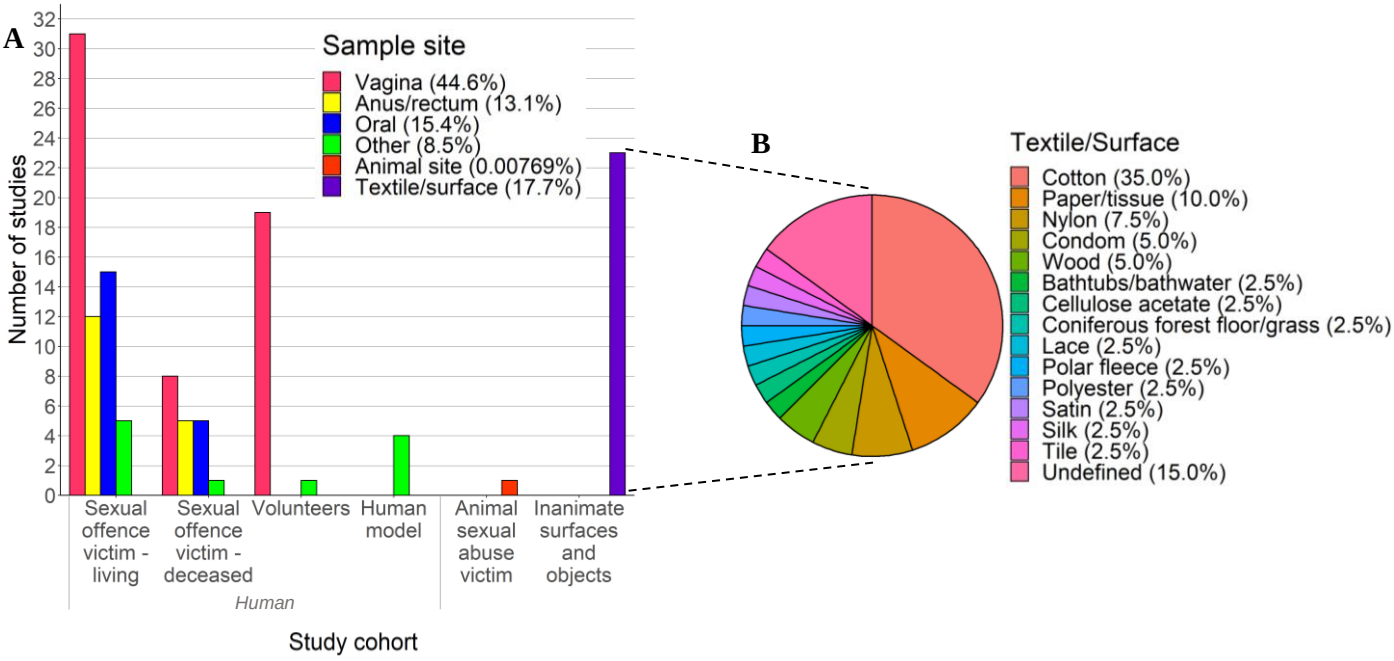

Figure 1.3: (A) The frequency of the various sample sites for each of the cohort types. The human vagina was the most frequently sampled site (44.6 %) and there was only one instance in which samples were obtained from an animal. **(B) The frequency (%) of the various textiles and surfaces (inanimate surfaces and objects) from which semen was sampled**. Cotton materials were the most commonly sampled inanimate object (35.0 %).

1.4.3 Techniques used

The number of studies which incorporated the use of either PT and/or CT for semen and/or, the generation of a DNA profile from semen is represented as a Venn diagram below (Figure 1.4). Most of the included articles reported the use of PT (n = 49/78; 62.8 %) and CT (n = 68/78; 87.2 %) whereas only 22 (28.2 %) studies reported the use of DNA profiling. Of 78 studies, only 12 (15.4 %), including the ASA case report, incorporated all three types of analyses. In more than half the studies (n = 42; 53.8 %), PT was followed by CT. Murray *et al.* represented the single study that performed PT with DNA profiling (no CT), as their volunteer subjects were mainly azoospermic.[52]

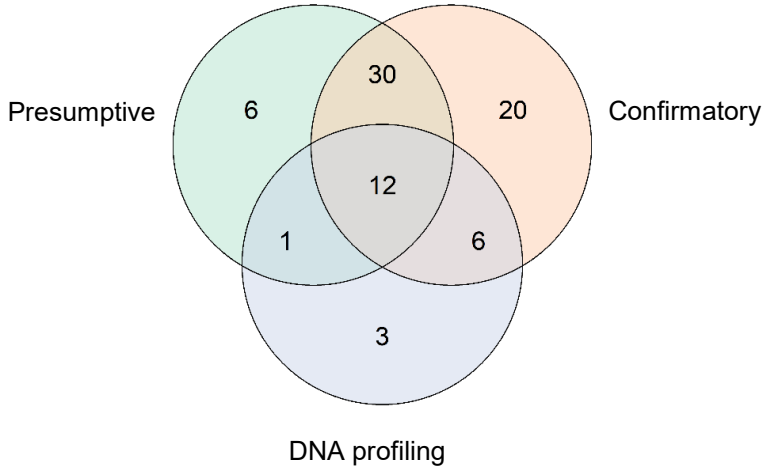

Figure 1.4: A Venn diagram illustrating the number of studies that reported the use of either one, two or all three of the various testing methods to detect and analyse semen evidence. Twelve studies utilised all three types of analyses.

Seven different PT methods were identified among the studies (Figure 1.5), with some studies incorporating more than one method. The detection of AP was the most frequently used PT (n = 43/69; 62.3 %),[11,52–93] followed by PSA (n = 13/69; 18.8 %).[51,56,58,63,65,70,73,74,82,86,88,94,95] While the use of alternative light sources (ALS) was the third most common (n = 7/69; 10.1 %),[63,74,85,90,96–98] it was only performed as a stand-alone test once, and twice as a stand-alone PT followed by CT.[96–98] The use of crime scene dogs, seminal vesicle-specific antigen (SVSA) and zinc, each appeared only once in the literature.[61,92,93]

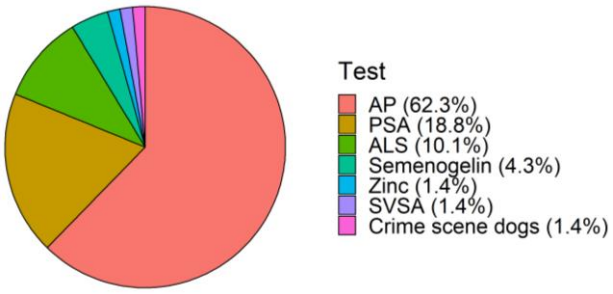

Figure 1.5: The frequency (%) of the different techniques used to presumptively test for semen. Acid phosphatase (AP) was the most commonly used test (62.3 %) followed by prostate specific antigen (PSA, 18.8 %); alternate light sources (ALS, 10.1 %) and; semenogelin (4.3 %). Crime scene dogs, seminal vesicle-specific antigen (SVSA) and, zinc (1.4 % each) were used only once.

The frequency of the different CT used to microscopically detect spermatozoa is illustrated in Figure 1.6. Identification of spermatozoa was usually performed using histochemical stains such as haematoxylin and eosin (H&E) (n = 17/78; 21.8 %),[48,49,59,62,64,79,81,88,91,93,99–105] Christmas tree (n = 13/78; 16.7 %)[51,54,56–58,65,70,77,85,92,99,100,106] and papanicolaou (n = 10/78; 12.8 %).[60,83,89,90,107–112] "Other" stains included crystal-violet, alkaline fuchsin, STIASNY/Lugol's reagent, baecchi, gram stain, Giemsa staining, florence iodine and erythrosine B.[11,50,55,67,69,79,94,99,113,114] Seven studies viewed spermatozoa without staining ("no stain") either through phase contrast, scanning electron microscopy (SEM) or unspecified techniques.[49,56,66,83,112,115,116] Immunohistochemical staining techniques included Sperm Hy-Liter™ (n = 3/78; 3.8 %)[48,63,74] and fluorescence *in situ* hybridisation (FISH) (n = 2/78; 2.6 %).[109,117] Sixteen studies did not specify which CT technique was used.[68,71,72,75,76,78,80,82,86,95,96,98,118–121]

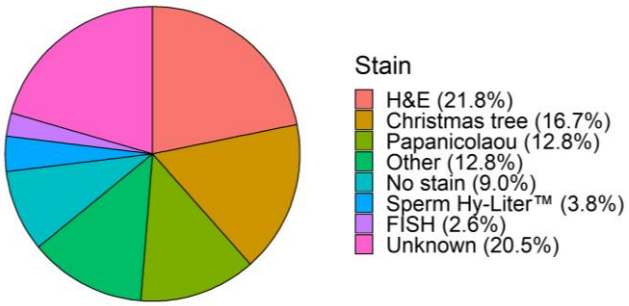

Figure 1.6: The frequency (%) of the different microscopy methods used to identify spermatozoa as a confirmatory test for semen. Haematoxylin and eosin (H&E) staining was the most commonly used method (21.8 %). Immunohistochemical staining techniques included Sperm Hy-Liter™ (3.8 %) and fluorescence *in situ* hybridisation (FISH) (2.6 %).

1.4.4 Recovery rate and persistence of semen and subsequent DNA

Of the 78 articles, seven presented data regarding the recovery of semen and/or subsequent DNA *after exposure to water only*. The remaining 71 studies pertained to the persistence of semen/DNA *over time* from humans (n = 55/71; 77.5 %), animals (n = 1/71; 1.4 %), non-living objects (n = 12/71; 16.9 %), or any combination thereof (n = 3/71; 17.6 %). Non-living objects also included semen stains on cotton that were exposed to water over or after a period of time (n = 3). All data extracted from the articles are presented in Appendix C, Tables C1 – C4. The persistence of semen *over time* was assessed for (i) the most common presumptive test, *i.e.* AP test, (ii) all CTs, and (iii) STR DNA profiling results (Table 1.1).

Table 1.1: Range of TSIs up to which AP, spermatozoa and DNA profiles were obtained at the various sample sites per cohort as reported in the literature. The number of studies that reported the presence of semen or DNA profiles within that time frame are also indicated. Each study is only represented once per test, per cohort. (TSI = time since intercourse, AP = acid phosphatase, h = hours).

Cohort	Sample site	AP		Spermatozoa		DNA profiles	
		Number of articles	Up to TSI of	Number of articles	Up to TSI of	Number of articles	Up to TSI of
Sexual offence victim - living	Vagina	12	3 - 168 h	22	3 - 179 h	2	< 72 h
	Anus/rectum	0	-	7	9 - 113 h	0	-
	Oral	0	-	5	6 - 48 h	0	-
	Other	1	8 h	3	9 - 17 h	0	-
Sexual offence victim - deceased	Vagina	3	48 h – 75 days	7	48 h - 75 days	1	48 h
	Anus/rectum	3	48 h - 30 days	3	48 h – 30 days	1	48 h
	Oral	3	48 h – 30 days	3	48 h, 30 days	0	-
	Other	0	-	1	60 h	0	-
Volunteers	Vagina	9	24 - 60 h	14	24 h - >21 days	2	38 h, 84 h
	Anus/rectum	0	-	0	-	0	-
	Oral	0	-	0	-	0	-
	Other	1	28 h	1	28 h	0	-
Human model	Other	0	-	4	4 h - 110 days	0	-
Animal sexual abuse victim	Animal site	1	< 24 h	1	< 24 h	1	< 24 h
Inanimate surfaces and objects	Textile/surface	5	48 h - 56 years	8	8 h - 56 years	5	96 h - 56 years

1.4.4.1 Recovery rate of AP over time

In living humans (victims and volunteers), AP was detected within seven days, with majority of studies typically detecting AP up to 96 hours post-coitus. In comparison, longer times were obtained for deceased individuals (up to 75 days) and inanimate surfaces and objects (up to 56 years) with some of these samples being exposed to different environmental conditions (*e.g.* low temperatures or water immersions).[59,68,94,102,112] The single ASA case had a TSI of less than 24 hours, and AP was detected in the vagina and anus of a sheep.

The longitudinal recovery rate of AP for different sample sites in living humans, per 24-hour intervals, is represented in Figure 1.7. At a TSI "greater than 48 hours", the success of AP recovery from the vagina ranged from 0 % - 31.6 %.[60,61,65,77,78,84,87] Recovery of AP showed a significant inverse correlation with time ("TSI") (Spearman's rho = - 0.645, $p < 0.001$).

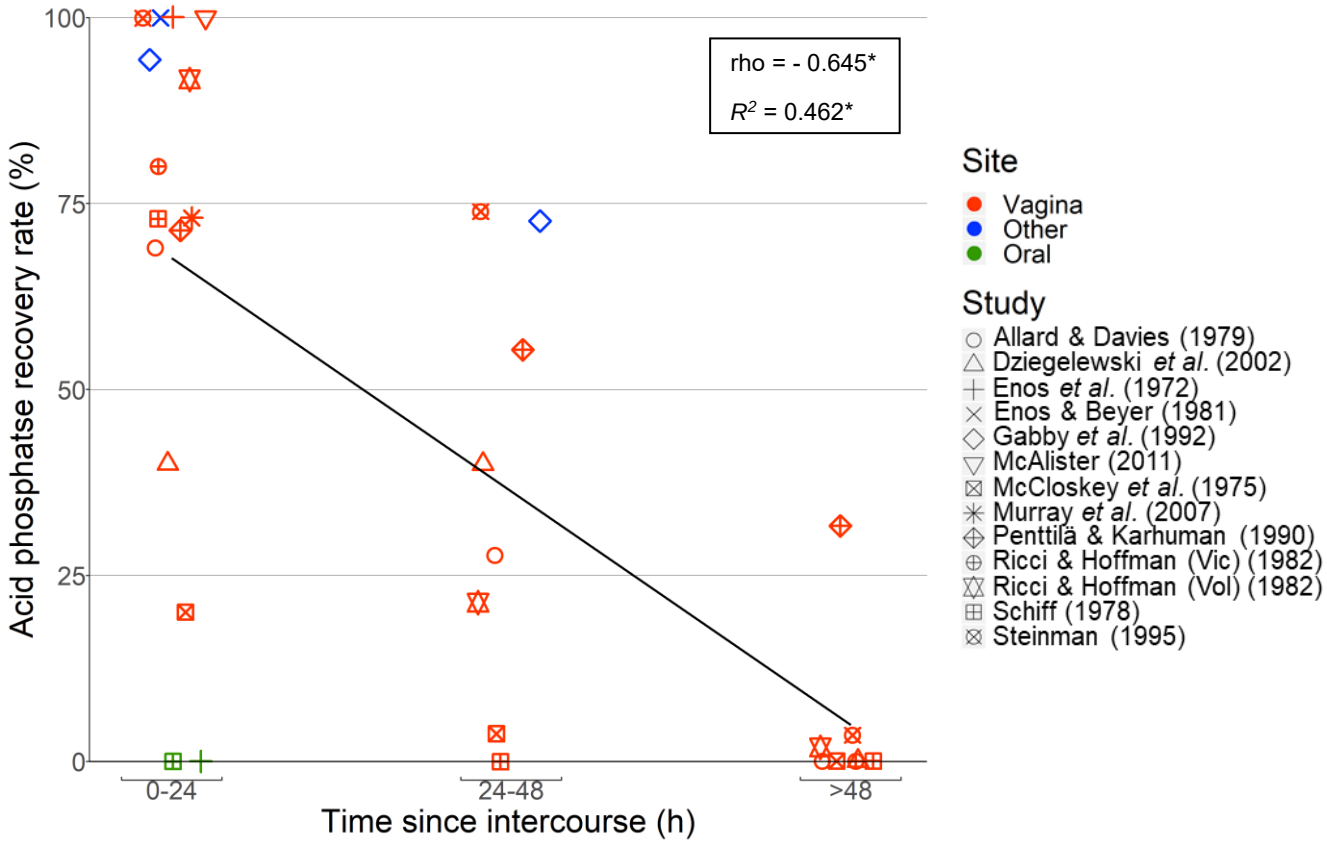

Figure 1.7: The recovery rate (%) of AP from various body regions on living humans per TSI interval with a linear best fit line (y = - 31.4x + 99). Each symbol represents one cohort from one study and each colour represents a different sample site. Ricci and Hoffman provided data for living sexual offence victims ("Vic") and volunteers ("Vol").[60] Each data point represents the reported rate for that interval. The Spearman's rank correlation coefficient rho and R^2 value are indicated in the top right hand corner and the asterisk (*) denotes a statistically significant value. (AP = acid phosphatase, TSI = time since intercourse, h = hours).

1.4.4.2 Recovery rate of spermatozoa over time

Spermatozoa were typically detected up to fourteen days in vaginal samples obtained from living humans with one study reportedly obtaining positive results after 21 days (Table 1.1).[108] Recovery from other body sites on living humans was only from a few hours to five days (Table 1.1). Deceased victims (various sites), human models and inanimate objects showed longer detection times (from one month up to 56 years) compared to living humans, despite the varying environmental conditions reported in these studies. For animals, the presence of spermatozoa was found in a sheep's vagina less than 24-hours post-assault.[11]

The longitudinal recovery rates of spermatozoa for different sample sites in living humans over a range of TSI intervals are represented in Figure 1.8. For vaginal samples, a recovery rate of 25.0 % – 33.3 %[64,108] was observed ten days post-assault and, Silverman and Silverman reported a 4 % detection rate for a TSI greater than 13 days.[108] A significant inverse correlation between 'TSI' and spermatozoa recovery rates was observed (Spearman's rho = -0.471, $p < 0.001$).

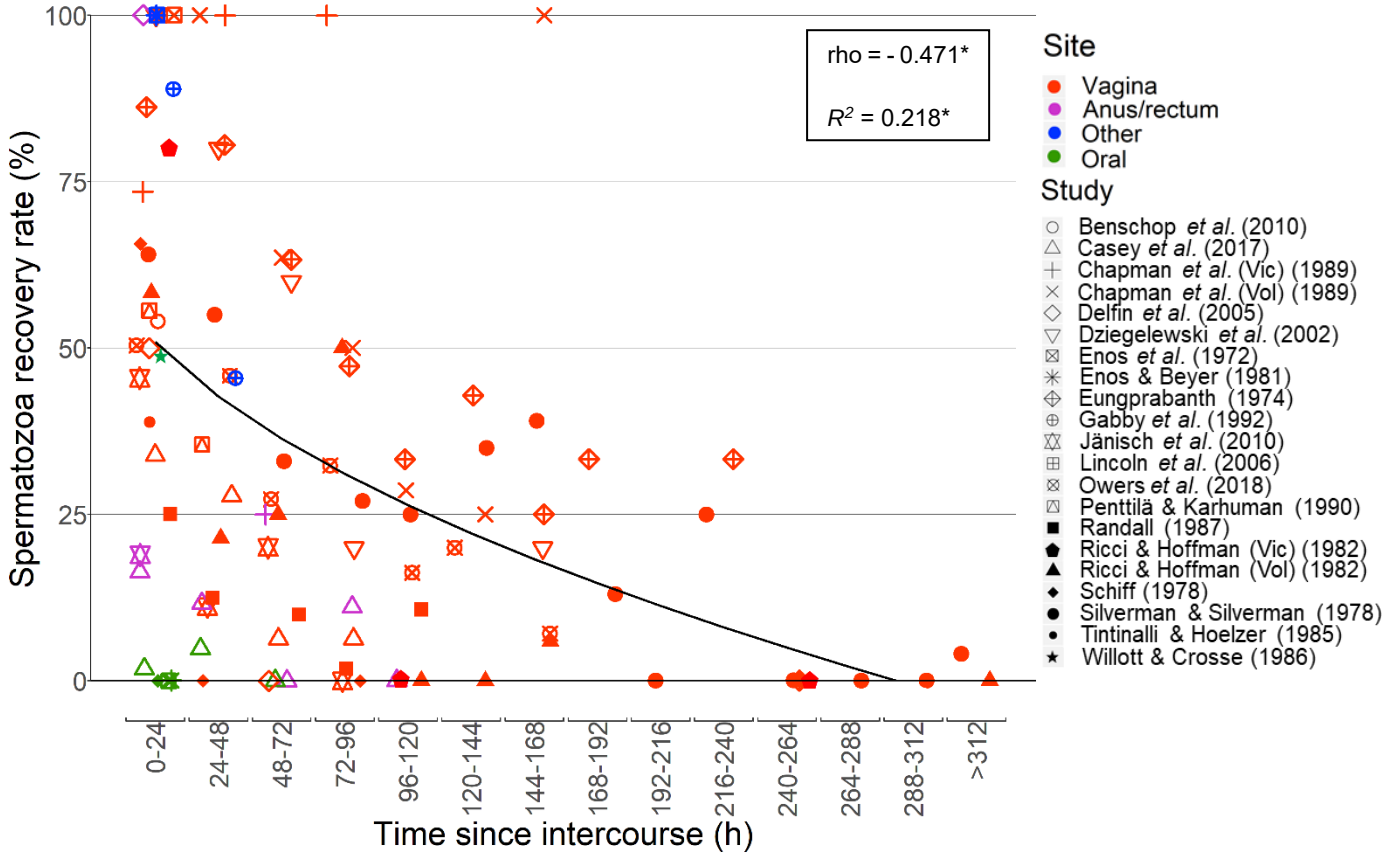

Figure 1.8: The recovery rate (%) of spermatozoa from various body regions on living humans per TSI interval with a best fit line ($y = -19.8\sqrt{x} + 70.7$). Each symbol represents one cohort from one study and each colour represents a different sample site. Chapman *et al.* and Ricci and Hoffman both provided data for living sexual offence victims ("Vic") and volunteers ("Vol").[60,105] Each data point represents the reported rate for that interval. The Spearman's rank correlation coefficient rho and R^2 value are indicated in the top right hand corner and the asterisk (*) denotes a statistically significant value. (TSI = time since intercourse, h = hours)

1.4.4.3 Recovery of semen from unaged stains on textiles exposed to water

Spermatozoa, with or without AP, was successfully recovered from various fabric types which had been laundered (Appendix C, Table C3).[56–58,63,71,74,85] Generally, the recovery rate of AP

and spermatozoa decreased with laundering (Figure 1.9) and spermatozoa typically had a higher recovery rate than AP.[56–58,63,71,74,85] Semen retention varied between different fabric types, with cotton-based materials reportedly retaining semen better.[56,71,85] Semen was also recovered from bathwater and bathtub surfaces.[91]

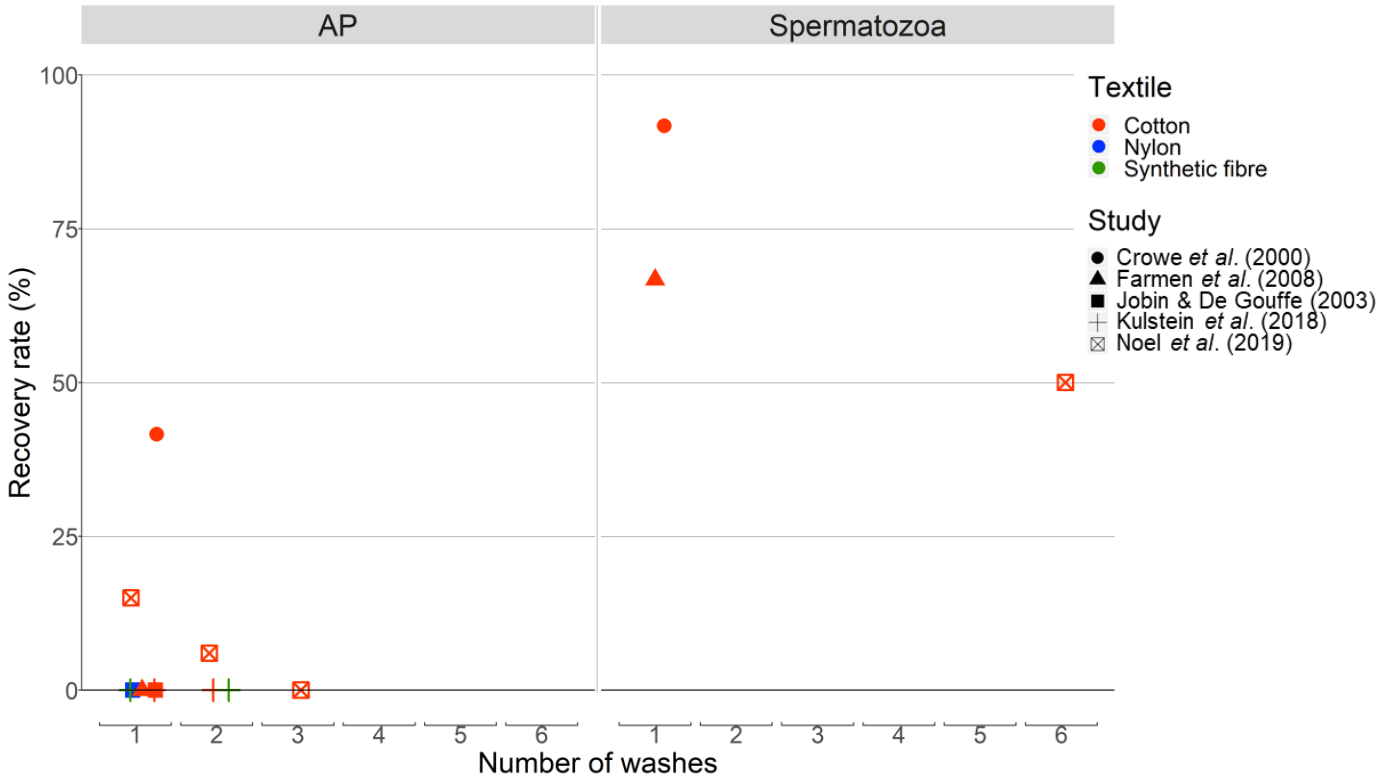

Figure 1.9: The recovery rate (%) of acid phosphatase (AP) and spermatozoa from different textiles after a certain number of washes. Each symbol represents one study and each colour represents a different textile. Each data point represents the reported rate after a specific number of washes.

1.4.4.4 Persistence of DNA from semen over time and/or after laundering

Fifteen studies detailed STR DNA profiling results obtained from semen with reference to time and/or after laundering (Appendix C, Table C4). Forensically informative autosomal DNA profiles were typically obtained within 72 hours when samples originated from the vagina (human and animal).[11,54,79,95,104,114,122] Benschop *et al*. produced a DNA profile from vaginal samples up to a TSI of 72 - 84 hours by using Y- chromosomal STR (Y-STR) profiling.[95] Although the success of DNA profiling typically decreased over time,[55,95,104,123] DNA appeared to persist longer (up to 56 years) on different fabrics and surfaces compared to samples obtained from the body – even when fabric was exposed to water and other environmental

conditions.[55,74,92,123,124] However, DNA profiling success varied between different textile types[74,92,124] and environmental conditions.[123] Laundering of unaged semen stains did not hinder the ability to produce DNA profiles.[56,57,63]

1.5 Discussion

During the medical examination of a human sexual offence victim, three major types of evidence are collected, namely; proof of force, proof of recent sexual contact and, evidence to identify the assailant.[125–127] While proof of force is dependent on the documentation of physical injury,[125] evidence of sexual contact and the ability to identify the assailant is based on molecular investigations such as BFI and DNA analyses. Although blood and saliva are good sources of DNA that can be used for identification, semen has the additional ability to prove some form of sexual activity occurred. It is thus plausible that semen evidence should be valuable in ASA investigations. To this extent, this review aimed to explore the retention and recovery of human semen and resultant DNA over time in a medico-legal context, with particular attention to ASA.

1.5.1. Existing literature regarding semen recovery in relation to time in ASA

From the systematic literature review, only one ASA case study was found which reported the successful detection of human semen in reference to TSI (Figure 1.1).[11] The minimal literature describing instances of ASA either lacked any reference to the medical or forensic examination of the animal or, where such examinations occurred, no reference to the TSI was provided. A reason for this may be that an animal cannot report being abused and as a consequence, the TSI often remains an unknown factor. Veterinary examinations where samples were collected for molecular forensic analysis was typically prompted by severe or fatal injury of the animal.[5,11,14] However, injury may not always occur during ASA, which may further contribute to the under-reporting of the crime, consequently resulting in fewer molecular forensic investigations being conducted.

Since the TSI will often be unknown in ASA, results from analyses of evidence can provide a guideline as to when the act occurred, thereby further aiding in corroboration of witness and suspect testimonies. It would thus be valuable for forensic analysts to understand the possibility and extent of the retention and analyses of human semen in the context of ASA. However, this

is challenged by the severe paucity of literature pertaining to this topic and warrants much needed research.

1.5.2 Retention and recovery of semen and subsequent DNA over time and/or post exposure to water

In order to evaluate the retention times and recovery rates of human semen from animals, the persistence of AP, spermatozoa and DNA, were assessed over time. Based on only one ASA case report,[11] no inference could be reasonably made regarding the relationship of human semen recovery from animals over time. Due to the larger amount of data available for human- and textile-based subjects, the typical persistence of the various semen components in these sample cohorts were assessed in more detail. However, in the absence of empirical data in the ASA context, it is not clear if these data can be extrapolated for animals.

1.5.2.1 Change in recovery of seminal constituents, including DNA, over time

Substantial research has been conducted on semen recovery from the vagina of living human sexual offence victims and volunteers (Figure 1.3 A). Literature indicates that, although AP has been detected in the vagina up to six days to seven days post-coitus for these cohorts (Table 1.1), the successful recovery of AP beyond 48 hours is extremely low (Figure 1.7). If evidence recovery in a sexual assault case had to occur after two days, CT by means of spermatozoa identification may be more informative of the presence of semen. Spermatozoa can be detected up to two weeks to three weeks in the living (Table 1.1) and although the success of recovering spermatozoa also decreases with time (Figure 1.8), it is less rapid than AP (Figure 1.7). In turn, the short retention time of AP can be a useful indicator of recent sexual intercourse compared to spermatozoa.

Based on the literature available, full or partial autosomal STR profiles derived from spermatozoa could be obtained from the body up to 72 hours post-coitus (Appendix C, Table C4). Given that DNA is derived from spermatozoa, it is expected that DNA profiles should be produced at periods longer than this, to commensurate with the fortnight to three weeks recovery period of spermatozoa. It is unclear as to why STR profiling was infrequently performed and/or reported in the literature, given its power in individual identification. STR typing through polymerase chain reaction (PCR) was only developed in the early 1990's.[128,129]

Therefore, this technology may not have been available at the time when most of the studies included in this review were conducted.

Semen, including DNA, tended to persist longer in non-living and non-human cohorts and this could be attributed to the stationary nature of non-living substrates, as well as the influence of different intrinsic biological factors (or lack thereof) and external exposures. Animals are biological beings whose activity is generally uncontrolled, and these numerous factors would likely affect the retention and recovery of semen from an ASA victim.

1.5.2.2 Factors that may influence recovery rate

In addition to time, the three main factors noted in the literature that contributed to the retention and recovery of semen from humans and inanimate objects were: intrinsic factors, external exposure and analytical methodology.

(i) Intrinsic differences between sample sites and subjects

The nature of the substrate upon which semen is deposited can influence the persistence of a biological fluid. In humans, survival of the various semen components will differ between anatomical sites due to physiological differences at that site (*e.g.* different microbiomes and chemistries). Furthermore, semen deposited within the body (*i.e.* vagina, anus/rectum and oral cavity) is subjected to the body's natural drainage, possible pathology, immune system and, normal physiological processes (*e.g.* urination and defecation)[130] compared to samples deposited on the skin and hair that are more exposed to external insults. With animals being complex biological beings interacting with the environment, semen deposited internally will possibly also display different retention abilities to that on the external fur and skin.

Inanimate objects are void of intrinsic biological factors, which may then allow semen to be detectable at extended periods of time. In deceased victims, decomposition processes may also affect semen detection.[48,49] Given the biological differences between these substrates, it is possible that semen retention times on animals may not be similar to what is observed for humans and inanimate objects.

An animal's movement is generally uncontrolled and post-coital activity can also largely dictate the persistence of semen on/in the victim.[66,78,101,102,131] A living individual may be mobile after the incident, perhaps attempting to flee or report for medical examination, which

may promote drainage of semen from various sites. In contrast, stationary femicide victims, skin models and fabrics experience far less movement, which may explain why semen and subsequent DNA persisted for much longer periods of time on these substrates (Table 1.1).[48,49,55,61,68,97,115,116,123]

(ii) External exposures

Human victims are usually advised not to wash before medical examinations as it can possibly reduce semen recovery.[108,111] This review also highlighted that, although success of recovery decreased, semen, including DNA, appeared to be able to withstand washing and treatment with detergents to a certain extent when deposited on fabric (Figure 1.9 and Appendix C, Table C3 – C4).[56–59,63,71,74,85,124] Animals can easily come into contact with water, for example in grooming, and the extent to which evidence of semen on these exposed areas may be removed is unknown and should be investigated. In the instance of water exposure, research studies suggest that spermatozoa detection would be more successful than AP, as AP is water-soluble.[56–58,63,71,74,85]

Animals are also exposed to micro-climate changes and conditions such as temperature and humidity were shown to affect the retention of semen. Colder temperatures can preserve semen for longer periods of time,[68,94,112] while an increase in humidity results in DNA degradation.[123]

(iii) Analytical methodology

Different sample collection and processing methods as well as different analytical techniques may also influence recovery rate of semen and should thus be carefully considered when investigating ASA cases. Effective protocols should be established and implemented to ensure optimal evidence recovery.

Swabs were usually used to collect evidence in sexual offence cases, but cervicovaginal scrapings allowed spermatozoa to be detected at a TSI of 21 days and longer in volunteers.[108] Different types of swabs and pre-processing of the samples also influenced spermatozoa abundance.[95,105]

As AP testing is presumptive in nature, confirmation of semen is dependent on spermatozoa identification. However, different CT techniques demonstrated varying levels of sensitivity. Spermatozoa could be detected for longer periods of time with advanced microscopy, such as

SEM, and staining techniques such as FISH and the spermatozoa specific stain, Sperm Hy-Liter™.[48,49,99,109] In the instance of an azoospermic offender, semen identification may have to rely on detection of seminal fluid proteins, such as AP, PSA or semenogelin, rather than spermatozoa. DNA degradation can still occur over time in morphologically intact spermatozoa through endogenous nucleases and protocol optimisation generally improves DNA profiling results.[55,104,132] Y-STR profiling may also be more sensitive over time than autosomal profiling as only loci on the Y-chromosome are targeted.[95]

1.5.3 Limitations

Analyses in this literature review were challenged by several limitations and it is important to note that inferences on retention times and changes in recovery were made on the available data. When comparing results, caution needs to be taken as the number of studies providing data for the different sample sites, cohort types and tests were imbalanced. Therefore, the inferences on retention times may be dependent on fewer studies compared to others. Due to the limited number of studies, statistical analyses could not be performed to assess the differences in semen retention between different sample sites, different cohorts, as well as after multiple washes.

Furthermore, not all data made in reference to time could be included for analyses as the sample site and/or test was not specified. Retention times of semen was also dependent on the cohort being studied, thus, cross-sectional studies and case reports could not report a maximum time point at which semen and/or subsequent DNA could no longer be detected. Although 47 articles provided data for living humans regarding recovery of AP and spermatozoa, the longitudinal change in recovery success per 24 hour intervals could only be obtained from 24 articles (51.1 %). When assessing recovery rates, differences in the studies' sample sizes both within and between time intervals could also introduce bias into the results. The broad ranges in retention times could exist because published data represents samples of different semen volumes, which may have been exposed to different conditions (*e.g.* different temperatures), and data may be based on self-reported and/or estimations of TSI.

1.6 Conclusion and rationale for project

Demonstration of human semen on/in animals can confirm an incident of ASA and may aid in the prosecution and conviction of a perpetrator. During legal proceedings, suspect and witness

testimonies and alibies may have to be corroborated. This would rely on knowing the time at which assault occurred - a hurdle likely to be encountered in ASA.

Data regarding the persistence and recovery of human semen over time can provide guidelines for estimating TSI. Based on the interpretation of the forensic evidence obtained, a timeline of events can be constructed. As a result of large-scale cohort data, guidelines for determining the most probable TSI have been published for use in human sexual offence investigations, but no such data exists for use in ASA investigations.[62,102]

There is a paucity of data regarding human semen persistence on/in animals and it remains unknown as to whether the typical retention times observed for humans and textiles can be extrapolated to animals. Thus, baseline data first needs to be established prior to in-depth research of this particular topic. To this end, a suitable animal model is needed on which to conduct such research. Despite the numerous sample sites and extensive textiles analysed, the analysis of semen retention over time on animal fur has not been previously explored. Just as it is possible for semen to be deposited on a victim's clothing during a sexual offence, semen can also be deposited on an animal's fur in ASA. Thus, the current project was designed as a pilot study using animal fur as a starting point, and incorporated molecular techniques to detect and analyse semen evidence over time in the application of understanding forensic evidence in investigations of ASA.

1.7 Research project aims and objectives

The aim of this project was to assess the retention and recovery of human semen, and subsequent DNA, on animal fur over time. The three main objectives were to:

- Longitudinally conduct PT and CT on human semen obtained from fur over a defined two-week time period.
- Assess DNA quantity and quality through quantitative real time PCR (qPCR) and DNA profiling by means of STR typing.
- Contribute to the development of a suitable animal fur model for use in ASA research.

Chapter 2: Methods and Materials

2.1 Study and experimental design

2.1.1 Research paradigm

This was a pilot study conducted with a quantitative and longitudinal study design. The dependant variables of BFI success (*i.e.* PT and CT outcomes) and DNA metrics (*i.e.* DNA concentration, degradation and STR profiling success) from human semen were measured against the independent variables of time and animal fur model.

2.1.2 Fur models

In order to prospectively study the retention of human semen on animal fur, a suitable fur model was needed. Although a living animal would represent an accurate ASA victim, ethical considerations motivated for a fur model to test the study objectives. Due to the absence of literature, no guidance pertaining to an all-rounded representative fur model was available.

As a first attempt at contributing towards this research gap, three fur models were assessed: (i) untreated fur from deceased animals; (ii) treated and semi-processed fur from deceased animals (*i.e*. real animal fur from the hunting industry which has been preserved with salt) and (iii) synthetic (faux) fur that has been manufactured for use in for example, clothing, upholstery and carpeting.

It was acknowledged upfront that each model carried its own limitations: while fur from deceased animals would best mimic the characteristics of interest in this study, such fur would decompose and limit the length of time which longitudinal experiments could take place. It was also hypothesised that the chemical preservation of fur (*i.e.* the salt) may act to preserve the DNA in these experiments. Lastly, the faux fur may not be a suitable proxy for animal fur and would be devoid of microorganisms and epidermis layer. However, as a pioneer study, these models were used as a first attempt, upon which future research could build.

For convenience, the untreated fur was obtained from Chacma baboon heads (*Papio ursinus*) (Figure 2.1 A) which were already available and being stored in the laboratory freezer for other research conducted in the Division.[133,134] The use of baboon fur is not necessarily representative of all animal fur, but was deemed suitable for this pilot project as it is maintained the authenticity of fur from a real animal. The fur was intact on the scalp and no decomposition of the tissue that could possibly compromise the integrity of the fur was present. Furthermore, the

morphology of the fur remained a constant variable as sufficient specimens of the same species was available to provide ample fur for the experimentation phase.

The semi-processed fur that was used was a nyala hide (*Tragelaphus angasii*) preserved with salt (Figure 2.1 B) and the faux fur (Figure 2.1 C) was bought from a local home decor store. Approval was obtained from the Faculty of Health Science Animal Ethics Committee to utilise the baboon and nyala fur models (AEC: 019_016, Appendix D).

Figure 2.1: The three fur models used in this study. (A) untreated, real animal fur from a Chacma baboon (*Papio ursinus*); (B) treated and semi-processed fur from nyala (*Tragelaphus angasii*) and; (C) synthetic (faux) fur.

2.1.3 Experimental setup

To determine retention times of human semen on animal fur, 28 aliquots of human semen were spotted onto each of the three fur models. Every day, two semen stains were swabbed from each fur type for a period of 14 days. One swab underwent PT and CT, while the second underwent DNA analysis. The experiment was optimised (Appendix F, Table F1) prior to carrying it out on two biological replicates (*i.e.* semen from two human donors). The experimental design overview is summarised in Figure 2.2 and each step is explained in detail below.

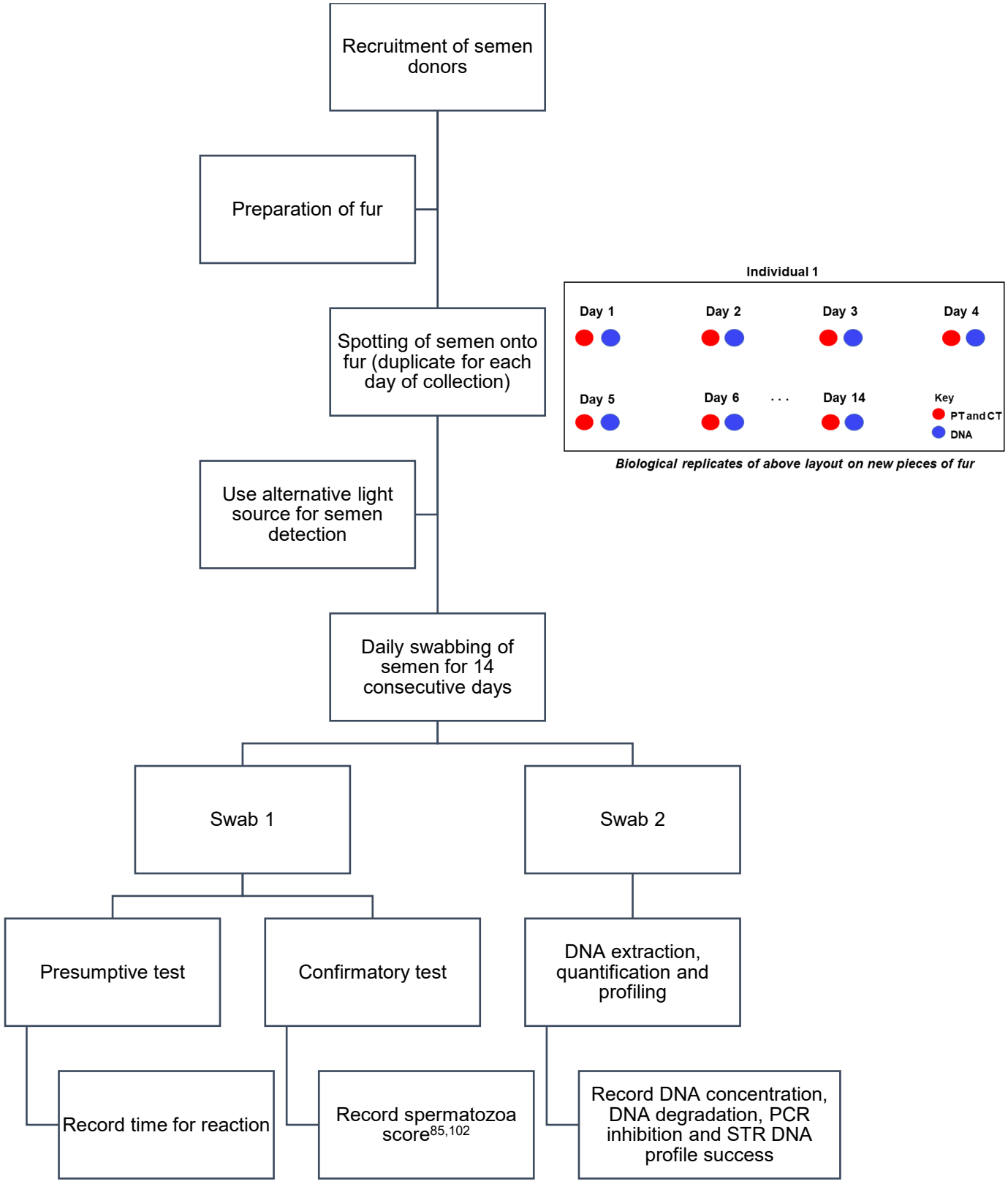

Figure 2.2: Flow diagram of the experimental design overview that was followed in the conduction of this project. The image to the right of the flow chart illustrates the layout of semen spotting for presumptive and confirmatory testing (red dots) and DNA analyses (blue dots) on the three fur models.

2.2 Experimental phase

2.2.1 Semen donors

Two human semen donors were recruited with written informed consent (Appendix E). Participants who had a vasectomy or were oligo/azoospermic were excluded. Donors who had or may have had sexually transmitted diseases were also excluded. Approximately 10 mL of semen from each donor was collected into a 50 mL conical centrifuge tube stored at 4 °C. Ethical approval from the Faculty of Health Science Human Research Ethics Committee was obtained (HREC: 190/2019, Appendix D).

2.2.2 Preparation of fur

Fur from the baboon heads were removed from the lateral aspect of the frontal process to the nuchal crest. The integrity of the baboon fur was visually assessed in terms of being most representative of living animals' fur, *i.e.* void of putrefaction. For the nyala hide, as much salt as possible was brushed off. The faux fur did not require any preparation prior to spotting. Furs were kept at room temperature in nets to prevent colonisation by flies and other insects. The fur models were stationary and not exposed to harsh conditions such as direct sunlight and water.

2.2.3 Spotting of semen

Semen ejaculates from the same donor were homogenised prior to aliquoting onto each fur type. A total of 28 aliquots, each comprising 100 µL of semen, were spotted onto each fur model, and allowed to air dry.

2.2.4 Alternative light sources

Prior to each swabbing, an ALS was used to assist in locating the semen spots on the fur models. A Crime-lite™ (Foster and Freeman, Evesham) blue light (430 – 470 nm) was used in combination with orange goggles to visualise semen stains on the nyala and faux fur, whereas blue-green (460 – 510 nm) was used with orange goggles for the baboon fur. A different ALS was used to locate semen on the baboon fur, as this wavelength allowed for better visualisation of the semen stains on this fur model compared to the blue light (430 – 470 nm).

2.2.5 Swabbing

Nylon flocked swabs (Copan, Brescia) were moistened with two drops of MilliQ water prior to swabbing of the respective semen stains. The swab was rotated, with the application of a gentle downward force, over the semen stain four times. Swabs for PT and CT were allowed to air dry for 20 minutes prior to testing, while the swabs for DNA analyses were immediately processed. The stains for "Day 1" were swabbed after one hour, and subsequent swabs were taken at 24 hour intervals until "Day 14". Background swabs from areas absent of semen were also taken from each of the furs – one for PT and CT and, one for DNA analyses.

2.2.6 Body fluid identification

2.2.6.1 Swab preparation

For PT and CT, the semen needed to be extracted from the swab. Swabs were immersed in 350 µL of 1 X phosphate buffered saline solution and left to incubate at room temperature (21 °C – 23 °C) for five minutes. The sample was then vortexed for 15 seconds and centrifuged at 13 000 rpm for one minute using an Eppendorf 5417c centrifuge (Eppendorf, Hamburg). The swab was removed from the solution, pressed against the sides of the tube to squeeze out the liquid and then discarded. The extract was centrifuged again at 13 000 rpm for one minute using the above mentioned centrifuge. Supernatant (200 µL) was removed from the pellet and stored in a clean microcentrifuge tube for PT, whereas the pellet in the remaining supernatant was retained for CT. Background swabs for PT and CT were extracted in the same manner.

2.2.6.2 Presumptive testing

PT to screen for the possible presence of semen was conducted by directly adding Brentamine Fast Blue (BFB) to the supernatant of the semen extract. The BFB reagent was made fresh on the day of PT. To make the BFB reagent, two solutions were prepared. Solution A consisted of 0.4 g sodium acetate (anhydrous) (Merck, New Jersey) and 0.2 g Fast Blue B salt (ortho-dianisidine) (Sigma-Aldrich, Missouri) dissolved in 2 mL MilliQ water. To this, 200 µL glacial acetic acid was added. Solution B consisted of 0.08 g 1-naphthyl phosphate monosodium salt monohydrate (α-naphthyl) (Sigma-Aldrich, Missouri) dissolved in 1 mL MilliQ water. The BFB reagent was then made up of 1 mL of Solution A, 100 µL of Solution B and 8.9 mL of MilliQ water.

Subsequently, 50 µL of BFB was added to an aliquot (60 µL) of the supernatant from the extracted swabs. The time it took for a colour change to purple was recorded, with the cut-off time for a positive reaction being ten minutes. This was done in triplicate for each of the extracted semen and background swabs along with positive (neat human semen) and negative controls.

2.2.6.3 Confirmatory testing

The pellet from the extracted swabs was resuspended in the remaining supernatant (approximately 25 µL) and 5 µL of this was spotted onto microscopy slides (Sysmex, Norderstedt). The slides were heat fixed and subsequently stained with H&E. One slide per swab extract as well as a positive control from each donor were prepared according to the internal standard operating procedures which can be made available upon request.

Visualisation of spermatozoa was achieved using a Leica DM500 microscope (Leica, Wetzlar) with a ICC50 HD camera attachment (Leica, Wetzlar) and 400X magnification. The slides were scored according to Allard[102] and Nolan *et al.*[85]

2.2.7 DNA analyses

2.2.7.1 DNA extraction

Immediately following swabbing, DNA was extracted from swabs using the QIAamp® DNA Investigator Kit (Qiagen, Hilden) and following the manufacturer's protocol (page 13 – 16).[135] However, 0.1 % (6.5 mM) dithiothreitol (DTT) was added to the first lysis step. Molecular biology grade pure ethanol (Sigma-Aldrich, Missouri) was used in the extraction process and the DNA was eluted in 100 µL of ATE buffer. Samples were centrifuged at the indicated rpm using an Eppendorf 5417c centrifuge (Eppendorf, Hamburg). A ThermoMixer® F2.0 with ThermoTop® (Eppendorf, Hamburg) was used to simultaneously shake and incubate the samples. DNA extraction was performed on all swabs as well as neat semen samples (100 µL) from each donor (positive controls).

2.2.7.2 DNA quantification

Quantification was achieved using Nanodrop spectrophotometry and qPCR. Nanodrop spectrophotometry was performed using the Nanodrop 2000 (Thermo Fisher Scientific, Massachusetts) and 2 µL of each sample (or control) was measured. This was done to screen if any sample had a DNA concentration above 50 ng/µL, which is greater than the dynamic range for qPCR. None of the samples quantified with spectrophotometry were above 25.25 ng/µL and thus, no dilutions were made prior to qPCR.

In order to determine the concentration of amplifiable human DNA, qPCR was performed for all samples and controls. This was conducted using the Investigator Quantiplex® Pro Kit (Qiagen, Hilden) following the manufacturers' protocol with no deviations (page 13 - 23)[136] and using the Applied Biosystems 7500 real-time PCR system (Foster City, USA). Data was captured on the HID Real-Time PCR Analysis software v1.2 (Applied Biosystems, Foster City, USA).

The Investigator Quantiplex® Pro assay makes use of TaqMan® probes and extrapolates the concentration of DNA by means of a standard curve prepared during the quantification process. Three target regions are amplified: two on the autosomal 4NS1C® marker (91 base pairs (bp) and 353 bp) and one Y-chromosome marker of 81 bp. To calculate the degradation index (DI), the concentration from the two regions on the autosomal marker were assessed as a ratio. This assay also simultaneously amplifies an internal control (IC, 434 bp) with every sample which is used to indicate PCR success as well as the presence of PCR inhibitors. No-template controls were included in the quantification assay.

2.2.7.3 STR DNA profiling

DNA was diluted to 0.5 ng/µL using molecular grade biology water based on the concentration of the 91 bp marker (small autosomal marker) quantified in qPCR for STR profiling. The PowerPlex® ESI 16 kit (Promega, Wisconsin) was used for multiplex PCR following the manufacturer's protocol (pages 6 – 9)[137] and performed in a T100 Thermal Cycler (Bio-Rad, California). Capillary electrophoresis was conducted on the Applied Biosystems 3130*xl* Genetic Analyzer (Applied Biosystems, California) following the aforementioned manufacturer's protocol (pages 27 – 29),[137] but using a 50 cm array. Other deviations included using a performance optimised polymer 7 (POP7) and conducting electrophoresis at 15kV. The GeneMapper® version 4.1 software (Applied Biosystems, California) was used to analyse the

electropherograms. The Promega PowerPlex® ESI 16 kit positive control and a no-template control were also included in the assay. The extracted neat semen reference samples from the donors were used to generate reference profiles.

2.3 Data analysis

All variables that were collected are detailed in Table G1 (Appendix G). For PT, the average time taken for a positive reaction to occur was calculated for each of the samples as well as the background swabs. For DNA analysis, the qPCR results were used to assess the quality and quantity of DNA extracted from the semen samples in terms of presence of PCR inhibitors, DI and DNA concentration. To assess the presence of PCR inhibitors, the cycle threshold (C_t) values of the IC in the samples were compared to the C_t values of the IC in the control standards. The DI was calculated by dividing the concentration of the small autosomal marker (91 bp) by the concentration of the larger autosomal marker (353 bp).

Shapiro-Wilk tests were performed to determine if the DNA concentration and DI were normally distributed in order to select the most appropriate statistical tests. Based on these results, non-parametric tests were conducted for all data regardless of the fur model. The change in DNA metrics over time was then assessed using a Spearman's Rank Correlation coefficient test. This test allowed for the linear relationship between DNA concentration and time since exposure (*i.e.* age of the sample) as well as between the DI and the time since exposure, for each of the fur models to be assessed.

To evaluate if any effect was caused by the type of fur model used on the detection of semen, a Kruskal-Wallis test was used to determine if a significant difference existed between the three fur models in terms of DNA concentration and DI. To assess where the significant difference in the DNA metrics existed between the three fur models, a post hoc Bonferroni correction followed by Wilcoxon signed rank tests were performed. All statistical analyses were conducted using R and R Studio Version 3.4 and a level of significance of $\alpha = 0.05$.

To assess the quality of the DNA profiles, electropherograms were compared to the reference DNA profile from the donors, as well as the profiles generated from the background swabs. The latter was done to evaluate possible interferences arising from animal DNA or other contaminants from the furs. The allele calling accuracy was calculated by comparing the alleles present in the DNA profiles obtained from swabs to those in the donor's reference DNA profile.

Chapter 3: Results

Data obtained from the BFI and DNA analyses for all samples and controls can be found in Appendix H, Table H1.

3.1 Presumptive and confirmatory testing

3.1.1 Presumptive testing

PT was achieved using the BFB reagent to detect the presence of AP. All semen samples from all fur models for the entire two-week period produced a positive reaction *i.e.* a colour change to purple (Figure 3.1 C). Majority of the samples reacted immediately with a maximum reaction time of 32.67 s (Day 5, baboon fur, donor 1). The time taken for a positive reaction to occur was not associated with the age of the sample, nor fur model. Positive and negative controls reacted as expected (Figure 3.1). None of the background fur swabs yielded a false positive result within the ten-minute cut-off time.

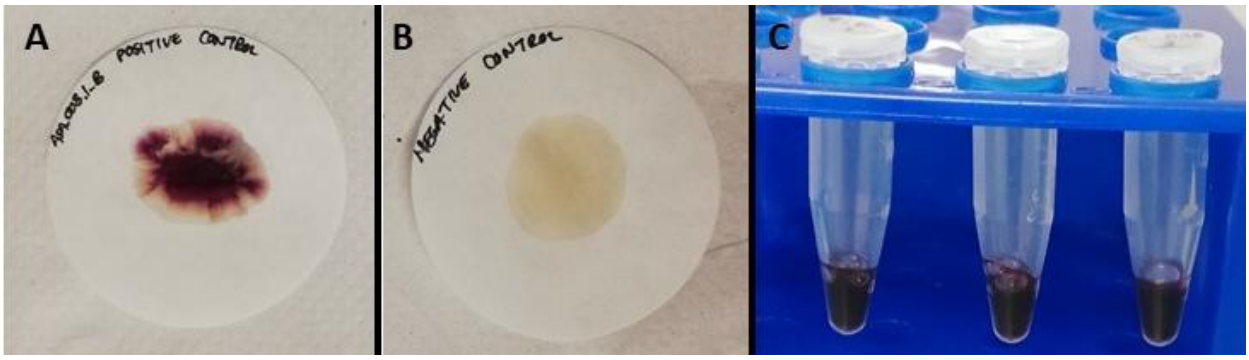

Figure 3.1: Examples of the (A) positive and (B) negative controls and, (C) the purple colour change seen in all presumptively tested samples.

3.1.2 Confirmatory testing

Using microscopy, spermatozoa were observed for all semen samples from all fur models across the entire two-week period (Figure 3.2 D-F). Dirt and other cellular material were observed from the baboon background swabs (Figure 3.2 A) and salt crystals were observed in the background swabs taken from the nyala fur (Figure 3.2 B). A background sample from the faux fur only showed small flecks of dust (Figure 3.2 C). These observations were taken into

consideration when scoring the subsequent slides. Both positive controls (one for each donor) had an abundance of spermatozoa present.

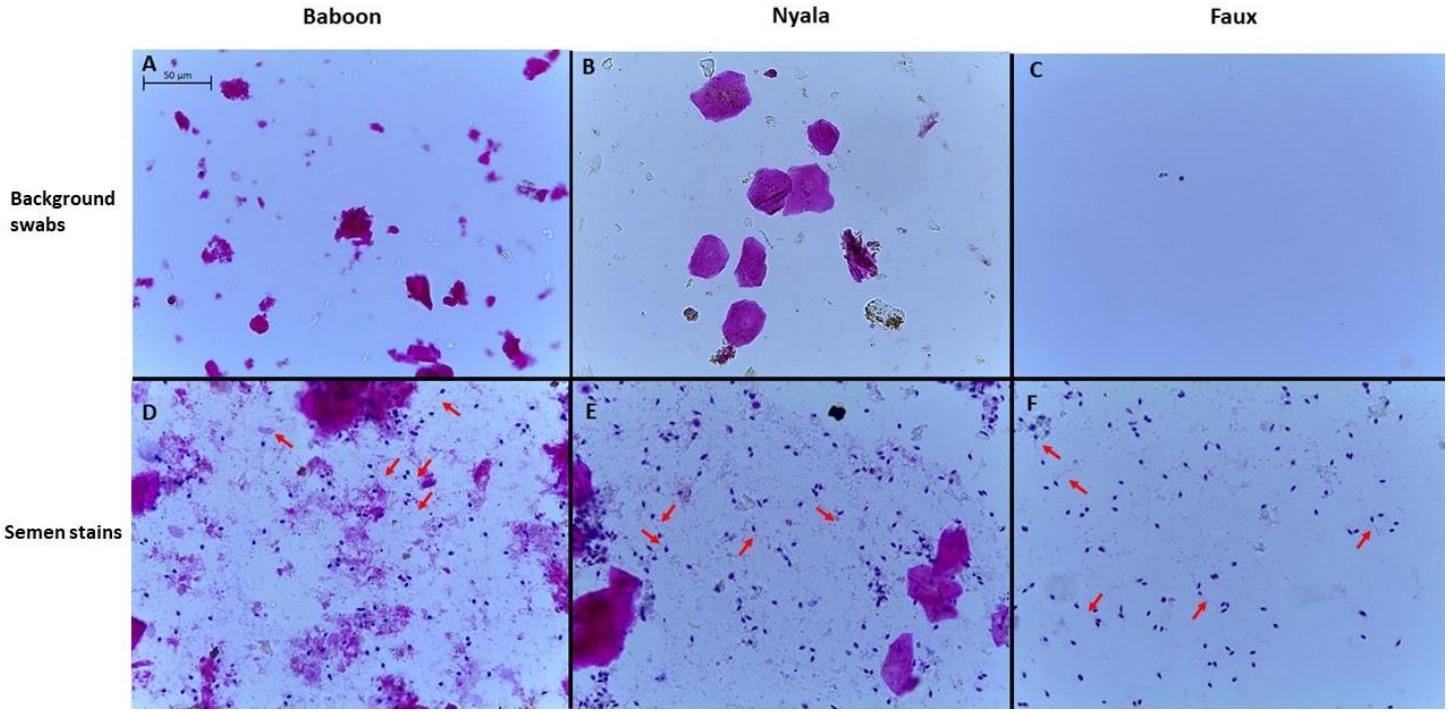

Figure 3.2: Examples of microscopic analyses on extracted background swabs (A-C) and swabbed semen stains (D-F) observed on the different fur types. Red arrows indicate some of the spermatozoa tails. Slides were viewed using a Leica DM500 microscope and 400X magnification. Images were captured with a Leica ICC50 HD camera attachment. Scale bar (A) is equal to 50 µm and is applicable to all images.

Spermatozoa were scored for each sample, the distribution of which can be seen in Figure 3.3. A score of 4+ (T) was obtained for all samples taken from the nyala and faux furs. Samples from the baboon fur showed more variation with lower scores obtained at random time points. Three out of the six samples with scores lower than 4+ (T) correlated with non-immediate AP reaction times (Appendix H, Table H1). For example, the lowest score of 1+ (T) was obtained from baboon fur on Day 5 from donor 1.

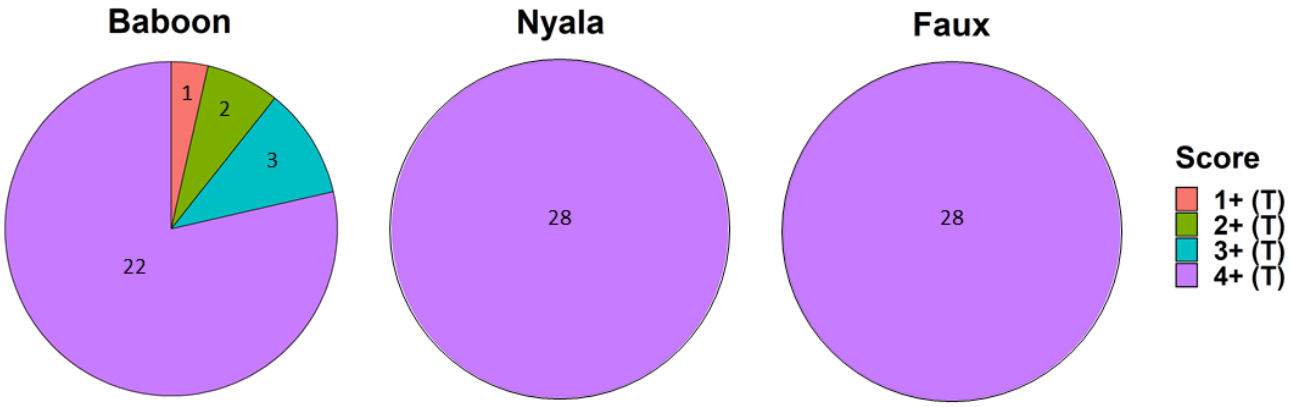

Figure 3.3: The frequency of spermatozoa scores for each of the fur models, for the entire two-week period (n = 28). Purple indicates a score of 4+ (T), blue represents a score of 3+ (T), green for 2+ (T) and pink for 1+ (T).

3.2 DNA analyses

3.2.1 DNA quantification

The concentration of DNA was measured using qPCR. Figure 3.4 illustrates the distribution of DNA concentrations (of the 91 bp marker) per fur model for the two-week period. DNA concentrations obtained from the baboon and faux furs were not normally distributed ($p < 0.05$).

DNA concentrations fluctuated over time for each of the models (Figure 3.4). When combining the data from both donors, the minimum DNA concentration was 0.728 ng/µL (Day 7, baboon fur) while the maximum DNA concentration was 17.282 ng/µL (Day 12, nyala fur). Only the faux fur showed a significant inverse correlation between "Day" and "DNA concentration" ($rho = -0.622$, $p < 0.05$).

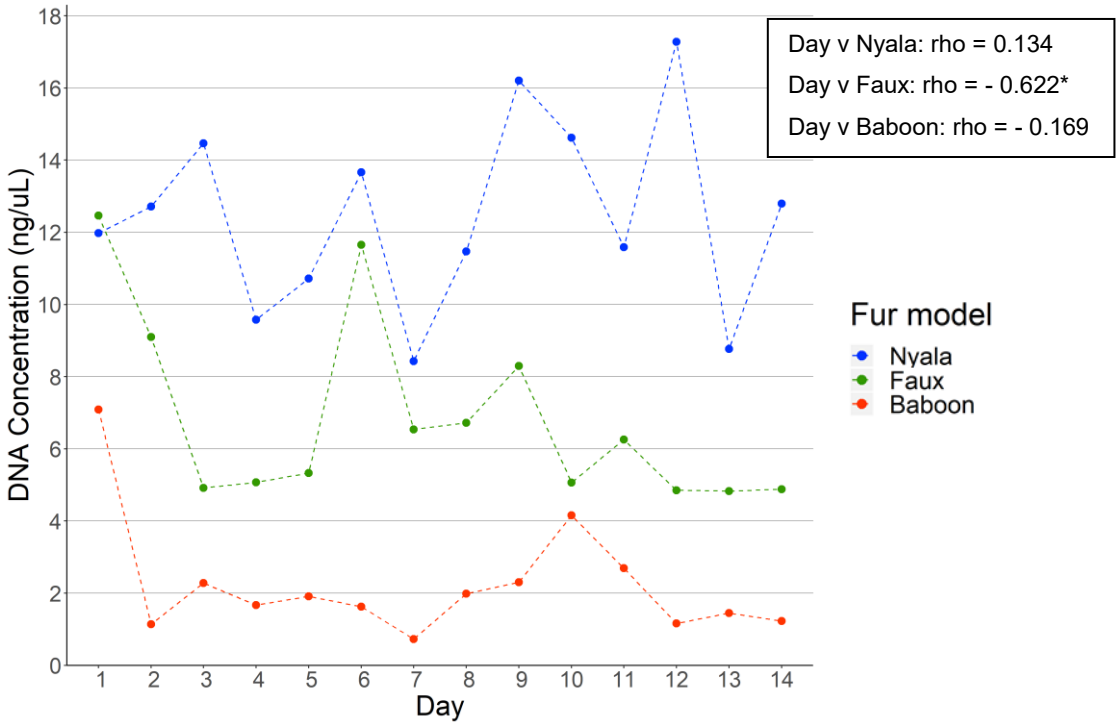

Figure 3.4: qPCR DNA concentrations (ng/µL) of the small autosomal marker (91 bp) obtained from nyala (blue), faux (green) and baboon (pink) furs from the semen stains that were swabbed over 14 days. Rho factors from the Spearman's rank correlation coefficient test are indicated in the top right-hand corner and the asterisk (*) denotes a statistically significant relationship between day and fur model with regards to DNA concentration. (qPCR = quantitative real time polymerase chain reaction, bp = base pairs, v = versus).

A Kruskal-Wallis test indicated that a significant difference between the fur models existed for the DNA concentrations ($p < 0.001$). When the concentrations for each model were compared, significant differences were found between each pairwise model ($p < 0.001$). Significantly higher DNA concentrations were obtained from samples swabbed from nyala fur, followed by faux fur and lastly, baboon fur.

The background swabs of the various furs yielded DNA of extremely low concentrations, the maximum being 0.114 ng/µL (from baboon fur). This was not unexpected for the nyala and baboon fur, due to the models comprising DNA themselves. When DNA was detected in background swabs, it was mostly from the small autosomal marker only. The positive and negative controls performed as expected. Figure H1 (Appendix H) illustrates an example of the standard curves generated in the qPCR assay.

3.2.2 PCR inhibition and DNA degradation

The qPCR data was also assessed in terms of the presence of PCR inhibitors and DNA degradation. No PCR inhibition was observed in any of the samples.

Figure 3.5 illustrates the DI per fur model over the two-week period. The DI from the nyala fur was not normally distributed ($p < 0.05$). When combining data from both donors, the minimum DI obtained was 0.902 (Day 3, faux fur) and the maximum DI was 1.159 (Day 10, baboon fur).

A trend of increasing DI over time can be seen in Figure 3.5, and a significant relationship could be observed between 'day' and the 'DI' for each fur model ($p < 0.05$), with the rho factors indicating strong, positive linear correlations (Figure 3.5).

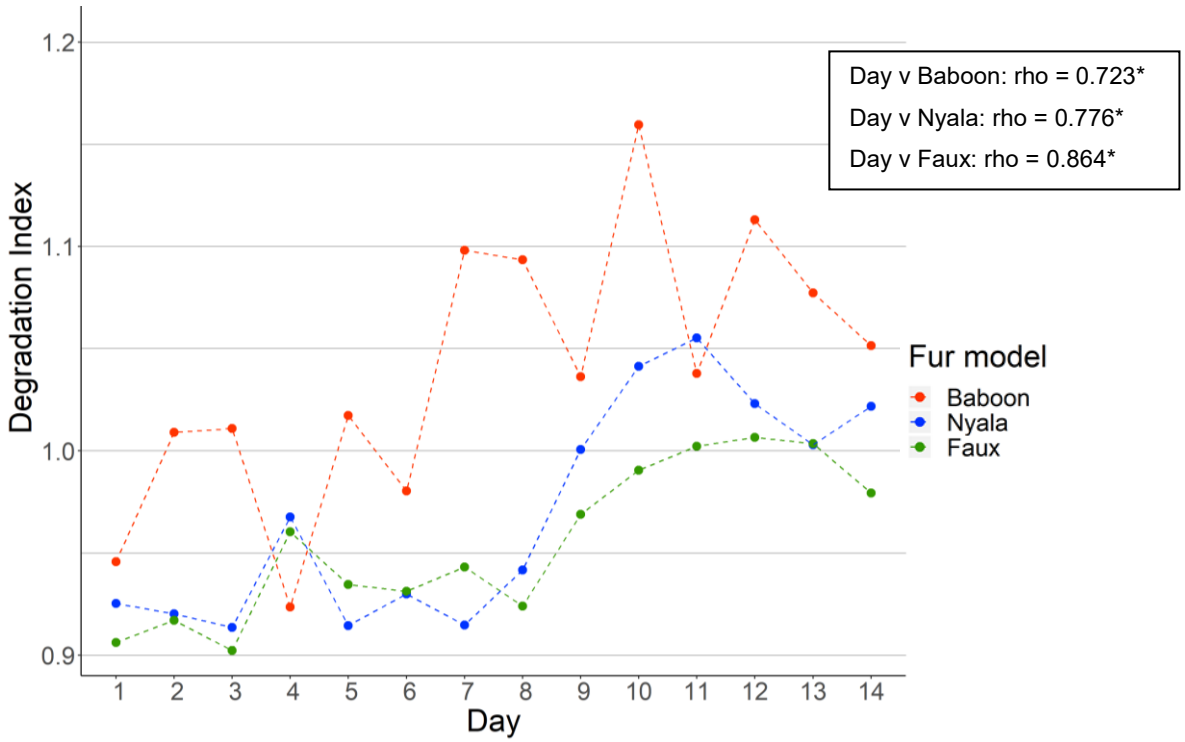

Figure 3.5: Calculated degradation index (DI) for baboon (pink), nyala (blue) and faux (green) furs for the semen stains that were swabbed over 14 days. Rho factors from the Spearman's rank correlation coefficient test are indicated in the top right-hand corner and the asterisk (*) denotes a statistically significant relationship between day and fur model with regards to DI. (v = versus).

A Kruskal-Wallis test indicated that a significant difference between the fur models existed for the DI. When the DI for each model was compared, significant differences were found between baboon and nyala fur ($p = 0.00170$), as well as between baboon and faux fur ($p < 0.001$).

3.2.3 DNA profiling

The ability to obtain good quality, informative STR DNA profiles from semen stains on animal fur was assessed. A good quality DNA profile has easily discernible peaks at all loci which meet the internally validated analytical and stochastic thresholds. A DNA profile would be regarded forensically informative if it holds enough discriminatory power. Internally, this is defined as twelve or more fully typed STRs.

Majority of the generated DNA profiles gave a 100 % allele call rate. Four samples had a dropout of one to three alleles but were still considered forensically informative. All DNA profiles corresponded to the respective donor's reference DNA profile. These results indicated that sufficient, good quality DNA to produce informative DNA profiles was obtained from the three types of fur for the full two-week period.

Positive and negative controls provided results as expected (Figure H2 and H3 respectively, Appendix H). Background swabs from the baboon fur produced several peaks that did not match the donor's reference profiles, nor any 'elimination' DNA profile from researchers in the laboratory (Figure 3.6 B). Figure 3.6 illustrates these peaks, namely; two off-ladder peaks at 237 bp and 241 bp at marker D2S1338, an allele 7 at 305 bp at marker D22S1045 and, an allele 15 at 124 bp at marker D16S5539. One to four of these additional peaks were observed in some of the profiles generated from samples obtained from the baboon fur (Figure 3.6 A and C), but they were substantially lower in relative fluorescent units (RFU). These additional peaks were attributed to amplification from the baboon fur itself and could thus be eliminated based on the profiles obtained from the background swabs of the fur.

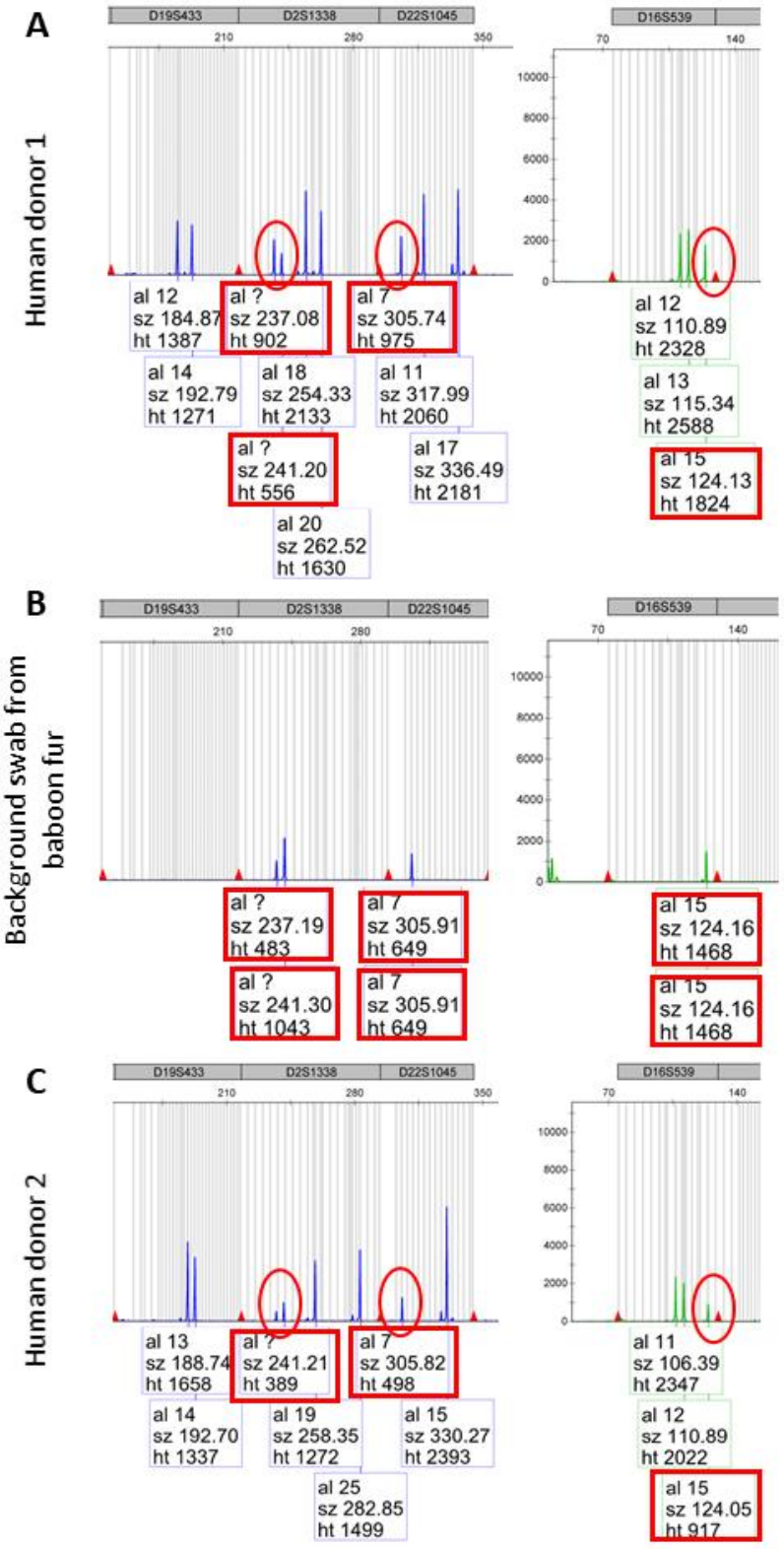

Figure 3.6: Example of the additional peaks in the electropherograms (red circles and red squares) observed from semen stains on the baboon fur (A and C) that corresponded to those observed in background swabs from the baboon fur (B). Marker size is represented on the x-axis and relative fluorescent units (RFUs) on the y-axis, and each marker is labelled on the top.

Chapter 4: Discussion

4.1 Importance of molecular forensics in ASA investigations

ASA involves the abuse of animals for sexual gratification which often poses a threat to an animal's wellbeing. ASA is illegal in many countries, including South Africa as it contravenes Section 13 of the Criminal Law (Sexual Offences and Related Matters) Amendment Act 32 of 2007.[5,21,38–43]

Currently, investigation and confirmation of ASA relies on confessions, eyewitness reports and veterinary forensic examination of abused animals.[5,8] The principle of *Corpus delicti* implies that a confession alone is insufficient to establish a conviction,[3] and ASA may not always be witnessed and/or injury may not always occur. The vital role that semen serves in investigations of human sexual assault would be valuable in ASA investigations as it is a forensically and legally accepted indicator that some form of sexual activity has taken place and provides DNA evidence for identification purposes.[60,95,101,138,139]

In legal proceedings, the timing of incidents may be crucial in the prosecution of an offender, but this is challenged in ASA as the TSI often remains unknown. Interpretation of the results obtained from presumptive and confirmatory testing of semen can provide an indication as to when ASA may have occurred. However, the systematic literature review (Chapter 1) showed that no empirical data previously existed regarding semen retention over time on/in animals. Thus, the aim of this pilot project was to provide baseline data pertaining to the detection and recovery of human semen and the resulting DNA from animal fur over time.

The current study showed that it was possible to obtain human semen, and subsequently DNA, from animal fur over a two-week period. AP and abundant spermatozoa were recovered from the different types of fur for all 14 days (Figure 3.1 - 3.3). Informative DNA profiles that could easily be matched to the reference donor profile were also successfully produced for the entire two-week period for all fur models. Since the fur models were stationary and not exposed to harsh environments (*e.g.* the immune system or extreme temperatures), these results were expected as previous studies have illustrated the persistence of semen at extended periods of time on substrates in similar conditions.[48,49,55,115,116,123,140–144] The results also showed that DNA from the animal itself could also be obtained and highlights the importance of background swabs to eliminate extraneous peaks on DNA profiles.

4.2 The influence of the fur model on semen and DNA retention and recovery

Selecting the appropriate fur model to test the study objectives was challenged by the absence of supporting literature as well as the ethical use of live animals in research. Observing the retention of human semen on a living animal's fur would have been the most representative of an instance of ASA, but would be illegal and unethical. Therefore, the suitability of untreated baboon fur, semi-processed nyala fur and synthetic faux fur was assessed.

Although the presence of semen was confirmed and DNA profiles were successfully generated, some differences between the models were seen in terms of spermatozoa abundance as well as DNA concentration and degradation (Figure 3.3 – 3.5). This may be explained by the different structural morphologies as well as the intrinsic biotic and abiotic factors of the fur models which can play a role in the retention and recovery of biological forensic evidence such as semen.

4.2.1 The structure of animal fur and its effect on semen and DNA recovery

Animals typically have two layers of fur. The ground layer, or undercoat, is soft and primarily present for insulation.[145] The guard layer, or topcoat, is much coarser and protects the animal from the elements. Fur varies across the body of a single animal and also displays both intra- and interspecies differences in terms of texture (smooth or coarse), thickness, number of layers, length and style (straight or curly).[146] These morphological differences between furs can then affect the retention and recovery of semen.

In this project, when aliquoting semen onto the fur, the semen was readily absorbed into the baboon fur while it appeared to remain at surface level for the other two models. Absorption into the deeper layers of the fur can make semen recovery more challenging, which explains the random variation seen in the baboon fur model regarding AP reaction times and spermatozoa scores over the two weeks. The same recovery method was used across all fur types and the structure of the fur may warrant for alternative sampling methods.

Inhibition of sufficient spermatozoa recovery inevitably means that DNA recovery would also be affected. Statistical analyses showed that a significant difference existed between all fur models in terms of DNA concentration, with the baboon fur having lower DNA yields than the other models (Figure 3.4). This could be because of semen seeping into the deeper layers of the fur.

Although, semen remained on the surface of the nyala and faux furs, sampling dry stains from the latter proved slightly problematic as strands of the fur would come off. The faux fur was thicker than the nyala hide and some absorption may have occurred but not to the extent of baboon fur. These reasons explain why DNA yields from the faux fur were lower than that from the nyala fur, but higher than from the baboon fur (Figure 3.4).

For the nyala hide, apart from semen not absorbing into the fur, spermatozoa recovery was easier as spermatozoa were seen to aggregate on the salt crystals. These two factors in conjunction could attribute to the higher DNA yields obtained from this model.

Just as previous studies illustrated varying recovery of semen (post-laundering) between different textiles, possibly due to their various weaving patterns,[56,71,85] different fur structural morphologies can contribute to the extent of semen retention and recovery. Retention times of semen on other fur types *e.g.* curly or long hair, from animal species that are more likely to be abused can be tested. In order to accomplish this, the necessary approval can be secured to obtain furs from euthanised animals from abattoirs or a Society for the Prevention of Cruelty to Animals (SPCA).

Mammalian fur strands comprise of three morphological regions – the medulla, cortex and cuticle, with the cuticle being composed of a layer of keratinised scales.[147] There are three types of scales: coronal (crown-like) scales, spinous (petal-like) scales and, imbricate (flattened) scales.[147] Primates, including humans, have imbricate scales, whereas other animals have one of the three types.[147] Although beyond the scope of the study, analysing and investigating these microscopic differences between furs and the influence thereof on semen retention would be worthwhile.

4.2.2 Biological factors on the fur that affect semen and DNA recovery

In a pairwise-comparison of DNA degradation between models, a significant difference was only observed between baboon and nyala furs as well as between baboon and faux furs.

Any biological material (*e.g.* tissue or body fluids) is susceptible to degradation by endogenic and microbial enzymes, as well as spontaneous chemical reactions.[148–150] Microorganisms (bacteria and fungi) are likely to be present on animal fur, such as the baboon and nyala furs, and their exoenzymes and general hydrolytic reactions cause DNA degradation.[123,148]

The baboon fur was untreated and, over time, tissue degradation by endogenic enzymatic and microbial action was bound to occur. Not only does the decomposition process of biological tissue encompass and promote microbial growth,[151] but the endogenous enzymatic reactions that are also involved would contribute to loss of DNA integrity.

However, although the nyala hide originated from a real animal, the furs were already dehydrated and preserved in salt prior to the application of semen. Salt is known to inhibit cellular and tissue degradation and thereby preserves DNA integrity.[152–154] Dehydration provides a protective effect where protein denaturation suppresses the action of endogenous enzymes.[148,155] The chemical processing of the nyala hide may also remove microbiomes to a certain extent.

The faux fur on the other hand, was composed of 100% acrylic (synthetic) fibres and the absence of biological factors would prevent DNA degradation from occurring in a natural manner. These reasons can explain why DNA degradation was significantly higher on the baboon fur when compared to the other models, and why the nyala and faux fur showed no significant difference in DNA degradation (Figure 3.5).

Forensically informative DNA profiles could easily be matched to the donor samples, but some additional peaks not attributed to instrumental artefacts, positive controls or other researchers in the laboratory were observed for some samples obtained from the baboon fur (Figure 3.6). The baboon specimens were obtained from a national reserve and it is possible that these additional peaks could be attributed to DNA transfer as a result of people handling the baboon heads. However, the nyala and faux furs were also items that would typically be handled by multiple people prior to being used in this project as well. Yet, samples obtained from the nyala and faux furs (both semen and background samples) did not indicate the presence of DNA contamination from a second individual. Furthermore, not all of the additional peaks observed in the semen and background samples obtained from the baboon fur aligned with the allele bins. Thus, the additional peaks can reasonably be attributed to amplification of primate DNA rather than DNA contamination from an unknown person.

The DNA profiling kit used in this study (Promega PowerPlex® ESI 16) is specific to higher primates and it has previously demonstrated DNA amplification for gorillas and spider monkeys.[156] However, no validation data exists for the *Papio ursinus* species,[156] and it thus remains likely that the additional peaks originated from baboon DNA. The detection of DNA from higher primate species is due to high DNA sequence similarity to humans.[157] However,

in ASA, domestic, docile animals are more likely to be victims of abuse[2,5–7] and these species usually do not cause any major interferences in the DNA analytical techniques used in this project.[156,158–161]

4.2.3 A suitable fur model for ASA research

Although it is unlikely that baboons would be ASA victims and it is acknowledged to not be representative of all animal fur variations, the fur was a readily available sample that still maintained the authenticity of using real animal fur. Due to the legal and ethical issues with simulating experiments that require intimate sampling and/or recovery of human semen from live animals, obtaining this type of data would have to rely on such fur models or casework samples.

While preserved animal hides and synthetic materials are convenient, it would be more valuable to use untreated fur from deceased animals to represent a more biologically realistic scenario. The preserved nyala hide is not ideal as the salt creates a bias in molecular analyses. The faux fur may be a more suitable model to simulate a stationary, living animal. However, this model lacks the inherent biological factors that real animal furs may have such as microorganisms, natural oils and moisture and, other biological contaminants (*e.g.* biological fluids, excretes and plant material). On the other hand, due to the decomposition component, the baboon fur model, *i.e.* fur from deceased animals, may likely represent what can be expected from fatal ASA cases. Due to the lack of data from ASA cases, it is unclear which model best represents authentic living animals. To this end, veterinarians, forensic laboratories and researchers are encouraged to publish BFI and DNA profiling data from ASA casework.

4.3 Recovery of semen over time

Apart from assessing differences between fur models, the change in the study's dependent variables over time was also evaluated. The recovery of AP and spermatozoa were generally consistent over time with differences in AP reaction times and spermatozoa scores only varying at a few random time points. The robustness of these seminal constituents has previously been demonstrated in dry, extremely aged semen stains kept in controlled environments.[48,49,55,61,115,116,142]

In terms of DNA, degradation was seen to increase over time for all fur models (Figure 3.5). Morphological changes of spermatozoa over time compromise the integrity of the cellular membrane and lysis of the cell can be accomplished much easier.[49,115,116,162] As a consequence of this cellular lysis, DNA is then exposed to a multitude of factors which can influence the rate and extent of degradation over time. These factors can include endogenous enzymatic activity as well as environmental insults such as ultraviolet light from the sun, heat, water, variation in pH, and microbial action.[148–150,163,164]

Even though the furs were kept in controlled environments, DNA degradation could still have occurred as previous research has demonstrated this longitudinal change with increasingly aged seminal stains (32 years – 62 years old) also maintained in controlled conditions.[55,142] Cellular integrity was not assessed but, based on light microscopy, abundant spermatozoa were obtained in majority of the samples, even after 14 days of exposure on the fur. Previous research has shown that degradation can still occur over time in morphologically intact spermatozoa through endogenous nucleases.[104] However, dehydration of the semen stain itself, can delay degradation due to suppressed endogenous enzymatic activity.[148,155]

An additional protective factor is offered by the difference in DNA chromatin structure in spermatozoa compared to somatic cells. Spermatozoa DNA is packaged around protamines instead of histones which are found in somatic cell nuclei and oxidation of cysteine-rich residues and the formation of disulphide bridges link the protamines together.[165,166] This type of DNA packaging can make spermatozoa DNA more resistant to isolation than DNA from somatic cells and can delay the degradation process. Although degradation increased over time in this project, it was not extensive enough over the two weeks to prevent good quality, forensically informative DNA profiles from being obtained.

Despite an increase in degradation, only the faux fur model showed a decrease in DNA concentration over time (Figure 3.4). DNA concentrations from semen aliquoted on swabs and kept in controlled environments have been shown to not differ significantly over a time period of 28 days[167] and it is unclear as to why only the faux fur model showed a significant change in DNA concentration over the two weeks. However, the effect of time on semen recovery should be assessed in conjunction with the morphological, biotic and abiotic characteristics of the furs. Thus, the significant change in DNA concentration over time could also be attributed to the semen absorbing deeper into the fur. Sampling of the dry semen stains from the synthetic

faux fur may also warrant a different approach such as taking fur cuttings as opposed to swabbing.

Although justified by the pilot nature of this project, the small sample size of two semen donors was a limitation of the study. By expanding the sample size, the relationships between time and the various tested variables (*i.e.* BFI outcomes and DNA metrics) of the project can be confirmed.

4.4 Additional challenges in ASA and future research

Given that baseline data has been produced by this project, additional variables can now be introduced into the model to represent a more realistic ASA scenario. Changes in movement, temperature and humidity can be introduced to assess the effect of these variables on semen persistence over time. The fur can also be brushed or washed with water and shampoos after semen deposition to simulate grooming, licking and bathing.

This study established a suitable methodology for molecular forensic investigations of ASA and, specificity and sensitivity assays can be done on the fur models in order to validate these analytical methods for use in an ASA casework scenario.

Method specificity to detect human semen can be assessed by applying mixtures of different body fluids - either with other human body fluids or those of animal origin. An analyst can be trained to identify the morphological and histological staining differences between animal and human spermatozoa.[168–170]

By mixing body fluids from humans and animals, the various DNA metrics can be assessed. The detection of additional peaks in samples obtained from the baboon furs highlights the need for assay validation. If needed, mitochondrial DNA genes such as the *cytochrome b* and *cytochrome c oxidase I* can be used to differentiate between human and animal DNA.[171,172] In the instance of multiple offenders (*i.e.* mixtures of human semen from different donors) Y-STR profiling will be more useful in differentiating the different donors' DNA profiles.[132]

DNA arising from non-perpetrators can easily be encountered in ASA and by extracting DNA specifically from spermatozoa, the identification of the semen donor can be ensured. This can be achieved through differential lysis and extraction, laser capture microdissection (LCM), and SpermElution©.[54,56,57,63,79,92,95,100,104] In cases of oligo/azoospermia, isolating male DNA through FISH, LCM and/or Y-STR typing can be more useful.[52] Sensitivity of the methods can

be investigated by adjusting the volume of semen and spermatozoa abundance applied to the fur to simulate donor-dependent factors such as the volume of ejaculate and, oligo/azoospermic males.

As estimating the TSI in sexual offence cases is crucial, the use of RNA degradation patterns to estimate sample age is being researched.[173–177] Such methodologies can be further explored in an ASA context using animal fur as the substrate. Trace evidence is commonly encountered in forensic science and with the added probability of delayed reporting, evidence from ASA is no exception to the rule. Advancements in BFI and analyses such as direct PCR prevents potential sample loss and commercial kits targeting shorter amplicons are more useful in DNA profiling of degraded samples.[141–144,162,178–180]

Specific mRNA or miRNA markers as well as DNA methylation profiles are being introduced as more sensitive and less subjective methodologies for BFI.[181–192] Some research has even shown that simultaneous BFI and DNA profiling can be achieved, thereby allowing for optimal use of trace evidence.[193,194] Other techniques for BFI make use of automation or spectroscopy which allow for quick sample analyses turnaround time – a benefit to forensic investigations.[195–199]

Additionally, it would also be beneficial to obtain consensus data on the management and scope of ASA nationally and this can be achieved through surveys distributed to veterinarians, animal welfare and care centres and even the police services. Effective protocols, necessary training and resources can then be established and implemented for the forensic investigation of ASA.

4.5 Conclusion

ASA involves the sexual molestation of animals that can lead to injury or even death. ASA is illegal in many countries and thus warrants forensic investigation. Semen provides legally and forensically accepted proof of sexual activity. Apart from confirming the nature of the crime, understanding the extent to which evidence of semen can be obtained over time can aid in inferring a timeline of events which are important details in legal prosecutions.

The paucity of research revealed by the systematic literature review prompted this study, which was the first study to prospectively investigate the detection and recovery of semen (AP and spermatozoa) and DNA from animal fur.

The majority of semen samples obtained from the fur models produced immediate AP reactions and provided abundant intact spermatozoa for all 14 days. Good quality, forensically informative DNA profiles that easily matched the semen donors were also obtained from all fur models.

Through the assessment of the suitability of various fur models for ASA research, this study illustrated that intrinsic components of the fur such as morphology and biological factors (or lack thereof) can influence the retention and recovery of semen and DNA. Pre-treated animal furs may introduce bias in molecular analyses while fur from deceased animals may provide a more suitable model for simulating fatal ASA cases.

This research has successfully provided baseline measurements and further exploration into semen persistence on animal fur can provide forensic analysts with useful guidelines to estimate the time since assault. Therefore, this project also aims to pioneer further research into the molecular forensic investigations of ASA and encourages the incorporation thereof into standard investigative protocols which can be applied in a court of law.

References

1. Miletski H. A history of bestiality. In: Beetz AM, Podberscek AL, eds. *Bestiality and Zoophilia: Sexual Relations with Animals*. Oxford: Berg; 2005:1-22.

2. Williams CJ, Weinberg MS. Zoophilia in men: A study of sexual interest in animals. *Arch Sex Behav*. 2003;32(6):523-535.

3. Holoyda B, Sorrentino R, Friedman SH, Allgire J. Bestiality: An introduction for legal and mental health professionals. *Behav Sci Law*. 2018;36(6):687-697.

4. Aggrawal A. References to the paraphilias and sexual crimes in the Bible. *J Forensic Leg Med*. 2009;16(3):109-114.

5. Stern AW, Smith-Blackmore M. Veterinary forensic pathology of animal sexual abuse. *Vet Pathol*. 2016;53(5):1057-1066.

6. Miletski H. Zoophilia: Another sexual orientation? *Arch Sex Behav*. 2017;46(1):39-42.

7. Beetz A. *Love, Violence and Sexuality in Relationships between Humans and Animals*. Aachen, Germany: Shaker Verlag GmbH; 2002.

8. Munro HMC, Thrusfield M V. "Battered pets": Sexual abuse. *J Small Anim Pract*. 2001;42(7):333-337.

9. Sendler DJ. Contemporary understanding of zoophilia — A multinational survey study. *J Forensic Leg Med*. 2019;62(May 2018):44-51.

10. Amoo G, Abayomo O, Olashore AA. Zoophilic recidivism in schizophrenia: A case report. *Afr J Psychiatry*. 2012;15(4):223-225.

11. Imbschweiler I, Kummerfeld M, Gerhard M, Pfeiffer I, Wohlsein P. Animal sexual abuse in a female sheep. *Vet J*. 2009;182(3):481-483.

12. Kind SS. The use of the acid phosphatase test in searching for seminal stains. *J Crim Law Criminol*. 1957;47(5):597-600.

13. Chee KT. A case of bestiality. *Singapore Med J*. 1974;15(4):287-288.

14. Satapathy S, Swain R, Pandey V, Behera C. An adolescent with bestiality behaviour: Psychological evaluation and community health concerns. *Indian J Community Med*. 2016;41(1):23-26.

15. Holoyda B. Bestiality in forensically committed sexual offenders: A case series. *J Forensic Sci*. 2017;62(2):541-544.

16. Hvozdík A, Bugarský A, Kottferová J, et al. Ethological, psychological and legal aspects of animal sexual abuse. *Vet J*. 2006;172(2):374-376.

17. Chandradasa M, Rathnayake LC. Zoophilia in an adolescent with high-functioning autism from Sri Lanka. *Australas Psychiatry*. 2017;25(5):486-488.

18. Beetz A. Bestiality and zoophilia - Associations with sex and violence offending. In: Beetz A, Podberscek AL, eds. *Bestiality and Zoophilia: Sexual Relations with Animals*. Oxford, United Kingdom: Berg; 2005:46-70.

19. Schenk AM, Cooper-Lehki C, Keelan CM, Fremouw WJ. Underreporting of bestiality among juvenile sex offenders: Polygraph versus self-report. *J Forensic Sci*. 2014;59(2):540-542.

20. Fleming WM, Jory B, Burton DL. Characteristics of juvenile offenders admitting to sexual activity with nonhuman animals. *Soc Anim J Human-Animal Stud*. 2002;10(1):31-45.

21. Holoyda B, Newman W. Zoophilia and the law: Legal responses to a rare paraphilia. *J Am Acad Psychiatry Law*. 2014;42(4):412-420.

22. Alvarez WA, Freinhar JP. A prevalence study of bestiality (zoophilia) in psychiatric in-patients, medical in-patients, and psychiatric staff. *Int J Psychosom Off Publ Int Psychosom Inst*. 1991;38(1-4):45-47.

23. Kinsey AC, Pomeroy WB, Martin CE, Gebhard PH. *Sexual Behaviour in the Human Female*. Philadephia, United States of America: W.B Saunders company; 1953.

24. Kinsey AC, Pomeroy WB, Martin CE. *Sexual Behavior in the Human Male*. Philadephia, United States of America: W.B Saunders company; 1948.

25. Story MD. A comparison of university student experience with various sexual outlets in 1974 and 1980. *Adolescence*. 1982;17(68):737-747.

26. Abel GG, Osborn CA, Twigg DA. Sexual assault through the lifespan: Adult offenders with juvenile histories. In: Barbee H, Marshall W, Hudson S, eds. *The Juvenile Sex Offender*. New York, United States of America: Guilford Press; 1993:104-117.

27. Miller KS, Knutson JF. Reports of severe physical punishment and exposure to animal cruelty by inmates convicted of felonies and by university students. *Child Abus Negl*. 1997;21(1):59-82.

28. Hensley C, Tallichet SE, Singer SD. Exploring the possible link between childhood and adolescent bestiality and interpersonal violence. *J Interpers Violence*. 2006;21(7):910-923.

29. Hensley C, Tallichet SE, Dutkiewicz EL. Childhood bestiality - A potential precursor to adult interpersonal violence. *J Interpers Violence*. 2010;25(3):557-567.

30. Emerick RL, Dutton WA. The effect of polygraphy on the self-report of adolescent sex offenders: Implications for risk assessment. *Ann Sex Res*. 1993;6(2):83-103.

31. Frazier MR. Physically and sexually violent juvenile offenders: A comparative study of victimisation history variables [dissertation]. Utah State Univeristy; 1998.

32. Simons DA, Wurtele SK, Durham RL. Developmental experiences of child sexual abusers and rapists. *Child Abus Negl*. 2008;32(5):549-560.

33. Aggrawal A. A new classification of zoophilia. *J Forensic Leg Med*. 2011;18(2):73-78.

34. Virgilio E, Franzese E, Caterino S. Zoosexuality: An unusual cause of colorectal injury. *Acta Chir Belg*. 2016;116(5):316-318.

35. American Psychiatric Association. Paraphilic disorders. In: *Diagnostic and Statistical Manual of Mental Disorders (DSM-5®)*. Fifth. Arlington, United States of America: American Psychiatric Association Publishing; 2013.

36. Beirne P. Rethinking bestiality: Towards a concept of interspecies sexual assault. *Theor Criminol*. 1997;1(3):317-340.

37. Levy N. What (if anything) is wrong with bestiality? *J Soc Philos*. 2003;34(3):444-456.

38. Denmark passes law to ban bestiality. BBC newsbeat website. http://www.bbc.co.uk/newsbeat/article/32411241/denmark-passes-law-to-ban-bestiality. Published 2015. Accessed March 26, 2019.

39. Glass J. All countries where bestilaity is legal but same sex marriage isn't. Pinknews website. https://www.pinknews.co.uk/2018/03/22/where-is-bestiality-legal-still/. Published 2018. Accessed March 26, 2019.

40. Geldenhuys K. The NSPCA: Bestiality and animal sex crimes. https://nspca.co.za/wp-content/uploads/2016/05/Beastiality.pdf. Published 2014. Accessed March 26, 2019.

41. Project on Extra-Legal Executions in Iran. Table of Capital Offenses in the Islamic Republic of Iran , and their Sources in Statute Law and Islamic Law. https://tbinternet.ohchr.org/Treaties/CCPR/Shared Documents/IRN/INT_CCPR_NGO_IRN_103_9087_E.pdf. Published 2011. Accessed March 20, 2019.

42. Mittal A, Shenoi SD, Kumar KB, Sharma PV. Genital lesions following bestiality. *Indian J Dermatology, Venereol Leprol*. 2000;66(2):95-96.

43. South Africa. Criminal Law (Sexual Offences and Related Matters) Amendment Act 32 of 2007.

44. Raven PH, Johnson GB, Mason KA, Losos JB, Singer SR. Animal form and function: The reproductive system. In: *Biology*. New York, Unites States of America: McGraw-Hill Education; 2014:1092.

45. Sherwood L. The reproductive system. In: *Fundamentals of Human Physiology*. Fourth. Belmont, United States of America: Cengage Learning; 2011:561.

46. Harbison S, Fleming R. Forensic body fluid identification: State of the art. *Res Reports Forensic Med Sci*. 2016;6:11-23.

47. Pinheiro M. Advances in DNA typing in sexual assault casework. In: Gonzalez-Andrade F, ed. *Forensic Genetics Research Progress*. New York, United States of America: Nova Science Publishers, Inc.; 2009:117-132.

48. Elshama SS, Aly SM, Abdalla ME, Hassan WA. Postmortem identification of spermatozoa on human skin based on fluorescent monoclonal antibody method. *Arch Med Sadowej Kryminol*. 2017;67(2):121-133.

49. Gibelli D, Mazzarelli D, Rizzi A, Kustermann A, Cattaneo C. Persistence of spermatozoa on decomposing human skin: A scanning electron microscopy study. *Int J Legal Med*. 2013;127(5):975-979.

50. Hooft P, van de Voorde H. In vitro changes in human spermatozoa exposed to gastric juice: Laboratory findings as a support for forensic practice. *Zeitschrift für Rechtsmedizin*. 1988;101(1):41-44.

51. McWilliams S, Gartside B. Identification of prostate-specific antigen and spermatozoa from a mixture of semen and simulated gastric juice. *J Forensic Sci*. 2009;54(3):610-611.

52. Murray C, McAlister C, Elliott K. Identification and isolation of male cells using fluorescence in situ hybridisation and laser microdissection, for use in the investigation of sexual assault. *Forensic Sci Int Genet*. 2007;1(3-4):247-252.

53. Schumann BG, Badaway S, Peglow A, Henry JB. Prostatic acid phosphatase - Current assessment in vaginal fluid of alleged rape victims. *Am J Clin Pathol*. 1976;66(6):944-952.

54. Hellerud BB, Bouzga M, Hoff-olsen P, Mevåg B. Semen detection: A retrospective overview from 2010. *Forensic Sci Int Genet Suppl Ser*. 2011;3(1):e391-e392. doi:10.1016/j.fsigss.2011.09.057

55. Nakanishi H, Hara M, Takahashi S, Takada A, Saito K. Evaluation of forensic examination of extremely aged seminal stains. *Leg Med*. 2014;16(5):303-307.

56. Jobin RM, De Gouffe M. The persistence of seminal constituents on panties after laundering: Significance to investigations of sexual assault. *J Can Soc Forensic Sci*. 2003;36(1):1-10.

57. Crowe G, Moss D, Elliot D. The effect of laundering on the detection of acid phosphatase and spermatozoa on cotton t-shirts. *J Can Soc Forensic Sci*. 2000;33(1):1-5.

58. Farmen RK, Cortez P, Skårland Frøyland E. Spermatozoa recovered on laundered clothing. *Forensic Sci Int Genet Suppl Ser*. 2008;1(1):418-420.

59. Joshi UN, Subhedar SK, Saraf DK. Effect of water immersion on seminal stains on cotton cloth. *Forensic Sci Int*. 1981;17(1):9-11.

60. Ricci LR, Hoffman SA. Prostatic acid phosphatase and sperm in the post-coital vagina. *Ann Emerg Med*. 1982;11(10):530-534.

61. Steinman G. Rapid spot tests for identifying suspected semen specimens. *Forensic Sci Int*. 1995;72(3):191-197.

62. Casey DG, Domijan K, MacNeill S, Rizet D, O'Connell D, Ryan J. The persistence of

sperm and the development of time since intercourse (TSI) guidelines in sexual assault cases at Forensic Science Ireland, Dublin, Ireland. *J Forensic Sci*. 2017;62(3):585-592.

63. Noël S, Lagacé K, Raymond S, et al. Repeatedly washed semen stains: Optimal screening and sampling strategies for DNA analysis. *Forensic Sci Int Genet*. 2019;38(October 2018):9-14.

64. Eungprabhanth V. Finding of the spermatozoa in the vagina related to elapsed time of coitus. *Zeitschrift für Rechtsmedizin*. 1974;74(4):301-304.

65. Dziegelewski M, Simich JP, Rittenhouse-Olsen K. Use of a Y-chromosome probe as an aid in the forensic proof of sexual assault. *J Forensic Sci*. 2002;47(3):601-604.

66. Rupp J. Sperm survival and prostatic acid phosphatase activity in victims of sexual assault. *J Forensic Sci*. 1969;14(2):177-183.

67. Soules MR, Pollard AA, Brown KM, Verma M. The forensic laboratory evaluation of evidence in alleged rape. *Am J Obstet Gynecol*. 1978;130(2):142-147.

68. Standefer JC, Street EW. Postmortem stability of prostatic acid phosphatase. *J Forensic Sci*. 1977;22(1):165-172.

69. Dahlke MB, Cooke C, Cunnane M, Chawla J, Lau P. Identification of semen in 500 patients seen because of rape. *Am J Clin Pathol*. 1977;68(6):740-746.

70. Gabby T, Winkleby MA, Boyce WT, Fisher DL, Lancaster A, Sensabaugh GF. Sexual abuse of children. The detection of semen on skin. *Am J Dis Child*. 1992;146(6):700-703.

71. Spector J, Von Gemmingen D. The effect of washing on the detection of blood and seminal stains. *J Can Soc Forensic Sci*. 1971;4:3-9.

72. Enos W, Beyer J. The importance of examining skin and hair for semen in sexual assault cases. *J Forensic Sci*. 1981;26(3):605-607.

73. Graves HCB, Sensabaugh GF, Blake ET. Postcoital detection of a male specific semen protein: Application to the investigation of rape. *N Engl J Med*. 1985;312(6):338-343.

74. Kulstein G, Schacker U, Wiegand P. Old meets new: Comparative examination of conventional and innovative RNA-based methods for body fluid identification of laundered seminal fluid stains after modular extraction of DNA and RNA. *Forensic Sci*

Int Genet. 2018;36(June):130-140.

75. Evrard JR, Gold EM. Epidemiology and management of sexual assault victims. *Obstet Gynecol*. 1979;53(3):381-387.

76. Brauner P, Gallili N. A condom - the critical link in a rape. *J Forensic Sci*. 1993;38(5):1233-1236.

77. Penttilä A, Karhumen PJ. Medicolegal findings among rape victims. *Med Law*. 1990;9(1):725-737.

78. Schiff AF. Reliability of the acid phosphatase test for the identification of seminal fluid. *J Forensic Sci*. 1978;23(4):833-844.

79. Tsuji A, Ishiko A, Ikeda N, Yamaguchi H. Personal identification using Y-chromosomal short tandem repeats from bodily fluids mixed with semen. *Am J Forensic Med Pathol*. 2001;22(3):288-291.

80. Rambow B, Adkinson C, Frost TH, Peterson GF. Female sexual assault: Medical and legal implications. *Ann Emerg Med*. 1992;21(6):727-731.

81. Chiasson D, Vigorito R, Lee Y, Smialek J. Interpretation of postmortem vaginal acid phosphatase determinations. *Am J Forensic Med Pathol*. 1994;15(3):242-246.

82. McAlister C. The use of fluorescence in situ hybridisation and laser microdissection to identify and isolate male cells in an azoospermic sexual assault case. *Forensic Sci Int Genet*. 2011;5(1):69-73.

83. Enos W, Beyer J, Mann G. The medical examination of cases of rape. *J Forensic Sci*. 1972;17(1):50-56.

84. Allard JE, Davies A. Further information on the use of p-nitrophenyl phosphate to quantitate acid phosphatase on vaginal swabs examined in cases of sexual assault. *Med Sci Law*. 1979;19(3):170-172.

85. Nolan A, Speers SJ, Murakami J, Chapman B. A pilot study : The effects of repeat washing and fabric type on the detection of seminal fluid and spermatozoa. *Forensic Sci Int*. 2018;289:51-56.

86. Bryson CK, Garlo AM, Piner SC. Vaginal swabs: Endogenous and postcoital components. *J Forensic Sci Soc*. 1989;29(3):157-171.

87. McCloskey KL, Muscillo GC, Noordewier B. Prostatic acid phosphatase activity in the postcoital vagina. *J Forensic Sci*. 1975;20(4):630-636.

88. Kamenev L, Leclercq M, Francois-Gerard C. Detection of p30 antigen in sexual assault case material. *J Forensic Sci Soc*. 1990;30(4):193-200.

89. Collins KA, Bennett AT. Persistence of spermatozoa and prostatic acid phosphatase in specimens from deceased individuals during varied postmortem intervals. *Am J Forensic Med Pathol*. 2001;22(3):228-232.

90. Christian CW, Lavelle JM, De Jong AR, Loiselle J, Brenner L, Joffe M. Forensic evidence findings in prepubertal victims of sexual assault. *Pediatrics*. 2000;106(1):100-104.

91. Page H, Sarna A, Watts L, Ward E, Hodgson C, Mckenzie M. The recovery of semen from bathwater using the Evidence Recovery System (ERS). *Sci Justice*. 2014;54(1):89-94.

92. Skalleberg AG, Bouzga MM. Detecting and collecting traces of semen and blood from outdoor crime scenes using crime scene dogs and presumptive tests. *Forensic Sci Int*. 2016;264:146-152.

93. Keil W, Bachus J, Tröger H. Evaluation of MHS-5 in detecting seminal fluid in vaginal swabs. *Int J Legal Med*. 1996;108(4):186-190.

94. Montagna CP. The recovery of seminal components and DNA from the vagina of a homocide victim 34 days postmortem. *J Forensic Sci*. 1996;41(4):700-702.

95. Benschop CCG, Wiebosch DC, Kloosterman AD, Sijen T. Post-coital vaginal sampling with nylon flocked swabs improves DNA typing. *Forensic Sci Int Genet*. 2010;4(2):115-121.

96. Lynnerup N, Hjalgrim H, Eriksen B. Routine use of ultraviolet light in medicolegal examinations to evaluate stains and skin trauma. *Med Sci Law*. 1995;35(2):165-168.

97. Miranda GE, Prado FB, Delwing F, Daruge E. Analysis of the fluorescence of body fluids on different surfaces and times. *Sci Justice*. 2014;54(6):427-431.

98. Lincoln C, McBride P, Turbett G, Garbin C, MacDonald E. The use of an alternative light source to detect semen in clinical forensic medical practice. *J Clin Forensic Med*.

2006;13(4):215-218.

99. Allery J-P, Telmon N, Mieusset R, Blanc A, Rogué D. Cytological detection of spermatozoa: Comparison of three staining methods. *J Forensic Sci*. 2001;46:349-351.

100. Owers R, Davidson G, McDonald A, Morgan R, O'Rourke P. Time since intercourse (TSI) data from a large-scale casework study of penile–vaginal penetration allegations using Sperm Elution™. *Forensic Sci Int*. 2018;288:10-13.

101. Willott G, Allard JE. Spermatozoa - Their persistence after sexual intercourse. *Forensic Sci Int*. 1982;19(2):135-154.

102. Allard JE. The collection of data from findings in cases of sexual assault and the significance of spermatozoa on vaginal, anal and oral swabs. *Sci Justice*. 1997;37(2):99-108.

103. Willott GM, Crosse MA. The detection of spermatozoa in the mouth. *J Forensic Sci Soc*. 1986;26(2):125-128.

104. Elliott K, Hill DS, Lambert C, Burroughes TR, Gill P. Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides. *Forensic Sci Int*. 2003;137(1):28-36.

105. Chapman RL, Brown NM, Keating SM. The isolation of spermatozoa from sexual assault swabs using proteinase K. *J Forensic Sci Soc*. 1989;29(3):207-212.

106. Costa MJ, Tadros T, Tackett E, Naib Z. Vaginocervical cytology in victims of sexual assault. *Diagn Cytopathol*. 1991;7(4):337-340.

107. Schmidt Astrup B, Lange Thomsen J, Lauritsen J, Ravn P. Detection of spermatozoa following consensual sexual intercourse. *Forensic Sci Int*. 2012;221(1-3):137-141.

108. Silverman EM, Silverman AG. Persistence of spermatozoa in the lower genital tracts of women. *JAMA - J Am Med Assoc*. 1978;240(17):1875-1877.

109. Rao PN, Collins KA, Geisinger KR, et al. Identification of male epithelial cells in routine postcoital cervicovaginal smears using fluorescence in situ hybridization: Application in sexual assault and molestation. *Am J Clin Pathol*. 1995;104(1):32-35.

110. Enos WF, Beyer JC. Spermatozoa in the anal canal and rectum and in the oral cavity of female rape victims. *J Forensic Sci*. 1978;23(1):231-233.

111. Randall B. Persistence of vaginal spermatozoa as assessed by routine cervicovaginal (Pap) smears. *J Forensic Sci*. 1987;32(3):678-683.

112. Wilson E. Sperm's morphological survival after sixteen days in the vagina of a dead body. *J Forensic Sci*. 1974;19(3):561-564.

113. Smith DA, Webb LG, Fennell AI, Nathan EA, Bassindale CA, Phillips MA. Early evidence kits in sexual assault: An observational study of spermatozoa detection in urine and other forensic specimens. *Forensic Sci Med Pathol*. 2014;10(3):336-343.

114. Jänisch S, Meyer H, Germerott T, Albrecht U-V, Schulz Y, Debertin AS. Analysis of clinical forensic examination reports on sexual assault. *Int J Legal Med*. 2010;124(3):227-235.

115. Lachica E, García-Ferrer R. Study of semen stains by scanning electron microscopy. Influence of their ageing. *Forensic Sci Int*. 1998;91(1):35-40.

116. Concheiro L, Carracedo A, Guitián F. The use of scanning electron microscopy in the examination of seminal stains. *Forensic Sci Int*. 1982;19(2):185-188.

117. Cina SM, Collins KA, Fitts M, Pettenati MJ. Isolation and identification of male and female DNA on a postcoital condom. *Arch Pathol Lab Med*. 2000;124(7):1083-1086.

118. Delfin FC, Madrid BJ, Tan MP, De Ungria MC. Y-STR analysis for detection and objective confirmation of child sexual abuse. *Int J Legal Med*. 2005;119(3):158-163.

119. Grossin C, Sibille I, Lorin de la Grandmaison G, Banasr A, Brion F, Durigon M. Analysis of 418 cases of sexual assault. *Forensic Sci Int*. 2003;131(2-3):125-130.

120. Tintinalli JE, Hoelzer M. Clinical findings and legal resolutions in sexual assault. *Ann Emerg Med*. 1985;14(5):447-453.

121. Morrison AI. Persistence of spermatozoa in the vagina and cervix. *Br J Vener Dis*. 1972;48(2):141-143.

122. Cerri N, Ricci U, Sani I, Verzeletti A, De Ferrari F. Mixed stains from sexual assault cases: Autosomal or Y-chromosome short tandem repeats? *Croat Med J*. 2003;44(3):289-292.

123. Sirker M, Schneider PM, Gomes I. A 17-month time course study of human RNA and DNA degradation in body fluids under dry and humid environmental conditions. *Int J*

Legal Med. 2016;130(6):1431-1438.

124. Brayley-Morris H, Sorrell A, Revoir AP, Meakin GE, Court DS, Morgan RM. Persistence of DNA from laundered semen stains: Implications for child sex trafficking cases. *Forensic Sci Int Genet*. 2015;19:165-171.

125. Ledray LE, Arndt S. Examining the sexual assault victim: a new model for nursing care. *J Psychosoc Nurs Ment Health Serv*. 1994;32(2):7-12.

126. Ledray LE. Sexual assault nurse clinician: An emerging area of nursing expertise. *AWHONNS Clin Issues Perinat Womens Health Nurs*. 1993;4(2):180-190.

127. Ferris LE, Sandercock J. The sensitivity of forensic tests for rape. *Med Law*. 1998;17(3):333-350.

128. Edwards A, Civitello A, Hammond HA, Caskey CT. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *Am J Hum Genet*. 1991;49(4):746-756.

129. Roewer L. DNA fingerprinting in forensics: Past, present, future. *Investig Genet*. 2013;4(1):22-31.

130. Culhane JF, Nyirjesy P, McCollum K, Casabellata G, Di Santolo M, Cauci S. Evaluation of semen detection in vaginal secretions: Comparison of four methods. *Am J Reprod Immunol*. 2008;60(3):274-281.

131. Davies A, Wilson E. The persistence of seminal constituents in the human vagina. *Forensic Sci*. 1974;3(1):45-55.

132. Hall A, Ballantyne J. Novel Y-STR typing strategies reveal the genetic profile of the semen donor in extended interval post-coital cervicovaginal samples. *Forensic Sci Int*. 2003;136(1-3):58-72.

133. Mole C, Heyns M, Cloete T. Determination of the force associated with blunt force blows to the lateral portion of the cranium: A baboon (Papio ursinus) model. In: A selection of abstracts presented at the 45th annual conference of the Anatomical Society of Southern Africa (ASSA), 23 - 36 April 2017. *Clin Anat*. 2018;31:e25-e39. doi:https://doi.org/10.1002/ca.23050

134. Mole CG, Coetze L, Burgers AL, Cloete T. Investigation of the force associated with lateral skull fracture through the use of the Hopkinson pressure bar. In: A selection of

abstracts presented at the 43rd annual conference of the Anatomical Society of Southern Africa (ASSA), 9 – 13 May 2015. *Clin Anat*. 2018;31:e1-e10. doi:https://doi.org/10.1002/ca.23041.

135. Qiagen. *QIAamp® DNA Investigator Handbook*.; 2012.

136. Qiagen. *Investigator® Quantiplex® Pro Handbook*.; 2017.

137. Promega. *PowerPlex® ESI 16 System*.; 2016.

138. Collins KA. The laboratory's role in detecting sexual assault. *Lab Med*. 1998;29(6):361-365.

139. Collins KA, Rao PN, Hayworth R, et al. Identification of sperm and non-sperm male cells in cervicovaginal smears using fluorescence in situ hybridization: Applications in alleged sexual assault cases. *J Forensic Sci*. 1994;39(6):1347-1355.

140. Hara M, Nakanishi H, Takahashi S, Kido A, Saito K, Takada A. STR and Y-STR genotyping of 30-50-year-old semen stains. *Forensic Sci Int Genet Suppl Ser*. 2013;4(1):e99-e100. doi:10.1016/j.fsigss.2013.10.051

141. Mameli A, Scudiero CM, Delogu G, Ghiani ME. Successful analysis of a 100 years old semen stain generating a complete DNA STR profile. *J Forensic Leg Med*. 2019;61(November 2018):78-81.

142. Hara M, Nakanishi H, Takahashi S, et al. Relationship between DNA degradation ratios and the number of loci detectable by STR kits in extremely old seminal stain samples. *Leg Med*. 2015;17(5):391-393.

143. Bini C, Ceccardi S, Trento C, et al. Analysis of aged seminal stains by current forensic DNA approach. *Forensic Sci Int Genet Suppl Ser*. 2015;5:e248-e249. doi:10.1016/j.fsigss.2015.09.099

144. Kido A, Hara M, Yamamoto Y, Kameyama H. Nine short tandem repeat loci analysis in aged semen stains using the AmpFLSTR Profiler Kit and description of a new vWA variant allele. *Leg Med*. 2003;5(2):93-96.

145. Hutchinson S. What's the difference between hair and fur? Mental Floss website. http://mentalfloss.com/article/58251/whats-difference-between-hair-and-fur. Published 2014. Accessed August 22, 2019.

146. Weisberger M. Fur, wool, hair: What's the difference? Live Science website. https://www.livescience.com/54701-fur-hair-wool-whats-the-difference.html. Published 2016. Accessed November 4, 2019.

147. Deedrik DW, Koch SL. Microscopy of hair part II: A practical guide and manual for animal hairs. Forensic Science Communications. https://archives.fbi.gov/archives/about-us/lab/forensic-science-communications/fsc/july2004/research/2004_03_research02.htm. Published 2004. Accessed August 13, 2019.

148. Dissing J, Søndervang A, Lund S. Exploring the limits for the survival of DNA in blood stains. *J Forensic Leg Med*. 2010;17(7):392-396.

149. Gates KS. An overview of chemical processes that damage cellular DNA: Spontaneous hydrolysis, alkylation and, reactions with radicals. *Chem Res Toxicol*. 2009;22(11):1747-1760.

150. Eglinton G, Logan GA. Molecular preservation. *Philos Trans R Soc Lond B Biol Sci*. 1991;30(333):315-327.

151. Parkinson RA, Dias KR, Horswell J, et al. Microbial community analysis of human decomposition on soil. In: Ritz K, Dawson L, Miller D, eds. *Criminal and Environmental Soil Forensics*. 1st ed. Dordrecht: Springer Netherlands; 2009:379-394.

152. Caputo M, Bosio LA, Corach D. Long-term room temperature preservation of corpse soft tissue: An approach for tissue sample storage. *Investig Genet*. 2011;2:17-22.

153. Dawson MN, Raskoff KA, Jacobs DK. Field preservation of marine invertebrate tissue for DNA analyses. *Mol Mar Biol Biotechnol*. 1998;7(2):145-152.

154. Carrió E, Rosselló JA. Salt drying: A low-cost, simple and efficient method for storing plants in the field and preserving biological repositories for DNA diversity research. *Mol Ecol Resour*. 2014;14(2):344-351.

155. Hall A, Sims LM, Ballantyne J. Assessment of DNA damage induced by terrestrial UV irradiation of dried bloodstains. *Forensic Sci Int Genet*. 2014;8(1):24-32.

156. Tucker VC, Hopwood AJ, Sprecher CJ, et al. Developmental validation of the PowerPlex® ESI 16 and PowerPlex® ESI 17 systems: STR multiplexes for the new European standard. *Forensic Sci Int Genet*. 2011;5(5):436-448.

157. Rogers J, Gibbs RA. Comparative primate genomics: Emerging patterns of genome content and dynamics. *Nat Rev Genet*. 2014;15(5):347-359.

158. Qiagen. *Developmental Validation of The Investigator® Quantiplex® Pro Kit*.; 2018.

159. Qiagen. *Developmental Validation of the Investigator® IDplex Plus Kit*.; 2012.

160. Holt A, Wootton SC, Mulero JJ, Brzoska PM, Langit E, Green RL. Developmental validation of the Quantifiler® HP and Trio Kits for human DNA quantification in forensic samples. *Forensic Sci Int Genet*. 2016;21:145-157.

161. Wang DY, Chang CW, Lagacé RE, Calandro LM, Hennessy LK. Developmental validation of the AmpFℓSTR® Identifiler® Plus PCR Amplification Kit: An established multiplex assay with improved performance. *J Forensic Sci*. 2011;57(2):453-465.

162. Thanakiatkrai P, Raham K, Pradutkanchana J, Sotthibandhu S, Kitpipit T. Direct-STR typing from presumptively-tested and untreated body fluids. *Forensic Sci Int Genet*. 2017;30:1-9.

163. Hughes-Stamm S. DNA typing methods for highly degraded samples [presentation]. 2012;(July).

164. Fondevila M, Phillips C, Naverán N, et al. Challenging DNA: Assessment of a range of genotyping approaches for highly degraded forensic samples. *Forensic Sci Int Genet Suppl Ser*. 2008;1(1):26-28.

165. Balhorn R. The protamine family of sperm nuclear proteins. *Genome Biol*. 2007;8(9):227-234.

166. Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR. Distinctive chromatin in human sperm packages genes for embryo development. *Nature*. 2009;460(7254):473-478.

167. Al-Kandari N., Singh J, Sangar VC. Time-dependent effects of temperature and humidity on quantity of DNA in samples of human saliva, blood and semen in Kuwait. *Int J Pharm Sci Res*. 2016;7(7):2852-2873.

168. Martinez P, Santiago B, Heinrichs B, Rosello J, Gil-Carreno I. Confirmation of dog semen fluid in an abuse scenario by morphometrics and mtDNA. *SciFed J Forensic Sci*. 2018;1. http://scifedpublishers.com/fulltext/confirmation-of-dog-semen-fluid-in-an-

abuse-scenario-by-morphometrics-and-mtdna/21970.

169. Vintiner S., Stringer P, Kanagasundaram S. Alleged sexual violation of a human female by a Rottweiler dog. *J Forensic Sci Soc*. 1992;32(4):357-362.

170. Prakash S, Prithiviraj E, Suresh S, et al. Morphological diversity of sperm: A mini review. *Iran J Reprod Med*. 2014;12(4):239-242.

171. Dawnay N, Ogden R, McEwing R, Carvalho GR, Thorpe RS. Validation of the barcoding gene COI for use in forensic genetic species identification. *Forensic Sci Int*. 2007;173(1):1-6.

172. Hird HJ, Brown MK. Design, optimisation and preliminary validation of a human specific loop-mediated amplification assay for the rapid detection of human DNA at forensic crime scenes. *Sci Justice*. 2017;57(6):409-414.

173. Simard A, DesGroseillers L, Sarafian V. Assessment of RNA stability for age determination of body fluid stains. *J Can Soc Forensic Sci*. 2012;45(4):179-194.

174. Weinbrecht KD, Fu J, Payton M, Allen R. Time-dependent loss of mRNA transcripts from forensic stains. *Res Reports Forensic Med Sci*. 2017;7:1-12.

175. Srettabunjong S, Betset P, Limawongpranee S, Ekpo P. The stability of prostate-specific antigen in semen under various temperatures. *J Forensic Sci*. 2015;60(6):1577-1581.

176. Weinbrecht K. RNA-seq of biological fluids for tthe evaluation of mRNA degradation in relation to sample age [dissertation]. Oklahoma State University; 2014.

177. Alshehhi S, Haddrill PR. Estimating time since deposition using quantification of RNA degradation in body fluid-specific markers. *Forensic Sci Int*. 2019;298:58-63.

178. Ogawa H, Hiroshige Y, Yoshimoto T, Ishii A, Yamamoto T. STR genotyping from a dry-cleaned skirt in a sexual assault case. *J Forensic Sci*. 2018;63(4):1291-1297.

179. Tobe SS, Swaran YC, Dennany L, et al. A proof of principal study on the use of direct PCR of semen and spermatozoa and development of a differential isolation protocol for use in cases of alleged sexual assault. *Int J Legal Med*. 2017;131(1):87-94.

180. Verheij S, Harteveld J, Sijen T. A protocol for direct and rapid multiplex PCR amplification on forensically relevant samples. *Forensic Sci Int Genet*. 2012;6(2):167-175.

181. Lee HY, Jung SE, Lee EH, Yang WI, Shin KJ. DNA methylation profiling for a confirmatory test for blood, saliva, semen, vaginal fluid and menstrual blood. *Forensic Sci Int Genet*. 2016;24:75-82.

182. Frumkin D, Wasserstrom A, Budowle B, Davidson A. DNA methylation-based forensic tissue identification. *Forensic Sci Int Genet*. 2011;5(5):517-524.

183. Zubakov D, Boersma AW, Choi Y, van Kuijk PF, Wiemer EA, Kayser M. MicroRNA markers for forensic body fluid identification obtained from microarray screening and quantitative RT-PCR confirmation. *Int J Legal Med*. 2010;124(3):217-226.

184. Wang Z, Zhang J, Luo H, Ye Y, Yan J, Hou Y. Screening and confirmation of microRNA markers for forensic body fluid identification. *Forensic Sci Int Genet*. 2013;7(1):116-123.

185. Lindenbergh A, De Pagter M, Ramdayal G, et al. A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces. *Forensic Sci Int Genet*. 2012;6(5):565-577.

186. Sakurada K, Ikegaya H, Fukushima H, Akutsu T, Watanabe K, Yoshino M. Evaluation of mRNA-based approach for identification of saliva and semen. *Leg Med*. 2009;11(3):125-128.

187. Juusola J, Ballantyne J. mRNA profiling for body fluid identification by multiplex quantitative RT-PCR. *J Forensic Sci*. 2007;52(6):1252-1262.

188. Haas C, Muheim C, Kratzer A, Bär W, Maake C. mRNA profiling for the identification of sperm and seminal plasma. *Forensic Sci Int Genet Suppl Ser*. 2009;2(1):534-535.

189. Juusola J, Ballantyne J. Multiplex mRNA profiling for the identification of body fluids. *Forensic Sci Int*. 2005;152(1):1-12.

190. Hanson E, Ingold S, Haas C, Ballantyne J. Targeted multiplexed next generation RNA sequencing assay for tissue source determination of forensic samples. *Forensic Sci Int Genet Suppl Ser*. 2015;5:e441-e443. doi:10.1016/j.fsigss.2015.09.175

191. Fleming RI, Harbison S. The development of a mRNA multiplex RT-PCR assay for the definitive identification of body fluids. *Forensic Sci Int Genet*. 2010;4(4):244-256.

192. Hanson E, Lubenow H, Ballantyne J. Identification of forensically relevant body fluids

using a panel of differentially expressed microRNAs. *Forensic Sci Int Genet Suppl Ser*. 2009;387(2):303-314.

193. Li Y, Zhang J, Wei W, Wang Z, Prinz M, Hou Y. A strategy for co-analysis of microRNAs and DNA. *Forensic Sci Int Genet*. 2014;12:24-29.

194. Zubakov D, Kokmeijer I, Ralf A, et al. Towards simultaneous individual and tissue identification: A proof-of-principle study on parallel sequencing of STRs, amelogenin, and mRNAs with the Ion Torrent PGM. *Forensic Sci Int Genet*. 2015;17:122-128.

195. Koukouvinos G, Metheniti A, Karachaliou CE, et al. White light reflectance spectroscopy biosensing system for fast quantitative prostate specific antigen determination in forensic samples. *Talanta*. 2017;175(May):443-450.

196. Kamanna S, Henry J, Voelcker NH, Linacre A, Kirkbride KP. Direct identification of forensic body fluids using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Int J Mass Spectrom*. 2016;397-398:18-26.

197. Muro CK, Doty KC, de Souza Fernandes L, Lednev IK. Forensic body fluid identification and differentiation by Raman spectroscopy. *Forensic Chem*. 2016;1:31-38.

198. Vandewoestyne M, Van Hoofstat D, Van Nieuwerburgh F, Deforce D. Automatic detection of spermatozoa for laser capture microdissection. *Int J Legal Med*. 2009;123(2):169-175.

199. Mitchell A. Validation study of KPICS SpermFinder™ by NicheVision Forensics, LLC for the identification of human spermatozoa. *J Forensic Sci*. 2012;57(4):1042-1050.

200. Shenken LI. Some clinical and psychopathological aspects of bestiality. *J Nerv Ment Dis*. 1964;139(2):137-142.

201. Holden TE, Sherline DM. Bestiality, with sensitization and anaphylactic reaction. *Obstet Gynecol*. 1973;42(1):138-140.

202. Schneck JM. Zooerasty and incest fantasy. *Int J Clin Exp Hypn*. 1974;22(4):299-302.

203. McNally RJ, Lukach BM. Behavioral treatment of zoophilic exhibitionism. *J Behav Ther Exp Psychiatry*. 1992;22(4):281-284.

204. Wiegand P, Schmidt V, Kleiber M. German shepherd dog is suspected of sexually

abusing a child. *Int J Legal Med*. 1999;112(5):324-325.

205. Munro HM, Thrusfield M V. "Battered pets": Features that raise suspicion of non-accidental injury. *J Small Anim Pract*. 2001;42(5):218-226.

206. Munro HMC, Thrusfield M V. "Battered pets": Non-accidental physical injuries found in dogs and cats. *J Small Anim Pract*. 2001;42(6):279-290.

207. Earls CM, Lalumière ML. A case study of preferential bestiality (zoophilia). *Sex Abus A J Res Treat*. 2002;14(1):83-88.

208. Kirov GK, Losanoff JE, Kjossev KT. Zoophilia: A rare cause of traumatic injury to the rectum. *Injury*. 2002;33(4):367-368.

209. Bhatia M, Srivastava S, Sharma S. 1. An uncommon case of zoophilia: A case report. *Med Sci Law*. 2005;45(2):174-175.

210. Ergun UG, Celik M, Ozer HTE. Reactive arthritis due to zoophilic (canine) sexual intercourse. *Int J STD AIDS*. 2007;18(4):285-286.

211. Blevins RO. A case of severe anal injury in an adolescent male due to bestial sexual experimentation. *J Forensic Leg Med*. 2009;16(7):403-406.

212. De Giorgio F, Polacco M, Rossi R, Lodise M, Rainio J. Fatal blunt injuries possibly resulting from sexual abuse of a calf: A case report. *Med Sci law*. 2009;49(4):307-310.

213. Raina G, Cersosimo MG, Micheli F. Zoophilia and impulse control disorder in a patient with Parkinson disease. *J Neurol*. 2012;259(5):969-970.

214. Almeida KJ, de Oilveira Filho MCS, Lopes Nery PC, Guimarães Silva JS, Campos Sousa RN. Zoophilia and Parkinson's disease. *Park Relat Disord*. 2013;19(12):1167-1168.

215. Sendler DJ. Similar mechanisms of traumatic rectal injuries in patients who had anal sex with animals to those who were butt-fisted by human sexual partner. *J Forensic Leg Med*. 2017;51:69-73.

216. Almeida DC, Torres SMF, Wuenschmann A. Retrospective analysis of necropsy reports suggestive of abuse in dogs and cats. *J Am Vet Med Assoc*. 2018;252(4):433-439.

217. Rodríguez Almada H, García Maggi I, Rodríguez M, Stewart J. Anal tear in a girl due to the penetration of a dog's penis. Unusual case report. *J Forensic Leg Med*.

2019;62(July 2018):112-114.

218. Rogers D, Newton M. Evidence-based forensic sampling - More questions than answers. *J Clin Forensic Med*. 2006;13(4):162-163.

219. Enos W. Treatment of rape victims. *J Forensic Sci*. 1977;22(1):3-4.

220. American College of Obstetricians and Gynecologists. Sexual assault - ACOG technical bulletin number 172 - September 1992. *Int J Gynecol Ostet*. 1993;42(1):67-72.

221. Breen J, Greenwald E, Gregori C. The molested young female. Evaluation and therapy of alleged rape. *Pediatr Clin North Am*. 1972;19(3):717-725.

222. Fisher RS. Acid phosphatase tests as evidence of rape. *N Engl J Med*. 1949;240:738-739.

223. Wickenheiser RA. Trace DNA: A review, discussion of theory, and application of the transfer of trace quantities of DNA through skin contact. *J Forensic Sci*. 2002;47(3):442-450.

224. Dupre AR, Hampton HL, Morrison H, Meeks GR. Sexual assault. *Obstet Gynecol Surv*. 1993;48(9):640-648.

225. Keating SM, Allard JE. What's in a name? - Medical samples and scientific evidence in sexual assault. *Med Sci Law*. 1994;34(3):187-201.

226. Healy DA, Hayes CJ, Leonard P, McKenna L, O'Kennedy R. Biosensor developments: Application to prostate-specific antigen detection. *Trends Biotechnol*. 2007;25(3):125-131.

227. Jeffreys AJ. DNA typing: Approaches and applications. *J Forensic Sci Soc*. 1993;33(4):204-211.

228. Hochbaum SR. The evaluation and treatment of the sexually assaulted patient. *Emerg Med Clin North Am*. 1987;5(3):601-622.

229. Newton M. The forensic aspects of sexual violence. *Best Pract Res Clin Obstet Gynaecol*. 2013;27(1):77-90.

230. Young WW, Bracken AC, Goddard MA, Matheson S. Sexual assault: Review of a national model protocol for forensic and medical evaluation. New Hampshire Sexual Assault Medical Examination Protocol Project Committee. *Obstet Gynecol*.

1992;80(5):878-883.

231. Vandewoestyne M, Deforce D. Laser capture microdissection in forensic research : a review. *Int J Legal Med*. 2010;124(6):513-521.

232. Cotton RW, Fisher MB. Review: Properties of sperm and seminal fluid, informed by research on reproduction and contraception. *Forensic Sci Int Genet*. 2015;18(September):66-77.

233. Virkler K, Lednev IK. Analysis of body fluids for forensic purposes: From laboratory testing to non-destructive rapid confirmatory identification at a crime scene. *Forensic Sci Int*. 2009;188(1-3):1-17.

234. Stefanidou M, Alevisopoulos G, Spiliopoulou C. Fundamental issues in forensic semen detection. *West Indian Med J*. 2010;59(3):280-283.

Appendix A: Literature review search queries

Table A1: Systematic literature review search queries and the results obtained from the various databases. (ASA = animal sexual abuse).

Database	Search query	Results
	Search date: 03-04-2019	
PubMed	((retain[All Fields] OR retaining[All Fields]) OR retained[All Fields]) OR retains[All Fields]) OR persist[All Fields]) OR persistence[All Fields]) OR persisted[All Fields]) OR persisting[All Fields]) OR persists[All Fields]) OR detect[All Fields]) OR detection[All Fields]) OR detecting[All Fields]) OR detected[All Fields]) OR detects[All Fields]) OR recover[All Fields]) OR recovery[All Fields]) OR recovered[All Fields]) OR recovering[All Fields]) OR recovers[All Fields]) OR deposit[All Fields]) OR deposits[All Fields]) OR deposition[All Fields]) OR deposited[All Fields]) OR depositing[All Fields]) OR degrade[All Fields]) OR degraded[All Fields]) OR ("metabolism"[Subheading] OR "metabolism"[All Fields] OR "degradation"[All Fields] OR "metabolism"[MeSH Terms] OR "degradation"[All Fields])) OR degrading[All Fields]) OR degrades[All Fields]) OR ("retention (psychology)"[MeSH Terms] OR ("retention"[All Fields] AND "(psychology)"[All Fields]) OR "retention (psychology)"[All Fields] OR "retention"[All Fields])) OR preserve[All Fields]) OR preserves[All Fields]) OR ("preservation, biological"[MeSH Terms] OR ("preservation"[All Fields] AND "biological"[All Fields]) OR "biological preservation"[All Fields] OR "preservation"[All Fields])) OR ("preservation, biological"[MeSH Terms] OR ("preservation"[All Fields] AND "biological"[All Fields]) OR "biological preservation"[All Fields] OR "preserved"[All Fields])) OR preserving[All Fields]) OR resilience[All Fields]) OR identify[All Fields]) OR ("identification (psychology)"[MeSH Terms] OR ("identification"[All Fields] AND "(psychology)"[All Fields]) OR "identification (psychology)"[All Fields] OR "identification"[All Fields])) OR identifies[All Fields]) OR identified[All Fields]) OR identifying[All Fields]) OR obtain[All Fields]) OR obtains[All Fields]) OR obtaining[All Fields]) AND ((((((((("humans"[MeSH Terms] OR "humans"[All Fields] OR "human"[All Fields]) OR ("humans"[MeSH Terms] OR "humans"[All Fields])) OR ("men"[MeSH Terms] OR "men"[All Fields] OR "man"[All Fields])) OR ("male"[MeSH Terms] OR "male"[All Fields])) OR ("male"[MeSH Terms] OR "male"[All Fields] OR "males"[All Fields])) OR ("persons"[MeSH Terms] OR "persons"[All Fields] OR "person"[All Fields])) OR ("persons"[MeSH Terms] OR "persons"[All Fields])) OR (("hominidae"[MeSH Terms] OR "hominidae"[All Fields] OR "homo"[All Fields]) AND sapien[All Fields])) OR ("humans"[MeSH Terms] OR "humans"[All Fields] OR ("homo"[All Fields] AND "sapiens"[All Fields]) OR "homo sapiens"[All Fields])) AND (((((((("semen"[MeSH Terms] OR "semen"[All Fields]) OR (seminal[All Fields] AND fluid[All Fields])) OR (seminal[All Fields] AND ("Eur J Mech B Fluids"[Journal] OR "fluids"[All Fields]))) OR ("spermatozoa"[MeSH Terms] OR "spermatozoa"[All Fields])) OR ("spermatozoa"[MeSH Terms] OR "spermatozoa"[All Fields] OR "spermatozoon"[All Fields])) OR ("semen"[MeSH Terms] OR "semen"[All Fields] OR ("seminal"[All Fields] AND "plasma"[All Fields]) OR "seminal plasma"[All Fields])) OR ("semen"[MeSH Terms] OR "semen"[All Fields] OR ("plasma"[All Fields] AND "seminal"[All Fields]) OR "plasma, seminal"[All Fields])) OR ("spermatozoa"[MeSH Terms] OR "spermatozoa"[All Fields] OR "sperm"[All Fields]))))) AND ((((((((((((((((((((((((("semen"[MeSH Terms] OR "semen"[All Fields]) AND ("staining and	**50**

Database	Search query	Results
	labeling"[MeSH Terms] OR ("staining"[All Fields] AND "labeling"[All Fields]) OR "staining and labeling"[All Fields] OR "stain"[All Fields])) OR (("semen"[MeSH Terms] OR "semen"[All Fields]) AND ("coloring agents"[Pharmacological Action] OR "coloring agents"[MeSH Terms] OR ("coloring"[All Fields] AND "agents"[All Fields]) OR "coloring agents"[All Fields] OR "stains"[All Fields]))) OR (("semen"[MeSH Terms] OR "semen"[All Fields]) AND stained[All Fields])) OR (seminal[All Fields] AND fluid[All Fields] AND ("staining and labeling"[MeSH Terms] OR ("staining"[All Fields] AND "labeling"[All Fields]) OR "staining and labeling"[All Fields] OR "stain"[All Fields]))) OR (seminal[All Fields] AND fluid[All Fields] AND ("coloring agents"[Pharmacological Action] OR "coloring agents"[MeSH Terms] OR ("coloring"[All Fields] AND "agents"[All Fields]) OR "coloring agents"[All Fields] OR "stains"[All Fields]))) OR (seminal[All Fields] AND fluid[All Fields] AND stained[All Fields])) OR (("spermatozoa"[MeSH Terms] OR "spermatozoa"[All Fields]) AND ("staining and labeling"[MeSH Terms] OR ("staining"[All Fields] AND "labeling"[All Fields]) OR "staining and labeling"[All Fields] OR "stain"[All Fields]))) OR (("spermatozoa"[MeSH Terms] OR "spermatozoa"[All Fields]) AND ("coloring agents"[Pharmacological Action] OR "coloring agents"[MeSH Terms] OR ("coloring"[All Fields] AND "agents"[All Fields]) OR "coloring agents"[All Fields] OR "stains"[All Fields]))) OR (("spermatozoa"[MeSH Terms] OR "spermatozoa"[All Fields]) AND stained[All Fields])) OR ((("semen"[MeSH Terms] OR "semen"[All Fields] OR ("seminal"[All Fields] AND "plasma"[All Fields]) OR "seminal plasma"[All Fields]) AND ("staining and labeling"[MeSH Terms] OR ("staining"[All Fields] AND "labeling"[All Fields]) OR "staining and labeling"[All Fields] OR "stain"[All Fields]))) OR ((("semen"[MeSH Terms] OR "semen"[All Fields] OR ("seminal"[All Fields] AND "plasma"[All Fields]) OR "seminal plasma"[All Fields]) AND ("coloring agents"[Pharmacological Action] OR "coloring agents"[MeSH Terms] OR ("coloring"[All Fields] AND "agents"[All Fields]) OR "coloring agents"[All Fields] OR "stains"[All Fields]))) OR ((("semen"[MeSH Terms] OR "semen"[All Fields] OR ("seminal"[All Fields] AND "plasma"[All Fields]) OR "seminal plasma"[All Fields]) AND stained[All Fields])) OR (("spermatozoa"[MeSH Terms] OR "spermatozoa"[All Fields] OR "spermatozoon"[All Fields]) AND ("staining and labeling"[MeSH Terms] OR ("staining"[All Fields] AND "labeling"[All Fields]) OR "staining and labeling"[All Fields] OR "stain"[All Fields]))) OR (("spermatozoa"[MeSH Terms] OR "spermatozoa"[All Fields] OR "spermatozoon"[All Fields]) AND ("coloring agents"[Pharmacological Action] OR "coloring agents"[MeSH Terms] OR ("coloring"[All Fields] AND "agents"[All Fields]) OR "coloring agents"[All Fields] OR "stains"[All Fields]))) OR (("spermatozoa"[MeSH Terms] OR "spermatozoa"[All Fields] OR "spermatozoon"[All Fields]) AND stained[All Fields])) OR (("spermatozoa"[MeSH Terms] OR "spermatozoa"[All Fields] OR "sperm"[All Fields]) AND ("staining and labeling"[MeSH Terms] OR ("staining"[All Fields] AND "labeling"[All Fields]) OR "staining and labeling"[All Fields] OR "stain"[All Fields]))) OR (("spermatozoa"[MeSH Terms] OR "spermatozoa"[All Fields] OR "sperm"[All Fields]) AND ("coloring agents"[Pharmacological Action] OR "coloring agents"[MeSH Terms] OR ("coloring"[All Fields] AND "agents"[All Fields]) OR "coloring agents"[All Fields] OR "stains"[All Fields]))) OR (("spermatozoa"[MeSH Terms] OR "spermatozoa"[All Fields] OR "sperm"[All Fields]) AND stained[All Fields])) OR (seminal[All Fields] AND ("staining and labeling"[MeSH Terms] OR ("staining"[All Fields] AND "labeling"[All Fields]) OR "staining and labeling"[All Fields] OR "stain"[All Fields]))) OR (seminal[All Fields] AND ("coloring agents"[Pharmacological Action] OR "coloring agents"[MeSH Terms] OR ("coloring"[All Fields] AND "agents"[All Fields]) OR "coloring agents"[All Fields] OR "stains"[All Fields]))) OR (seminal[All Fields] AND stained[All Fields])) OR (("semen"[MeSH Terms] OR "semen"[All Fields] OR ("plasma"[All Fields] AND "seminal"[All Fields]) OR "plasma, seminal"[All	

Database	Search query	Results
	Fields]) AND ("staining and labeling"[MeSH Terms] OR ("staining"[All Fields] AND "labeling"[All Fields]) OR "staining and labeling"[All Fields] OR "stain"[All Fields])))) OR (("semen"[MeSH Terms] OR "semen"[All Fields] OR ("plasma"[All Fields] AND "seminal"[All Fields]) OR "plasma, seminal"[All Fields]) AND ("coloring agents"[Pharmacological Action] OR "coloring agents"[MeSH Terms] OR ("coloring"[All Fields] AND "agents"[All Fields]) OR "coloring agents"[All Fields] OR "stains"[All Fields]))) OR (("semen"[MeSH Terms] OR "semen"[All Fields] OR ("plasma"[All Fields] AND "seminal"[All Fields]) OR "plasma, seminal"[All Fields]) AND stained[All Fields])) OR ((((((("animals"[MeSH Terms:noexp] OR animal[All Fields]) OR ("animals"[MeSH Terms:noexp] OR animals[All Fields])) OR ("mammals"[MeSH Terms] OR "mammals"[All Fields] OR "mammal"[All Fields])) OR ("mammals"[MeSH Terms] OR "mammals"[All Fields])) OR ("mammals"[MeSH Terms] OR "mammals"[All Fields] OR "mammalian"[All Fields])) AND (((((((((fur[All Fields] OR furs[All Fields]) OR ("hair"[MeSH Terms] OR "hair"[All Fields])) OR ("hair"[MeSH Terms] OR "hair"[All Fields] OR "hairs"[All Fields])) OR pelt[All Fields]) OR pelts[All Fields]) OR hide[All Fields]) OR hides[All Fields]) OR coat[All Fields]) OR coats[All Fields])))) AND ((((((("time"[MeSH Terms] OR "time"[All Fields]) OR ("time"[MeSH Terms] OR "time"[All Fields] OR "times"[All Fields])) OR ("Age"[Journal] OR "Age (Omaha)"[Journal] OR "Age (Dordr)"[Journal] OR "Adv Genet Eng"[Journal] OR "age"[All Fields])) OR ("aged"[MeSH Terms] OR "aged"[All Fields])) OR ("aging"[MeSH Terms] OR "aging"[All Fields])) OR old[All Fields])) AND ((((((((((((((((((((((((("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("sexual"[All Fields] AND "abuse"[All Fields]) OR "sexual abuse"[All Fields]) OR (("sexual behavior"[MeSH Terms] OR ("sexual"[All Fields] AND "behavior"[All Fields]) OR "sexual behavior"[All Fields] OR "sexual"[All Fields]) AND abused[All Fields])) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("sexual"[All Fields] AND "abuses"[All Fields]) OR "sexual abuses"[All Fields])) OR (("sexual behavior"[MeSH Terms] OR ("sexual"[All Fields] AND "behavior"[All Fields]) OR "sexual behavior"[All Fields] OR "sexual"[All Fields]) AND assault[All Fields])) OR (("sexual behavior"[MeSH Terms] OR ("sexual"[All Fields] AND "behavior"[All Fields]) OR "sexual behavior"[All Fields] OR "sexual"[All Fields]) AND assaulted[All Fields])) OR (("sexual behavior"[MeSH Terms] OR ("sexual"[All Fields] AND "behavior"[All Fields]) OR "sexual behavior"[All Fields] OR "sexual"[All Fields]) AND assaults[All Fields])) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("sexual"[All Fields] AND "offense"[All Fields]) OR "sexual offense"[All Fields])) OR (("sexual behavior"[MeSH Terms] OR ("sexual"[All Fields] AND "behavior"[All Fields]) OR "sexual behavior"[All Fields] OR "sexual"[All Fields]) AND offenses[All Fields])) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("sex"[All Fields] AND "offense"[All Fields]) OR "sex offense"[All Fields])) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields])) OR ("rape"[MeSH Terms] OR "rape"[All Fields])) OR ("rape"[MeSH Terms] OR "rape"[All Fields] OR "rapes"[All Fields])) OR ("rape"[MeSH Terms] OR "rape"[All Fields] OR "raped"[All Fields])) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("sexual"[All Fields] AND "violence"[All Fields]) OR "sexual violence"[All Fields])) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("sexual"[All Fields] AND "violences"[All Fields]) OR "sexual violences"[All Fields])) OR (("sexual behavior"[MeSH Terms] OR ("sexual"[All Fields] AND "behavior"[All Fields]) OR "sexual behavior"[All Fields] OR "sexually"[All Fields]) AND	

Database	Search query	Results
	abused[All Fields])) OR (("sexual behavior"[MeSH Terms] OR ("sexual"[All Fields] AND "behavior"[All Fields]) OR "sexual behavior"[All Fields] OR "sexually"[All Fields]) AND assaulted[All Fields])) OR (("sexual behavior"[MeSH Terms] OR ("sexual"[All Fields] AND "behavior"[All Fields]) OR "sexual behavior"[All Fields] OR "sexually"[All Fields]) AND offended[All Fields])) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("sexual"[All Fields] AND "offence"[All Fields]) OR "sexual offence"[All Fields])) OR (("sexual behavior"[MeSH Terms] OR ("sexual"[All Fields] AND "behavior"[All Fields]) OR "sexual behavior"[All Fields] OR "sexual"[All Fields]) AND offences[All Fields])) OR (("sex"[MeSH Terms] OR "sex"[All Fields]) AND offence[All Fields])) OR (("sex"[MeSH Terms] OR "sex"[All Fields]) AND offences[All Fields])) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("offense"[All Fields] AND "sex"[All Fields]))) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("offenses"[All Fields] AND "sex"[All Fields]))) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("violence"[All Fields] AND "sexual"[All Fields]) OR "violence, sexual"[All Fields])) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("violences"[All Fields] AND "sexual"[All Fields]))) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("abuse"[All Fields] AND "sexual"[All Fields]) OR "abuse, sexual"[All Fields])) OR ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("abuses"[All Fields] AND "sexual"[All Fields])))	
Search date: 30-09-2019		
Scopus	((TITLE-ABS-KEY (retain* OR persist* OR detect*) OR TITLE-ABS-KEY (recover* OR deposit* OR degrad*) OR TITLE-ABS-KEY (retention OR preserv* OR resilience) OR TITLE-ABS-KEY (identif* OR obtain*))) AND (((TITLE-ABS-KEY (human* OR man OR male*) OR TITLE-ABS-KEY (person* OR homo AND sapien* OR men))) AND (TITLE-ABS-KEY (semen OR seminal* OR sperm*))) AND (((TITLE-ABS-KEY (semen AND stain* OR seminal AND fluid AND stain* OR seminal AND plasma AND stain*) OR TITLE-ABS-KEY (spermatozoa AND stain* OR spermatozoon AND stain* OR sperm AND stain*) OR TITLE-ABS-KEY (seminal AND stain*))) OR ((TITLE-ABS-KEY (animal* OR mammal*)) AND ((TITLE-ABS-KEY (fur* OR hair* OR pelt*) OR TITLE-ABS-KEY (hide* OR coat*))))) AND ((TITLE-ABS-KEY (time* OR age* OR old) OR TITLE-ABS-KEY (aging))) AND ((TITLE-ABS-KEY (sexual AND abuse* OR sexual AND assault* OR sexual AND offense*) OR TITLE-ABS-KEY (sex AND offense* OR rape* OR sexual AND violence*) OR TITLE-ABS-KEY (sexually AND abused OR sexually AND assaulted OR sexually AND offended) OR TITLE-ABS-KEY (sexual AND offence* OR sex AND offence*)))	**19**
Search date: 01-10-2019		
Web of Science™	Used same strategy as Scopus. Full search query unattainable from site.	**201**

Database	Search query	Results
	Search date: 26-12-2019	
PubMed (specific search for ASA case reports)	((((((((((((("animals"[MeSH Terms:noexp] OR animal[All Fields]) AND ("sex offenses"[MeSH Terms] OR ("sex"[All Fields] AND "offenses"[All Fields]) OR "sex offenses"[All Fields] OR ("sexual"[All Fields] AND "abuse"[All Fields]) OR "sexual abuse"[All Fields])) OR bestiality[All Fields]) OR zoophilia[All Fields]) OR zoophile[All Fields]) OR zoophiles[All Fields]) OR zoosadism[All Fields]) OR zoophilic[All Fields]) OR zoophilism[All Fields]) OR zoorasty[All Fields]) OR zooerastia[All Fields]) OR zooerasty[All Fields]) OR zoosexuality[All Fields]) OR (zoophilic[All Fields] AND ("sadism"[MeSH Terms] OR "sadism"[All Fields]))) OR necrobestiality[All Fields]) OR buggery[All Fields]	**1115**

Table A2: The themes and additional key words that were used to build search queries.

Theme	Additional key words
Persistence	Retain, retaining, retained, retains, persist, persistence, persisted, persisting, persists, detect, detection, detecting, detected, detects, recover, recovery, recovered, recovering, recovers, deposit, deposits, deposition, deposited, depositing, degrade, degraded, degradation, degrading, degrades, retention, preserve, preserves, preservation, preserved, preserving, resilience, identify, identification, identifies, identified, identifying, obtain, obtained, obtains, obtaining
Human	Human, humans, man, men, male, males, person, persons, homo sapien, homo sapiens
Semen	Semen, seminal fluid, spermatozoa, seminal plasma, spermatozoon, seminal fluids, sperm
Semen stain	Semen stain, semen stains, semen stained, seminal fluid stain, seminal fluid stains, seminal fluid stained, seminal plasma stain, seminal plasma stains, seminal fluid stained, spermatozoa stain, spermatozoa stains, spermatozoa stained, spermatozoon stain, spermatozoon stains, spermatozoon stained, sperm stain, sperm stains, sperm stained, seminal stain, seminal stains, seminal stained
Animal	Animal, animals, mammal, mammals, mammalian
Fur	Fur, furs, hair, hairs, pelt, pelts, hide, hides, coat, coats
Time	Time, times, age, aged, aging, old
Sexual offence	Sexual abuse, sexual abused, sexual abuses, sexual assault, sexual assaulted, sexual assaults, sexual offense, sexual offenses, sex offense, sex offenses, sexual offence, sexual offences, sex offence, sex offences, rape, rapes, raped, sexual violence, sexual violences, sexually abused, sexually assaulted, sexually offended,
Animal sexual abuse	Animal sexual abuse, bestiality, zoophilia, zoosadism, zoophilic, zoophilism, zoophile, zoophiles, zoorasty, zooerastia, zooerasty, zoosexuality, bestiosexuality, zoophilic sadism, bestialsadism, necrozoophilia, necrobestiality, leptozoosexuality, baryzoosexuality, buggery

Table A3: Applicable MeSH terms that were included in the PubMed search. The remaining key words presented no additional MeSH terms. (MeSH = medical subject headings).

Term	**MeSH term**
Human	Humans
Man, Men	Men
Male, Males	Male
Person, Persons	Persons
Homo sapien, homo sapiens	Hominidae
Semen	Semen; plasma, seminal
Spermatozoa	Spermatozoa
Seminal plasma	Semen; plasma, seminal
Sperm	Spermatozoa
Animal, animals	Animals
Mammal, mammals, mammalian	Mammals
Fur, furs	Animal fur (combined in the search)
Hair, hairs	Hair
Time, time	Time
Aging	Aging
Sexual abuse, sexual abused, sexual abuses, sexual offense, sexual offenses, sex offense, sex offenses, sexual offence, sexual violence, sexual violences	Sexual abuse; sexual abuses; offenses, sex; offense, sex; sex offense; sexual violence; sexual violences; abuse, sexual; abuses, sexual; violence, sexual; violences, sexual
Rape, rapes, raped	Rape

Appendix B: Animal sexual abuse case reports

Table B1: The titles and relevant references of the 29 animal sexual abuse (ASA) case reports that were obtained from the specific literature search thereof in PubMed.

Reference	Title
Shenken (1964)[200]	Some clinical and psychopathological aspects of bestiality.
Holden & Sherline (1973)[201]	Bestiality, with sensitization and anaphylactic reaction.
Chee (1974)[13]	A case of bestiality.
Schneck (1974)[202]	Zooerasty and incest fantasy.
McNally & Lukach, (1992)[203]	Behavioral treatment of zoophilic exhibitionism.
Vintiner, Stringer & Kanagasundaram (1992)[169]	Alleged sexual violation of a human female by a Rottweiler dog.
Wiegand, Schmidt & Kleiber (1999)[204]	German shepherd dog is suspected of sexually abusing a child.
Mittal *et al*. (2000)[42]	Genital lesions following bestiality.
Munro & Thrusfield (2001a)[205]	'Battered pets': Features that raise suspicion of non-accidental injury.
Munro & Thrusfield (2001b)[206]	'Battered pets': Non-accidental physical injuries found in dogs and cats.
Munro & Thrusfield (2001c)[8]	‘Battered pets’: Sexual abuse.
Earls & Lalumière (2002)[207]	A case study of preferential bestiality (zoophilia).
Kirov, Losanoff & Kjossev (2002)[208]	Zoophilia: a rare cause of traumatic injury to the rectum.
Bhatia, Srivastava & Sharma (2005)[209]	1. An uncommon case of zoophilia: A case report.
Hvozdík *et al*. (2006)[16]	Ethological, psychological and legal aspects of animal sexual abuse.
Ergun, Celik & Ozer (2007)[210]	Reactive arthritis due to zoophilic (canine) sexual intercourse.
Blevins (2009)[211]	A case of severe anal injury in an adolescent male due to bestial sexual experimentation.

Table B1 cont.

De Giorgio *et al*. (2009)[212]	Fatal blunt injuries possibly resulting from sexual abuse of a calf: A case report.
Imbschweiler *et al*. (2009)[11]	Animal sexual abuse in a female sheep.
Amoo, Abayomo & Olashore (2012)[10]	Zoophilic recidivism in schizophrenia: A case report.
Raina, Cersosimo & Micheli (2012)[213]	Zoophilia and impulse control disorder in a patient with Parkinson disease.
Almeida *et al*. (2013)[214]	Zoophilia and Parkinson's disease.
Satapathy *et al*. (2016)[14]	An adolescent with bestiality behaviour: Psychological evaluation and community health concerns.
Virgilio, Franzese & Caterino (2016)[34]	Zoosexuality: An unusual cause of colorectal injury.
Chandradasa & Champika (2017)[17]	Zoophilia in an adolescent with high-functioning autism from Sri Lanka.
Holoyda (2017)[15]	Bestiality in forensically committed sexual offenders: A case series.
Sendler (2017)[215]	Similar mechanisms of traumatic rectal injuries in patients who had anal sex with animals to those who were butt-fisted by human sexual partner.
Almeida, Torres & Wuenschmann (2018)[216]	Retrospective analysis of necropsy reports suggestive of abuse in dogs and cats.
Rodríguez Almada *et al*. (2019)[217]	Anal tear in a girl due to the penetration of a dog's penis. Unusual case report.

Appendix C: Data obtained from articles included in the literature review

Table C1: Data from studies from which *only* the maximum TSIs could be derived for positive PT and CT. The type of test/stain is represented in italics. In certain instances, only data from subsets of the cohort or specific cases were presented. (Ref = reference, TSI = time since intercourse, PT = presumptive testing, CT = confirmatory testing, h = hours, AP = acid phosphatse, SVSA = semen vesicle-specific antigen, PSA = prostate specific antigen, H&E = haematoxylin and eosin, Pap = papanicolaou, SEM = scanning electron microscopy).

		TEST							
		Maximum recovey TSI for PT				**Maximum recovery TSI CT**			
Cohort	**Ref**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**
SEXUAL OFFENCE VICTIM - LIVING	Rupp (1969)[66]	*AP:* 34.5 h				*No stain* : 14 h			
	Eungprabhanth (1974)[64]					*H&E:* 144 h			
	Dahlke *et al.* (1977)[69]	*AP:* 18 h				*Giemsa:* 48 h			
	Enos & Beyer (1978)[110]					*Pap* : 4 h (*highlighted cases*)		*Pap:* 6 h	
	Willott & Allard (1982)[101]					*H&E:* 179 h	*H&E:* 65 h	*H&E* : 9 h	
	Keil, Bachus & Tröger (1996)[93]	*AP:* 38 h *SVSA:* 47 h				*H&E:* 47 h			
	Christian *et al* . (2000)[90]				*AP and/or Papanicolaou:* 9 hrs (*site undefined*)				
	Hellerud *et al* . (2011)[54]					*Christmas tree:* 96 h			
	Smith *et al* . (2014)[113]					*Unknown:* 9 h (*highlighted cases*)	*Unknown:* 9 h (*highlighted cases*)		*Unknown:* 9 h (*highlighted cases*)
						Unknown : 36 h (*site undefined for full evidence kit*)			
	Casey *et al.* (2017)[62]	*AP:* >96 h							
SEXUAL OFFENCE VICTIM - DECEASED	Chiasson *et al.* (1994)[81]					*H&E* : 48 h			
	Collins & Bennett (2001)[89]	*AP:* 2.5 months	*AP:* 14 days	*AP:* 120 h		*Pap:* 2.5 months	*Pap:* 48 h		
VOLUNTEERS	Morrison (1972)[121]					*Unknown:* 12 days			
	Schumann *et al* . (1976)[53]	*AP:* 48 h							
	Soules *et al* . (1978)[67]	*AP:* 24 h				*Other:* 72 h			
	Randall (1987)[111]					*Pap:* 168 h			
	Bryson, Garlo & Piner (1989)[86]	*AP:* 48 h *PSA:* 18 h				*Unknown:* 72 h			
	Allery *et al* . (2001)[99]					*H&E, Christmas tree, Alkaline fuchsin:* 72 h			
	Elliott *et al.* (2003)[104]					*H&E* : 100 h			
HUMAN MODEL	Gibelli *et al* . (2013)[49]								*H&E* : 12 days *SEM* : 84 days
	Elshama *et al* . (2017)[48]								*H&E* : 12 days *Sperm Hy-liter™* : 110 days
MIXED	Allard (1997)[102]					Sexual offence victims - living *H&E* : 168 h	*H&E* : 113 h	*H&E:* 31 h	
						Sexual offence victims - deceased *H&E* : 17.5 days (*highlighted cases*)		*H&E* : 21 days (*highlighted cases*)	

Table C2: Recovery rates (%) for PT and CT for given TSI intervals per sample site that were derived from a number of studies. The type of test/stain is represented in italics. In certain instances, only data from subsets of the cohort or specific cases were presented. (Ref = reference, PT = presumptive testing, CT = confirmatory testing, TSI = time since intercourse, h = hours, AP = acid phosphatse, PSA = prostate specific antigen, Sg = semenogelin, ALS = alternative light source, H&E = haematoxylin and eosin, Pap = papanicolaou, ∴ = therefore, FISH = fluorescence *in situ* hybridisation, CIE = counterimmuno-electrophoresis, ELISA = enzyme-linked immunosorbent assay).

			TEST							
			Recovery rate (%) for PT				**Recovery rate (%) for CT**			
Cohort	**Ref**	**TSI (h)**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**
SEXUAL OFFENCE VICTIM - LIVING	Enos, Beyer & Mann (1972)[83]	Case report (3h)		*AP*				*No stain & Pap*		
		0-24	100.0		0		100.0		0	
	Schiff (1978)[78]		*AP*							
		0-24	72.9 (14 h max)		0		65.7 (14 h max)		0	
		>24	0				0			
	Allard & Davies (1979)[84]		*AP*							
		0-24	69.0							
		24-48	27.8 (42 h max)							
		> 48	0							
	Evrard & Gold (1979)[75]	0-168	*AP:* 36.1				*Unknown:* 41.2			
	Tintinalli & Hoelzer (1985)[120]						*Unknown*			
		0-24					38.9			
		>24					5.0			
	Willott & Crosse (1986)[103]	0-24							*Unknown:* 48.8 (13 h max)	
	Penttilä & Karhuman (1990)[77]		*AP*				*Christmas tree*			
		0-24	71.4				55.6			
		24-48	55.3				35.5			
		48-96	35.7				20.0			
		>96	20.0				20.0			
	Costa *et al* . (1991)[106]						*Christmas tree*			
		0-72					*Laboratory:* 56.2			
		0-72					*Crime state lab* : 69.8			
	Rambow *et al.* (1992)[80]			*AP*				*Unknown*		
		0-24		64.3 (*site undefined*)				63.1 (*site undefined*)		
		24-48		50 (*site undefined*)				50 (*site undefined*)		
	Lynnerup, Hjalgrim & Eriksen (1995)[96]	Case report (3-40 h)								
		0-48	*ALS:* 100.0				*Unknown:* 100.0			
	Grossin *et al.* (2003)[119]	0-72						*Unknown:* 30.3 (*site undefined*)		
	Delfin *et al.* (2005)[118]							*Unknown*		
		0-24					50.0	100.0		
		24-48					-	-		
		48-72					0	-		
		0-24						20 (*site undefined*)		
		24-48						100 (*site undefined*)		

Table C2 cont.

			TEST							
			Recovery rate (%) for PT				Recovery rate (%) for CT			
Cohort	**Ref**	**TSI (h)**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**
SEXUAL OFFENCE VICTIM - LIVING	Lincoln *et al.* (2006)[98]	Case report (4 h & 12 h)						Case at 4 h: 75.0 (*site undefined*)		
		0-24	*ALS*: 100.0 (4 h)			*ALS:* 100.0 (12 h)				*Unknown* 100.0 (12 h)
	Jänisch *et al*. (2010)[114]							*Unknown*		
		0-24					45.5	18.9		
		24-48					11.1			
		48-72					20			
		>72					0			
	Hellerud *et al.* (2011)[54]		*AP (subset of data available, vaginal & textile, ∴ site undefined)*							
		0-24	46.6							
		>24	9.1							
	McAlister (2011)[82]	Case report (4 h)								
		0-24	*AP:* 100.0							
		0-24	*PSA:* 100.0							
	Casey *et al*. (2017)[62]							*H&E*		
		0-24					33.9	16.3	1.8	
		24-48					27.8	11.6	4.8	
		48-72					6.3	0	0	
		72-96					6.3	11.1		
		> 96					1.9	0 (96-120 h)		
	Owers *et al*. (2018)[100]						*H&E or Christmas tree*			
		0-24					50.4			
		24-48					45.8			
		48-72					27.3			
		72-96					32.3			
		96-120					16.2			
		120-144					20			
		144-168					7.1			
SEXUAL OFFENCE VICTIM - DECEASED	Wilson (1974)[112]	Case report (16 days)					*Unknown* 100.0			
	Standefer & Street (1977)[68]			*AP*				*Unknown*		
		0-24	100.0	100.0	100.0		100.0	100.0	100.0	
		24-48	100.0	-	100.0		100.0	-	100.0	
		48-72	-				-			
		72-96	-				-			
		96-120	100.0				0			
		120-144	100.0				100.0			
		144-168	-				-			
		168-192	100.0				0			
		30 days	100.0	100.0	100.0		100.0	100.0	100.0	

Table C2 cont.

			TEST							
			Recovery rate (%) for PT				Recovery rate (%) for CT			
Cohort	**Ref**	**TSI (h)**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**
SEXUAL OFFENCE VICTIM - DECEASED	Montagna (1996)[94]	Case report (34 days)	*PSA:* 100.0				*Other stain:* 100.0			
	Tsuji *et al.* (2001)[79]	Case report		*AP*				*H&E and Baecchi*		
		24-48	100.0	100.0	100.0		100.0	100.0	100.0	
VOLUNTEERS	McCloskey, Muscillo & Noordewier (1975)[87]		*AP*							
		0-24	20.0							
		24-48	3.8							
		>48	0							
	Silverman & Silverman (1978)[108]						*Pap*			
		0-24					64.0			
		24-48					55.0			
		48-72					33.0			
		72-96					27.0			
		96-120					25.0			
		120-144					35.0			
		144-168					39.0			
		168-192					13.0			
		192-216					0			
		216-240					25.0			
		240-264					0			
		264-288					0			
		288-312					0			
		312-504					5.0			
		>504					4.0			
	Graves, Sensabaugh & Blake (1985)[73]	0-72	*AP:* 35.3 (27 h max)							
			PSA: 69.6 (33 h max)							
	Randall (1987)[111]						*Papanicolaou*			
		0-24					25.0			
		24-48					12.5			
		48-72					9.9			
		72-96					1.8			
		96-120					10.7			
	Gabby *et al.* (1992)[70]					*ALS*				*Christmas tree*
		0-24				Unclear				88.9
		24-48				54.5 (28 h max)				45.5 (28 h max)
						AP				
		0-24				94.4				
		24-48				72.7 (28 h max)				
						PSA				
		0-24				89.8				
		24-48				63.6 (28 h max)				

Table C2 cont.

			TEST							
			Recovery rate (%) for PT				Recovery rate (%) for CT			
Cohort	**Ref**	**TSI (h)**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**
VOLUNTEERS	Rao *et al* . (1995)[109]						*Pap*			
		0-72					76.9			
		72-168					37.5			
		168-336					20.0			
		2-3 weeks					0			
							FISH			
		0-72					76.9			
		72-168					62.5			
		168-336					40.0			
		2-3 weeks					0			
	Steinman (1995)[61]		*AP*							
		0-24	100.0							
		24-48	73.9							
		>48	3.6							
	Dziegelewski, Simich & Rittenhouse-Olsen (2002)[65]		*AP*				*Christmas tree*			
		0-24	40.0				100.0			
		24-48	40.0				80.0			
		48-72	0				60.0			
		72-96	0				20.0			
		96-120	-				-			
		120-144	-				-			
		144-168	0				20.0			
			PSA							
		0-24	60.0							
		24-48	20.0							
		48-72	0							
		72-96	20.0							
		96-120	-							
		120-144	-							
		144-168	0							
	Murray, McAlister & Elliott (2007)[52]	0-24	*AP:* 73.1							
	Benschop *et al* . (2010)[95]		*PSA*				*Unknown*			
		0-24	88				54			
		24-48	42.3							
		48-72	40							
		72-96	0							
			Sg							
		0-24	88							
		24-48	61.5							
		48-72	60							
		72-96	0							

Table C2 cont.

			TEST							
			Recovery rate (%) for PT				Recovery rate (%) for CT			
Cohort	**Ref**	**TSI (h)**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**
VOLUNTEERS	Schmidt Astrup *et al* . (2012)[107]	0-48					*Pap* : 87.5			
HUMAN MODEL	Hooft & van de Voorde (1988)[50]									*No stain*
		0-24								100.0
		24-48								100.0
		48-72								100.0
		72-96								100.0
		96-120								100.0
		120-144								100.0
		144-168								100.0
	McWilliams & Gartside (2009)[51]					*PSA*				*Christmas tree*
		0-24				74.1				100.0
ANIMAL SEXUAL ABUSE VICTIM	Imbschweiler *et al* . (2009)[11]	Case report (<24 h)		*AP*			*Other*			
		0-24	100.0	100.0			100.0			
MIXED	Eungprabhanth (1974)[64]	Sexual offence victim - living	*AP (subset of data available)*							
		0-24	100.0							
		24-48	80.0							
		48-72	66.7							
		72-96	-							
		96-120	-							
		120-144	100.0							
		Volunteers					*H&E*			
		0-24					86.2			
		24-48					80.5			
		48-72					63.3			
		72-96					47.3			
		96-120					33.3			
		120-144					42.9			
		144-168					25.0			
		168-192					33.3			
		192-216					-			
		216-240					33.3			
		>240					0			

Table C2 cont.

			TEST							
			Recovery rate (%) for PT				Recovery rate (%) for CT			
Cohort	**Ref**	**TSI (h)**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**
MIXED	Enos & Beyer (1981)[72]	Sexual offence victim - living: Case reports (8 h & 17 h)								
		0-24				*AP:* 100.0 (8 h)			0	100.0 (17 h)
		Sexual offence victim - deceased: Case report (60 h)								
		48-72		*AP:* 0 (*site undefined*)			0	0	0	100.0
	Ricci & Hoffman (1982)[60]	Sexual offence victim - living								
		0-24	80.0 (20 h max)				80.0 (20 h max)			
		24-48	-				-			
		48-72	-				-			
		72-96	-				-			
		96-120	0				0			
		120-144	-				-			
		144-168	-				-			
		168-192	-				-			
		192-216	-				-			
		216-240	-				-			
		240-264	0				0			
		Volunteers	*AP*				*Pap*			
		0-24	91.7				58.3			
		24-48	21.4				21.4			
		48-72	12.5 (60 h max)				25.0			
		72-96	0				50.0			
		96-120	0				0			
		120-144	0				0			
		144-168	0				5.9			
		>312	0				0			

Table C2 cont.

			TEST							
			Recovery rate (%) for PT				Recovery rate (%) for CT			
Cohort	**Ref**	**TSI (h)**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**	**Vagina**	**Anus/rectum**	**Oral**	**Other body region/fluid**
MIXED	Chapman, Brown & Keating (1989)[105]	Sexual offence victim - living					*H&E*			
		0-24					73.5			
		24-48					100.0			
		48-72					-	25.0		
		72-96					100.0			
		Volunteers					*H&E*			
		0-24					100.0			
		24-48					100.0			
		48-72					63.6			
		72-96					50.0			
		96-120					28.6			
		120-144					25.0			
		144-168					100.0			

Table C3: PT and CT recovery from inanimate surfaces and objects. In certain instances, only data from subsets of the cohort or specific cases were presented. (Ref = reference, PT = presumptive testing, CT = confirmatory testing, TSI = time since intercourse, h = hours, min = minutes, AP = acid phosphatase, PSA = prostate specific anitgen, ALS = alternative light source, Sg = semenogelin, SEM = scanning electron microscopy, FISH = fluorescence *in situ* hybridisation, H&E = haematoxylin and eosin, N/A = not applicable).

				Test			
				PT		CT	
Ref	**Textile/surface**	**Age**	**Treatment**	**Technique**	**Recovery**	**Technique**	**Recovery**
Concheiro, Carracedo & Guitián (1982)[116]	Cotton, linen, paper, wood, silk	Few days to 1 year	Different volumes of semen			SEM	100% for all times
Kamenev, Leclercq & Francois-Gerard (1990)[88]	Various	2 days; 1 week; 1 & 3 months; 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6 & 10 years	Unknown	PSA	2 days: 100% 1 week: 66.7% 1 month: 66.7% 3 months: 100% 1 year: 14.3% 1.5 years: 0% 2 years: 100% 2.5 years: 90% 3 years: 70% 3.5 years: 50% 4 years: 0% 4.5 years: 100% 5 years: 75% 6 years: 100% 10 years: 0%		
Brauner & Gallili (1993)[76]	Condom	TSI = ~48 h	N/A	AP	Detected	Unknown	Detected
Steinman (1995)[61]	Cotton, tissue	230 days old	N/A	AP Zinc	100% on cotton 100% on tissue 100% on cotton 100% on tissue		
Lachica & García-Ferrer (1998)[115]	Cotton	2, 4, 6, 8, 10, 15, 25 days; 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 6, 7, 8, 9, 10 and 12 months	N/A			SEM	100% for all times
Christian *et al*. (2000)[90]	Clothing, linen	TSI = 24 - 44 h	N/A	*AP and/or Papanicolaou:* Detected			
Cina et al. (2000)[117]	Condom	TSI = 8 h	N/A			FISH	Detected
Hellerud *et al*. (2011)[54]	Clothing	Various	N/A	AP	(*subset of data available, vaginal & textile ∴ site undefined*) 0-24 h: 46.6 >24 h: 9.1	Christmas tree	Detected up to 96 hrs

Table C3 cont.

				Test			
				PT		CT	
Ref	**Textile/surface**	**Age**	**Treatment**	**Technique**	**Recovery**	**Technique**	**Recovery**
Miranda *et al*. (2014)[97]	Wood, cotton, paper, tile	1 min; 1 and 24 h; 10, 35, 60 days	N/A	ALS	100% for all times		
Nakanishi *et al.* (2014)[55]	Cotton	33, 41, 44 and 56 years old	N/A	AP	100% for all times	Baecchi	100% for all times
				Sg	100% for all times		
Skalleberg & Bouzga (2016)[92]	Coniferous and grass ground	*Coniferous ground*: 1, 4, 9, 24, 32, 50 hours *Grass ground*: 1, 4, 24, 96, 120, 144 hours	Different volumes of semen	Crime scene dogs	*Coniferous:* 0-24 h: 94.4% 24-48 h: 100% 48-72 h: 50% *Grass:* 0-24 h: 72.2% 24-48 h: - 48-72 h: - 72-96 h: 33.3% 96-120 h: 66.7% 120-144 h: 0%	Christmas tree	*Coniferous:* 0-24 h: 83.3% 24-48 h: 66.7% 48-72 h: 33.3% *Grass:* 0-24 h: 87.5% 24-48 h: - 48-72 h: - 72-96 h: 100%
				AP	*Coniferous:* 0-24 h: 100% 24-48 h: 83.3% 48-72 h: 100% *Grass:* 0-24 h: 91.3% 24-48 h: - 48-72 h: - 72-96 h: 100% 96-120 h: 0%		
Joshi, Subhedar & Saraf (1981)[59]	Cotton	12, 24, 48, 72, 96, 120, 144 h	Immersed in water	AP	100% for all times	H&E	100% for all times
Kulstein, Schacker & Wiegand (2018)[74]	Cotton and synthetic cloths	30 days on cotton (1 sample)	Different washing conditions & number of washes Different volumes of semen	ALS	0%	Sperm Hy-Liter™	Detected on cotton and synthetic fabric washed once and twice.
				AP	0%		
				PSA	0%		
				Sg	58.3% from cotton washed once. 100% from cotton washed twice. 100% from synthetic fabric washed once and twice. 100% from 30 day old stains washed once.		

Table C3 cont.

				Test			
				PT		CT	
Ref	**Textile/surface**	**Age**	**Treatment**	**Technique**	**Recovery**	**Technique**	**Recovery**
Spector & Von Gemmingen (1971)[71]	Cellulose acetate, cotton	N/A	Different washing conditions Some with semen/blood mixtures	AP	*Semen only* Detected in 1 out of 12 wash conditions for cellulose acetate. Detected in 11 out of 12 wash conditions for cotton. *Semen and blood* Detected in 1 out of 12 wash conditions for cellulose acetate. Detected in 3 out of 12 wash conditions for cotton.	Unknown	*Semen only* Detected in 10 out of 12 wash conditions for cellulose acetate. Detected in 11 out of 12 wash conditions for cotton. *Semen and blood* Detected in 4 out of 12 wash conditions for cellulose acetate. Detected in 12 out of 12 wash conditions for cotton.
Crowe, Moss & Elliot (2000)[57]	Cotton	N/A	Different washing conditions	AP	41.6%	Christmas tree	91.7%
Jobin & De Gouffe (2003)[56]	Cotton and nylon	N/A	Washed	AP PSA	0% 100% for cotton 0% for nylon	No stain Christmas tree	Detected on cotton and nylon Detected only on cotton
Farmen, Cortez & Skårland Frøyland (2008)[58]	Cotton	N/A	Different washing conditions	AP PSA	0% 33.3%	Christmas tree	66.7%
Page *et al* . (2014)[91]	Bathwater and bathtubs	N/A	Body washes and dust/dirt added to water.	AP	Recovered from bathwater with 3 of the 4 body washes. Recovered from bathwater with dust/dirt. Not recovered from tub.	H&E	Recovered from bathwater for all body washes and dust/dirt. Recovered from bathtub for 1 body wash and with dust/dirt
Nolan *et al* . (2018)[85]	Cotton, nylon, terry towel (cotton based), polar fleece, satin, lace	N/A	Repeatedly washed stains (6 washes)	ALS AP	Detected after one wash on cotton, terry towel and polar fleece. 0% in subsequent washes Detected after one wash on cotton only. 0% in subsequent washes	Christmas tree	Detected after one wash on all Detected after three washes on cotton, terry towel and satin. Detected after six washes on cotton and terry towel.
Noël *et al* . (2019)[63]	Cotton	N/A	Different washing conditions Repeatedly washed stains (6 washes)	ALS AP PSA	100% after all washes 15% after first wash 6% after second wash 0% after third wash 92.5% after first wash 45% after fourth wash 25% after sixth wash	Sperm Hy-Liter™	50% after six washes

Table C4: DNA profiling results from the included literature for various cohort types. (h = hours, n = sample size, Y-STR = Y-chromosomal short tandem repeat, ASA = animal sexual abuse, μL = microliter).

			DNA	
Cohort	**Ref**	**Sample site**	**Nature of (male) profile**	**Male DNA profiling success**
SEXUAL OFFENCE VICTIM - LIVING	Cerri *et al.*(2003)[122]	Vagina (Case report, <24 h)	Autosomal	100%
	Jänisch *et al.*(2010)[114]	Vagina (0-72 h)	Autosomal	Specific cases (n = 2): 100%
SEXUAL OFFENCE VICTIM - DECEASED	Tsuji *et al.* (2001)[79]	Vagina and anus/rectum (Case report, 24-48 h)	Autosomal Y-STR	100% 100%
VOLUNTEERS	Elliott *et al.* (2003)[104]	Vagina	Autosomal	0-24 h: 91.4% 24-48 h: 47.1% Maximum persistence: 38 h
	Benschop et al. (2010)[95]	Vagina	Autosomal Y-STR	0-24 h: 86% 24-48 h: 76.9% 48-72 h: 100% 72-84 h: 0% 0-24 h: 14.3% 24-48 h: 33.3% 48-72 h: - 72-84 h: 100%
MIXED	Hellerud *et al.* (2011)[54]	Vagina and inanimate objects and surfaces	Autosomal	81.3% Maximum persistence: 67 h
ANIMAL SEXUAL ABUSE VICTIM	Imbschweiler *et al.* (2009)[11]	Vagina and anus/rectum (ASA case report, < 24 h)	Autosomal	Forensically useable (human) profile obtained
INANIMATE SURFACES AND OBJECTS	Nakanishi *et al.* (2014)[55]	Aged (33, 41, 44 and 56 years old) semen stains on cotton fabric	Autosomal	33 years: 56.3 - 100% 41 years: 62.5 - 93.8% 44 years: 18.8 - 31.3% 56 years: 6.3 - 31.3%

Table C4 cont.

			DNA	
Cohort	**Ref**	**Sample site**	**Nature of (male) profile**	**Male DNA profiling success**
INANIMATE SURFACES AND OBJECTS	Sirker, Schneider & Gomes (2016)[123]	Different volumes of semen deposited on filter paper, exposed to either dry or humid conditions and tested at various times.	Autosomal	*Dry: 5 and 0.5 µL* 0-70 weeks: 90-100% *Dry: 0.05 µL* 7-8 weeks: 40-60% 18-19 weeks: 80-100% 29-30 weeks: 0-30% 34-35 weeks: 0-90% 44-45 weeks: 40-90% 70 weeks: 30-90% *Humid: 5 µL* 8-33 weeks: 90-100% 47 weeks: 60-70% 53-55 weeks: 50-100% 70-72 weeks: 0-90% *Humid: 0.5 µL* 8-10 weeks: 90-100% 19-20 weeks: 40-90% 33 weeks: 20-30% 47 weeks: 30-40% 47-55 weeks: 0-40% 70-72 weeks: 0-30% *Humid: 0.05 µL* 8-10 weeks: 50-60% 19-47 weeks: 0-10% 53-55 weeks: 10-20% 70-72 weeks: 10-20%
	Skalleberg & Bouzga (2016)[92]	Different volumes of semen deposited on coniferous and grass ground and tested at different time points	Autosomal	*Coniferous:* 0-24 h: 81.8% 24-48 h: 100% 48-72 h: 100% *Grass:* 0-24 h: 78.6% 24-48 h: - 48-72 h: - 72-96 h: 50%

Table C4 cont.

			DNA	
Cohort	**Ref**	**Sample site**	**Nature of (male) profile**	**Male DNA profiling success**
INANIMATE SURFACES AND OBJECTS	Brayley-Morris *et al*. (2015)[124]	Laundered, 8-month old semen stains (one or two donors) on cotton, polyester and nylon fabrics. Exposed to different washing conditions and number of washes.	Autosomal	100% after one, two or three washes Mixed donor stain on cotton washed once: 100% for both donors Mixed donor stain on polyester washed once: 100% for donor 2 and 6.7% for donor 1
	Kulstein, Schacker & Wiegand (2018)[74]	Laundered semen stains of different volumes on cotton and synthetic cloths. Some washed multiple times. One aged to 30 days prior to washing.	Autosomal	100% from cotton washed once and twice. 100% from synthetic fabric washed once. 75% from synthetic fabric washed twice. 100% from 30 day old stains on cotton washed once.
	Crowe, Moss & Elliot (2000)[57]	Laundered semen stains on cotton fabric exposed to different washing conditions.	Autosomal	100%
	Jobin & De Gouffe (2003)[56]	Laundered semen stains on cotton and nylon fabric	Autosomal	Profiles obtained
	Noël *et al*. (2019)[63]	Repeatedly washed semen stains on cotton fabric (6 washes). Different washing conditions.	Autosomal	100% after one wash 100% after six washes 100% after six washes and bleach treatment

Appendix D: Project ethical approval

Ethical approval letter from the University of Cape Town's Animal Ethics Committee (AEC):

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Animal Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: sumayah.ariefdien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/animalethics/forms

23 April 2019

Mr C Mole /Ms L Heathfield
Forensic Medicine
Room 5.07, Level 5
Falmouth Building, Entrance 2
FHS

Dear Mr Cole /Ms Heathfield

PROTOCOL TITLE: Molecular Forensic Investigations into Animal Sexual Abuse in South Africa

FHS AEC REF NO: 019_016

Thank you for submitting your request for approval of use of animal material for scientific purposes to the Faculty of Health Sciences (FHS) Animal Ethics Committee (AEC).

I am pleased to inform you that the FHS AEC EXCO has **approved** your request, which will terminate on **30 April 2022.**

Number of animal material & species: 10 specimens from the baboon heads & 10 specimens from hunted animals

Please quote the FHS AEC REF NO (above) in all future correspondence.

Please note that the approval of this protocol imposes the following obligations on the principal investigator (PI):

1. To submit an annual mandatory progress report. The first annual report for this protocol is due on **28 February 2020.** The forms can be accessed from http://www.health.uct.ac.za/fhs/research/animalethics/forms
2. To submit a final mandatory report on the **30 April 2022,** please access the final report form from: http://www.health.uct.ac.za/fhs/research/animalethics/forms
3. Ensuring that all study participants perform within the confines of the procedures and experimental design of the protocol as approved, or as amended.

AEC letter cont.

4. Ensuring that all study participants comply with all applicable national legislation, UCT policies, FHS AEC policies and standard operating procedures (SOPs) and national standards (SANS 10386: 2008).

My best wishes for a successful research and /or teaching endeavour.

Yours sincerely

Signature Removed

PROF PJ COMMERFORD
CHAIR, FHS AEC

AEC REF# 019_016

Ethical approval letter from the University of Cape Town's Human Research Ethics Committee (HREC):

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6626
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

15 April 2019

HREC REF: 190/2019

Ms Laura Heathfield
Forensic Medicine & Toxicology
Room 5.10, Entrance 2 Level 5
Falmouth Building

Dear Ms Heathfield

PROJECT TITLE: MOLECULAR INVESTIGATIONS INTO ANIMAL SEXUAL ABUSE IN SOUTH AFRICA (MPHIL CANDIDATE: MS K NATHA)

Thank you for submitting your response to the Faculty of Health Sciences Human Research Ethics Committee.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until 30 April 2020.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

The HREC acknowledge that the student, Khilona Natha will also be involved in this study.

Yours sincerely

Signature Removed

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
NHREC-registration number: REC-210208-007

HREC 190/2019

HREC letter cont.

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI), and Declaration of Helsinki (2013) guidelines. The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

HREC 190/2019

Appendix E: Informed consent for human semen donors

INFORMATION FORM and INFORMED CONSENT

Project Title: **Molecular Forensic Investigations into Animal Sexual Abuse in South Africa**

Researcher: Khilona Natha

Supervisor: Laura Heathfield

Division of Forensic Medicine and Toxicology of the University of Cape Town

We would like to invite you to participate in a research study that involves forensic presumptive and confirmatory testing for semen, as well as DNA analysis. The recruitment of this study will end on the date agreed upon with the researcher.

The decision to participate is entirely your own. If you decide not to participate in the study this will not disadvantage you in any way. There is no obligation on you to participate in this study. In addition, at any point during the study you are free to withdraw without having to provide any reason for this, and with no consequences to you. There is no monetary incentive for this study.

Background:

Animal sexual abuse (ASA) involves the harm of animals by humans for sexual gratification. The current prevalence of this issue is unknown, yet is possible to be more common that perceived. ASA can involve acts such as fondling of genitals, penetration of genitals or the mouth and, the injuring or killing of an animal for sexual gratification. ASA is seen as a criminal offence in South Africa and the perpetrators can be charged and sentenced. Animals do not have a voice of their own, and therefore rely on forensic investigations to provide scientific evidence that injustice has been done to them.

During sexual penetration of animals, semen from the male perpetrators will often be deposited on to the animal's fur. The detection and recovery of semen from the fur provides the necessary evidence to confirm an act of ASA. However, no literature is available to describe the time that semen can still be detected and recovered from fur. This study aims to test the detection and recovery of semen, as well as DNA, from animal fur. This data will be used to determine a

statistical relationship between detectability of semen and DNA and the time since it was deposited on to the fur. The quality and quantity of DNA that can be obtained from these fur exposed semen samples will also be assessed.

The study will involve collecting semen from male volunteers. The donated semen samples will be used to investigate the ability to detect and confirm the presence of semen over time from animal fur as well as assess the quality and quantity of DNA obtained from the semen samples given the time it was present on the fur. This will allow for baseline literature to be provided regarding the retention and recovery of human semen samples from animal fur. DNA analysis will not be used to obtain any information regarding your physical appearance, health status, heritage, or familial relationships. The DNA analysis will only be used to determine the quality and quantity of DNA that can be obtained from the semen samples over time. The results will not be used as an identifying tool.

This project is aimed to be completed within one year. Should you agree, the semen samples will be retained for further analysis pertaining to the field of this project, otherwise they will be destroyed once analysis is completed.

What we need from you:

To participate, you will need to donate one semen sample. You will be required to masturbate and ejaculate into the sample jar and record the time at which this was done. This can be done in the comfort of your own home. There will be no risk your health.

The donation must be done in the morning to ensure a fresh sample to be used in the study. The sealed specimen jar with ejaculate must be returned to the researcher as soon as possible after collection. If there is any time delay between sample collection and handing it to the researcher, the sample must be kept in the fridge.

You will be required to remain abstinent for 36 hours before making a donation, i.e. no ejaculations 36 hours before the donation. You will also be required to keep the semen from making any contact with any other bodily fluids. The samples will be anonymised and coded, and stored in a 4° C fridge within an access controlled laboratory in the Division of Forensic Medicine and Toxicology until the project is complete. If you should decide to withdraw from the study at any time, your samples will be destroyed.

All information about you will be kept strictly private and confidential. You will not be given the results of any tests done.

Contact details:

Ethics approval has been obtained for this project from the Faculty of Health Science Human Research Ethics Committee. If you may have any questions or require referral to a grief centre or psychological support, please do not hesitate to ask the person taking the consent. If you may have any questions with regards to the rights and welfare of a research subject in the study, please contact the Chairperson of the University of Cape Town Faculty of Health Science Human Research Ethics Committee, **Professor Marc Blockman** on (021) 406 6496 or alternatively on (021) 406 6338. If you require any further information about this study, please contact **Laura Heathfield** at (021) 406 6569 or email at laura.heathfield@uct.ac.za

Please answer the participant questionnaire by ticking yes or no before giving informed consent to donate. **Thank you for your participation and your valuable time.**

PARTICIPANT QUESTIONNAIRE:

	YES	NO	UNSURE
To the best of your knowledge, do you have any sexually transmitted infection or diseases?			
Have you had a vasectomy?			
To the best of your knowledge, are you azoospermic i.e. “no sperm count”?			
To the best of your knowledge, are you oligospermic i.e. “low sperm count”?			
If you have answered “Yes” to any of the questions above, you are not eligible to donate. Thank you for your willingness to participate.			
Are you willing to donate semen more than once?			

Full name:……

Date of birth:.....

PLEASE CONTINUE TO NEXT PAGE TO PROVIDE INFORMED CONSENT. COMPLETE THE INFORMED CONSENT FORM BEFORE DONATING.

INFORMED CONSENT

Read the following terms and choose an option below.

- I have read the above/ the above has been read to me, and I understand what this study entails.
- I have had the opportunity to discuss the study and my questions have been answered to my satisfaction.
- I know what is required of me, and I understand and accept the requirements.
- I understand that I will not receive any compensation for my participation.
- I understand that I may withdraw from the research study at any time without giving a reason and without any consequence to me whatsoever.
- I understand that is I withdraw from participation at any time, my samples will be destroyed.
- I consent to participate in this study and I understand that my consent is entirely voluntary.

□ I accept the terms and give my informed consent to participate in the study.

□ I do not accept the terms and no longer wish to continue.

I understand the samples will be stored for the duration of the project in the Division of Forensic Medicine and Toxicology at the University of Cape Town. After the project, I would like (please tick one option):

□ The samples to be stored for future research for any research project which is approved by the Faculty Health Science Human Research Ethics Committee.

□ The samples to be stored for future research that stems directly from this research project and which is approved by the Faculty Health Science Human Research Ethics Committee.

□ The samples to be destroyed.

If you sign this form, it means that you voluntarily give permission to participate in the study.

Participant:

Print Name: …….

Signature: ……..…….……

Date: |__|__|/|__|__|/|__|__|__|__|

Person obtaining consent:

I have explained the nature, demands and foreseeable risks of the above study to the volunteer:

Print Name: ……....

Signature: …….……

Date: |__|__|/|__|__|/|__|__|__|__|

Witness

Print Name: ……....

Signature: …….……

Date: |__|__|/|__|__|/|__|__|__|__|

Donation

Date & time of donation: |__|__|/|__|__|/|__|__|__|__| Time:

Number of samples donated: _____

Appendix F: Method optimisation

Table F1: Details of the optimisation of various variables prior to conducting experimentation. Semen stains were prepared on faux fur which were then swabbed. (qPCR = quantitative real time polymerase chain reaction, DTT = dithiothreitol, BFB = Brentamine Fast Blue, T = tails).

Variable	Tests	Constant conditions	Results: DNA concentration based on qPCR	Better performing variable	Comments
Volume of DTT	- 20 µL DTT - 3 mL DTT	- Cotton swabs - 100 µL semen stains	20 µL DTT: 2.394 ng/µL 3 mL DTT: 0.556 ng/µL	20 µL DTT	Constant shaking of samples with 3 mL of DTT was not possible.
Swab type	- Cotton (Copan, Brescia) - Flocked (Copan, Brescia)	3 different sets: - 3 day old stains (100 µL semen, 20 µL DTT) - 3 mL DTT (100 µL semen) - 17-hour incubation (100 µL semen, 3 mL DTT)	Cotton *3 day old stain:* 2.394 ng/µL *3 mL DTT:* 0.556 ng/µL *17- hour incubation:* 0.894 ng/µL Flocked *3 day old stain:* 3.916 ng/µL *3 mL DTT:* 23.822 ng/µL *17- hour incubation:* 6.522 ng/µL	Flocked	Swabbing of dry stains was easier with flocked swabs.
Incubation period during DNA extraction	- 1 hour (as per protocol) - Overnight (~ 17 hours)	- Flocked swabs - 3 mL DTT - 100 µL semen stains	1 hour: 23.822 ng/µL Overnight (~17 hours): 6.522 ng/µL	1 hour	

Table F1 cont.

Variable	Tests	Constant conditions	Results: DNA concentration based on qPCR	Better performing variable	Comments
Volume of semen	- 50 µL - 100 µL	- Flocked swabs - 3 mL DTT - 17-hours incubation	50 µL: 0.934 ng/µL. 100 µL: 6.522 ng/µL	100 µL	
Method of presumptive testing: Direct or indirect	- Direct method = using an aliquot of swab extract. - Indirect method = Blotting semen stain with filter paper and testing the paper.	Two hours old semen stains. Stains were either swabbed or blotted with filter paper.	Instant colour change to purple for both methods of testing.	Direct method	Convenient to take one swab for presumptive and confirmatory testing.
Method of presumptive testing: Volume of supernatant to be tested.	- Aliquot volume (20, 50, or 100 µL), or add BFB directly to supernatant	Two-hours old semen stains.	Instant colour change to purple for all volumes and directly adding BFB to supernatant.	Extract swab and add BFB directly to supernatant	
Method of slide fixing	- Candle - Air dry	- Flocked swabs - 100 µL semen	Candle: 100 µL: 4+ (T) Air dry: 100 µL: 4+ (T)	Candle	Candle fixing is more efficient.

Appendix G: Variable table

Table G1: The variables, and their classifications, that were addressed in this study. (C_t = cycle threshold, IC = internal control)

Variable	In/dependent	Type
Fur model	Independent	Categorical nominal
Time since exposure	Independent	Numerical discrete
Presumptive tests		
Time for positive reaction	Dependent	Numerical continuous
Confirmatory tests		
Score[85,102]	Dependent	Categorical ordinal
DNA analyses		
Concentration	Dependent	Numerical continuous
C_t of IC	Dependent	Numerical continuous
Degradation index	Dependent	Numerical continuous
Accuracy of allele calling	Dependent	Numerical continuous

Appendix H: Data from experimental analyses

Table H1: Results obtained from all experimental analyses conducted on samples, including background samples, obtained over the 14 day period from both semen donors for all fur models. Where applicable, data presented here has been rounded off to four decimal places, however statistical analysis was conducted on raw data. (qPCR = quantitative real time polymerase chain reaction; PT = presumptive testing; CT = confirmatory testing; bp = base pairs; DI = degradation index; IC = internal control; * = average time from three replicates; ✓ = immediate colour change to purple; × = no colour change within ten minutes; s = seconds (T) = tails).

					DNA concentration (ng/µL)					
						qPCR				
Day	**Model**	**Participant**	**PT***	**CT**	**Nanodrop**	**91 bp marker**	**353 bp marker**	**DI**	**C_t of IC**	**Profiling success (% of alleles called)**
	Positive control	1	✓	4+ (T)	25.25	26.8661	30.7012	0.8751	20.8196	100
		2	✓	4+ (T)	21.1	24.5621	24.6654	0.9958	20.9556	100
	Baboon	Blank 1	×	Dirt/Cell material	2.95	0.0094	0.0012	7.8245	21.2147	Some peaks called, few off-ladder peaks
		Blank 2	×	Dirt/Cell material	3.3	0.0091	-	-	21.2251	Some peaks called
		Blank 3	×	Dirt/Cell material	5.5	0.1138	-	-	21.1901	Some peaks called, few off-ladder peaks
		Blank 4	×	Dirt/Cell material	2.25	0.0078	-	-	21.1026	Some peaks called, few off-ladder peaks
		Blank 5	×	Dirt/Cell material	1.8	0.0078	-	-	21.1933	Some peaks called, few off-ladder peaks
		Blank 6	×	Dirt/Cell material	2.25	0.0125	-	-	21.1323	Some peaks called, few off-ladder peaks
		Blank 7	×	Dirt/Cell material	2.3	0.0163	-	-	21.1123	Some peaks called, few off-ladder peaks
	Nyala	Blank 1	×	Salt	1.9	0.0033	0.0029	1.1426	21.1608	-
		Blank 2	×	Salt	2.2	0.0004	-	-	21.1031	-
	Faux	Blank	×	Dirt	2.3	0.0008	0.0005	1.7287	21.3472	-

Table H1 cont.

					DNA concentration (ng/µL)					
						qPCR				
Day	**Model**	**Participant**	**PT***	**CT**	**Nanodrop**	**91 bp marker**	**353 bp marker**	**DI**	**C_t of IC**	**Profiling success (% of alleles called)**
1	Baboon	1	✓	4+ (T)	9.3	6.7105	7.6746	0.8744	21.1089	100
		2	✓	4+ (T)	8.9	7.4703	7.3452	1.0170	20.7935	100
	Nyala	1	✓	4+ (T)	13.85	13.4195	15.5937	0.8606	21.0138	100
		2	✓	4+ (T)	10.85	10.5426	10.6502	0.9899	20.9068	100
	Faux	1	✓	4+ (T)	16.55	16.7190	20.2841	0.8242	20.9408	100
		2	✓	4+ (T)	10.7	8.2130	8.3128	0.9880	21.0504	100
2	Baboon	1	✓	4+ (T)	4.6	1.2174	1.2039	1.0112	21.2423	100
		2	✓	3+ (T)	3.55	1.0670	1.0599	1.0067	21.1269	100
	Nyala	1	✓	4+ (T)	17.45	17.7550	19.7749	0.8979	20.9334	100
		2	✓	4+ (T)	9.0	7.6667	8.1344	0.9425	20.9404	100
	Faux	1	✓	4+ (T)	12.5	12.6843	14.2267	0.8916	20.9194	100
		2	✓	4+ (T)	6.4	5.5165	5.8534	0.9424	20.8952	100
3	Baboon	1	12.33 s	2+ (T)	3.1	0.4475	0.4355	1.0274	21.1207	100
		2	✓	4+ (T)	5.2	4.1115	4.1343	0.9945	20.7425	100
	Nyala	1	✓	4+ (T)	14.35	13.4221	15.7895	0.8501	20.9536	100
		2	✓	4+ (T)	12.65	15.5142	15.8774	0.9771	20.5972	100
	Faux	1	✓	4+ (T)	6.15	4.0355	4.9097	0.8219	21.1000	96.88
		2	✓	4+ (T)	6.7	5.8002	5.9030	0.9826	20.8639	100

Table H1 cont.

					DNA concentration (ng/µL)					
						qPCR				
Day	**Model**	**Participant**	**PT***	**CT**	**Nanodrop**	**91 bp marker**	**353 bp marker**	**DI**	**C_t of IC**	**Profiling success (% of alleles called)**
4	Baboon	1	✓	4+ (T)	3.3	0.8704	1.0401	0.8368	21.1548	93.75
		2	✓	4+ (T)	4.9	2.4723	2.4470	1.0104	20.9664	100
	Nyala	1	✓	4+ (T)	14.2	11.3358	13.3741	0.8476	21.0114	100
		2	✓	4+ (T)	9.35	7.8210	7.1910	1.0876	21.1036	100
	Faux	1	✓	4+ (T)	6.75	4.5317	5.0884	0.8906	21.0964	93.75
		2	✓	4+ (T)	6.7	5.6168	5.4521	1.0302	21.0709	100
5	Baboon	1	32.67 s	1+ (T)	4.65	2.0008	1.9806	1.0102	21.0646	100
		2	✓	4+ (T)	3.65	1.8095	1.7727	1.0208	20.9471	100
	Nyala	1	✓	4+ (T)	17.65	17.0619	19.3621	0.8812	20.9406	100
		2	✓	4+ (T)	6.0	4.3791	4.6210	0.9476	20.9686	100
	Faux	1	✓	4+ (T)	6.1	3.8278	4.4424	0.8617	21.0794	100
		2	✓	4+ (T)	6.9	6.8309	6.7796	1.0076	20.8797	100
6	Baboon	1	✓	3+ (T)	5.15	1.8090	1.9402	0.9324	21.1002	90.63
		2	✓	4+ (T)	4.45	1.4441	1.4043	1.0284	20.9532	100
	Nyala	1	✓	4+ (T)	24.05	24.6967	29.0240	0.8509	20.7856	100
		2	✓	4+ (T)	4.75	2.6337	2.6104	1.0089	21.0350	100
	Faux	1	✓	4+ (T)	17.8	17.7694	21.6746	0.8198	20.9412	100
		2	✓	4+ (T)	7.05	5.5346	5.3089	1.0425	20.9951	100

Table H1 cont.

					DNA concentration (ng/µL)					
						qPCR				
Day	**Model**	**Participant**	**PT***	**CT**	**Nanodrop**	**91 bp marker**	**353 bp marker**	**DI**	**C_t of IC**	**Profiling success (% of alleles called)**
7	Baboon	1	✓	4+ (T)	2.95	0.2996	0.2704	1.1082	21.1001	100
		2	✓	4+ (T)	4.95	1.1557	1.0621	1.0881	21.2673	100
	Nyala	1	✓	4+ (T)	17.3	14.4237	17.5192	0.8233	20.9295	100
		2	✓	4+ (T)	4.8	2.4281	2.4131	1.0062	21.1284	100
	Faux	1	✓	4+ (T)	7.5	5.3158	5.8562	0.9077	20.9163	100
		2	✓	4+ (T)	8.1	7.7610	7.9306	0.9786	20.9628	100
8	Baboon	1	10.67 s	3+ (T)	8.1	3.5637	3.2642	1.0918	21.1286	100
		2	✓	4+ (T)	2.65	0.4017	0.3668	1.0953	21.0928	100
	Nyala	1	✓	4+ (T)	20.3	15.8181	17.1841	0.9205	20.8908	100
		2	5.33 s	4+ (T)	8.0	7.1194	7.3936	0.9629	20.9876	100
	Faux	1	✓	4+ (T)	9.75	8.4206	9.2886	0.9065	21.0369	100
		2	✓	4+ (T)	6.5	5.0128	5.3250	0.9414	21.0706	100
9	Baboon	1	✓	4+ (T)	7.6	3.9569	3.9918	0.9913	20.9096	100
		2	✓	4+ (T)	3.5	0.6531	0.6041	1.0812	21.0920	100
	Nyala	1	✓	4+ (T)	18.35	17.8829	19.8590	0.9005	20.8666	100
		2	7.33 s	4+ (T)	12.45	14.5225	13.1917	1.1009	20.9054	100
	Faux	1	✓	4+ (T)	8.25	5.6594	5.8469	0.9679	20.8881	100
		2	✓	4+ (T)	12.45	10.9352	11.2755	0.9698	21.0841	100

Table H1 cont.

					DNA concentration (ng/µL)					
						qPCR				
Day	**Model**	**Participant**	**PT***	**CT**	**Nanodrop**	**93 bp marker**	**353 bp marker**	**DI**	**C_t of IC**	**Profiling success (% of alleles called)**
10	Baboon	1	5.33 s	4+ (T)	13.05	7.6441	7.1335	1.0716	20.9598	100
		2	✓	2+ (T)	4.75	0.6696	0.5368	1.2474	21.1992	100
	Nyala	1	✓	4+ (T)	24.15	20.9788	21.7813	0.9632	20.9715	100
		2	9 s	4+ (T)	9.1	8.2612	7.3800	1.1194	21.0464	100
	Faux	1	✓	4+ (T)	8.75	7.2426	7.3794	0.9815	21.0192	100
		2	✓	4+ (T)	5.0	2.8829	2.8841	0.9996	21.0627	100
11	Baboon	1	✓	4+ (T)	7.45	3.3469	3.1790	1.0528	21.0103	100
		2	✓	4+ (T)	4.95	2.0354	1.9898	1.0229	21.1118	100
	Nyala	1	✓	4+ (T)	12.1	10.8534	10.2948	1.0543	20.9559	100
		2	✓	4+ (T)	9.25	12.3176	11.6630	1.0561	20.9001	100
	Faux	1	✓	4+ (T)	5.75	3.5801	3.5376	1.0120	20.9234	100
		2	✓	4+ (T)	8.25	8.9271	8.9947	0.9925	21.0487	100
12	Baboon	1	✓	4+ (T)	2.95	0.6725	0.6186	1.0871	21.0016	100
		2	✓	4+ (T)	4.1	1.6539	1.4521	1.1390	21.2032	100
	Nyala	1	✓	4+ (T)	21.6	23.1135	22.8680	1.0107	20.7141	100
		2	✓	4+ (T)	13.05	11.4514	11.0609	1.0353	21.2186	100
	Faux	1	✓	4+ (T)	8.7	7.0829	7.9428	0.8917	20.9642	100
		2	✓	4+ (T)	4.25	2.6186	2.3352	1.1214	21.2320	100

Table H1 cont.

					DNA concentration (ng/µL)					
						qPCR				
Day	**Model**	**Participant**	**PT***	**CT**	**Nanodrop**	**93 bp marker**	**353 bp marker**	**DI**	**C_t of IC**	**Profiling success (% of alleles called)**
13	Baboon	1	5.33 s	4+ (T)	4.95	1.1471	1.2324	0.9308	21.1560	100
		2	✓	4+ (T)	3.5	1.7519	1.4317	1.2236	21.2092	100
	Nyala	1	✓	4+ (T)	9.25	6.6389	6.7063	0.9899	21.0773	100
		2	✓	4+ (T)	11.1	10.8896	10.7203	1.0158	21.0643	100
	Faux	1	✓	4+ (T)	8.2	6.3135	6.5213	0.9681	20.8671	100
		2	✓	4+ (T)	5.25	3.3477	3.2222	1.0389	21.1699	100
14	Baboon	1	✓	4+ (T)	4.05	0.9678	1.0015	0.9664	21.0175	100
		2	✓	4+ (T)	3.35	1.4801	1.3024	1.1364	21.1218	100
	Nyala	1	✓	4+ (T)	13.8	12.3760	12.5261	0.9880	20.8420	100
		2	✓	4+ (T)	14.35	13.2071	12.5126	1.0555	21.1500	100
	Faux	1	✓	4+ (T)	8.4	5.8194	6.6025	0.8814	20.8295	100
		2	✓	4+ (T)	5.2	3.9380	3.6553	1.0773	21.0771	100

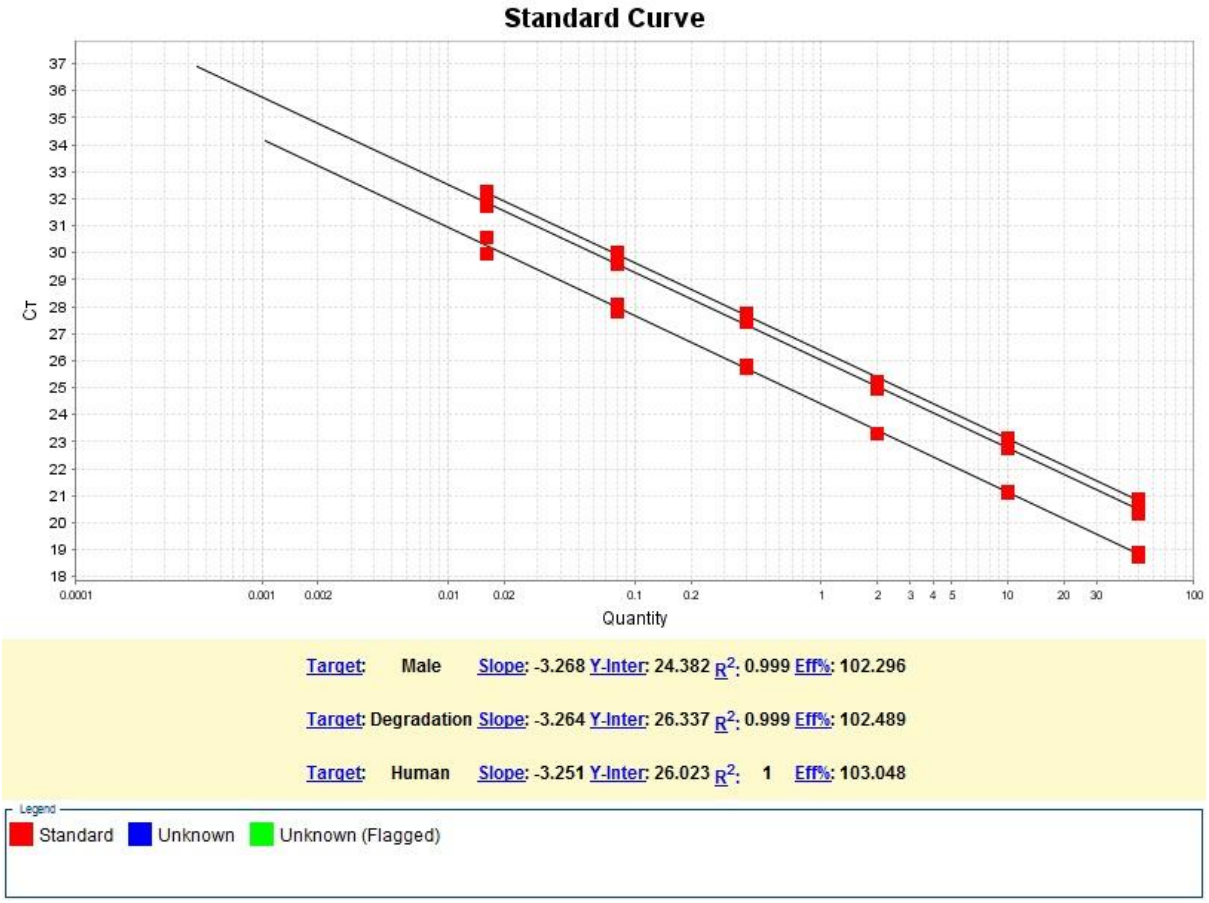

Figure H1: Example of the standard curves generated in the quantitative real time polymerase chain reaction (qPCR) assay. Red symbols represent the DNA standard. DNA quantity (ng/µL) is represented on the x-axis and the cycle threshold value (C_t) on the y-axis.

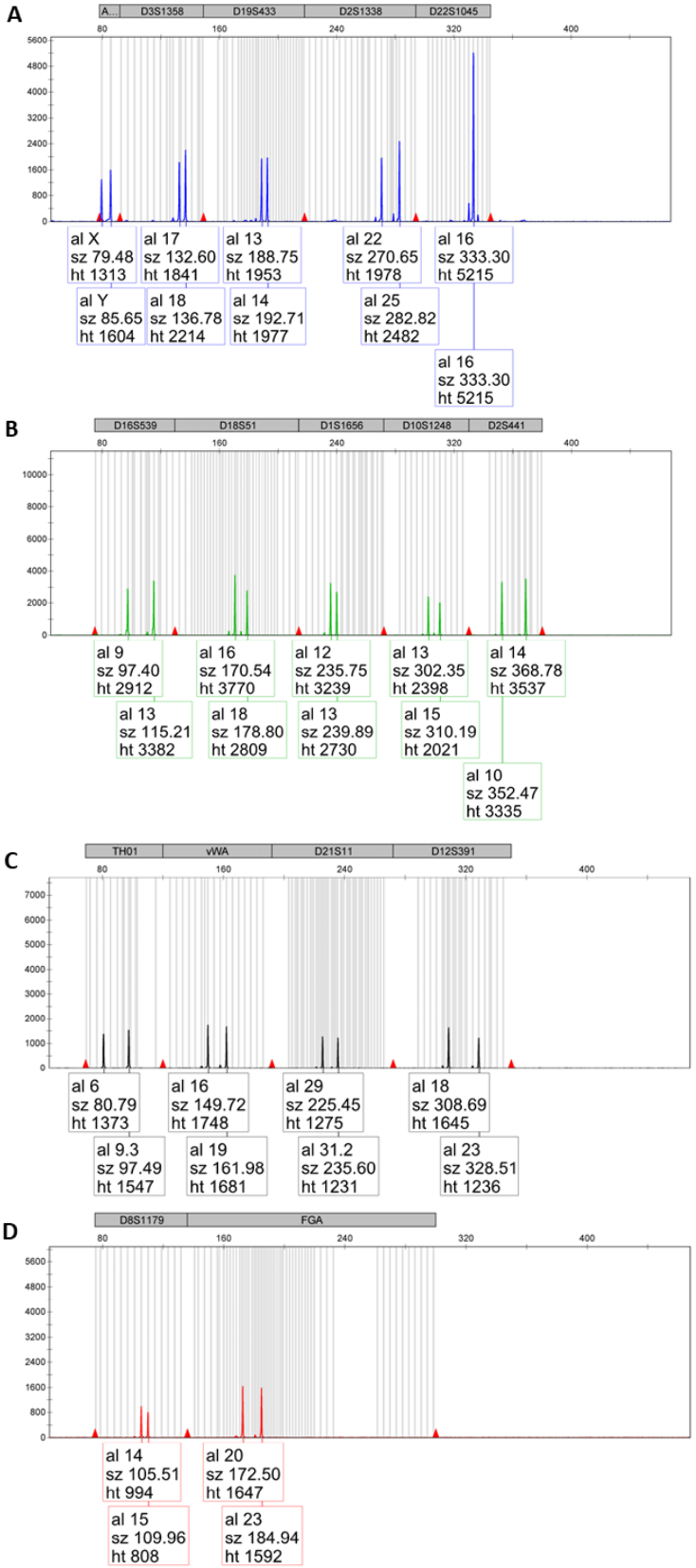

Figure H2: Electropherogram for the (A) fluorescein-, (B) JOE-, (C) TMR-ET-, (D) CXR-ET-labelled loci of the positive control of the Promega PowerPlex® ESI 16 kit. Marker size is represented on the x-axis and relative fluorescent units (RFUs) on the y-axis.

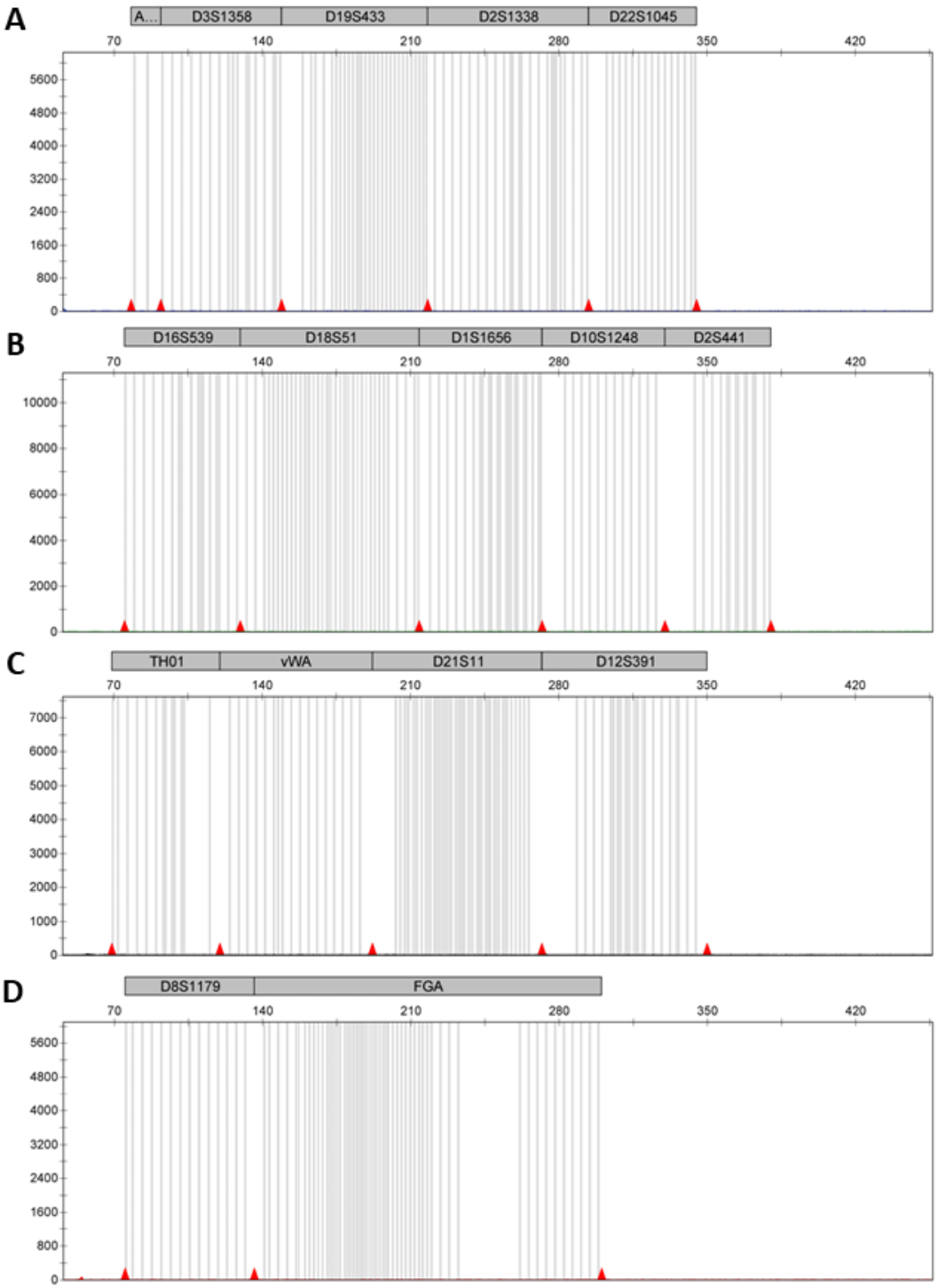

Figure H3: Electropherogram for the (A) fluorescein-, (B) JOE-, (C) TMR-ET-, (D) CXR-ET-labelled loci of the negative control of the Promega PowerPlex® ESI 16 kit. Marker size is represented on the x-axis and relative fluorescent units (RFUs) on the y-axis.