MALE REPRODUCTIVE TRENDS IN THE NOVEL EQUINE MODEL: INVESTIGATIONS INTO ANTHROPOGENIC ENVIRONMENTAL CHEMICAL EXPOSURE.

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A thesis submitted in partial fulfilment of the requirements of the University of the West of England, Bristol for the degree of Doctor of Philosophy.

This research programme was carried out in collaboration with Hartpury University and Hartpury College.

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The research carried out during my doctorate would not have been possible without the willingness and support of industry partners and collaborators. I would like to acknowledge the generosity of Tullis Matson and Pamela Humphreys at Stallion AI Services, the veterinarians at Three Counties Equine Hospital and Rossdales Veterinary Surgeons, and all the stallion and yard owners who helped with my research. I would like to thank the library department at Hartpury University for their support in developing my systematic review.

Over the last three years, there have been many processes and concepts that I have found challenging regarding researcher development. I would like to thank Helen Frisby and all others involved in the researcher skills development programme for providing a range of workshops that have helped me to build confidence and develop as a researcher.

Finally, I would like to thank all of my family and friends for being there every step of the way. The endless mental support you have given me has enabled me to push on with my doctorate, even through the difficult parts. I am very lucky to have such an incredible support system surrounding me.

CONTENTS PAGE

ABSTRACT

Introduction: Adverse trends are reported in human, canine, and bovine reproductive health, associated with exposure to environmental chemicals (ECs). Poor testicular function in stallions poses an economic, health and welfare concern. Assessing equine testicular EC accumulation initiates this species as a sentinel for human reproduction and a biomonitor for terrestrial environmental health. Trends in semen quality in global and UK-based equines and the potential aetiological involvement of EC exposure are investigated within this thesis.

Methods: A comprehensive evidence synthesis and meta-regression analysis determined trends (1984-2019) in objectively analysed fresh sperm progressive motility (PMOT; %; n=230 articles) from global equine populations. Trends were analysed in sperm motility (TMOT; %), concentration (million/ml), volume (ml), and total sperm output (TSO; million) in a UK-based equine population from a single breeding facility (2001-2020; 11,387 samples; 1,036 stallions). A linear mixed model (REML) accounted for predetermined variables. For EC analysis; testes (n=6), soils (n=2), grass (n=2), and feedstuffs (n=6) were analysed for Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP (GC-MS). EC concentration statistics incorporated an ANOVA and Tukey's post hoc, or an independent t-test.

Results: PMOT declined by 31.89% between 1984 and 2019, irrespective of sensitivity analyses ($p<0.05$). In the UK-based equine population, TMOT declined by 10.10% (2001 and 2010), whilst concentration, TSO, and volume increased. All chemicals analysed were detected in testicular samples, feedstuffs, and pastures.

Conclusions: Adverse trends in two sperm motion characteristics raises concern regarding the reproductive health and fertility of the equine population. This research provides novel data on equine testicular EC accumulation and suggests ingestion as a key exposure route. The research initiates the use of the novel equine model as a sentinel species for reproductive trends and as a biomonitor species for terrestrial ecosystems. Further research is required to determine whether EC exposure is associated with declining equine motility trends.

ETHICS AND DATA MANAGEMENT

Ethical acceptance was granted by Hartpury University Ethics Committee for the entire project prior to the commencement of this research. The ethical acceptance code is ETHICS2019-52. A low-risk amendment was accepted by the committee in December 2021 to accommodate for alterations within the methods (Appendix A1; page 247).

A data management plan was created in the first year of doctoral study and updated at the beginning of each year to maintain relevance to the research. The final data management plan can be found within the appendices (Appendix A2; pages 248-250).

ABBREVIATIONS

AhR	*Aryl hydrocarbon receptor*
AI	*Artificial insemination*
AIC	*Akaike information criterion*
Alf	*Alfalfa*
ANOVA	*Analysis of variance*
APHA	*Animal and Plant Agency*
AR	*Androgen receptor*
ART	*Artificial reproductive techniques*
AV	*Artificial vagina*
B	*Regression slope coefficient*
B-C	*Backward citation (search)*
BPA	*Bisphenol A*
BTB	*Blood-testis-barrier*
Ca^{2+}	*Calcium ion*
Cam	*Cambridgeshire*
cAMP	*Cyclic adenosine monophosphate*
CAQDAS	*Computer-Assisted Qualitative Data Analysis Software*
CASA	*Computer Assisted Semen Analysis*
CatSper	*Cation channel of sperm*
CEE	*Collaboration for Environmental Evidence*
COOH	*Carboxylic acid*
DEET	*N, N-Diethyl-meta-toluamide*
Defra	*Department for Environment, Food and Rural Affairs*
DEHP	*Di (2-ethylhexyl) phthalate*

DHT	*Dihydrotestosterone*
DW	*Dry weight*
E_2	*Oestrogen*
ECs	*Environmental chemicals*
EDCs	*Endocrine disrupting chemicals*
ER	*Oestrogen receptor*
ERα	*Oestrogen receptor alpha*
ERβ	*Oestrogen receptor beta*
EU	*European Union*
FC	*Foal cubes*
F-C	*Forward citation (search)*
FSH	*Follicle stimulating hormone*
g	*Gram*
GC-MS	*Gas chromatography-mass spectrometry*
Glouc	*Gloucestershire*
GnRH	*Gonadotropin-releasing hormone*
GPCR	*G-Protein-Coupled receptor*
H_0	*Null hypothesis*
H_1	*Hypothesis*
H-B	*Baled haylage*
H-CB	*Commercially baled haylage*
HPG	*Hypothalamic-pituitary-gonadal (axis)*
ISO	*International Organisation for Standardization*
kg	*Kilogram*
L	*Litre*

L-C	*Leydig cell*
LH	*Luteinizing hormone*
Log	*Logarithmic*
mg	*Milligram*
ml	*Millilitre*
mm	*Millimetre*
MOOSE	*Meta-Analyses of Observational Studies in Epidemiology*
mol	*Mole*
MPPs	*Micro plastic particles*
ND	*Not detected*
ng	*Nano gram*
OCP	*Organochlorine pesticides*
PAHs	*Polyaromatic hydrocarbons*
PBDEs	*Polybrominated diphenyl ethers*
PCDDs	*Polychlorinated dibenzo-dioxins*
PCDFs	*Polychlorinated dibenzo-furans*
PCBs	*Polychlorinated biphenyls*
PFCs	*Perfluorinated compounds*
PFOS	*Perfluorooctanesulfonic acid*
PMOT	*Progressive motility*
PO	*Population, Outcome*
POPs	*Persistent organic pollutants*
ppm	*Parts per million*
PRISMA	*Preferred Reporting Items for Systematic Reviews and Meta-Analyses*
RC	*Race cubes*

REML	*Restricted maximum likelihood*
ROS	*Reactive oxygen species*
ROSES	*RepOrting standards for Systematic Evidence Syntheses*
S-C	*Sertoli cell*
SC	*Stud cubes*
SD	*Standard deviation*
SEM	*Standard error of the mean*
STR	*Straightness*
T_4	*Testosterone*
TCa	*Testicular cancer*
TCDD	*2,3,7,8-Tetrachlorodibenzo-P-dioxin*
TDS	*Testicular dysgenesis syndrome*
TMOT	*Total motility*
TSO	*Total sperm output*
UK	*United Kingdom*
USA	*United States of America*
VAP	*Average Path Velocity*
<LOD	*Below limit of detection*
µl	*Microlitre*
µm	*Micrometre*
Σ	*Sigma, sum of*
<	*Less than*
>	*More than*
±	*Plus or minus*

LIST OF FIGURES

LIST OF TABLES

ACADEMIC PUBLICATIONS

Harris, I. T., Lea, R. G. and Sumner, R. N. (2022) Exposure to environmental contaminants and the impact on reproductive health, *Clinical Theriogenology Conference Proceedings, Seattle.* 22nd July 2022.

**Attached in Appendix B, pages 264-271.*

Perrett, J., **Harris, I. T.**, Maddock, C., Farnworth, M., Pyatt, A. Z. and Sumner, R. N. (2021) Systematic Analysis of Breed, Methodological, and Geographical Impact on Equine Sperm Progressive Motility, *Animals,* **11**(3088), pp. 1-13.

**Attached in Appendix C, pages 272-284.*

Sumner, R. N.*, **Harris, I. T**.*, Van Der Mescht, M., Byers, A., England, G. C. W. and Lea, R. G. (2020) The dog as a sentinel species for environmental effects on human fertility, *Reproduction,* **159**(6), pp. R265-R276.

**Sumner, R. N. and Harris, I. T. contributed equally to this publication.*

**Attached in Appendix D, pages 285-296.*

LAY AUDIENCE PUBLICATIONS

Harris, I. T. (2022) Environmental chemicals: A concern for stallion fertility?, *Stallion AI Services,* The Directory, a Breeders Guide. [online] https://www.stallionai.co.uk/serviceoption/the-directory-a-breeders-guide (Last accessed: 05 September 2022).

**The Directory is available in hardcopy format and online, having a reach of 50,000 readers in over 100 countries.*

**Attached in Appendix E, page 297.*

ACADEMIC CONFERENCE ORAL PRESENTATIONS

Fertility; Belfast; Oral presentation; January 2023 (abstract accepted):

- ‘Reproductive trends in the equine model: the question of declining semen quality and environmental aetiologies revisited’.

Research and Knowledge Exchange Committee; Hartpury University; Oral presentation; 2022:

- ‘Temporal trends in equine sperm progressive motility (1984-2019): a systematic review and meta-regression’.

British Society of Animal Sciences annual conference; Virtual conference; Oral presentation; 2021:

- ‘Temporal trends in stallion semen quality: The development of the horse as a biomonitor species’; (Harris, Pyatt and Sumner, 2021).

Hartpury Equestrian Performance Research Seminar; Virtual conference; Oral presentation; 2021:

- ‘Temporal trends in stallion semen quality and associated environmental aetiologies’.

ACADEMIC CONFERENCE POSTER PRESENTATIONS

Fertility; Virtual conference; Poster presentation; 2022:

- ‘Reprotoxic environmental chemicals (ECs) in the herbivorous equine model: testicular contamination and routes of exposure’; Appendix F (page 298).

Fertility; Virtual conference; Poster presentation; 2021:

- ‘Temporal trends in stallion sperm quality: furthering the debate on declining male fertility through the equine model’; Appendix G (page 299).

INVITED SPEAKER LECTURES

Developments in Equestrian Science (BSc module); Hartpury University; 2022; ‘Stallion fertility and the environment’.

Reproductive Sciences Faculty Meeting; School of Veterinary Medicine and Science, The University of Nottingham; 2021; ‘Temporal trends in stallion semen quality: The development of the horse as a biomonitor species’.

Investigating Equestrian Research (MSc module); Hartpury University; 2021; ‘Temporal trends in stallion semen quality: the aetiological role of environmental chemicals and mechanisms of reprotoxicity’.

Investigating Equestrian Research (MSc module); Hartpury University; 2020; ‘Declining male fertility: environmental chemicals and their plausible impacts on stallion fertility’.

Developments in Equestrian Science (BSc module); Hartpury University; 2020; ‘The environment and sex’.

The Society of Reproduction; Hartpury University; 2019; ‘Environmental influences on sperm quality and subsequent assessment’.

TEACHING

To accompany my professional development, and as a regulation of my doctorate, I undertook a number of teaching responsibilities. My involvement with teaching on undergraduate modules are listed below.

Functional anatomy (BSc level):

- Assisted and led practical sessions in the laboratory, involving teaching basic anatomy concepts through presentation, dissection, and other practical based skills.
- Led drop-in sessions for students to ask questions on topics covered in lectures.

Undergraduate research process (BSc level):

- Led and assisted with seminars for the undergraduate research process, which involved teaching students about quantitative and qualitative research methods.
- Taught students how to use quantitative research software, including SPSS.
- Taught students basic concepts of qualitative research, including thematic analysis.

Structure and function (Foundation level):

- Assisted with practical dissections of equine cadavers to enable learning of anatomy and physiology.

Supervision (BSc level):

- In my final year, I provided supervision for two BSc students' dissertations in topics associated with equine reproductive science.

MASTERS MODULES UNDERTAKEN

In total, I have been accredited the required 60 credits for the completion of three MSc level modules at Hartpury University and the University of the West of England.

Postgraduate independent study

I have been accredited a total of 15 credits for Postgraduate Independent Study, Hartpury University (UINVL4-15-M), achieving 85%. My project was titled 'A review: *in utero* exposure to emerging Bisphenol analogues and associated reproductive perturbations'.

Investigating Equestrian Research

I was awarded 15 credits for Investigating Equestrian Research (Hartpury University; HEQV6Y-15-7), for which I achieved a grade of 88%. My submission was a grant proposal titled 'A preliminary investigation on the effects of di(2-ethylhexyl) phthalate on key reproductive genes in Thoroughbred testes'.

Research in Contemporary Context

I successfully completed Research in Contemporary Context (USSKM3-30-M) at the University of the West of England, being awarded 30 credits in total for this pass/fail module. My portfolio contained 11 reflective pieces on topics including epistemology, doctoral research, grant writing, public engagement, project management and research governance, integrity, and data management. My portfolio also contained one case study on the topic of transdisciplinary research, specifically evaluating the importance of the involvement between the science and creative arts sectors. Topics undertaken as part of this module align with the Vitae Researcher Development Framework (Vitae, 2010), designed to further skills integral to the successful synthesis, execution, and dissemination of high quality research.

CERTIFIED TRAINING COURSES

Certified training course in NVivo 12 Fundamentals; QSR International NVivo Academy.

Advanced learning course in control of substances hazardous to health (COSHH) awareness; The Royal Society for the Prevention of Accidents (ROSPA).

COMMITTEE INVOLVEMENT AND RESPONSIBILITIES

Early Career Representative; The Society of Reproduction and Fertility; 2022 – present.

- Involved in producing and running early career events and promoting the society's social media account (Twitter).

Early Career Representative; Public Engagement Committee; The Society of Reproduction and Fertility; 2022 – present.

- Active in promoting the society through social media (Twitter).
- Assisted in the progression of the society's public lecture series.
- Involved in reviewing applications for award schemes.
- Involved with reviewing abstracts for the society's annual international conference.

Committee Secretary; Natures SAFE; 2022 – present.

- Responsible for recording the minutes for the charity's scientific committee meetings.

AIMS AND OBJECTIVES

The overarching aim of the research presented within this thesis was to assess temporal trends in stallion semen quality and to analyse the plausible aetiological involvement of anthropogenic environmental chemicals. To address the overall aim, the PhD was formulated around three key objectives.

Objective A:

Assess temporal trends in semen quality across global domesticated equine populations through systematic review and meta-regression analysis.

Objective A aligns with chapter 3 of this thesis (Temporal trends in sperm progressive motility in the novel equine model (1984-2019): a systematic review and meta-regression; pages 68-93).

Objective B:

Determine temporal trends in semen quality from retrospective data collected on a population of stallions at a single breeding facility in the UK.

Objective B aligns with chapter 4 of this thesis (Temporal trends in semen quality from a population of stallions at a single breeding facility in the UK (2001-2020); pages 94-126).

Objective C:

Determine the presence or absence and concentrations of key environmental chemicals in stallion testes and typical equine management feedstuffs as potential exposure routes.

Objective C aligns with chapter 5 of this thesis (Anthropogenic environmental chemicals in the novel equine model: testicular contamination and routes of oral exposure; pages 127-165).

HYPOTHESES

A scientific hypothesis (H_1) and null hypothesis (H_0) were produced for the overall research project and for each individual study. The research was centred around three studies, the data for which was used to address each hypothesis. The overall scientific hypothesis and null hypothesis are presented below:

H_1 Male equine populations are at risk of declines in reproductive health and are exposed to reprotoxic anthropogenic environmental chemicals as reported in alternative species.

H_0 Male equine populations present no trends in reproductive health or function and are not exposed to anthropogenic environmental chemicals.

Hypotheses were further broken down to address specific studies associated with objectives A, B and C, of this research project:

Objective A:

H_1 Sperm progressive motility is declining across global domesticated equine populations.

H_0 No trends are observed in sperm progressive motility across global domesticated equine populations.

Objective B:

H_1 Sperm quality, including total motility, concentration, semen volume, and total sperm output, are declining in an equine population from a single breeding facility in the UK.

H_0 No trends are observed in sperm quality parameters within an equine population from a single UK-based breeding facility.

Objective C:

H_1 Anthropogenic environmental chemicals, including PCBs, PBDEs, PAHs, and DEHP accumulate in stallion testis, and exposure includes the ingestion of contaminated pastures and feedstuffs.

H_0 Anthropogenic environmental chemicals, including PCBs, PBDEs, PAHs, and DEHP do not accumulate in stallion testicular tissue, and the equine species are not exposed to contaminants through the ingestion of contaminated pastures or feedstuffs.

CHAPTER 1

A GENERAL LITERATURE REVIEW

1.1 The equine industry and associated breeding sector

Male reproductive function is fundamental for the sustainability of a population. High importance is placed on stallion fertility due to heritable traits passed on to sired offspring (Gottlieb *et al.*, 2020). Reproductive abnormalities and impaired fertility are associated with compromised animal health and welfare. A reduction in fertilising potential could also place greater strain on animals due to increased coverings and collections required to achieve conception. The equine industry promotes stallions to be selected as sires based on pedigree, performance, and conformation, with little concern given to fertility (Maziero *et al.*, 2019; Varner, Gibb and Aitken, 2015). Within European breeding organisations, only three out of 19 organisations included fertility as a breeding objective, with the remaining groups focussing on conformation and performance (Koenen, Aldridge and Philipsson, 2004). It is vital to promote stallion health, including that of the reproductive system, to maintain high animal welfare standards and sustain the equine population.

From an economic perspective, the equine sector is a lucrative global industry. In the UK in 2019, the estimated equine population stood at 847,000, with the sector's economic contribution reported at £4.7 billion (British Equestrian Trade Association, 2019). In the USA in 2018, the equine industry directly supported 686,000 jobs and contributed $36.8 billion to the national economic market (Lord, 2019). Considering counterparts of the equine industry, in 2012, the Thoroughbred racing sector reached £3.45 billion in total expenditure, contributed £275 million in tax, and supported 85,200 employees in the UK through direct, indirect, and associated employment routes (British Horseracing Authority, 2013). High economic importance is placed on the reproductive performance of stallions, given the high numbers of offspring each individual can sire (Gottlieb *et al.*, 2020). The stud fee for a retired champion Thoroughbred stallion stands at £200,000 per live foal (Juddmonte Farm, 2022). The stud fee for a world-class show jumping stallion is quoted at £1,750 for the 2022 breeding season (Stallion AI Services, 2022). A reduction in the fertilising potential of stallions could have significant implications for breeding efficiency, compromising the stallion's economic and industrial profile (Darr *et al*., 2017).

Stud fees for champion stallions in the Thoroughbred racing sector are substantially higher than stallions competing at equivalent levels in alternative disciplines. Economically, the

Thoroughbred breeding industry may be significantly impacted by poor stallion fertility, given the significant monetary values of breeding stock associated with this sector. Breeding legislation in the Thoroughbred racing industry do not permit artificial reproductive techniques (ART), relying on natural coverings (Campbell and Sandøe, 2015). The inability to implement assistive breeding techniques in the management of subfertile and infertile stallions may further reduce their potential to producing offspring. However, the sustainability of equine populations and the economics of alternative disciplines are still likely to be impacted. In addition, regardless of the stallion's economic value, it is vital to promote stallion health, including that of the reproductive system, in order to maintain high animal welfare standards. Assessing temporal trends in reproductive parameters, including semen quality, enables a comprehensive assessment of the past and current fertility statuses of a population. Whilst equine specific research in this area is limited, trends in alternative species are indicative of declining reproductive health and function.

1.2 Reproductive trends and testicular dysgenesis syndrome

Adverse trends in reproductive health and function have become an increasing concern in human populations (Skakkebaek *et al*., 2015), companion animals (Sumner *et al*., 2020), livestock (Wahl and Reif, 2009), and wildlife species (Rodríguez-Estival and Mateo, 2019). Despite reports of the high prevalence of subfertility and infertility in the male equine population (Boer, 2007; Oristaglio Turner, 2007), research concerning trends specific to this species is limited (Multigner *et al*., 2000, 1999). Declining reproductive function became a topic of contentious debate following the publication of an evidence synthesis in human semen quality (Carlsen *et al*., 1992). Results indicated a global decline of 50% in sperm concentration and decrease in seminal volume from 3.40 ml to 2.75 ml between 1938 and 1990 (Carlsen *et al.*, 1992). Results were heavily scrutinised due to heterogeneity and developments in semen analysis methods (Pacey, 2013). A reanalysis of the dataset continued to show a decline in sperm concentration between 1938 and 2013 (Johnson *et al.*, 2015). However, as individual parameters, volume and concentration do not provide a comprehensive understanding of testicular output. Seminal fluids are produced by the accessory glands, and so volume is not a direct measure of testicular health. Given that concentration is the number of sperm per millilitre of seminal fluid, concentration is also directly related to volume (World Health Organization, 2021). Whilst trends required a

comprehensive re-evaluation to gain scientific value, the initial evidence synthesis acted as a necessary stimulus in the discussion associated with reproductive trends (Fisch, 2008).

Updated analyses of the metadata continue to indicate declines in semen quality, including sperm count (Levine *et al*., 2022, 2017), a more accurate predictor of testicular output (World Health Organization, 2021). Following the application of stricter inclusion criteria, accepted evidence synthesis protocols (Moher *et al*., 2009; Stroup *et al*., 2009), and stringent analytical methodologies (Borenstein *et al*., 2009), current meta-analyses continue to suggest a 50% to 60% decline in sperm concentration and sperm count between 1973 and 2011 (Levine *et al*., 2017). Declines show no plateau, raising substantial concern for future male fertility in human populations. Geospatial variability in semen quality trends analysed through systematic methodologies has also been reported (Swan, Elkin and Fenster, 2000, 1997). Temporal declines are more significant in the Western world, including Europe, North America, Australia, and New Zealand, compared to South Africa, Asia and South America (Levine *et al.*, 2017).

Whilst the current evidence syntheses in human sperm quality trends (Levine *et al.*, 2022, 2017) avoid many of the limitations associated with the systematic methodologies, some limitations were not addressed. Limitations included the calculation of semen collection year, differences in semen analysis training protocols, and laboratory quality control (Boulicault *et al*., 2021). Research based on data collected from single laboratories with consistent analytical and quality control schemes mitigates methodological limitations associated with evidence syntheses (Pacey, 2013).

Retrospective studies support the hypothesis of adverse semen quality trends. Declines in sperm motility, concentration, count, and semen volume have been reported in data from sperm bank donors in China from 2008 to 2014 (Wang *et al*., 2017) and 2001 to 2015 (Huang *et al*., 2017). In the USA, sperm concentration and count, motility, and normal morphology declined by 37%, 42%, 15%, and 16%, respectively between 2000 and 2017 (Mínguez-Alarcón *et al*., 2018). In France, sperm count, motility, and normal morphology declined in a population of men presenting for couple infertility assistance between 1988 and 2007 (Geoffroy-Siraudin *et al*., 2012). Retrospective research also supports the

hypothesis of geographic-sensitive declines reported in semen quality. Significant national geospatial differences exist in the prevalence of male factor infertility in patients at an ART centre in the USA (Odisho *et al.*, 2014). Distinct geographical variability in human semen trends could be a result of genetic, environmental, or lifestyle factors. In men, only 15 to 30% of infertility is accounted for by genetic disorders (Neto *et al*., 2016), which would indicate that alternative aetiologies are involved in declining semen quality.

Adverse trends in semen quality in human populations also result in significant clinical implications. Approximately 8-12% of couples on a global scale are impacted by infertility and impaired reproductive function, 40-50% of which is associated with the male factor (Kumar and Singh, 2015). The accessibility of ART may impact the natural selection of hereditary traits, reducing evolutionary pressures for reproduction (Perrett *et al*., 2021). This phenomenon is likely to impact both human populations (Tavalaee, Razavi and Nasr-Esfahani, 2009) and the equine breeding sector and should therefore be considered when interpreting reproductive trends.

Whilst there is a significant body of published research indicating that reproductive parameters are declining, results in humans are not consistent across literature. In a population of andrology laboratory patients in Argentina, the incidence of impaired semen quality in men did not increase between 2001 and 2020 (Ramírez *et al.*, 2022). However, data resulting from monitoring trends in incidence rates, as opposed to semen quality parameters, could be impacted by differential diagnostics and increasing awareness across the study period. It is therefore more accurate to monitor semen quality parameters with consistent analysis methods to develop a deeper understanding of true reproductive trends. From 1996 to 2010, sperm concentration and count increased in a group of Danish men (Jørgensen *et al*., 2012). Sperm concentration presented an overall adverse trend between 1994 and 2005 in a Scottish population, although the research group suggested that fluctuations in the parameter were evident across the study period (Sripada *et al*., 2007). As opposed to the current sperm count decline hypothesis, the inconsistencies in trends are supportive of the sperm count biovariability hypothesis, a proposed framework that suggests dynamic, non-linear time trends in sperm count with social and non-pathological aetiologies (Boulicault *et al*., 2021). However, this argument is associated with

the debate on sperm count and does not consider alternative semen quality parameters. In humans, male-factor infertility was not associated with sperm count or volume, and instead motility, morphology, and vitality (Eric *et al*., 2017), indicating the importance of analysing trends in a range of reproductive parameters to form a holistic understanding of true reproductive trends.

Trends in semen quality are associated with the proliferation of testicular cancer (TCa) and congenital genitourinary abnormalities such as cryptorchidism and hypospadias, which are collectively termed testicular dysgenesis syndrome (TDS) (Ghazarian *et al.*, 2018; Skakkebaek *et al*., 2015). The incidence rates of TCa are increasing globally, although the highest rates are reported in Northern European and western countries (Park *et al*., 2018; Znaor *et al.*, 2014; Richiardi *et al.*, 2004). Increasing TCa prevalence in developing industrial regions is indicative of an anthropogenic environmental aetiology. Incidence rates of TCa in adolescent and young adult patients are also reported to be highest in European regions (Kusler and Poynter, 2018). Increasing trends in TCa in young patients raises significant concern over their future reproductive potential, given the association with perturbed semen quality (Girasole *et al*., 2006).

A risk factor associated with TCa is cryptorchidism, defined as the failure of a single or both of the testis to descend into the scrotum (Ferguson and Agoulnik, 2013). The incidence rate of cryptorchidism increased from 2% to 9% from 1959 to 1961 and 1997 to 2001, respectively (Nordkap *et al*., 2012). In France, cryptorchidism incidence rates increased for all age groups between 2002 and 2014, although the steepest incline was for boys under the age of two (Le Moal *et al*., 2021). Cryptorchidism in boys is linked to impaired semen quality later in life, raising concern over their fertility potential (Ferguson and Agoulnik, 2013). Geospatial differences in cases of cryptorchidism were also reported, which were suggested to be linked to levels of industrialisation and associated variation in the spatial distribution of anthropogenic environmental chemicals (ECs) (Le Moal *et al*., 2021).

Increases in TCa and cryptorchidism are coupled with the proliferation of hypospadias, one of the most common congenital abnormalities in human male infants (Nordkap *et al*., 2012). Clinically, hypospadias is defined as the proximal displacement of the opening to the

urethra on the penis (van der Horst and de Wall, 2017). Between 1968 and 1993, hypospadias rates doubled in the USA (Paulozzi, Erickson and Jackson, 1997). Whilst a variety of factors could give rise to such changes, these trends are suggested to be a result of toxicant exposure at vital periods of sexual development (Brenner *et al*., 2019; Pishgar *et al*., 2019). However, trends in cryptorchidism and hypospadias must be interpreted with caution due to the collection of data from birth registries, which introduce limitations, including differential diagnostic criteria (Nordkap *et al.*, 2012). In addition, an increase in awareness of disorders may be associated with trends in incidence rate measurements, as reported in alternative conditions (Morinville, Barmada and Lowe, 2010). Such limitations emphasise the importance of analysing trends in direct semen quality measurements, which are not reflective of variability in awareness levels.

Despite the extensive analyses of reproductive trends in human populations, there is still some dispute regarding the hypothesis of declining semen quality in the research community. Evidence syntheses in human semen quality recommend the utilisation of alternative species as comparative models for tracking temporal trends in reproductive parameters (Olsen *et al.*, 1995), a hypothesis that is supported in current literature (Sonne *et al*., 2020; Sumner *et al*., 2020; Basu *et al*., 2007). Cross-comparisons of animal and human-based semen quality trends have the ability to add significant data to the debate surrounding declining fertility across species and promote a deeper understanding of associated aetiologies. Species comparisons of research determining temporal trends in semen quality parameters are presented in table 1.

Table 1: Summary of the research reporting semen quality trends across species discussed within the current chapter.

Species	Location	Date range	Parameters	Trend	Reference
Equine	France	1981 - 1996	Concentration Volume Sperm count	Increase Decline Unchanged	Multigner *et al.,* 1999; 2000
Bovine	USA	1965 - 1995	Volume Concentration Daily sperm output Normal morphology	Decline Decline Decline Increase	Wahl and Reif, 2009
Bovine	Netherlands	1977 - 1996	Sperm output	Unchanged	Van Os *et al.*, 1997
Bovine	Global	1932 -1995	Concentration	Unchanged	Setchell, 1997
Porcine	Global	1932 -1995	Concentration	Unchanged	Setchell, 1997
Ovine	Global	1932 -1995	Concentration Total sperm output	Unchanged Increase	Setchell, 1997
Canine	UK	1988 - 2014	Motility Normal morphology Total sperm output	Decline Decline Increase	Lea, Byers, *et al.,* 2016
Human	Global	1973 - 2011	Concentration Sperm count	Decline Decline	Levine *et al*., 2017
Human	China	2008 - 2014	Concentration Sperm count Motility Normal morphology	Decline Decline Decline Decline	Wang *et al*., 2017
Human	China	2001 - 2015	Concentration Normal morphology Progressive motility Motile sperm count	Decline Decline Unchanged Decline	Huang *et al*., 2017
Human	USA	2000 - 2017	Concentration Sperm count Motility Normal morphology	Decline Decline Decline Decline	Mínguez-Alarcón *et al*., 2018
Human	France	1988 - 2007	Sperm count Motility Normal morphology	Decline Decline Decline	Geoffroy-Siraudin *et al*., 2012
Human	Copenhagen	1996 - 2010	Concentration Sperm count Motility Abnormal spermatozoa	Increase Increase Unchanged Unchanged	Jørgensen *et al*., 2012

1.2.1 Semen quality trends in current sentinel species

Sentinel species are biological indicators used as comparative models for humans in the assessment of health and exposure to potentially toxic chemicals (Amadi, Frazzoli and Orisakwe, 2020; Rabinowitz, Scotch and Conti, 2010). Current research fails to evaluate the suitability of the equine species as a comparative model for reproductive trends. Currently, the dog is the most common sentinel species used as a comparative model for human reproductive health and function (Sumner *et al.*, 2020). This is due to the dog sharing close

environmental conditions to humans (Venier and Hites, 2011), and being exposed to similar household and outdoor stressors. Parallel adverse reproductive trends to those in humans have been reported in the dog sentinel, with an overall decline of 30% in progressive sperm motility (PMOT) between 1988 and 2014 (Lea, Byers, *et al*., 2016). An updated analysis of the dataset continued to show a declining trend in PMOT from 2014 to 2020 in the population of stud dogs (Harris *et al.,* 2020; Lea, Byers, *et al.,* 2016). Data were collated from a singular laboratory with consistent analytical methods, thereby adding to the weight of evidence exhibited in humans and limiting the criticism over semen quality assessments.

Researchers have proposed the use of bovine species as an additional herbivorous comparative model for human reproductive trends. In a population of Holstein dairy bulls in the USA, seminal volume declined (p>0.05), and sperm concentration, total daily sperm output, and normal morphology declined significantly (p<0.001) between 1965 and 1995 (Wahl and Reif, 2009). Considering species-specific factors that may be involved with trends, inbreeding in dairy bulls, a result of selectively breeding for the phenotypic gain of high milk yield, is associated with reproductive aberrations (Walsh, Williams and Evans, 2011; Lucy, 2001) and may therefore be involved in declining semen quality in this breed. The effects of selective breeding on reproductive performance are also reported to impact the equine population (Pirosanto *et al*., 2019) and should be considered when observing trends in semen quality within this species. Research in a population of dairy bulls from the Netherlands presented no adverse trends in sperm count between 1977 and 1996 (Van Os *et al.*, 1997). Whilst differences in trends could be a result of geographical variability, as reported in human populations, the semen analysis methods utilised for the bulls in the Netherlands were subject to changes across the study period (Van Os *et al.*, 1997), which could impact the results. Alterations in analytical methodologies highlight a limitation of assessing trends in semen quality (Pacey, 2013), with further research utilising consistent datasets required to provide a more accurate representation of trends.

In other livestock animals including, bovine, porcine, and ovine species, there was no correlation with the year of publication, with the latter two species presenting a slight positive correlation (Setchell, 1997). Systematic approaches assumed within these analyses did not meet current criteria for the interpretation of accurate results, as demonstrated

within human studies (Levine *et al.*, 2017). Further research utilising the year of sample collection instead of the year of publication may provide a more accurate representation of reproductive trends. Considering the ovine species, whilst research analysing semen quality trends is limited, this species is an established sentinel model regarding reproductive aberrations associated with exposure to environmental factors (Viguié *et al.*, 2020). When compared to alternative livestock species, the equine population is reported to have lower fertility rates (Mucha, Wolc and Szwaczkowski, 2011), which raises significant concern about the reproductive status of the male equine population. It is possible that lower fertility rates in horses are partially due to selective breeding regimes focussing on conformational and performance traits (Maziero *et al.*, 2019; Varner, Gibb and Aitken, 2015). However, additional aetiologies associated with perturbed reproductive health and function warrant further investigation.

1.2.2 Semen quality trends in the equine species

Horses share similar terrestrial environmental conditions to humans (Yavuz *et al*., 2022) and thus could pose as an important sentinel model for the comparison of trends in semen quality across species. Reproductive health assessment in stallions includes the analysis of key semen quality parameters (Hernández-Avilés *et al*., 2021) such as semen volume, sperm concentration, sperm count, total motility, progressive motility, and vitality (Appendix H; page 300). Additional parameters include DNA fragmentation (Love and Kenney, 1998), Acrosome Reaction Assay and the Hypoosmotic swelling test. Within the equine breeding sector, semen collection methods vary, although standard techniques include the use of artificial vaginas with a teaser mare, a phantom, or ground collection. Whilst collection method is reported to impact certain semen characteristics, this is not considered as a concern if consistency is maintained for collections of individual stallions (Jeannerat *et al*., 2018; Burger *et al*., 2015). The stallion will ejaculate in three fractions, with the intermediate portion being the ‘sperm-rich’ fraction. Ejaculate can be cleaned via density gradient centrifugation to remove cellular debris, or analysed as a raw sample. Colloid centrifugation is commonly used to improve stallion sperm quality, isolating spermatozoa with higher quality from the sample (Morrell and Nunes, 2016). Semen is typically diluted using pre-warmed extender medium, such as INRA 96, to preserve semen quality (Giaretta *et al*, 2017).

Equine semen quality parameters could be used to determine trends specific to the equine population and develop the horse as a novel comparative model. With any sentinel model, there are habitat and species-specific differences that must be acknowledged. For example, environmental differences exist between outdoor and stabled equine settings and indoor-human surroundings. The equine model has recently been presented as a sentinel model for neonatal health risks resulting from pollutant exposure from unconventional natural gas development systems (Mullen *et al*., 2020). However, the potential of the equine model as a sentinel species from a male reproductive perspective has not yet been fully explored. In 1999, a research group in France analysed secular trends in Breton Draught stallions from 1981 to 1996, reporting a 1.8% decline in semen volume per year, although no changes were observed in sperm count, and there was a slight increase in sperm concentration (Multigner *et al*., 1999). The increase in concentration was suggested to be attributed to the decline in volume due to the relationship between these two parameters and the fact that sperm count did not change appreciably. Further research determining trends in semen quality parameters, including motion characteristics, is warranted to develop a greater understanding of testicular health and function specifically.

Whilst there are evident limitations of the current study in equine semen trends (Multigner *et al*., 1999), the research group suggested that exposure to chemicals with anti-androgenic properties could be associated with the declining volume of seminal fluids, an androgen-mediated process (Multigner *et al*., 2000, 1999). Subsequent research has reported mean seminal volumes below that of the artificial insemination (AI) referencing ranges (60-120 ml) in the UK (Wilson and Flesner, 2017; Multigner *et al*., 1999). Secretions from the male accessory glands during ejaculation initiates essential alterations to the sperm cell membrane and surface proteins, resulting in important changes required for the processes of capacitation and fertilisation (Leeb, Sieme and Töpfer-Petersen, 2005). Exposure to factors that impair the functionality of the accessory glands may therefore result in the reduced fertilising ability of spermatozoa. Given the limited applicability of results to the 21st century associated with the equine study on semen trends (Multigner *et al*., 1999), further research is required to develop an understanding of current stallion fertility statuses. In analysing equine semen quality trends, results could also initiate investigations

into the suitability of the species as a comparative model for human reproductive research and contribute vital data to the debate surrounding declining semen quality. It is essential to account for specific industry and species-specific factors when determining semen quality trends in sentinel species. From an industry practice perspective, horses are used for sporting and competition purposes, the intensity and discipline of which may influence semen quality and other reproductive parameters (Wilson *et al.*, 2019). Such confounders could be a potential limitation of utilising the equine species as a comparative model for reproductive trends, so should be included in analyses where possible.

1.2.3 Reproductive comparisons of current sentinels and the equine species

In order to determine the full potential of sentinel species for trends in semen quality and their suitability for further investigation into the involvement of potential environmental aetiologies, differences in reproductive function must be considered. A fundamental difference is that the equine species are seasonal breeders (Shakeel *et al*., 2022), whereas humans are defined as continuous breeders. Considering the production of sperm, total spermatogenesis is defined as the time in which it takes for an immature germ cell to develop into a mature spermatozoa. The spermatogenic cycle is defined as the complete phase it takes for a spermatogonial stem cell to appear at a given section of the seminiferous epithelium (Zeng *et al*., 2006). Spermatogenesis, the process resulting in the production of spermatozoa from germ cell multiplication and differentiation, is formed of three distinct stages defined as spermatocytogenesis, meiosis, and spermiogenesis (Staub and Johnson, 2018). Sperm development is regulated by complex endocrine pathways, including that associated with the hypothalamic-pituitary-gonadal-axis (figure 1).

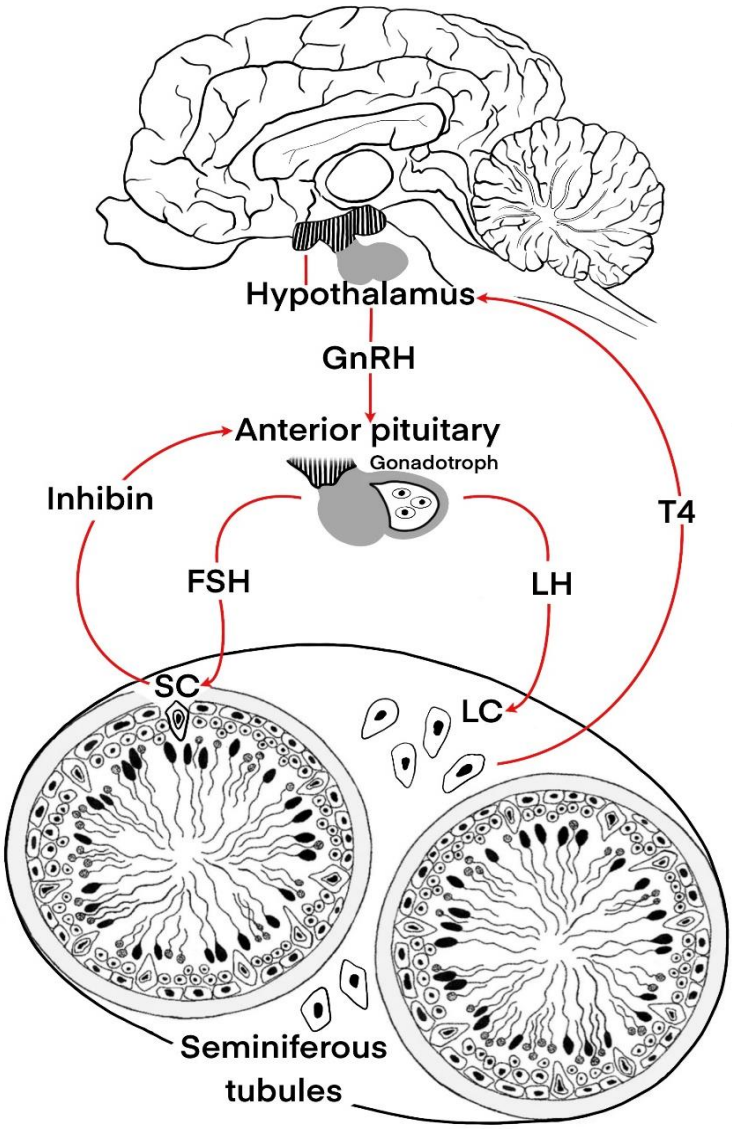


Figure 1 Hypothalamic-pituitary-gonadal (HPG) axis associated with endocrine control of the testis. GnRH: Gonadotropin releasing hormone; LH: Leutinising hormone; FSH: Follicle stimulating hormone; S-C: Sertoli cell; L-C: Leydig cell; T4: Testosterone.

In mammals, total spermatogenesis takes approximately 4.5 spermatogenic cycles (Zeng *et al*., 2006). When comparing the length of the spermatogenic cycle and total spermatogenesis of current herbivorous sentinels such as the ovine model, the equine species is closer to that reported in humans (Table 2) (Misell *et al*., 2006; Zeng *et al*., 2006; Johnson *et al.*, 1997; Heller and Clermont, 1963). This would indicate that the equine model could be a more effective sentinel for human spermatogenesis compared to the already established ovine species. Spermatogenesis cycle lengths in bovines are reportedly similar to that of horses (Staub and Johnson, 2018; Clément *et al*., 2010; Johnson *et al*., 1997), suggesting that the equine model could act as an important herbivorous sentinel for human-based reproductive trends and the analysis of environmental aetiologies.

Table 2: Species differences in the time period of a spermatogenic cycle and total spermatogenesis.

Species	Spermatogenic cycle	Total spermatogenesis	Reference
Humans	16.0 days	64.0 days (42-76 days)	(Misell *et al*., 2006; Heller and Clermont, 1963)
Equine	12.2 days	57.0 days	(Johnson *et al.*, 1997)
Canine	13.6 days	56.0 to 63.0 days	(Goericke-Pesch *et al*., 2009; Foote, Swierstra and Hunt, 1972)
Ovine	10.6 days	47.0 – 48.0 days	(Zeng *et al*., 2006)
Bovine	13.5 days	54.0 days	(Staub and Johnson, 2018; Clément *et al*., 2010)

In developing a comparative species for the assessment of reproductive trends and associated environmental aetiologies, ethical implications must also be considered. The ejaculate constitutes of spermatozoa and seminal plasma, which is produced by the accessory glands and contains a multitude of factors including fructose, prostaglandins, alpha-glucosidase, citric acid, and bicarbonate. Such factors are essential in supporting spermatozoa physiology and maintaining sperm quality (Ricardo, 2018). As such, analysing parameters of semen quality can provide an important material to measure the function and health of the testis and the accessory glands. The ability to harvest surplus semen samples from stallions as part of breeding soundness examinations provides accessibility to samples through non-invasive means (Oristaglio Turner, 2007).

Epididymal flushing from testes collected as part of routine castration procedures is an additional means to collect sperm reservoirs for further analysis (Boye *et al.*, 2019). When comparing the collection of equine testis with routine procedures in alternative herbivorous species, methods of castration may impact the ability to collect surplus tissue for analytical purposes. In cattle, castration protocols commonly include surgical removal or the use of rings or clamps (Stafford *et al.*, 2002). In ovine practice, lambs are castrated using a rubber ring or Burdizzo procedure (Melches *et al*., 2007). Such practices could prevent the harvesting and use of reproductive samples for analysis due to tissue destruction. This is opposed to common surgical equine castration procedures by which the testes remain intact, providing samples suitable for extensive research purposes.

1.2.4 Testicular dysgenesis in sentinel species

Cryptorchidism is a common congenital disorder reported in the equine population (Han *et al.*, 2020), although trends in prevalence remain unknown. In the dog sentinel model, increases in cryptorchidism have been reported in male pups, of which the sires had reduced sperm quality (Lea, Byers, *et al*., 2016). Preliminary evidence within the canid also suggests an increased incidence of testicular tumours over a 40-year period (Grieco *et al*., 2008). In sledge dogs, hypospadias has been linked to the exposure of anthropogenic ECs (Sonne *et al*., 2008). However, incidence rates of hypospadias in the equine population specifically are unreported (Bleul *et al*., 2007). Species-specific differences in reproductive aberrations introduces the limitations of cross-species comparisons and the utilisation of sentinel models. However, in general, there is limited research available regarding hypospadias in horses. Most equine hypospadias cases do not result in veterinary intervention (Bleul *et al*., 2007), so the true incidence rate of the malformation may be underestimated. Testicular degeneration, a common cause of subfertility and infertility in the stallion, has many of the same clinical signs and histopathological observations as that of TDS in human populations and has been linked to the exposure of anthropogenic ECs (Oristaglio Turner, 2007). Therefore, leading investigations into the initiation of the novel equine model as a sentinel for reproductive trends is warranted. A possible common aetiology further emphasises the need for research assessing the potential interactions of ECs within the equine species.

1.3 Environmental chemical aetiologies

Exposure to environmental chemicals (ECs), often endocrine disruptive in nature, is a key aetiological factor resulting in reproductive perturbations, including TDS (Skakkebaek *et al*., 2015). Anthropogenic ECs are typically utilised in a range of industrial and agricultural processes (Chen *et al.*, 2019). Uses include plasticisers, flame-retardants, solvents, preservatives, additives, coatings, and agrochemicals, including insecticides, pesticides, herbicides, fungicides, and fertilisers. An increase in industrialisation, consumerism, urbanisation, economic growth, and demands placed on agricultural production systems has led to the rapid increase in the synthesis and environmental deposition of contaminants (Guvvala *et al*., 2020). Under the Toxic Substances Control Act in the USA, approximately 82,000 chemicals have been regulated, given associated environmental

impacts and toxicity (Egeghy *et al.*, 2011). Common anthropogenic chemical classes include; bisphenols, dioxins, biphenyls, phthalates, parabens, organobromine compounds, polycyclic aromatic hydrocarbons (PAHs), and perfluorinated compounds (PFCs) (Pelch *et al.*, 2019; Ruzzin *et al*., 2012; Woodruff, Zota and Schwartz, 2011). Despite many chemicals being of anthropogenic origin, other inorganic sub-types of ECs include trace elements (Glorennec *et al*., 2016) and heavy metal ions (Nazir and Khan, 2015).

Considering organic anthropogenic ECs, natural sources of PAH deposition include volcanic eruptions and forest fires. Given their presence in organic matter, a secondary level of release through anthropogenic means includes the incomplete combustion of organic matter through burning wood, coal, and petroleum (Ramesh *et al*., 2004). Anthropogenic sources of PAH deposition are of primary concern (Samanta, Singh and Jain, 2002) given their extensive uses in processes of industrialisation. Polybrominated diphenyl ethers (PBDEs) are halogenated flame-retardants utilised in commercial products (Kurt-Karakus *et al.,* 2019). Polychlorinated biphenyls (PCBs) were historically utilised in the manufacturing of electronic equipment but incurred a statutory ban in the 1970s (Melymuk *et al*., 2022). Under the regulations of the Stockholm Convention in 2004, global restrictions and prohibition on the manufacture of PCBs and PBDEs were implemented due to their environmental persistence and toxicity (United Nations Environment Programme, 2001). PAHs, PBDEs, and PCBs, as well as several other halogenated compounds, are categorised as persistent organic pollutants (POPs), defined as toxic organic contaminants that are resistant to biodegradation, resulting in their persistence and accumulation within the environment (Antignac *et al*., 2016; Mobegi, Nyambaka and Nawiri, 2016). Whilst restricted pollutants such as PCBs are considered historical given ceased production, many remain persistent within the current environment (Lee *et al.*, 2017).

Industry led pressures result in the progressive development of alternative EC congeners, which often have equal or higher levels of toxicity compared to their predecessors (Wei *et al.*, 2015; Delfosse *et al*., 2012). Alternative flame retardant chemicals include organophosphate tri-esters, the environmental proliferation for which is commonly reported (Wei *et al.*, 2015). Another example is Bisphenols, with the primary congener, Bisphenol A (BPA), incurring stringent regulations (Ullah *et al.*, 2018). This has led to an

influx in the use and deposition of alternative analogues, of which there are now 15 (Zhang *et al.*, 2019), in addition to multiple halo-substituted derivatives (Kitamura *et al*., 2005). Evolving regulations in the manufacture and use of ECs sees an increasing number of chemical congeners released into the surrounding environment. With the ability of impacting the 'environmental pollutome', chemical mixtures are becoming more complex (Sumner *et al*., 2020). As such, concern over exposure to a combination of a growing number of toxic EC congeners is steadily increasing.

1.4 Environmental deposition and exposure routes of EC contaminants

Environmental deposition of ECs occurs through direct industrial and agricultural emission and migration from product matrices in which contaminants originate (Wania, 2003). Chemical levels surrounding areas of high industrialisation are reported to have elevated levels of contamination (Wang *et al*., 2018) when compared to more rural areas (Peng *et al*., 2013). Given the production of PAHs through burning organic materials, including fossil fuels, contamination of industrial areas is of primary concern (Xu *et al.*, 2021; Zhang *et al.*, 2006). The ability of ECs to leach from product matrices into surrounding environments has led to their ubiquitous deposition, now considered a global health concern (Lenoir *et al*., 2016; Ike *et al*., 2006). An important source of environmental deposition of regulated or prohibited chemicals is municipal landfill sites due to the presence of contaminants in previously discarded waste materials (Sibiya, Olukunle and Okonkwo, 2017). As such, waste sites act as sources of environmental contamination to both historical ECs with ceased or restricted production lines and current ECs. Recycling of industrial resources, including electronic waste, acts as a secondary level of environmental deposition (Zhang *et al*., 2020; Chakraborty *et al*., 2019).

The reuse of biosolids as sewage sludge fertiliser is reported to be an additional route for ECs to enter the environment (Fernandes, Lake, *et al*., 2019). Sewage sludge is produced through the recycling of wastewater from treatment plants and organic biosolids. The nutrient-rich properties of sewage sludge result in the reported effectiveness of this material as an agricultural fertiliser. It is considered a sustainable method for fertilising agricultural land (Lamastra, Suciu and Trevisan, 2018; Inglezakis *et al*., 2011), reducing the need for synthetic fertilisers (Seleiman, Santanen and Mäkelä, 2020). However, sewage

sludge is also reported to be contaminated with numerous ECs (Inglezakis *et al*., 2011), acting as a method of redistributing contaminants into terrestrial ecosystems.

Sewage sludge is treated through processes of disinfection, anaerobic- and bio-stabilisation, composting, and incineration (Cieślik, Namieśnik and Konieczka, 2015). Contaminants can be deposited into terrestrial and aquatic environments via aqueous effluent or volatilisation, adsorption to sludge, and biotic and abiotic degradation (Zuloaga *et al*., 2012). In 2015, incineration was the most commonly used method for the disposal of sewage sludge in the EU, with 61.5% burnt, compared to 38.2% used for agricultural land application (Hudcová, Vymazal and Rozkošný, 2019). Given that PAHs are associated with burning organic matter, the incineration of sewage sludge could provide a route for contaminants to enter the atmosphere. Direct land application is associated with EC accumulation within terrestrial ecosystems and contaminates aquatic ecosystems through water run-offs (Zhang, Barron and Sturzenbaum, 2021). Given the use of sewage sludge in pasture management and the subsequent contamination of agricultural and terrestrial ecosystems, this method of environmental deposition is likely to act as a key exposure route to grazing species, including horses.

The national legislative European Union (EU) Directive 86/278/EEC (CEC 1986) enforced a set of maximum permissible limits for the land application of sewage sludge concerning heavy metal contamination. However, the legislation contained no concentration limits for organic chemicals and failed to consider the long-term accumulation rates of ECs in top-level soils (Hudcová, Vymazal and Rozkošný, 2019; Inglezakis *et al.*, 2011). Further research initiated by the European commission suggested threshold values for ECs including, Di (2-ethylhexyl) phthalate (DEHP), PAHs, PCBs, polychlorinated dibenzo-dioxins (PCDDs), and polychlorinated dibenzo-furans (PCDFs) (Zuloaga *et al*., 2012). Concentration limits for DEHP, PAHs, and PCBs are regulated by the EU (European Commission, 2000) at 100 mg/kg/DM, 6 mg/kg/DM, and 0.8 mg/kg/DM of sewage sludge, respectively, although national differences exist (Hudcová, Vymazal and Rozkošný, 2019). Whilst regulations are in place, current treatment methods fail to effectively remove ECs from sewage sludge, which accumulate in agricultural, terrestrial, and aquatic ecosystems, resulting in exposure to a range of species.

Sewage sludge land application also results in the dissemination of micro plastic particles (MPPs) into surrounding environments (Weithmann *et al*., 2018). Regulations in the EU Directive 86/278/ECC and national regulations associated with European states fail to include recommendations on maximum values of sewage sludge MPPs (Milojevic and Cydzik-Kwiatkowska, 2021; Stubenrauch and Ekardt, 2020). MPPs are now reported to contaminate agricultural soil samples (Piehl *et al*., 2018; Zhang and Liu, 2018), raising concern over the risks associated with environmental deposition. In soils with a history of sewage sludge application, MPPs were detected at levels 256% greater than in lands with no history of sludge treatment (van den Berg *et al*., 2020). Such levels of soil contamination and presence within biota at the lower levels of the trophic infrastructure raises concern regarding MPP ingestion by herbivorous species including, the equine model, and biomagnification at higher trophic levels (Morris *et al*., 2018).

MPPs are defined as plastics with a diameter of less than 5 mm. Primary MPPs are those that are commercially produced in smaller particles including, in cosmetics and synthetic textile fibres. Secondary MPPs are produced by the environmental biodegradation of larger pieces of plastic debris (Zhang and Liu, 2018). In 2019, the global yearly production of plastic reached approximately 370 million tonnes (Plastics Europe, 2021). DEHP and Bisphenols are two ECs, which are utilised in plastics to give them their malleable properties (Amir *et al*., 2021; Zhao *et al*., 2020). DEHP is a low molecular weight (LMW) chemical, which, along with other phthalates, accounts for 70% of plasticisers within the 2014 market (Pivnenko *et al*., 2016). The ability of chemicals originating from polymer matrices to leach into the surrounding environment or digestive tract if ingested acts as an initial exposure route to biota. Additionally, MPPs act as vectors to other hydrophobic organic chemicals (Caron *et al*., 2018). In simulated aquatic environments, MPPs act as both sinks and sources to the toxic BPA (Liu *et al*., 2019). Thus, MPPs act as transport vectors, facilitating the widespread dissemination and uptake of a complex range of toxic ECs. It is thus concerning that research surrounding MPP contamination of terrestrial and agroecosystems is scarce.

Considering equine management practices, horses are fed feedstuffs packaged in plastics, haylages wrapped in plastic films, hay bound in polyethylene twines, and are often provided feeds in plastic containers and hay nets made of polypropylene, polyester or nylon materials (Lind, 2020). In alternative species such as cattle, concentrate feeds are reported to be a primary route of EC exposure (Mclachlan, 1993), likely a result of chemical leaching from plastic wrappings. Equine feed management practices may also result in the contamination of feedstuff materials from chemical leaching and MPP contamination. MPPs have been detected in equine manure samples (Lind, 2020), confirming the ingestion of plastic debris and raising concern over the effects of exposure. Currently, research has not addressed the topic of equine feedstuff MPP and EC contamination, with potential implications on the health and welfare of the species and industry relevance for feeding management practices.

Plastic films for haylages and silages are reported to be a key source of MPP deposition in terrestrial and agroecosystems (Harms *et al.*, 2021). Due to the presence of agrochemicals, it can be difficult to recycle film materials (Steinmetz *et al*., 2016). The disposal of plastics within the agricultural sector specifically requires further policy change to prevent excess environmental contamination. On-farm plastic burning, an illegal yet reported process, results in the deposition of dioxins at levels 20 times higher than controlled incineration (Levitan and Barros, 2003), raising serious concern regarding the atmospheric deposition of ECs. The EU has not yet defined procedures for the disposal or recycling of plastic wastes produced from agricultural production lines (Steinmetz *et al.*, 2016). Considering plastic disposal strategies in general industries, current policies are not yet effective. In the UK, between 10 and 50% of plastics end up in landfill (Plastics Europe, 2015), the leachate of which is likely to result in a continuous source of EC deposition. In southern and eastern European countries, plastic disposal in landfills is reported to be more than 50% (Plastics Europe, 2015), raising serious concern over this material as a source of EC and MPP deposition. Whilst recycling reduces the need for the synthesis of virgin plastics and raw chemical constituents (Pivnenko *et al*., 2016), solid waste recycling results in elevated environmental deposition of phthalates, including DEHP (Ding *et al*., 2022). Such research would suggest that recycling plastics is not an effective strategy in reducing chemical deposition, raising concern over the contamination of terrestrial ecosystems.

1.4.1 Pasture contamination

The ability of ECs to leach from products, including plastics, into the environment has resulted in the ubiquitous nature of contaminants; present in the air, water, soil, and vegetation (figure 2) (Eladak *et al.*, 2018; Lenoir *et al*., 2016). Here, ECs are available for uptake by animals and humans on a global scale (Terzaghi *et al*., 2018). Due to the outlined ubiquitous nature of ECs, exposure to the horse could arise through multiple means. Thus, there is a distinct need to assess typical stallion management practices, including feeding, housing, and turnout regimes, as potential routes of EC exposure. ECs have been detected in water, soil, pastures, feedstuff, and silage provided to cattle and sheep (Schulz *et al*., 2005), which are also likely to act as key exposure routes in the equine model. Ruminants, species sharing close environments to the horse, are exposed to ECs through the ingestion of contaminated pastures and water, percutaneous absorption, and inhalation of aerosolised EC particles (Rhind, Evans, *et al*., 2010). Highly chlorinated congeners, including PCBs, adsorb to soil organic matter, reducing uptake through root structures. Instead, high molecular weight (HMW) ECs are readily absorbed into vegetation from the surrounding air (Terzaghi *et al.*, 2018). When pasture levels are low, direct consumption of contaminated soils may result in an additional exposure route to grazing species. In horses, dry soil intake was estimated at 543 g to 648 g per animal per day (Jurjanz *et al.*, 2021). Ingestion of soils contaminated with complex mixtures of ECs is likely to result in significant equine bodily burdens.

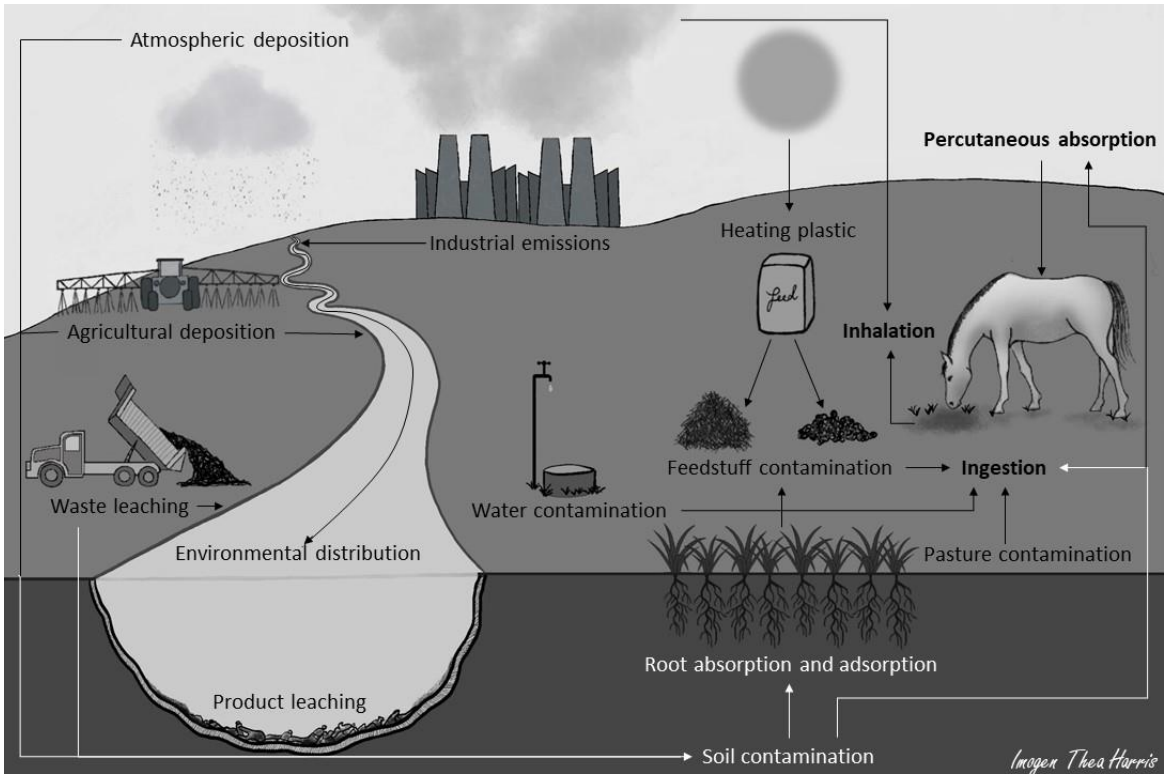


Figure 2: Environmental deposition and potential equine specific EC exposure routes (authors own).

PAHs are predominantly excreted through faecal deposition, whilst 10% are excreted in urine (Castano-Vinyals *et al.*, 2004). In wild horses grazing areas surrounding intensive agricultural areas in Galicia, Spain, PAHs were detected at 2.6 µg/kg in equine manure, similar levels to that detected in pig excrement (Rey-Salgueiro *et al.*, 2008). Geographical variations in PAH concentrations were noted, a direct result of the contamination levels in the surrounding environment. Contaminants present within equine manure, with an animal producing approximately 23 kg of manure per day, may be redistributed during manure storage and spreading (Westendorf *et al.*, 2012). Horse manure for stabled horses also contains bedding materials (Hadin and Eriksson, 2016). Treated wood sources used as animal bedding is reported to be a route of EC environmental deposition (Fernandes, Lake, *et al.*, 2019). Analysis of woodchip shavings at an equine breeding farm found concentrations of pentachlorophenol ranging from 630 to 9,810 mg/kg of bedding (Kerkvliet *et al.*, 1992). The analysis confirmed levels of pentachlorophenol and its degradation products; PCDDs, and PCDFs within tissue samples of individuals exposed to the bedding, confirming chemical bioavailability and equine uptake (Kerkvliet *et al.*, 1992).

Concentrations of PCDD and PCDF have also been detected in eggs extracted from hens reared on contaminated wood shavings, with an overall half-life of 3.8 weeks (Brambilla *et*

al., 2009), suggesting chemical localisation to the reproductive tract. In hen bedding made from untreated, unused wood sources as a control, levels of ECs including PCDD, PCDF, PCBs, PBDEs, Perfluorooctanesulfonic acid (PFOS), hexabrominated cyclododecane, and polychlorinated naphthalenes were detected between 0.01 µg/kg and 5.9 ng/kg of material indicating relatively low levels of exposure (Fernandes, Lake, *et al*., 2019). However, continuous exposure to persistent pollutants present within bedding materials could result in the bioaccumulation of ECs in animals, including horses. A lack of current research analysing equine bedding materials for ECs limits our understanding of contaminant levels specific to equine management practices. Contamination of bedding materials may act as a direct exposure route to horses through percutaneous absorption and inhalation of airborne dust particles (Xing *et al*., 2011). Equine staff handling contaminated bedding materials may be exposed to similar levels through equivalent pathways, acting as a potential occupational health risk. Terrestrial environmental deposition of contaminants present within bedding and manure could exist during storage, treatment and spreading of waste materials.

Considering alternative means of EC exposure through dermal contact, fabrics treated with waterproofing materials are suggested to be an exposure route to perfluoroalkyl substances in humans (Ragnarsdóttir, Abdallah and Harrad, 2022). In addition, chemicals such as PBDE have been reported to adsorb to synthetic and cotton textiles, acting as a sink of contaminants and potential route of exposure through dermal contact (Saini *et al.*, 2016). A current link between equine rug materials and exposure to contaminants through percutaneous absorption has not yet been evaluated. Given the toxicity of contaminants found present within waterproofing materials and synthetic and cotton textiles, further research analysing equine rugs as a potential exposure route to horses is warranted.

Research in horses has suggested the use of insecticide-treated rugs to deter biting midges in the UK (Baker *et al*., 2015). Pyrethroid and Cypermethrin are two insecticides used in commercial sprays designed to deter flies through direct application onto the skin of animals and have been recommended as a horse rug treatment (Baker *et al*., 2015). Both chemicals are associated with environmental pollution and impaired reproductive function through interaction with endocrinology pathways (Guvvala *et al*., 2020). In rats, N, N-

Diethyl-meta-toluamide (DEET), and permethrin, chemicals present in commercially available equine insect repellent sprays, are reported to impair testicular and sperm function through epigenetic mechanisms, resulting in transgenerational effects (Manikkam *et al*., 2012). Therefore, commercial fly deterrents could contribute to reproductive aberrations reported in stallions, although a current link has not yet been analysed. In addition, the action of applying fly deterrent treatments to horses could result in health risks associated with human exposure and lead to increased environmental deposition of contaminants, including into surrounding soil reservoirs.

During rainfall, EC particles present within the soil become available in surface water and migrate to aquatic ecosystems, including rivers, lakes, and oceans (Diringer *et al*., 2015). This means that EC exposure in animals, including the equine model, could be within drinking water (Rahman, Yanful and Jasim, 2009). At two Standardbred broodmare farms in the USA, 27 and 40 PAH congeners above the limit of detection were reported in water and air samples, respectively. ΣPAH levels ranged from 26.59 ng/L and 40.0 ng/L for water and 631 ng/m^3 and 789 ng/m^3 for air, depending on sample site (Mullen *et al*., 2020). Despite growing evidence to support equine exposure, this study failed to assess chemical concentrations within the equine tissues, with such research essential in understanding accurate equine contamination levels.

1.4.2 EC exposure and the utilisation of biomonitor species.

Whilst the current research base is indicative of equine EC exposure, our understanding of contamination specific to the male reproductive system remains unknown. Biomonitors are species utilised to monitor and predict habitat changes in potentially toxic environmental factors (Amadi, Frazzoli and Orisakwe, 2020). DEHP has been detected at a mean concentration of 7.85 ± 2.45 nM in the follicular fluid of equine ovaries (Marzano *et al.*, 2019), indicative of accumulation specific to the reproductive organs. However, physiological differences are likely to result in differing levels between the ovaries and testis, with further research required specific to the male equine population.

The value of using equine samples to biomonitor chemical contamination levels in environments has previously been recognised. However, research has focused on utilising hair samples (Yavuz *et al*., 2022; Te *et al.*, 2020; Madejón, Domínguez and Murillo, 2012, 2009). Median levels of ∑16PCBs were detected at 2 ng/g in horsehair, higher than that found in cattle and sheep's wool. Levels detected in horsehair were only 0.07 ng/g less than that found in human hair (Te *et al*., 2020). Such research would indicate similar levels of contaminant exposure in humans and horses. In horses stabled in Turkey, POPs including organophosphate pesticides, OCPs, PCBs, and PAHs were found in 85%, 98%, 58%, and 56% of the hair samples analysed at concentrations of 4.04 to 3234.89 ng/g, 2.52 to 3918.04 ng/g, 0.32 to 732.93 ng/g, and 1.57 to 66.07 ng/g, respectively (Yavuz *et al*., 2022). Elevated levels of all congeners detected in a high percentage of horse hair samples analysed (Yavuz *et al*., 2022) indicate that the equine species are exposed to ECs and potentially the health risks associated. ΣPAH levels in dog hair were substantially higher compared to horses, with concentrations ranging from 201 to 556 ng/g (Zheng *et al*., 2011). Species differences are likely to be a result of exposure levels through differential management processes, dietary factors, and potential variability in sampling regions.

An alternative methodological perspective of assessing EC contamination in equines is analysing horse meat as a commercially available food product for human consumption (Jurjanz *et al.*, 2021). Horsemeat sampled from Belgium was contaminated with Σ6PCB concentrations of up to 5.50 ng/g fat (Cimenci *et al*., 2013). In equine fat samples, Σ7PCB concentrations ranged from 5.35 to 140 ng/g lipid weight. The chemicals utilised within flame retardant materials, Σ7PBDEs, were detected in equine fat samples at concentrations ranging from below limit of detection (0.78 to 1.90 ng/g lipid weight dependent on congener) to 6.34 ng/g lipid weight (Naert and Van Peteghem, 2007). The presence of ECs within meat samples is indicative of bodily burdens of the animal source and poses a health risk to humans consuming contaminated meat (Guruge *et al.*, 2008). It is worth considering that contamination levels present within commercial meat products may also be representative of chemical leaching from the foodstuff packaging materials (Taylor and Sapozhnikova, 2022). Additionally, potential degradation of the chemicals may occur within the tissue, dependent on the physiochemical properties of the congener. Therefore, further analysis of biological levels of ECs specific to horses, including accumulation within the reproductive organs, is warranted.

Contamination levels of ECs specific to the male equine reproductive system remain unknown. Current understanding surrounding testicular contamination of ECs is extracted from the dog sentinel model. Mean levels of DEHP and PCB 153 in dog testis were reported at 182 ± 44 μg/kg and 0.063 ± 0.01 μg/kg, respectively (Lea, Byers, *et al.,* 2016). Levels of the PCB congener 153 was detected at a mean value of 13.05 ± 3.18 μg/g in canine ejaculates (Lea, Byers, *et al.*, 2016). Differences in concentrations between materials could partially be a result of differing lipid contents of the testicular tissue and ejaculate due to the lipophilic nature of ECs (Bourez *et al.*, 2013). Testicular tissue and ejaculate are two fundamental materials that provide accurate readings of EC contamination levels specific to the male reproductive system. Contamination of the testicular tissue represents the ECs that sperm are exposed to during spermatogenesis. The ejaculate comprises of the mature sperm originating from the testes and other seminal fluids that combine with the sperm portion during ejaculation (World Health Organization, 2021). Therefore, contaminant levels present within the ejaculate are likely to reflect acute EC exposure to spermatozoa.

In alternative grazing species sharing close environmental conditions to equine populations, a range of ECs have been detected. The mean concentration of Σ5PCBs in the seminal plasma of bulls (n=10), rams (n=30), goats (n=15) and boars (n=23) was measured at 1.06 ± 0.29 ng/ml, 1.21 ± 0.64 ng/ml, 1.44 ± 0.94 ng/ml and 0.64 ± 0.09 ng/ml, respectively (Kamarianos *et al.*, 2003). Whilst levels of PCB contamination are lower than concentrations reported in dog ejaculates (Lea, Byers, *et al*., 2016), results are still indicative of EC exposure and bioaccumulation in herbivorous species. Higher levels of contamination in the carnivorous dog compared to herbivorous species also exist in non-reproductive biological materials. The mean concentration of PFOS in dog blood serum from Japan was 57 ng/ml, 36 times greater than the concentration detected in horse sera (0.71 ng/ml) (Guruge *et al*., 2008). Consumption of contaminated meat is considered as a main exposure route in carnivorous and omnivorous species, given the lipophilic nature of many ECs (Fernandes, Mortimer, *et al.*, 2019). The ingestion of contaminated meat sources leads to EC biomagnification across trophic levels, with apex predators incurring the highest bodily burdens (Prince, Taylor and Angelini, 2020; Jepson *et al*., 2016). In apex predator species, population declines are associated with EC-induced reproductive aberrations. Species of concern include killer whales (Desforges *et al*., 2018), harbour porpoises (Williams *et al*., 2021), alligators, and crocodiles (Gonzalez-Jauregui *et al.*, 2012). However,

the presence of ECs in herbivorous species such as horses indicates that the consumption of contaminated meat is not the only route of exposure, with alternative routes specific to the equine model warranting further investigation.

The sewage sludge sheep model is a common herbivorous research tool utilised to analyse the exposure and biological contamination associated with complex mixtures of ECs (Viguié *et al*., 2020). This model is valuable in understanding real-life exposure given the extensive number of ECs in sewage sludge, which is representative of the current environmental contamination status. Considering the distribution of PCBs and 2,3,7,8-Tetrachlorodibenzo-P-dioxin (TCDD), contaminants were detected at varying concentrations in adipose tissue, liver, muscle, serum, faeces, and wool samples, indicative of accumulation throughout the body (Lerch *et al*., 2020). Increasing duration of EC exposure was associated with significant increases in EC concentrations detected in the liver samples of ewes grazing on sewage sludge treated pastures (Rhind *et al*., 2011). Phthalate concentrations were elevated in sheep grazing on both control and sewage sludge treated pastures, indicating that base-level environmental contamination is already of concern (Rhind *et al*., 2005). In another study, ECs accumulated within maternal and foetal liver samples of sheep, although concentrations of PAHs, DEHP, PBDEs, and PCBs between sewage sludge-treated and inorganic fertiliser-treated pastures did not differ significantly (Rhind *et al*., 2010). From a reproductive perspective, whilst research indicates an association between TDS and exposure to ECs, there is limited understanding of the specific testicular contamination levels in the ovine model. With reference to the utilisation of equines for future research, as with the dog sentinel, the ability to collect surplus testicular tissue from routine castrations creates an easily accessible tissue for analysis. The novel equine model could therefore pose as an invaluable sentinel to biomonitor environmental and human health and should be utilised within further research associated with environmental toxicology.

1.5 Effects of ECs on reproductive function

The current research base provides a limited understanding of the effects of ECs specific to equine reproductive function. In an equine *in vitro* study, sperm incubated for 72 hours at 5°C in TCDD did not display reduced motion characteristics, membrane integrity, lipid peroxidation, or chromatin status compared to the control group (Nowak *et al.*, 2018).

However, equine oocytes presented increased sensitivity to TCDD, with exposure resulting in aberrations to oocyte maturation and a higher degree of degradation compared to control groups (Nowak *et al*., 2018). Whilst the work by Nowak demonstrates perturbed reproductive potential in the female, the TCDD concentrations utilised were taken from previous research using 'toxicological' levels (Kimbrough *et al.,* 1977). This epidemiological study was associated with the treatment of riding arenas with waste sludge oils, resulting in high levels of TCDD and PCB exposure and subsequently 62 deaths, multiple illnesses, and abortions. Soil samples from the year of treatment reported 31.8 to 33.0 ppm and 1350 to 1590 ppm TCDD and PCB, respectively (Kimbrough *et al*., 1977). Such 'toxicological' levels are an inaccurate representation of contamination that the equine population face in the modern day. Further research is required to specify contamination levels in the equine species, including testicular accumulation, in order to initiate investigations into the true effects of ECs on reproductive parameters such as semen quality. In addition, *in vitro* research analysing the effects of a single EC in isolation has limited applicability to the true effects of ECs, given that exposure is to a complex mixture of contaminants.

To determine the effects of real life exposure to a broad range of ECs, the sewage sludge sheep model is most commonly utilised. Exposure to EC mixtures in sewage sludge is associated with aberrations to testicular development in sheep. Gestational exposure during an 80-day period resulted in reduced testis weight, whilst those exposed to EC contaminated sewage sludge during the last 60-140 days of pregnancy presented a reduced anogenital distance and reduced testosterone (T_4) levels (Lea *et al*., 2022). Testicular weight is directly associated with reproductive efficiency, with a reduction likely to have direct implications on a male's fertility status (Yuan *et al*., 2018). Neonatal sheep exposed to EC-contaminated biosolids during gestation presented a reduced number of gonocytes within the seminiferous tubules (Elcombe *et al*., 2022, 2021), which phenotypically is reflective of testicular atrophy and characteristic of TDS. Sewage sludge EC exposure during the gestational period was associated with reduced testicular weight and a reduction in the number of gonocytes, Sertoli cells (S-C), and Leydig cells (L-C) (Paul *et al.*, 2005). The L-Cs are associated with testosterone biosynthesis (Zirkin and Papadopoulos, 2018), whilst S-Cs contain T_4 and follicle-stimulating hormone (FSH) receptors (Walker and Cheng, 2005) and are primary targets of ECs with endocrine disrupting properties (Wang *et al*., 2021; Wan *et al*., 2013). The S-Cs form a specialised environment within the testicular seminiferous

tubules and secrete nutrients, growth factors, and hormones to support the development of germ cells (Walker and Cheng, 2005). Therefore, a reduction in S-C and L-C numbers, as reported in EC exposed sheep (Paul *et al.*, 2005), is likely to be associated with endocrine and testicular infrastructure alterations and subsequently compromised spermatogenesis.

1.5.1 Mechanisms of action associated with EC exposure

During spermatogenesis within the seminiferous tubules of the testis, ECs are reported to have reprotoxic effects through endocrinology interactions (Ianos *et al*., 2018; Daoud *et al*., 2017). In literature, ECs discussed are often referred to as endocrine disrupting chemicals (EDCs), given their ability to alter hormonal and homeostatic processes (De Coster and Van Larebeke, 2012). ECs interfere with the fundamental processes of hormonal secretion, synthesis, metabolism, and transportation, resulting in reproductive developmental and homeostatic abnormalities (Amir *et al.*, 2021). Interestingly, in a population of men presenting at andrology clinics, incidences of hypogonadism, a condition in which hormones fail to be produced at an appropriate level, increased significantly between 2008 and 2018 (Fallara *et al*., 2021). Hormonal alterations could be a result of increasing exposure to EC contaminants.

Contaminants are reported to exert actions through binding to nuclear hormone receptors, including oestrogen (ER) and androgen receptors (AR) (Delfosse *et al.*, 2012; Kitamura *et al.*, 2005), resulting in cellular alterations (figure 3). This is concerning as androgenic and oestrogenic pathways are fundamental in reproductive development and function (Amir *et al*., 2021). In stallions, oestrogens and androgens are closely involved in spermatogenesis (Lemazurier *et al*., 2002), with endocrine disruption likely to result in perturbed sperm production. ECs also elicit responses through alternative mechanisms, including orphan receptors (aryl hydrocarbon receptor; AhR), non-nuclear steroid receptors, non-steroid receptors (neurotransmitters), and enzymatic pathways (De Coster and Van Larebeke, 2012).

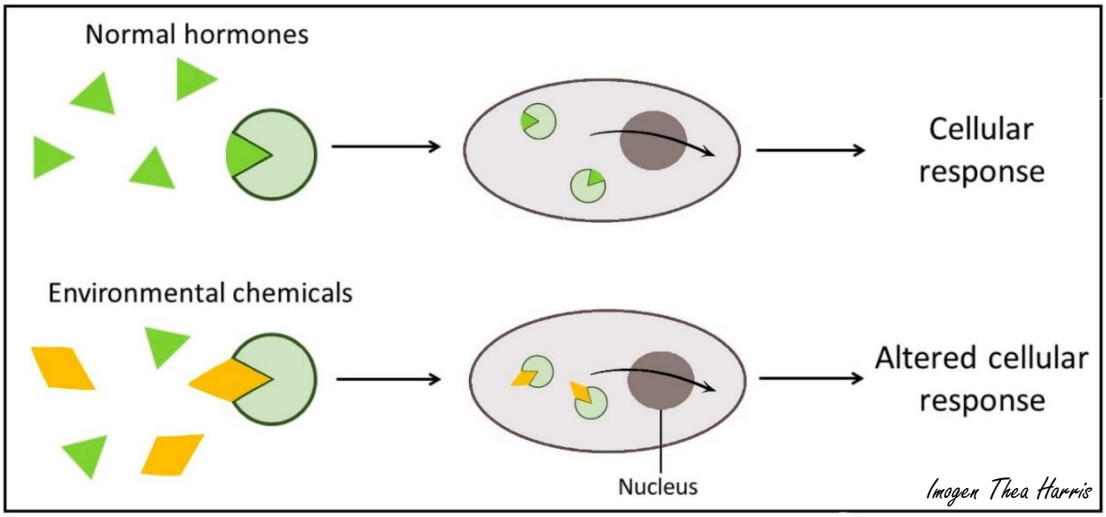


Figure 3: Docking of ECs with compatible biological receptors and cellular responses (authors own).

Depending on specific chemical composition, interruption of endocrine pathways are a result of affinities for different receptors. In murine models, Bisphenol-induced steroidogenesis alterations have been indicated to occur through antagonising or agonising the nuclear hormone ER and AR pathways, inducing oestrogenic, anti-oestrogenic, androgenic, or anti-androgenic alterations (Ziv-Gal *et al*., 2013; Delfosse *et al*., 2012; Kitamura *et al.*, 2005). An agonist binds to a receptor and activates a response, whilst an antagonist inhibits the action of the receptor. Through interaction with ER and AR pathways, EC exposure is associated with the interference of a number of mechanisms involved in reproductive health and function, including chromosomal aberrations and DNA damage (Amir *et al*., 2021).

Oestrogen (E_2) is essential for developmental processes and the maintenance of multiple physiological reproductive mechanisms (Cano-Nicolau *et al.*, 2016; Heldring *et al*., 2007). In ERs, the carboxylic acid (-COOH) terminal, a multifunctional ligand binding site, forms an open structure that enables the docking of ECs such as Bisphenols, resulting in the contaminant mimicking the oestradiol hormone (Heldring *et al.*, 2007). Oestrogenic activity through the nucleus occurs through ligand binding to ER-alpha (ERα) and ER-beta (ERβ) pathways. The secondary pathway, the membrane-mediated pathway, involves the interaction of the ligand with G-Protein-coupled receptors (GPCRs) and membrane-bound ERs, resulting in the activation of the insulin-like growth factor receptor and the epidermal growth factor receptor (Amir *et al.*, 2021).

Bisphenol A and PCBs are reported to act as agonists to ER pathways, whilst PBDE elicits both agonistic and antagonistic responses through ER binding (Amir *et al*., 2021). In a molecular docking programme using the human ERα, PBDEs presenting anti-oestrogenic activities extended into the receptor channel, where raloxifene and 4-hyroxytamoxifen, two ER antagonists, are usually bound (Yang *et al.*, 2010). The PBDE congeners that occupy the ERα docking channel are likely to result in anti-oestrogenic effects. The PBDE congeners that elicit no anti-oestrogenic activity are reported to bind to the cavity in a comparable way to oestrogen (Yang *et al.*, 2010). Phthalates, a category of EC esters, can bind to ERα and ERβ and elicit agonistic and antagonistic effects on the pathways (Engel *et al*., 2017). Considering the affinity of BPA to GPCRs, a group of transmembrane proteins involved in signal transduction pathways (Tang *et al.*, 2012), activation of this pathway results in the interference in the developmental programming of the proliferation and differentiation of male germ cells (Bouskine *et al*., 2009).

Stallions are reported to have particularly high levels of testicular oestrogens compared to other mammalian species (Arkoun *et al.*, 2014), which may result in reduced susceptibility to endocrinology aberrations resulting from oestrogenic compound exposure. Differences in reproductive physiology between humans and horses could act as a potential limitation of cross-species evaluations, although this is true of using any comparative species for human reproductive research. In addition, whilst some ECs are reported to act through oestrogenic mechanisms, contaminants also interact with alternative pathways, and so the effects of ECs on stallion reproductive function should not be discredited without further species-specific investigations.

As well as oestrogenic pathways, male mammal physiology requires androgenic mechanisms to sustain reproductive function. The AR interacts with testosterone T_4 and 5α-dihydrotestosterone (DHT), resulting in the transcription of target genes (Schrader and Cooke, 2003). The activation of the AR can occur through genomic and non-genomic pathways. Genomic responses of ligand activation involve modifications to the processes of transcription and protein synthesis, whilst non-genomic pathways are typically associated with membrane-initiated enzymatic modifications with other signalling molecules (De Coster and Van Larebeke, 2012; Watson *et al.*, 2007). Structurally, the AR

contains a -COOH terminal with a ligand binding domain, much like the ER structure (Amir *et al*., 2021). T_4 controls prenatal masculinisation, responsible for the development of the male genitalia (Kolatorova *et al*., 2018). Together with its derivative DHT, these hormones are essential in the sexual differentiation of the male phenotype (Kalfa *et al*., 2013). Exposure to ECs that interact with these pathways is therefore of significant concern for testicular development and, subsequently the ability of the testis to sustain an environment that supports spermatogenesis in adult individuals.

A range of ECs are reported to elicit reproductive toxicity through interaction with androgenic pathways, disrupting the sensitive homeostatic balance which is required for the health and function of the testis. BPA and PBDEs are reported to have antagonistic actions on the AR, whilst PCBs are reported to have both agonistic and antagonistic results (Amir *et al*., 2021). The 4-hydroxyl group in Bisphenol congeners is suggested to be involved in their anti-androgenic effects (Heldring *et al.*, 2007; Kitamura *et al.*, 2005). A range of PCB congeners, including 49, 66, 74, and 105, were reported to completely antagonise the production of DHT through interaction with the AR pathway *in vitro*, whilst alternative PCB congeners elicited a reduced antagonistic effect (Schrader and Cooke, 2003). Benzo[a]pyrene, a PAH congener, is reported to impair epididymal sperm quality through androgenic pathways, including the aberration of T_4 concentrations (Oh, 2014). Sperm quality aberrations induced by the interactions of ECs with androgenic mechanisms are likely to result in compromised male reproductive potential and success.

Given that real-life exposure is to a complex mixture of contaminants, the interactions of ECs with receptors in the presence of alternative congeners are essential in understanding the likely outcomes on reproductive health and function. In rats, exposure to DE-71, a PBDE mixture, inhibited DHT-induced transcriptional activation through interaction with the AR pathway, associated with the restricted growth of the testis (Stoker *et al*., 2005). A commercially available mixture of PCBs, referred to as Delour-103, was reported to initiate androgenic activity in an *in vitro* yeast assay model (Svobodová *et al.*, 2009). Exposure to Aroclor, an alternative PCB mixture, antagonised DHT activation of the AR pathway by 50% (Schrader and Cooke, 2003). In an *in vitro* assay, phthalate plasticisers are reported to have inhibitory effects on the AR pathway (Engel *et al.*, 2017), resulting in primarily anti-steroidal

activity (Sarath Josh *et al*., 2016). In relation to the dog sentinel model, DEHP and mixtures of PCBs at environmentally relevant levels were reported to have anti-androgenic and oestrogenic effects, respectively, resulting in ratio and concentration-dependent effects on sperm progressive motility and DNA fragmentation (Sumner *et al*., 2019). Whilst interaction with the AR and ER are fundamental in the mechanistic action of EC toxicity, research indicates that contaminants may elicit reproductive alterations through other pathways.

Dioxins and dioxin like-PCBs are reported to induce apoptosis of the seminiferous epithelium through the upregulation of AhR mRNA (Ghotbaddini and Powell, 2015). The AhR is essential for the development and maintenance of structurally functional seminiferous tubules. Male AhR knockout mice present histopathological abnormalities, including apoptosis of the seminiferous epithelium, multinucleated giant cells, and vacuoles, indicative of missing stages in germ cell development, retained spermatids, and the degradation of the seminiferous epithelium (Hansen *et al*., 2014). Apoptosis resulting from EC exposure has severe consequences on fertility potential. Interestingly, many of these histopathological signs are also observed in stallions presenting testicular degeneration (Oristaglio Turner, 2007). Histopathological similarities could indicate some sort of depletion of the AhR receptors within the stallion testis that could be induced by exposure to AhR ligand chemicals, including TCDD. Such interactions could begin to explain the pathology of idiopathic testicular degeneration within the stallion leading to infertility. Differences in congener-specific interactions with biological receptors highlight the complexity of EC exposure and their mechanisms of toxicity. Further research regarding mechanistic actions of mixtures of ECs within the equine model is warranted, although testicular contamination specific to this species must first be established.

ECs may elicit additional toxicity to the reproductive system via the cyclic adenosine monophosphate (cAMP)-dependent protein kinase pathway (Warner *et al.*, 2019) and the Cation channel of Sperm (CatSper) pathway (Tavares *et al.*, 2013). CatSper channels are important in the mechanisms of sperm motility, including the functionality of the flagellar and subsequent male fertilising ability (Leeb, Sieme and Töpfer-Petersen, 2005). The role of CatSper is to deliver an influx of calcium (Ca^{2+}) to the flagellum of the sperm, controlling

motility characteristics (Schiffer *et al*., 2014; Lishko and Kirichok, 2010). During fertilisation, CatSper responds to secretions from the female to initiate sperm hyperactivation and penetration of the zona pellucida (Marquez and Suarez, 2004). Studies suggest that such channels are impacted by EC exposure (Birch *et al.*, 2022; Njembele, Bailey and Tremblay, 2014), therefore EC perturbation of these pathways may result in impaired semen quality parameters and the reduced ability of the sperm to induce fertilisation.

An alternative pathway, which should be considered in further research, is the relationship between EC exposure, microbiomes, and testicular health. In alternative species, ECs and MPPs are associated with dysbiosis, the disruption of the gastrointestinal microbiome, altering microbial population diversity and abundance (Wan *et al*., 2019; Zhang *et al*., 2015). A current area of interest is the association of the gastrointestinal microbiome with reproductive health through endocrinology and immunological pathways (Wang and Xie, 2022). In the equine species, novel research has indicated that specific seminal flora of the family Peptoniphilaceae is associated with higher total sperm motility, whilst alternative bacterial families such as Incertae Sedis XI and Clostridiales are associated with reduced sperm PMOT (Salas-Huetos *et al.*, 2022). It is therefore possible that reproductive perturbations resulting from EC and MPP ingestion is linked to associated imbalances in the microbiome. The extensive actions of ECs on a range of physiological processes and biological receptors involved with the development and maintenance of testicular infrastructure, spermatogenesis, and overall reproductive health and function highlight the complexity of EC exposure. Within the breeding stallion, the potential health risk of EC exposure, including associations with reproductive aberrations, is yet to be deciphered. However, in alternative species, the determination of phenotypic outcomes of EC exposure is heavily associated with the timing of exposure.

1.6 Timing of EC exposure and the intrauterine environment

Whilst research specific to the equine model acknowledges the influence of ECs in disease aetiologies (Mullen *et al.*, 2020; Durward-Akhurst *et al*., 2019), understanding of EC toxicity specific to the male equine reproductive system remains unknown. Stallion EC exposure is likely to occur throughout life, beginning *in utero* and continuing throughout the postpartum, adolescent, and adulthood periods. The intrauterine environment is where

the foetus is most susceptible to EC exposure due to the endocrine-mediated developmental period (Kot *et al.*, 2019). The hypothesis of the 'foetal origins of adult disease' (Barker and Osmond, 1986) or the 'developmental origins of health and disease' (Robles *et al.*, 2017) states that events during gestation can have significant health implications in adult life. Whilst these hypotheses are centred around disease development, they hold relevance in the discussion of EC interactions with reproductive health (Wohlfahrt-Veje, Main and Skakkebæk, 2009).

The placenta, a dynamic endocrine organ, is a prime target of ECs with endocrine disrupting tendencies (Mao *et al.*, 2020). Perturbations to the placenta, playing essential roles in homeostasis, foetal growth, and sustaining pregnancy (Rosenfeld, 2015), may adversely impact foetal development, contributing to chronic health perturbations in adult life (Gabory *et al*., 2013). In mammalian species, including the equine model, the testes develop *in utero*, with the prenatal environment important in supporting these physiological processes (Chavatte-Palmer, Peugnet and Robles, 2017). It is thus concerning that ECs, including PAHs (Drwal, Rak and Gregoraszczuk, 2019), Bisphenols (Gingrich *et al.*, 2019), PCBs (Correia Carreira *et al*., 2011), PBDEs (Zhao *et al*., 2013), and DEHP metabolites (Mose *et al.*, 2007) have the ability to cross the placental barrier, exposing the developing foetus to a range of toxic chemicals. Relatively low concentrations of Bisphenol congeners are actively transported to the foetal compartment (Grandin *et al*., 2018). However, due to physiochemical specificities of Bisphenol metabolites, and their inability to be removed from the foetal compartment in their glucorono-conjugated forms, a back-metabolism cycle is initiated, by which the bioactive forms are resynthesized. This cycle increases foetal exposure significantly (Gauderat *et al.*, 2016). Such exposure may disrupt essential developmental processes of the male gonads, leading to chronic perturbations in adult life.

In the rabbit model, *in utero* and adolescent exposure to low doses of dibutyl phthalate induced significant aberrations to reproductive development. The number of morphologically abnormal sperm in such individuals significantly increased, with acrosomal and nuclear defects forming the majority of the population (Higuchi *et al*., 2003). Research in the equine model has found infertile stallions to display similar defects, including sperm nuclear-acrosomal defects, to those induced by chemical exposure within the rabbit

(Veeramachaneni, Moeller and Sawyer, 2006). It has therefore been suggested that possible EC exposure during the intrauterine life of a future breeding stallion could be a primary cause of idiopathic infertility within the species (Veeramachaneni, Moeller and Sawyer, 2006). Pre- and peri-natal exposure to DEHP and PCBs at environmentally relevant doses has the ability to impact male testicular health and function in adulthood (Fiandanese *et al.*, 2016). In a study in mice exposed to DEHP and PCBs through the dam, interactions between contaminants included synergistic effects on testicular morphology and antagonistic effects on the expression of genes responsible for pituitary-gonadal interactions associated with sperm quality and the production of T_4 (Fiandanese *et al*., 2016). With reference to the sheep model, foetal gonadal alterations were present in both females and males reared by ewes on pastures treated with EC-contaminated sewage sludge (Lea, Amezaga, *et al*., 2016; Paul *et al.*, 2005). Alterations to the foetal gonads are likely to result in developmental aberrations and impair the functionality of the testis later in life, associated with reduced reproductive potential and success.

EC exposure may manifest long after initial exposure due to epigenetic modifications originating from exposure at the critical window of genitourinary development in the foetus and new-born (Bommarito, Martin and Fry, 2017; Thankamony *et al*., 2016). Modifications include DNA methylation, microRNA, and histone alterations (Bommarito, Martin and Fry, 2017). Epigenetic interactions with adverse effects on reproductive potential, are considered to be transgenerational (Rattan and Flaws, 2019; Goldsby, Wolstenholme and Rissman, 2017). Chronic reproductive perturbations from epigenetic modifications induced by *in utero* and neonatal exposure play an important role in the effects of EC exposure. However, exposure occurs throughout an individual's life. Therefore, acute perturbations in reproductive potential from exposure during puberty and adult life are essential in fully understanding the reprotoxicity of ECs. Although susceptibility to the adverse effects of ECs *in utero* remains high, new-borns are still at risk of endocrinology alterations through EC exposure (Wineland *et al.*, 2019).

1.6.1 Postpartum exposure

Due to the lipophilic properties of some ECs, including PCBs, PCDDs, and PCDFs, primary deposition occurs in lipid droplets in adipose tissue. Having a high lipophilic affinity, PCB,

PBDE, and PAH exposure result in significant bioaccumulation within fatty tissues, according to research in other species (Lehmann *et al*., 2015). During milk production, free fatty acids and lipoprotein stores are utilised, along with the ECs that have accumulated there (Pratt *et al.*, 2012). Despite the lower affinity of chemicals such as BPA and other bisphenol analogues to adipose tissue, levels are still detected in human breast milk samples (Deceuninck *et al*., 2015). In ruminants, the rate of transfer from animal feedstuffs to milk during lactation is suggested to vary from 5% to 90% for PCBs and 1% to 40% for PCDD and PCDFs, associated with specific congener hydrophobicity (Rychen *et al.*, 2014). International geospatial variation has been reported in human breast milk samples analysed for POPs (Antignac *et al*., 2016), likely a reflection of lifestyle, environmental and dietary factors resulting in differing levels of contamination. Concern has been raised over the consumption of colostrum contaminated with lipophilic ECs such as PCBs and the potential impact this may have on future development (Schultz *et al*., 2003).

The equine gastrointestinal epithelium, remaining porous for three days postpartum, could allow for the absorption of ECs in the suckling foal (Ayad *et al.*, 2017). In killer whales, EC concentrations in calves are higher than in their lactating mothers (Haraguchi, Hisamichi and Endo, 2009). Comparably, in suckling polar bear cubs, PCB concentrations surpassed that of maternal contamination (Polischuk, Norstrom and Ramsay, 2002). Such research demonstrates the accumulation of toxic ECs within the developing neonate. Konik Polski mare milk is contaminated with chlorinated hydrocarbon insecticides, including Gamma-hexachlorocyclohexane (ɣ-HCH) and Dichlorodiphenyltrichloroethane (DDT), despite their statutory ban in the 1970s (Pietrzak-Fiećko *et al*., 2009). It is therefore likely that the transfer of ECs from maternal milk to developing offspring is a fundamental process in the aetiology of contaminant exposure and subsequent reproductive perturbations in the equine model. Despite research indicating the transfer of ECs from dam to foal, little is known regarding the bodily burdens of equine neonates, including accumulation within the reproductive organs.

1.7 Concluding statement and research to be carried out within this thesis

Despite the growing evidence base indicating geographic-sensitive adverse trends in the reproductive health and function of multiple species, research specific to the equine model

is minimal. In a range of alternative species, significant evidence indicates that exposure to anthropogenic ECs are a key aetiological factor in perturbed reproductive parameters. Given that research is indicative of chemical exposure and reproductive toxicity across species, and the ubiquitous nature of ECs across terrestrial, agricultural and aquatic ecosystems, it is clear that contaminants are a global problem. The deep interlinks between environmental, animal, and human health are presented within the One Health framework. The One Health approach is mainly focused around the prevention and control of cross-species transmission of zoonotic diseases (Gebreyes *et al*., 2014), although the key principles hold value when considering EC exposure and toxicity across species (Brack *et al.*, 2022). One Health addresses concepts that promote a holistic approach to optimising the health of the environment, humans, and animals (Bhattacharjee *et al*., 2022). Further research is required to determine reproductive trends and initiate investigations into the aetiological involvement of ECs in equines to provide a holistic understanding of environmental and health risks associated with contaminant exposure.

The overall research hypothesis of the current thesis is that equine semen quality is declining in a comparable way to that reported in alternative species and that the equine population are exposed to anthropogenic ECs through pastures and feedstuffs, resulting in the testicular accumulation of contaminants. The overarching aim of the research presented is to assess temporal trends in stallion semen quality and initiate investigations into the plausible aetiological involvement of environmental chemicals. To address the hypothesis, the PhD is formulated around three key objectives.

- Assess temporal trends in stallion semen quality across global domestic populations through systematic review and meta-analysis (Objective A; Chapter 3; pages 68-93).

- Determine temporal trends in semen quality from retrospective data collected on a population of stallions at a single breeding facility in the UK (Objective B; Chapter 4; pages 94-126).

- Determine the presence or absence and concentrations of key environmental chemicals in stallion testes and typical equine pastures and feedstuffs as potential exposure routes (Objective C; Chapter 5; pages 127-165).

In addressing these aims, the research seeks to contribute valuable data to the equine sector regarding the status of equine fertility and initiate discussion into the health risks posed by anthropogenic EC exposure. In addition, the research initiates investigations into the suitability of the equine model as a novel comparative species to monitor reproductive trends and EC contamination in terrestrial ecosystems. Furthering understanding of reproductive trends and EC toxicity within the equine model will also contribute to the One Health approach. This has global relevance due to the distinct need to increase public understanding and promote a more sustainable future in a human-led changing environment.

CHAPTER 2

RESEARCH METHODOLOGY AND METHODS

2.1 ETHICS AND DATA MANAGEMENT

The research for all studies (objective A; chapter 3, objective B; chapter 4 and objective C; chapter 5) was approved as a single submission by Hartpury University Ethics Committee (ETHICS2019-52; Appendix A; pages 246-263). The research project was considered as a low-risk ethical application, as the studies included the collection of publicly available meta-data, retrospective electronically stored data from a Defra (Department for Environment, Food and Rural Affairs) approved breeding facility, and the collection of environmental samples and surplus tissue materials from routine procedures. For the collection of retrospective data from breeding records, a site permission form was signed by the owner of the facility prior to data collection (Appendix A3; page 251). For the collection of testicular tissue, owner (Appendix A5; pages 257-259) and veterinarian (Appendix A4; pages 252-256) information sheets, data sheets, and consent forms were disseminated to study participants. Signed consent was obtained by both the stallion owners and attending veterinarians prior to sample collection. Yard owner information sheets, data sheets, and consent forms (Appendix A6; pages 260-263) were signed prior to the collection of soil, grass, and haylage from equine facilities. All documentation was accepted by Hartpury University Ethics Committee as part of the main research application.

The Control of Substances Hazardous to Health (COSHH) risk assessments and chemical safety sheets were completed for the use of laboratory chemicals to maintain safe working conditions throughout the research process. To ensure the ethical collection, storage, analysis, dissemination and disposal of data associated with the research project, a data management plan was completed and adhered to, in line with policy implemented by the University of the West of England (Appendix A2; pages 248-250).

2.2 RESEARCH EPISTEMOLOGY

This chapter introduces the onological and epistemological position of the research carried out within the thesis and provides justifications for methodological approaches and methods assumed. The ontological position that the research is founded on is the concept that at least one reality exists independently from human perception. Considering the epistemological position and theoretical perspective, this research aligns with the positivist

paradigm, by which knowledge is produced from empirical evidence (Al-Ababneh, 2020). All proposed studies originate from deductive reasoning, where the hypotheses are developed from a broader theory regarding stallion reproduction and environmental toxicology. Given the nature of a positivist epistemology, probabilistic justifications are required to statistically underline the plausibility of the belief proposed. Quantitative, statistical research methods were utilised to determine the probability of results. All studies are considered mono-method, consisting of only quantitative, empirical data (Goertz and Mahoney, 2012). A breakdown of the study aims, hypotheses, and methods of analysis employed within each chapter of this thesis are presented in table 3.

Table 3: A breakdown of studies addressed within this thesis, including aims, hypotheses, methodological, and statistical approaches utilised for each chapter. Full details of each methodological approach and methods utilised are available in individual chapters, the page numbers for which are provided below.

Chapter	Study aims	Hypothesis	Analysis method
Chapter 3; Objective A Pages 68-93	**To analyse temporal trends in stallion semen quality across global domestic populations through systematic review and meta-regression analysis.** 1. To systematically collect and analyse publicly available data on stallion sperm progressive motility; 2. To assess trends accounting for covariates including geographical location; 3. To investigate the suitability of the equine model as a novel comparative species for male reproductive trends in human populations.	It was hypothesised that sperm progressive motility has declined in recent years across global domesticated equine populations.	**Method:** Evidence synthesis **Statistical approach:** Simple linear, meta-regression, and sensitivity analyses **Software:** NVivo, GenStat, GraphPad, ImageJ
Chapter 4; Objective B Pages 94-126	**To determine temporal trends in semen quality from a population of stallions at a single breeding facility in the UK.** 1. To collect and analyse retrospective data on semen quality to assess trends in a single equine population at a breeding facility in the UK; 2. To determine time-trends in stallion semen quality whilst accounting for alternative variables including stallion age, breed, and abstinence period; 3. To further investigate the suitability of the equine model as a comparative species for human reproductive research.	It was hypothesised that semen quality in a UK-restricted equine population has declined in recent years.	**Method**: Retrospective data **Statistical approach:** Linear mixed model (REML) **Software:** GenStat, GraphPad
Chapter 5; Objective C Pages 127-165	**To analyse stallion testis and equine feedstuffs and pastures for anthropogenic environmental chemicals.** 1. To analyse Thoroughbred stallion (*Equus caballus*) testis for four categories of anthropogenic ECs; Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP; 2. To analyse equine pasture and commercial feedstuff materials for Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP as potential routes of oral exposure; 3. To evaluate the suitability of the equine species as a novel comparative model for reproductive EC contamination and terrestrial environmental health.	It was hypothesised that ECs accumulate in stallion testis and that exposure includes the ingestion of contaminated pastures and feedstuffs.	**Method:** Chemical analysis **Statistical approach:** One way ANOVA and Tukey's post hoc; independent t-test **Software:** GraphPad

2.3 OBJECTIVE A: A SYSTEMATIC REVIEW AND META-REGRESSION ANALYSIS.

Objective A aligns with research presented in chapter 3 of this thesis (Temporal trends in equine sperm progressive motility (1984-2019): a systematic review and meta-regression; pages 68-93). Objective A aimed to determine temporal trends in sperm quality across global domesticated equine populations through systematic review and meta-regression analysis. It was hypothesised that sperm progressive motility has declined in recent years across this population.

Methodological approaches enable the utilisation of comprehensive published datasets and the synthesis and analysis of evidence to answer a focused research question. Given that metadata is collected from public sources, it transposes high levels of transparency within the research and permits updates in line with newly published data. The importance of these abilities are highlighted in the progression of systematic reviews and meta-analyses assessing trends in human semen quality (Levine *et al*., 2017; Swan, Elkin and Fenster, 2000; Carlsen *et al*., 1992). Whilst regulations for registered racing Thoroughbred horses state that horses may only reproduce through natural coverings, there has been an increase in the use of artificial reproductive techniques (ART) across the rest of the equine industry (Campbell and Sandøe, 2015). Industry pressures have led to a significant volume of research aimed at optimising equine reproductive success. This comprehensive research base has not yet been utilised to determine temporal trends in stallion semen quality.

The methodological approach for the evidence synthesis was constructed in consultation with Dr Mark Farnworth (MF), a systematic review specialist. This systematic review aimed to determine temporal trends in sperm progressive motility (PMOT) in the global equine population through a systematic review and meta-regression analysis. Research questions were constructed to include both population and outcome terminology (PO; Table 4), as recommended by the RepOrting standards for Systematic Evidence Syntheses (ROSES; Haddaway *et al.*, 2018). A pilot-study and evidence synthesis was formulated in accordance with the Collaboration for Environmental Evidence guidelines (CEE, 2018). CEE recommendations enable narrative synthesis and quantitative analysis. Given that published data on stallion semen quality has not yet been utilised for systematic purposes, the CEE protocol was selected to provide scope for differing forms of analyses.

Table 4: Structuring of the research question around population and outcome ("PO") terminology.

Question Type	Question	Question Elements	Element Terms
"PO" structure	The evaluation of temporal trends in stallion sperm progressive motility.	Population	Stallions belonging to the *Equus caballus* genus.
		Outcome	Sperm progressive motility.

2.3.1 Protocol and pilot-study

In line with CEE recommendations, a protocol was constructed and critically reviewed by a team of researchers, including a methodological specialist (Dr Mark Farnworth). The protocol was formed of three stages; a test-search, literature scope, and the formation of a test-list, which acted as the pilot-study (CEE, 2018). Two primary reviewers were responsible for the development and piloting of the protocol. An additional aim of the protocol was to determine the methods to be employed during the evidence synthesis, including the use of specific software packages. NVivo (QSR International Pty Ltd., 2018; QSR International version 12) was selected as a management and screening software for the systematic review (Franzosi *et al*., 2013). NVivo is a computer-assisted qualitative data analysis software (CAQDAS), which is predominantly associated with qualitative data synthesis (Dhakal, 2022). Whilst it does not possess functions for statistical analysis, which were needed for the meta-regression, NVivo can be utilised for systematic and literature reviews to facilitate transparency, inter-reviewer comparisons, and analytical processes (Rylee and Cavanagh, 2021). It was therefore selected as a software to be used alongside the reference management software, Mendeley, for the systematic review.

2.3.2 Protocol stage one: The test-search

Two primary reviewers (IH and Jodie Perrett; JP) developed and tested a search strategy using key terminology produced through group discussion to determine literature volume and accessibility of returns, assess relevant literature formats and produce informed eligibility criteria. Developing a comprehensive search strategy is an essential step to minimise the risk of bias (CEE, 2018). As with the research question, the search strategy was structured incorporating both population and outcome terminology, used to define the primary elements of the research question. An advanced Boolean search was utilised

to improve the specificity and sensitivity of the search strategy (Shaffril, Samsuddin and Samah, 2020; CEE, 2018). The 'map term to search heading' function was utilised via the Ovid platform to determine keywords that had not been considered by the review team. Utilising these search functions enables greater access to research articles beyond the specific terminology in titles and associated abstracts (Gault, Shultz and Davies, 2002).

2.3.3 Protocol stage two: Literature scoping

An initial scoping of literature involved the comparison and analysis of database functionality, coverage, and relevance to the research question, in addition to the identification of relevant population and outcome terminology. CAB direct and Scopus are key databases for veterinary-based evidence syntheses (Grindlay, Brennan and Dean, 2012). Scopus and Medline (Ovid) were allocated as primary search databases. Embase (Ovid) was deemed suitable as a secondary database, a result of platform scoping and recommendations in literature (Gusenbauer and Haddaway, 2020; Bramer *et al*., 2017). CAB direct (VetMed) was selected as a subject specialist database, given the high coverage of veterinary-based research (Grindlay, Brennan and Dean, 2012). A limitation of systematic reviews is publication bias, and missing grey literature can contribute to this (Adams, Smart and Huff, 2017). Through consultation with a team of librarians (Hartpury University), CORE was selected as a grey literature database due to high levels of international coverage.

2.3.4 Protocol stage three: Test-list establishment

A test-list was established for each selected database, defined as a group of articles deemed relevant to answer a specific research question (CEE, 2018). Databases were divided between the two primary reviewers (IH and JP) to develop suitable search strings. Final refinements of the search strategy included an iterative process by which each population and outcome term was removed from the search consecutively to determine its impact on the relevance of returns. The test-list involved the selection of one research article from each database at 5-year intervals (2020, 2015, 2010, 2005, 2000, 1995, 1990, 1985, and 1980). A 'date of publication' limit was applied to each search, and the first article with both a population and outcome term in the title was selected. This created a test-list bibliography consisting of 50 research articles (1980 to 2020). For dates that failed to

produce a return, an article in the previous or subsequent year was utilised where possible. Full access was available for 39 articles, which were subject to full journal screening. One duplicate was excluded from the data set (Stradaioli *et al*., 2000). The title and abstract were screened for the remaining studies but were not included in the test-list bibliography. The final test-list bibliography constituted of 38 articles (Appendix I; pages 301-305), reviewed for the pilot study. Five further articles were selected based on eligibility uncertainties, brought forward for discussion with the entire research group to finalise the criteria (Table 5).

Table 5: Final criteria used to determine eligibility of articles within the review.

Question elements	**Eligibility criteria**	
Study-specific	Inclusion	English language documents only (both original and translated); Peer-reviewed published literature including; primary and retrospective data sets and case reports; Academic grey literature including; dissertations and theses, conference presentations and posters**.**
	Exclusion	Publications in a language other than English or without translation; Data presented in the format of a review article or opinion article; Duplicate data sets.
Population	Inclusion	Domesticated *Equus caballus* only.
	Exclusion	Alternative sub-species of the *Equus* genus.
Outcome	Inclusion	Semen collection utilising standard methods including an artificial vagina (AV); Semen quality analysis of parameters including; progressive motility and sperm concentration; Progressive motility assessment via Computer-Assisted Sperm Analysis or Microscopy; Sperm concentration analysis via the NucleoCounter; Fresh and cooled semen samples.
	Exclusion	Non-standard methods of semen collection; including epididymal retrieval; Semen quality parameters presented for sexed semen samples; Stimulation of ejaculation via pharmacological- and electro-methods; Stallions displaying signs of perturbed reproductive health including anatomical, seminal, and bacterial or poor libido; Parameters reported for cryopreserved or frozen-thawed semen.

2.3.5 The evidence synthesis: a systematic review

The systematic review was completed utilising the ROSES *pro forma* and flow diagram (Haddaway *et al.*, 2018), which is presented in the results section of chapter 2 (page 79). The second reviewer (JP) utilised the Meta-Analyses of Observational Studies in Epidemiology (MOOSE) *pro forma* and flow diagram (Stroup *et al*., 2009). Having two reviewers for literature screening stages is recommended to ensure all relevant studies are included within the synthesis (Stoll *et al*., 2019). Selected databases; Scopus, Medline (Ovid), Embase (Ovid), CAB Direct (VetMed), and CORE were screened using the final search string; (stallion* OR horse* OR equine* OR colt*) AND (sperm* OR (semen AND quality) OR insemin*) AND NOT (human* OR horseradish). The term 'insemin*' was used for Scopus, although was removed for subsequent databases, due to irrelevant returns. A Boolean search strategy was utilised for all databases, an integral part of developing a comprehensive, sensitive and precise search strategy (Gusenbauer and Haddaway, 2020).

Where possible, the search strategy was limited to the title, abstract, and keywords, and an English language filter was applied. Whilst this increases the risk of language bias, this was deemed to be a necessary limitation, given the lack of a translator within the review team. All returns were downloaded onto Mendeley. Database screening occurred between 13th January 2021 and 20th May 2021, with all databases reassessed on the final date for the inclusion of new publications. The second reviewer (JP) followed the same search strategy, utilising an alternative subset of databases; PubMed, BASE, and PubAg (Perrett *et al*., 2021). Results from all listed databases were combined for the current systematic review, although each selection met the requirements for an individual review (each constituted of a suitable primary, secondary, and subject specialist database).

2.3.6 Two-stage screening process for eligibility determination

All articles reporting primary or retrospective data on stallion (*Equus caballus)* sperm progressive motility (PMOT), and sperm concentration were considered eligible for screening. PMOT is defined as "sperm moving actively, either in a linear or large circle, regardless of speed" (World Health Organization, 2021). PMOT and concentration are important parameters for conception (Dcunha *et al*., 2020; Simon and Lewis, 2011; Zinaman *et al*., 2000) and reflect the reproductive health of the individual. PMOT values

analysed by both objective (computer assisted sperm analysis; CASA) and subjective (microscopy) methods were included in the study. PMOT values estimated via microscopy were collected as a comparison to the findings reported through CASA. Values given for both fresh and cooled semen samples were deemed eligible for inclusion within the systematic review. Whilst cooling semen can have detrimental impacts on sperm quality, during the development of the protocol, it was deemed necessary to include these values to obtain a sufficient volume of data for analysis. However, to account for differences in semen processing techniques, further data associated with cooling protocols, including time cooled, temperature, and extenders used, were collected.

Returned articles were critiqued through a two-stage screening process to determine eligibility based on the predefined criteria (Table 5) (Levine *et al*., 2017). Two reviewers were responsible for screening articles in both stage one and stage two (Stoll *et al.*, 2019). A third independent reviewer (Christy Maddock; CM) was included for stage two screening. Exact duplicates returned from multiple databases were removed prior to stage one screening to produce a collection of unique publications. A title and abstract screening formed the first stage of the process. Studies that were deemed eligible through stage one were subject to a full-text screening. Relevant review articles were accepted through stage one to be utilised within citation searching, a subsequent stage of this review. For articles with restricted access, a collaborating librarian (Hartpury University) worked to retrieve articles through the British Library archives. Articles were uploaded to QSR NVivo (version 12), used for all requirements of the review.

Stage two screening was completed using the 'case classifications' function on NVivo. Whilst the test group was not always suitable for inclusion, the control population was considered. Where articles were eligible but failed to provide a factor within the eligibility criteria (method of collection, for example), the corresponding author was contacted via email or ResearchGate where possible. Supplementary data from authors were then used to determine eligibility. Authors that did not respond to requests within a two-month period were excluded from the dataset due to time constraints of the project. Where articles cited previous methods, these references were cross-checked in relation to the original article to determine final eligibility. The 'memos' function on NVivo was used to

store emailed, cited, and supplementary data and linked to the original articles to maintain transparency (Hutchisona, Johnstonb and Breckona, 2009). The reasoning behind exclusion was categorised as due to population-related data, outcome data, and study specifics.

All articles accepted through to stage two screening underwent a citation searching process. Google Scholar was used to screen articles that had cited the original article, defined as forward citation (F-C) searching. The reference list of each eligible article was also screened, which formed the backward citation (B-C) search. Articles returned through citation searching were subject to a three-tiered screening process, where the initial title and abstract stage were separated, and a full article screening formed the final stage.

The second reviewer (JP) completed the same process of searching and screening independently, using their allocated databases (PubMed, BASE, and PubAg). Unique articles returned from this search were added and utilised for the current review. Opinions on eligibility of common articles were also considered. Where the two primary reviewers (IH and JP) displayed differences in opinions, the third independent reviewer was used (CM). Eligible returns and articles with eligibility uncertainties from both the main and citation stages were sent to an independent reviewer (CM). Irregularities in eligibility were dealt with through group discussion with the research team to determine final eligibility.

2.3.7 Critical appraisal of article validity

The predefined protocol, including the development of stringent eligibility criteria, the conduct of the systematic review, and the selection of statistical tests, sought to minimise bias. Two independent reviewers critically appraised all articles deemed eligible after full-text screening (n=522), to further limit reviewer bias (Bilotta, Milner and Boyd, 2014). Systematic errors within individual studies can significantly impact the results and interpretations of data in a systematic review. Following stringent quality appraisal methods is therefore an essential component. Systematic error for individual studies was measured by assessing the research based on six domains of bias (selection, performance, detection, attrition, reporting, and other bias). The key definitions for each domain of bias, as outlined by the CEE, are presented in table 6. For each domain, the article was deemed as high or low risk. If the risk level for any domain was high, the study was excluded. This

method of critical appraisal is recommended for evidence syntheses by the CEE (CEE, 2018). Justifications for the exclusion of articles due to high risk of bias included; lack of control groups and reporting of specific results, non-blinded microscopy analysis, and the removal of stallions or samples from analyses based on failing to meet specific fertility or sperm quality thresholds. Some duplicate datasets were also removed during this stage. Where a dataset was duplicated in thesis and published formats, the published article was included, given the assurance of the peer-review process.

Table 6: Bias domains assessed during critical appraisal, as defined by the CEE. The table was developed based on recommendations from the CEE (CEE, 2018).

Bias domain	Question
Selection bias	"Are the intervention/exposure and control groups comparable on all important potential effect modifiers?"
Performance bias	"Are there any factors, other than the intended intervention or exposure that may have influenced the outcome?"
Detection bias	"Was the outcome assessment method likely to have accurately measured the outcome, and was the method of outcome assessment the same across all relevant study groups?"
Attrition bias	"If there were missing data were these of minor numerical importance (relative to the total sample size and effect estimate), balanced across the study groups and unrelated to the intervention/exposure and outcome?"
Reporting bias	"Are all the measured outcomes fully reported; if not, are the missing data likely to be related to whether the results were positive or negative?"
Other bias	Are there any other forms of bias present?

2.3.8 Data extraction

Summary statistics, including mean, standard deviation (SD), standard error of the mean (SEM), range, median and interquartile range of PMOT, and concentration were extracted from all eligible articles (n=288). Additional data collected included; year of sample collection, stallion and ejaculate sample size, fertility status, country, season of collection, horse breed, horse age, semen form (fresh or cooled), temperature and time period for cooled semen only, use of extender and centrifugation, method of analysis (CASA, microscopy, NucleoCounter), CASA model and CASA settings. The CASA settings, velocity average path (VAP), and straightness (STR) thresholds for progressively motile sperm were

collected as both were suspected to influence the interpreted PMOT value. VAP (μm/s) is defined as "the time-averaged velocity calculated along the average path", and STR (%) is defined as "the linearity of the average path" (World Health Organization, 2021).

For missing data of key variables, estimations were made where possible. For studies that reported the mean and SD or SEM in graphical formats, ImageJ (Image Processing and Analysis, Java) software was used to calculate estimations for each value. Where studies provided raw data or mean values on an individual stallion basis, an overall mean was calculated, resulting in a single value per article. Each article was given a label to define the method of data collection that occurred; graph extraction, calculation, or given value. For studies that failed to provide a year of sample collection, the value was estimated by subtracting the mean difference between publication year and collection year in studies that provided both values (Levine *et al*., 2017). In the instance where a study reported the SD but not the SEM of PMOT, the SD was divided by the square root of the sample size for each estimate to calculate the SEM. When neither SD nor SEM were reported, the mean SD of studies that reported this value was divided by the square root of the sample size to estimate the SEM (Levine *et al*., 2017). When threshold values for CASA settings were given for motility and not PMOT, the mean difference between these values in studies that reported both was added to the motility values to calculate threshold CASA setting values for PMOT. Where studies failed to provide a value for the sample size, it was estimated that each stallion provided a single sample. For studies that failed to state the country of sample location, the author affiliations associated with the publication were used.

2.3.9 Narrative synthesis of descriptive data

Descriptive data that were not deemed relevant for quantitative analysis were observed and summarised as part of the narrative synthesis stage. Limited data on sperm concentration (n=3) resulted in this parameter being removed from further analysis. PMOT values for cooled semen (n=30) and analysis via subjective methods (n=22) were also removed from the dataset. Cooled sperm quality parameters are reflective of the semen treatment protocols, whereas fresh values are directly associated with testicular health and function. Whilst PMOT via subjective microscopy was to be included to support the parameter analysed via CASA, it is a highly subjective method resulting in significant intra-

and inter-observer variability (Whitesell *et al*., 2020). Values that were provided for 0 hour cooled samples were included within the fresh semen analysis. PMOT assessed via CASA was selected as an objective measure of sperm quality to extract and analyse within this systematic review. One factor highlighted during narrative synthesis was the variability in CASA settings utilised by differing research groups. CASA settings for the VAP and STR threshold values were included in the subsequent quantitative analysis.

Stallions were categorised as fertile or 'unselected for fertility' (Levine *et al.*, 2017) to assess the risk of selection bias and applicability to the wider equine population (Merzenich, Zeeb and Blettner, 2010). To determine geographic variability, articles were categorised as western or non-western, based on accepted methods (Levine *et al*., 2017) and data availability. Western was defined as Europe, North America, and Australasia, whilst non-western populations consisted of South American, African, and Asian regions. Given that horses are seasonal breeders (Walbornn *et al*., 2017), and global populations were included, season of collection was separated based on hemispheres. Collections were either categorised as occurring within the breeding season or non-breeding season. For northern hemispheres, breeding and non-breeding seasons were defined as February to June and September to December, respectively (Walbornn *et al*., 2017). For the southern hemisphere, breeding and non-breeding seasons were the opposite of those in the northern hemisphere, defined as September to December and February to June, respectively. Regardless of hemisphere, if semen collection occurred across the defined seasons, the article was categorised as occurring year-round. Breeds were grouped as warmbloods, hotbloods, coldbloods, mixed breeds, miniature and pony types, a recognised method of categorisation in equine reproductive research (Ebel *et al*., 2020). Definitions of breeds contributing to each category are provided in the supplementary information (Appendix J; page 306). There was no plausible way to include age within the statistical analysis given the method of reporting within the articles.

2.3.10 Rationale for statistical analysis

Data were analysed on GenStat 17th edition (VSN International, 2022b; VSN International Ltd, Hempstead, UK) and graphically interpreted on GenStat and GraphPad Prism 9 (GraphPad Prism, 2022; GraphPad Prism version 9.0, GraphPad Software, California, CA,

USA). Statistical methods for this objective were designed in consultation with Dr David Baird (Statistical Consultant and GenStat Developer; GenStat VASN (NZ) Limited) and informed by best practices (Borenstein *et al*., 2009). Sample size, which was defined as the number of articles included within the statistical analysis for this systematic review, was determined through the comprehensive search strategy reported. A total of 230 articles were included in the statistical analysis. For each paper, a single mean value and standard error of the mean (± SEM) were utilised. The mean parameter value and SEM was selected as the most appropriate method of representing the data. The SEM was selected as it indicates how the estimated means of different groups relate to each other and how accurately the sampled data represents the whole population (Pleil, 2016), which, for the purposes of this research question, is more relevant than looking at the spread of the data around the mean (SD). It was not appropriate to calculate an overall mean for a single paper assessing the impact of CASA settings on PMOT outcome, so two values were included for this paper. Subsequent statistical analyses were employed to determine the influence of including two values for a single article. The final dataset included 231 data points from 230 original articles (Appendix K, pages 307-324). Residual diagnostic plots were interpreted for each model to ensure that the assumptions of the selected statistical approach had been met (VSN International, 2020). The residual diagnostic plots include a histogram, a plot of the residuals fitted against the fitted values, and a normal (ordered residuals) and half-normal (residual absolute values) plot (VSN International, 2020).

Data on PMOT were first analysed through a simple linear regression weighted by article sample size to determine overall trends across year of collection. Data were then analysed through a multiple meta-regression approach, weighted by study SEM. The meta-regression accounted for a set of predetermined covariates to enable the reliable interpretation of results (Thompson and Higgins, 2002). Through a stepwise approach, the significance of each covariate was evaluated, to determine inclusion within the final model. Regardless of significance, year of collection was included in the final analyses. A fixed-effects regression model was determined as the most appropriate method compared to a random-effects regression model as the data did not have distinct population groups (Borenstein *et al*., 2010). The meta-regression was weighted by SEM as a predictor of study estimate reliability, to mitigate the associated methodological limitation of heterogeneity (Baker and Jackson, 2010). A higher number of independent readings contributing towards

a mean value are reported to provide a more accurate estimate (Pleil, 2016). Predicted mean PMOT (± SEM) values, calculated from the statistical models, and the slope of regression (*b*) were interpreted to determine time trends. The overall decline per year was calculated by dividing the overall decline by the number of collection years (Levine *et al*., 2017). Graphs were plotted so that each point represented the sample size (simple linear regression) and the SEM (meta-regression) of each article. Graphical formatting provides a visual presentation of the data that each article contributes toward the analysis (Thompson and Higgins, 2002).

Sensitivity analyses sought to determine trends specific to separate populations of the data. Cubic and quadratic functions were added to year of collection to assess non-linearity in the full, refined meta-regression model. Datasets were restricted to articles providing data with sample sizes of more than 10 stallions (Borenstein *et al*., 2009), articles providing values for all predetermined covariates, and date restricted to articles with year of collection falling between 2000 and 2019. Further analyses included determining trends specific to populations unselected for fertility potential and trends specific to western and non-western groups (Levine *et al*., 2017). Each article included within the dataset consisted of a single mean (± SEM) value. One article provided two PMOT data points in relation to differential CASA settings. It was therefore improper to calculate a single value for this article. Sensitivity analyses were carried out after sequentially removing each value, followed by the article itself, to determine the impact of these points on the overall model. A p value of <0.05 was considered statistically significant, whilst a value of <0.001 was statistically highly significant, and a 95% confidence interval was assumed for all analyses.

2.4 OBJECTIVE B: RETROSPECTIVE COHORT STUDY OF STALLION SEMEN QUALITY

Objective B aligns with research presented in chapter 4 of this thesis 'Temporal trends in semen quality from a population of stallions at a single breeding facility in the UK (2001-2020)' (pages 94-126).

Assessing temporal trends in semen quality provides a valuable understanding of the profile of testicular health and function and as a prediction of reproductive success (World Health Organization, 2021). Whilst evidence syntheses contribute valuable data towards understanding reproductive trends across a broad population, the methodological approaches are not without limitations (Levine *et al*., 2017; Pacey, 2013; Fisch, 2008). Utilising retrospective datasets with consistent methods provides an alternative approach of assessing trends in stallion semen quality, building on the limitations of systematic reviews. This retrospective cohort study aimed to assess temporal trends in semen quality from a single equine population at a breeding facility within the UK. It was hypothesised that semen quality in a UK-restricted equine population has declined in recent years.

2.4.1 Retrospective data collection and data classifications

Retrospective data on fresh stallion semen quality parameters were collected from records at a UK-based Defra and Animal and Plant Agency (APHA) approved breeding facility and associated laboratory. As reported within the ethics and data management plans (Appendix A1; A2; A3; pages 247-251), stallions were coded using a random allocation system to maintain full anonymity throughout. Number of stallions and ejaculates included within this objective was determined by the volume of data available through retrospective data collection at a single breeding facility. Data on 11,722 samples from 1,041 stallions of mixed ages and breeds were obtained between the years of 2001 and 2020. Given data was collected from retrospective records from an industry partner, the 20-year approach was determined by the data available. Fresh semen quality parameters included; total motility (TMOT; %), concentration (million per ml; $x10^6$/ml), and volume (ml). Volume is a result of seminal fluids produced by the accessory glands so, it is not directly related to testicular health, and concentration is a result of the amount of seminal fluid (World Health Organization, 2021). Total sperm output (TSO; million sperm; $x10^6$), calculated by

multiplying volume by concentration, is a more accurate parameter for measuring testicular output. TSO was calculated for each collection and analysed as a separate parameter (Hernandez-Aviles and Love, 2021; Hering *et al*., 2014). The final dataset contained four semen quality parameters as predictors of reproductive health and function in a single UK-based equine population to assess over time.

Data on date of collection, sample treatment, stallion date of birth, breed, country of birth and discipline were collected from corresponding stallion breeding documents and competition records of individual stallions. Raw data on date of collection was used to calculate season of collection and used in tandem with stallion date of birth to calculate age at collection. Previous evidence syntheses assessing trends in human semen quality have been critiqued for the failure to include abstinence period and geographic data (Fisch, 2008). Dates of collections were used to calculate abstinence period for stallions with multiple or consecutive ejaculations. Whilst all stallions were based in the UK for collections, to account for geographic variations, data on country of birth were collected for analysis. The original dataset consisted of stallions belonging to 71 breeds, 21 countries of birth, and 17 disciplines. Raw data on stallion breeds, country of birth, and discipline were categorised into related groups. Breeds were grouped using recognised categories into warmblood, coldblood, hotblood, crossbreed, and pony types (Ebel *et al*., 2020). Sporting discipline categories included; endurance, flatwork, jumping, harness, American style, and breeding. Definitions for each category of breed and discipline are presented in the supplementary information (Appendix M; page 327). Country of birth was grouped into Europe, North Americas and other. Season of collection was classified as occurring in winter (January to February), spring (April to May), summer (July to August), or autumn (October to November), a recognised method of categorising breeding season (Crespo *et al.*, 2020). Semen was either categorised as raw or extended (with a commercially available extender). Given the potential spermicidal properties of urine (Ellerbrock *et al.*, 2016) and blood contamination (Turner *et al.,* 2016), samples displaying haematospermia (blood contamination) or urospermia (urine contamination) were excluded from the dataset.

2.4.2 Semen collection and analysis

Data were collected from retrospective records on equine semen collections carried out at a Defra and APHA approved breeding facility by trained personnel. Training of collection

and semen analysis procedures were carried out by one of two individuals to ensure the same standard of analysis was met, contributing to the standardisation of methods. Protocols used by the facility were in accordance with recognised breeding management regimes. Collections were carried out using standard procedures with an AV and a teaser mare, phantom, or via ground collection. Given stallion preference to differing methods, technicians selected a suitable method to promote a successful collection for each individual stallion. Whilst there was variation in the technicians responsible for sample and stallion handling, training was standardised across the study period. Following successful collection, samples were taken to the associated laboratory at the same facility for immediate quantitative and qualitative analysis. For motility, a 10 µl drop of semen was placed onto a slide and observed subjectively by eye (microscope; x100 magnification). Whilst microscopy is subjective, standardised training for staff across the study period sought to optimise consistency and minimise variability. Microscopy is still commonly used for analysing raw semen, given that samples are usually diluted with an extender to a suitable concentration to obtain an accurate reading using objective methods of assessment such as CASA. Volume was calculated by weighing the samples, using a conversion of 1 g = 1 ml. Concentration was measured using a SpermaCue up to the year 2011 and a NucleoCounter SP-100 for post 2011 collections.

2.4.3 Rationale for statistical analysis

Data were analysed using GenStat 17th edition (VSN International, 2022b; VSN International Ltd, Rothampsted, Harpenden, UK) and graphically interpreted on GenStat and GraphPad Prism 9 (GraphPad Prism, 2022; GraphPad Prism version 9.0, GraphPad Software, California, CA, USA). Statistical analyses for this objective were carried out following consultation with Professor David Gardner (School of Veterinary Medicine and Science, University of Nottingham). Each dependent parameter was treated as an isolated variable to determine trends specific to TMOT, volume, concentration, and TSO. Year of collection acted as the independent variable to assess time trends. Additional variables accounted for within the statistical model included; season of collection, use of an extender, abstinence period and stallion age, breed, country of birth, and sporting discipline.

With large data sets such as that associated with this research, an approximate normal distribution is assumed (Curran-Everett and Benos, 2004). However, data were checked for approximate normality through graphical interpretations of histograms and normal probability plots. Extreme outliers were defined as those greater than three times that of the interquartile range and were removed from the datasets (Curran-Everett and Benos, 2004). For TMOT, concentration, volume and TSO, threshold values defined as extreme outliers were <4% (n=26 collections), >725 $x10^6$/ml (n=45 collection), >120 ml (n=143 collections) and >2100 $x10^6$ sperm (n=63 collections), respectively. Following outlier removal, the datasets for TMOT, concentration, volume and TSO consisted of 10,686, 5,185, 11,122 and 5,152 samples from 984, 524, 1,030 and 523 stallions, respectively.

Residual diagnostic plots for each model were interpreted to ensure that the assumptions of the test were met (VSN International, 2022a). Residual plots included a histogram of residuals, a fitted-value plot, a normal plot, and a half-normal plot (VSN International, 2022a). Data were analysed using a linear mixed model (restricted maximum likelihood; REML). The decline of PMOT per year of collection was calculated by dividing the overall decline by the number of collection years (Levine *et al*., 2017). The linear mixed model (REML) was selected as it allows for the assessment of the interplay between variables whilst accounting for stallion and sample numbers in relation to temporal trends (Ge, Smoller and Sabuncu, 2016). This technique permits the inclusion of stallion and sample number as random effects whilst accounting for other variables in a fixed model. The ability to account for inter-stallion variation was a necessary feature of the statistical test, as some stallions contributed multiple samples over the study period. The failure to account for potential clustering of data could have resulted in an overestimation of the statistical significance of observed trends (Wainwright, Leatherdale and Dubin, 2007). The model assumes that missing data are randomly distributed, enabling the inclusion of all data and preventing bias of estimated values.

Stallion age, breed, sporting discipline, country of birth, abstinence period, extender use, and season of collection were included as fixed effects, with year of collection fitted last as the independent variable of interest. The REML model initially contained all variables, the significance of which were determined through an iterative process, dropping and

removing terms from the fixed-model. Variable significance and the Akaike information criterion (AIC) value were interpreted to determine inclusion within the final refined model. The lowest AIC value represents the best model fit (Verbyla, 2019). Regardless of significance, year of collection was included within all models. As part of the statistical model, predicted mean (± SEM) values were calculated for each dependent parameter. Given that the predicted means account for all included variables, values were considered more representative in relation to the data and so were used for graphical interpretations. The SEM was selected as the most appropriate descriptive statistic for this data, indicating how well the predicted mean represents the true mean of the whole population (Pleil, 2016).

Whilst age at collection was included within the statistical models so that predicted means were calculated accounting for this variable, further analyses sought to determine trends accounting for the interaction between aging stallions and year of collection. Means plots were drawn up do determine age-based trends in each semen quality parameter. Based on these results, stallions were categorised as reproductively prime or senescent. Age-restricted trends were compared to confirm that time trends in semen quality parameters were a true representation of reproductive developments and not a result of aging stallions. For all analyses, a 95% confidence interval was assumed, and a p value of <0.05 and <0.001 were considered statistically significant and highly significant, respectively.

2.5 OBJECTIVE C: CHEMICAL ANALYSIS OF STALLION TESTIS AND EQUINE FEEDSTUFFS

Objective C aligns with research presented in chapter 5 of this thesis (Environmental chemicals in the thoroughbred stallion: testicular contamination and routes of oral exposure; pages 127-165). The study associated with chapter 5 was funded by the Horserace Betting Levy Board (Project SPjr050; Are thoroughbred breeding stallions exposed to reproductive perturbing environmental chemicals?).

The aim of the final objective was to assess testicular contamination and potential exposure routes to anthropogenic ECs; Polychlorinated biphenyls (Σ7PCBs), Polybrominated diphenyl ethers (Σ7PBDEs), Polycyclic aromatic hydrocarbons (Σ16PAHs) and Di (2-ethylhexyl) phthalate (DEHP), using the Thoroughbred stallion as a predictive model. It was hypothesised that ECs accumulate in stallion testis and that exposure includes the ingestion of contaminated pastures and feedstuffs.

The final study sought to evaluate equine-specific EC exposure to initiate investigations into their potential aetiological involvement with stallion reproductive aberrations. The research also sought to initiate the use of the novel equine model as a biomonitor species for environmental and human reproductive health. Testicular tissue was analysed based on the need to develop an understanding of the chemical concentrations that spermatozoa may be exposed to during the process of spermatogenesis. Castrations are routine procedures within the UK equine industry (Price *et al*., 2005), providing an important opportunity for the analysis of surplus material with relatively limited ethical implications. To determine plausible exposure routes of anthropogenic ECs specific to stallions, soil, grass, and feedstuffs including baled haylage, concentrate, and forage-based feeds commonly fed to breeding stock and the racehorse, were analysed for the same chemical congeners. All samples were stored in tin foil or glass containers cleaned with 70% alcohol to prevent contamination of samples through chemical leaching from plastic containers.

2.5.1 Sample collection of Thoroughbred testes

Sample sizes for this research objective were determined by the funding sources available. A range of contaminants and samples were analysed to provide a broad preliminary

understanding of contamination levels in horses and potential routes of exposure. Six clinically healthy Thoroughbreds within the racing industry were included in this research. Testes (n=6) were collected as surplus tissue from routine castrations completed by qualified veterinarians. Samples were collected from the Gloucestershire (n=3) and Cambridgeshire (n=3) areas in November 2020. Immediately post castration, the tunica vaginalis and epididymis were removed, and the right and left testis were weighed separately. Each testis was bisected down the axial plane, and a 4mm disc was cut (figure 4; sections B & C). From this disc, a biopsy punch (Stiefel; 4mm) was used to take a sample from the centre of the parenchyma and immediately submerged in Allprotect Tissue Reagent (figure 4; section B; Qiagen; Cat. No./ID: 76405) in line with manufacture guidelines. Remaining tissue from this section was wrapped in tin foil and transported to the laboratory on ice. Testicular samples treated in Allprotect Tissue Reagent were archived at -20°C to preserve the RNA for further genomic sequencing. A second 4 mm disc (figure 4; section C) was cut and treated with Bouin's fixative (Sigma-Aldrich; HT10132) for histological observation and stored in 70% ethanol. Tissue from section A and any remaining tissue was wrapped in tin foil and frozen at -80°C until chemical analysis to prevent chemical degradation.

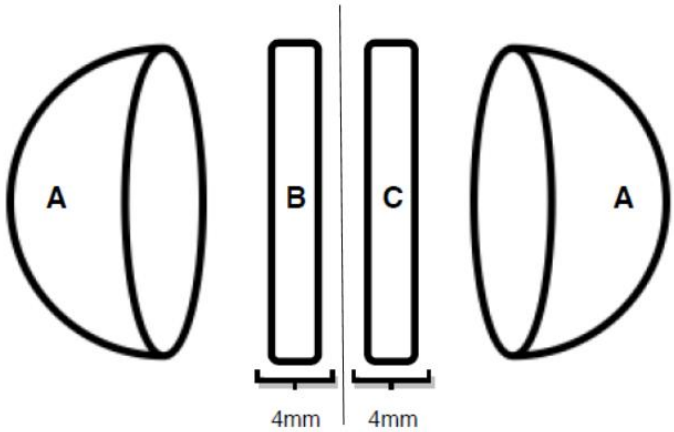


Figure 4: Equine testicular bisection diagram. Sections A were chemically analysed. Biopsies from section B were preserved for genetic analysis, with the surplus cuts chemically analysed with section A. Section C was stored for further histological observations (authors own).

2.5.2 Soil and grass collection

To determine plausible EC exposure routes specific to stallions, soil and grass were collected for analysis. Horses ingest a significant amount of soil directly (Jurjanz *et al.*,

2021), and soil reservoirs provide a source of ECs to growing vegetation, including grasses. As such, both could act as key exposure routes to grazing horses. Soil and grass were collected from Thoroughbred breeding facilities and racing yards in the Gloucestershire (n=2) and Newmarket, Cambridgeshire (n=3) areas. A list of Thoroughbred breeding and racing yards was generated from publicly available sources, to which requests were sent regarding study participation. The number of samples contributing to each of the Gloucestershire and Cambridgeshire soil and grass samples were determined by the participant response rate, based on convenience sampling.

Nine soil and grass samples from each sampled yard were taken, using a 'W' transect for the spatial distribution of sampling sites to provide a representative sample of each site (figure 5). The size of the transect was dependent on the size of the land available to sample. To give an accurate representation of contamination level within the grass samples, where possible, cuttings were taken from grasses presenting the whole blade. All appliances were cleaned with 70% alcohol prior to and in-between collections to prevent cross-contamination. Shears were used to take cuttings from ground level, just above the roots. Once the grass cutting was collected, the roots were cleared from the area, and a soil core was taken using a stainless steel probe (VOSAREA; 2.8 cm diameter by 10 cm depth). A sampling depth of 10 cm was used for the evaluation of surface-level contamination (Te *et al*., 2020). Sampling to this level enables an understanding of concentrations grasses are exposed to and levels that may be consumed through the direct ingestion of soils.

Soil types in Cambridgeshire (Newmarket) and Gloucestershire are classed as lime-rich loam and clay with impeded drainage, and loam with shallow lime-rich soil over chalk or limestone, respectively (Cranfield University, 2022). Due to available funding, samples for grass and soil analyses had to be pooled with samples from respective geographical locations. This resulted in one pooled grass sample and one pooled soil sample from both the Gloucestershire and Cambridgeshire sampling regions. All soil and grass samples were stored in glass jars, transported to the laboratory on ice, and subsequently frozen at -20°C until chemical analysis.

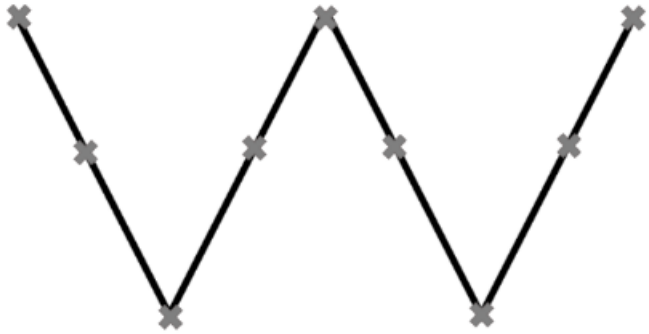


Figure 5: The 'W' transect used for the spatial distribution of sampling locations. Used for the collection of soil and grass. Each cross in the transect represents a sample site (authors own).

2.5.3 Feedstuff material collection

Samples from a small commercially wrapped bale (n=1) and a large farm baled haylage (n=1) were collected for chemical analysis. Samples were taken from the external and internal portions of the bag to provide a representative sample. Concentrate and forage-based feeds were selected based on exposure through differing stages of a stallion's life. Hard feeds (n=4), including a forage-based alfalfa with high oil content, a breeding cube fed to stallions and mares, a racehorse-specific cube, and a pellet recommended for weaning foals were also analysed. Specific ingredients for each commercial feedstuff material are presented in the supplementary information (Appendix P; page 333). Feed from internal and external portions of the bag were taken to provide a sample representative of the whole feed contents. Haylage and feed samples were stored in tin foil or glass jars at -20°C until analysis to prevent chemical degradation.

A high oil alfalfa-based fibre feed was analysed. Alfalfa has high nutritional content for breeding stock, including stallions (DeBoer *et al.*, 2018). Feed companies recommend the use of this feed for all breeding stock, given its nutritional benefits. Separately, alfalfa has been recognised for its phytoremediation abilities within contaminated soils (Bourceret *et al.*, 2018). Currently, a link between feeding this plant species to horses and chemical exposure to contaminants has not yet been explored. A high oil alfalfa was selected for analysis due to the lipophilic properties of many ECs. It was hypothesised that a high oil feed would be subjected to higher levels of EC accumulation compared to a low fat content material. A stud cube designed for stallions and broodmares was analysed to determine contaminant levels exposing all breeding stock. A pellet fed to weaning foals was analysed

to determine exposure during a prime period of reproductive development. Finally, given the simultaneous breeding and racing careers of Thoroughbred stallions, a hard feed pellet designed for the racing Thoroughbred was analysed as a potential route of exposure.

2.5.4 Chemical analysis (Gas chromatography-mass spectrometry analysis)

Samples were transported on ice to a collaborating laboratory. To prevent sample contamination, an ISO-17025 (International Organisation for Standardization) accredited laboratory (James Hutton Institute) carried out all chemical analyses. Samples were analysed for a selection of ECs including Polychlorinated biphenyl (PCB) congeners (28, 52, 101, 118, 138, 153, 180), Polybrominated diphenyl ether (PBDE) congeners (28, 47, 99, 100, 153, 154, 183), Di (2-ethylhexyl) phthalate (DEHP) and Polycyclic aromatic hydrocarbons (PAHs). A total of 16 priority PAHs, as listed by the US Environmental Protection Agency (EPA) and the European Union (EU) were analysed (Tang *et al*., 2005). Σ16PAHs analysed included; Naphthalene, Acenaphthalene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo[a]anthracene, Chrysene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[a]pyrene, Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene and Benzo[ghl]perylene (Appendix N; pages 328-331).

Given that there is a lack of research concerning testicular contamination and associated routes of exposure in stallions, a range of chemical congeners were analysed to provide a broad preliminary understanding of the chemical categories associated with the equine population specifically. Chemical analysis was undertaken using best practice, standardised extraction protocols, and gas chromatography-mass spectrometry (GC-MS) analysis. GC-MS is a sensitive and accepted method for the analysis of anthropogenic ECs, ensuring accurate and reliable readings of chemical burdens (Yavuz *et al*., 2022; Lea, Byers, *et al*., 2016; Ali *et al*., 2013). An extension of this analytical method is described in previous literature (Rhind, Kyle, *et al*., 2010; Rhind *et al*., 2005). The Σ16PAH limits of detection (LOD) ranged from 1.00-5.00 µg/kg. Limit of detection was 0.02 µg/kg for all Σ7PCBs, between 0.02 and 0.50 µg/kg for Σ7PBDEs and 0.05 µg/kg for DEHP. The LOD values for each congener specifically are presented within supplementary information (Appendix Q, page 334). Prior to the analysis of samples, standards and quality control were assessed for performance, resolution, contamination, identification, and quantification. The

identification of compounds was achieved by monitoring specific ions at their associated retention times. All results for analysis are reported on a dry-weight basis.

2.5.5 Rationale for statistical analysis

Data were analysed and graphically interpreted on GraphPad Prism 9 (GraphPad Prism, 2022; GraphPad Prism version 9.0, GraphPad Software, California, CA, USA), enabling the inclusion of statistical outputs within the graphical format. Initial descriptive statistics were utilised to determine the mean (± SD) Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP burdens for each individual stallion, followed by the mean (± SD) testicular concentrations of each congener in the sample population (n=6). Haylages (n=2) and commercial feeds (n=4) were analysed together, defined as feedstuff materials (n=6). Mean concentrations (± SD) were calculated for Σ7PCBs, Σ7PBDEs, Σ16PAHs and DEHP in separate feed types and specific congeners in grouped feeds (n=6). Means were also calculated for soil and grass based on geographic location and pooled samples.

Data were interpreted through a two-tiered system, first analysing Σ7PCBs, Σ7PBDEs, and Σ16PAHs burdens in individual testicular, soil, grass, and feedstuff samples, and secondly analysing specific congeners in respective grouped samples. The sample size for DEHP was too small for both these analyses, so only the summary statistics are presented for this chemical. For the analysis of geospatial differences in testicular EC concentrations between the Cambridgeshire and Gloucestershire regions, testis from respective areas were grouped. Testis one to testis three were defined as Cambridgeshire samples, and testis four to testis six were defined as originating from the Gloucestershire region.

Normality was initially tested using standard procedures, a Shapiro-Wilk test, and graphical interpretation of normal probability plots (Curran-Everett and Benos, 2004). Skewed data were normalised by applying a logarithmic (log) transformation to meet the assumption of the statistical test (Manikandan, 2010). Σ7PCBs, Σ7PBDEs, and Σ16PAHs were log-transformed for analyses of testicular contamination. For the analysis of geographical differences in testicular burdens, data for Σ7PCBs, Σ7PBDEs and Σ16PAHs were log-transformed to obtain a normal distribution and meet the assumptions of a parametric test prior to analysis. DEHP was normally distributed as determined by a Shapiro-Wilk test and

normality plot assessment. For feedstuffs, the data for Σ7PCBs and Σ16PAHs were log-transformed prior to analyses. Data for all other chemicals and soil and grass followed a normal distribution. All data were analysed using parametric tests to determine differences in chemical concentrations between samples and differences in specific congeners in all related grouped samples. For testicular and feedstuff samples, A one-way analysis of variance (one-way ANOVA) determined the statistical significance of EC differences. The one-way ANOVA is an omnibus statistic, meaning that it cannot specify where the differences lie within a test. A post hoc analysis (Tukey's post hoc) was included within the model to define where statistical differences occurred (Chen *et al*., 2018). For soil and grass samples, an independent t-test was used to analyse differences in Σ7PCB, Σ7PBDE, and Σ16PAH concentrations from Gloucestershire and Cambridgeshire.

To determine the contamination of differing chemical congeners in grouped respective samples, all data were analysed with a one-way ANOVA and Tukey's post hoc. For testicular samples, log-transformations were imposed on data for PCBs and PAHs prior to statistical analysis to obtain a normal distribution (Manikandan, 2010). To analyse the geographical variability in EC testicular burdens between the Cambridgeshire and Gloucestershire sampling regions, differences in DEHP and, following transformation, PCBs, PBDEs, and PAHs were analysed with an independent t-test. Data for all chemical categories analysed in feedstuff materials, soils, and grass samples followed normal distributions. Statistical significance was accepted with a p value <0.05, and a confidence interval of 95% was assumed.

CHAPTER 3

TEMPORAL TRENDS IN SPERM PROGRESSIVE MOTILITY IN THE NOVEL EQUINE MODEL (1984-2019): A SYSTEMATIC REVIEW AND META-REGRESSION.

3.1 INTRODUCTION

Current trends in reproductive health and function specific to the male equine population remain unknown. Trends in testicular dysgenesis syndrome (TDS) within human populations indicate a reduction in semen quality and parallel increases in reproductive malformations and congenital abnormalities (Skakkebaek *et al*., 2015). Declining semen quality in men is a topic of contentious debate (Tong *et al*., 2022), with comparative species required to contribute vital understanding towards trends in TDS (Sumner *et al.*, 2020; Lea, Byers, *et al*., 2016; Wahl and Reif, 2009). Despite growing concern regarding perturbed reproductive health, there is no plausible evidence evaluating equine semen quality trends. Results could contribute valuable data on the past and current reproductive statuses of stallions to support the fertility, monetary value, health and welfare of breeding stock in the equine industry. In testing the hypothesis of declining equine semen quality, data generated could initiate the utilisation of this species as an additional comparative model for human fertility. As such, this research has significant implications for the equine and human reproductive biology sectors.

3.1.1 The development of the declining semen quality hypothesis

Research in human populations presents an increasing body of published evidence indicating that human male fertility and reproductive health has declined over the last 40 to 60 years. Adverse trends in semen quality became a topic of contentious debate in 1992, initiated by a systematic review and meta-analysis indicating a significant decline in human sperm count and semen volume from 1940 to 1990 (Carlsen *et al.*, 1992). Limitations in the methodological approaches implemented within this research have been criticised for heterogeneity and the inclusion of data from historical sources, and with advancing semen analysis methods (Pacey, 2013). Whilst the research was heavily scrutinised (Fisch, 2008; Pacey, 2013), the initial systematic review of human semen quality (Carlsen *et al*., 1992) gained warranted public attention. The study operated as a stimulus for research furthering the debate surrounding adverse reproductive trends (Fisch, 2008).

An important factor of systematic reviews is the public availability of metadata, which provides scope for further exploration of a specific research question. Two further systematic reviews on human-based semen quality trends built upon the initial publication

(Carlsen *et al*., 1992), mitigating associated methodological issues (Levine *et al*., 2017; Swan, Elkin and Fenster, 2000). The addition of 63 studies to the original publication (Carlsen *et al*., 1992), whilst accounting for age, resulted in the observation of a significant decline in sperm concentration between 1983 and 2013 (Johnson *et al*., 2015). A further reanalysis continued to indicate significant declines of 52.4% and 59.3% in sperm concentration and total sperm count, respectively, between the years 1973 and 2011 (Levine *et al*., 2017). The slope of decline shows no sign of plateau (Levine *et al.,* 2022), a significant concern for the reproductive health and function of men. If equivalent declines were to exist in the equine population, there could be significant economic, health and welfare consequences for the breeding sector. To date, semen quality trends specific to the equine population in the 21st century remain unknown.

The term 'global declines' when referring to trends in semen quality is heavily used across literature, although this selection of terminology should be used and interpreted with caution (Fisch, 2008). Research suggests that temporal declines are present within specific populations, dependent on multiple factors, including geographical location (Swan, Elkin and Fenster, 2000, 1997; Carlsen *et al*., 1992). Adverse trends in human sperm concentration and count are more significant in populations based in North American, European, and Trans-Tasman regions (Levine *et al*., 2017). Whilst the declines observed in sperm count remains a topic of contentious debate, geospatial variability is a predominantly accepted hypothesis (Rahban and Nef, 2020). Researchers have suggested that geospatial differences in semen quality are likely reflective of lifestyle and environmental factors, including tobacco smoking, recreational drug use, alcohol consumption, physiological stress, and exposure to environmental contaminants (Rahban and Nef, 2020).

Current evidence syntheses do not explore temporal trends in motion characteristics in any species, including humans and equines. However, retrospective analyses support the results of adverse trends reported through systematic methodologies. In humans, adverse trends in total motile sperm count have been reported between 2002 and 2017 in Europe (Tiegs *et al*., 2019) and North America (Chang *et al.*, 2018). Over the same time period, the dog, which shares a close environment with human populations, has presented a 30%

decline in sperm motility (Lea, Byers, *et al*., 2016). Adverse trends in motion characteristics collected from single laboratories with consistent methods mitigates methodological limitations associated with evidence syntheses, further supporting the hypothesis of declining semen quality. Given that equivalent trends exist in both dog and human sperm motion characteristics, it is likely that a common aetiology associated with the shared environment they coexist in is involved in the declines observed. Adverse trends in sperm motility are concerning, given the importance of the parameter for reproductive health and function. Progressive motility (PMOT) is an important parameter for the prediction of male infertility (Dcunha *et al*., 2020). It is essential for the penetration of the zona pellucida and, subsequently the ability to achieve a successful pregnancy (Simon and Lewis, 2011). As such, PMOT is an important predictive parameter of fertility that can be utilised to help monitor trends in reproductive health and function.

Within the equine industry, a threshold PMOT value of less than 40% is associated with compromised fertility (Colenbrander, Gadella and Stout, 2003), whilst Warmblood stud books recommend a threshold PMOT value of 50% (Aurich *et al*., 2020; Parlevliet, Kemp and Colenbrander, 1994). PMOT readings below these threshold values have been reported in stallions throughout literature including those defined as fertile (Griffin *et al*., 2020; Ortiz-Rodriguez *et al*., 2019; Cabrera *et al.*, 2018; Florez-Rodriguez *et al*., 2014). Such research would suggest that equine sperm motility is not being maintained at an optimal level, a significant concern for the fertility potential of stallions in the breeding sector. Despite reports of suboptimal equine sperm motility, research analysing equine trends in this parameter remains limited. Such an understanding is vital to initiate a holistic approach in protecting the fertility, health and welfare of the male equine population.

3.1.2 Semen quality trends in herbivorous species

Subfertility and infertility are common concerns in stallions (Salas-Huetos *et al*., 2022; Oristaglio Turner, 2007), although research assessing trends in semen parameters remains limited. The current research base, reporting declines in equine semen volume, is associated with two horse breeds, the Breton draught and Anglo-Arab Thoroughbred, between 1981 and 1996 (Multigner *et al.*, 2000, 1999). Such results are unrepresentative of current trends, with limited applicability to the general equine population. Whilst

research in bovine semen quality is indicative of adverse trends, populations sampled are also isolated to single breeds (Wahl and Reif, 2009; Van Os *et al*., 1997). In dairy bulls, adverse time-trends are also reported in pregnancy rate (De Vries and Risco, 2005), incorporating both male and female factor fertility. The male factor contributes 50% towards fertilisation, so it is essential to evaluate the contribution of sperm quality to adverse pregnancy rates. In addition, horses have reduced fertility in comparison with other livestock species discussed (Mucha, Wolc and Szwaczkowski, 2011), which is likely to be partially associated with male factor infertility. Focus on artificial reproductive techniques (ART) in the equine industry reduces the natural selection of heritable reproductive traits (Colenbrander, Gadella and Stout, 2003). Selective breeding may be associated with declining semen quality reported in horses and bulls, a factor that must be acknowledged when interpreting results. Further analysis of comprehensive datasets is required in order to produce valuable data on reproductive trends that are applicable to wider current populations, to further understanding of fertility statuses in herbivorous species, including the equine model.

Given that ART is a common breeding strategy within the global equine industry (Campbell and Sandøe, 2015), an extensive amount of metadata is available that could contribute to our understanding of temporal trends in stallion semen quality. The equine species poses as an essential reproductive model whose full potential has not yet been harnessed. Determining trends in stallion semen quality could therefore support or contradict previous research in other species, adding significant weight to the debate surrounding declining reproductive parameters. The analysis of stallion PMOT trends could develop understanding on the proportion of stallions at risk of perturbed reproductive health and function, vital data for the breeding and wider equine industries. If the hypothesis of declining equine semen quality is accepted, research can focus on determining underlying aetiologies responsible for trends across species. Perturbed reproductive function could pose a threat to the health and welfare of stallions and hold ergonomic and economic consequences on the industry status of breeding stock. Systematic and meta-analytical approaches provide essential evidence and understanding that can be used in combination with primary datasets to contribute to our understanding of trends in semen quality.

Through systematic review and meta-regression analysis, this research aimed to assess temporal trends in stallion semen quality across global domesticated equine populations. The objectives to address the research aim are as follows:

1. To systematically collect and analyse publicly available data on stallion sperm PMOT.
2. To assess trends accounting for additional covariates, including geographical location.
3. To investigate the suitability of the novel equine model as a comparative species for male reproductive trends in human populations.

For this study, it was hypothesised that sperm PMOT has declined in recent years across global domesticated equine populations. The null hypothesis was that no trends are observed in equine sperm PMOT across global domesticated populations.

3.2 METHODS

An expansion and justification for the methodology and methods assumed within this study are presented in chapter 2 of this thesis (research methodology and methods; pages 40-67). Hartpury University Ethics Committee granted ethical acceptance (ETHICS2019-52) for all research associated with this study. A data management plan, in line with UWE regulations, was adhered to, ensuring the responsible collection, storage, and analysis of metadata.

3.2.1 Evidence synthesis protocol and pilot-study

Prior to the commencement of the systematic review, a protocol was constructed in accordance with the Collaboration for Environmental Evidence (CEE) recommendations (CEE, 2018). Two primary reviewers were involved in the formation of the protocol (IH and JP), which was critically reviewed by all researchers associated with the study, including a systematic review specialist (MF). The protocol acted as a pilot-study for the evidence synthesis, formed of a test search, literature scope, and the establishment of a test-list. These steps were carried out to evaluate the volume of relevant literature, produce informed eligibility criteria, and assess database applicability in relation to the research question. The final stage of the protocol included the establishment of a test-list. The test-list pilot study involved screening the title and abstract of 50 articles and screening full texts of those available (n=38; Appendix I; pages 301-305). Discrepancies were resolved through group discussion, resulting in the refinement of the final systematic review protocol.

3.2.2 The evidence synthesis: a systematic review

The systematic-review and meta-regression analysis was conducted in line with the CEE recommendations, following the RepOrting standards for Systematic Evidence Syntheses (ROSES) pro-forma and flow diagram (Haddaway *et al*., 2018). Scopus, Medline (Ovid), Embase (Ovid), CAB direct (VetMed), and CORE were searched utilising a Boolean search string [(stallion* OR horse* OR equine* OR colt*) AND (sperm* OR (semen AND quality) OR insemin*) AND NOT (human* OR horseradish)] between January and May 2021. No date restriction was applied to the search strategy. All English language returns were included for screening. A second reviewer (JP) utilised the same strategy to search PubMed, PubAg, and BASE.

3.2.3 Eligibility determination through a two-stage screening process

The review considered all articles reporting data on fresh and cooled equine sperm progressive motility (PMOT) assessed objectively (CASA) and subjectively (microscopy), and sperm concentration analysed via a NucleoCounter. Returns were critically evaluated by two independent reviewers (IH and JP) based on the predefined eligibility criterion. Article eligibility was determined through a two-stage screening process. The first and second stages involved title and abstract screening, and full-text screening, respectively. Supplementary data from the corresponding author or cited articles were collected for eligible articles that failed to provide a single factor. Based on the primary and supplementary information available, articles were included or excluded. Justification of exclusion was recorded as related to the study population, outcome, or article specifics.

All articles eligible for screening at stage two of the process were subject to citation searching. A forward citation (F-C) search was carried out on each eligible article via the Google Scholar platform. Backward citation (B-C) searching involved screening the reference list of each article. Citation search screening was carried out by two reviewers. The second reviewer (JP) completed the same screening process independently, using their allocated databases (PubMed, BASE, and PubAg). This preliminary dataset is available in published format (Perrett *et al.*, 2021). For the purposes of the current evidence synthesis, a third independent reviewer (CM) screened all 'include' and 'uncertain' articles from the primary and citation searches. Inconsistencies in opinions on article eligibility were resolved through group discussions with the research team.

3.2.4 Critical appraisal and bias assessment

The implemented protocol, methodological, and statistical approaches sought to mitigate associations of bias at all stages of this evidence synthesis. Additionally, the list of included studies were subject to a final stage of critical appraisal in-line with the CEE recommendations. Each article was assessed based on five domains of bias; selection bias, performance bias, detection bias, attrition bias, reporting bias, and other bias. For each domain, the articles were deemed as 'low risk' or 'high risk'. Articles that were subject to a high level of risk for any domain were excluded from the dataset. Two independent (IH and

CM) reviewers critically appraised all relevant articles following the same method of assessment, and any articles with eligibility discrepancies were removed from the dataset.

3.2.5 Narrative synthesis

Summary statistics (mean, SD, SEM, range, median, interquartile range) on PMOT and concentration were extracted from eligible articles. Year of sample collection, sample size, fertility group, geographical location, season of collection, horse breed, horse age and age range, semen form (fresh or cooled), temperature and time period cooled, use of extender and centrifugation, method of analysis (CASA, microscopy, NucleoCounter), CASA model, STR and VAP threshold values were recorded for statistical analysis. For key variables with missing data, estimations were made where possible. An expansion of calculations can be found in chapter 2 of this thesis (research methodology and methods; pages 40-67).

All data associated with concentration (n=3), microscopy PMOT values (n=22) and cooled semen (n=30) were removed from the dataset. This resulted in 230 original datasets on fresh PMOT values analysed via CASA. Descriptive data that were not included within quantitative analysis were summarised as part of narrative synthesis. Covariates, including breed, season, hemisphere, and CASA model, were grouped accordingly. Season was categorised as breeding, non-breeding or year-round, in reference to hemisphere. Western included Europe, North America, and Australasia, whilst those based in South America, Africa and Asia were defined as non-western. Stallions were categorised into warmbloods, coldbloods, hotbloods, miniature and pony types, and mixed breeds, definitions for which can be found in the supplementary information (Appendix J; page 306).

3.2.6 Statistical analysis

The statistical code utilised for this objective is provided in appendix L of this thesis (pages 325-326). Mean PMOT estimates for each eligible study were evaluated to model trends across time, as assessed by the predicted yearly percentage change and the regression coefficient (*b)*. A simple linear regression was run to assess time trends in PMOT, with each study weighted by sample size. Mean (± SEM) values were predicted from the model for each year of collection to determine overall trends. A fixed-effects meta-regression with a stepwise approach was utilised to determine the significance of covariates. Significant

covariates were included within the final full-refined model. Regardless of significance, year of collection was also included. The meta-regression was weighted by the SEM of each study, so more precise studies had more influence within the analysis (Thompson and Higgins, 2002). Accounting for covariates, year of collection was removed from the model to determine its significance. The strength of association was calculated utilising Cohen's d method, where b values of 0.10-0.29, 0.30-0.49, and greater than 0.50 are defined as having small, medium, and large effect sizes, respectively (Nieminen, 2022; Cohen, 1998).

Multiple sensitivity analyses were performed to evaluate trends in relation to different contexts and populations. Sensitivity analyses included a meta-regression model weighted by study SEM, initially containing all predefined covariates. The significance of covariates were evaluated for each individual sensitivity analysis to determine the final fixed model. Cubic and quadratic functions were added to year of collection individually in the refined meta-regression model to assess non-linearity. Sequential meta-regression analyses were run, excluding studies with less than a sample size of 10 (Borenstein *et al*., 2009), datasets with missing values for any variable, and articles before the collection year 2000.

Groups of stallions 'unselected for fertility' (n=152) were analysed to evaluate trends relevant to wider populations. Groups belonging to western (n=191) and non-western (n=32) regions were analysed separately to determine geographical differences. A single value was calculated for all articles, apart from one, which provided two values, given that there was no plausible way to calculate an overall mean. To assess the impact of this article on the dataset, the meta-regression was run sequentially, removing each value followed by the article altogether.

3.3 RESULTS

3.3.1 Systematic review output

Using Scopus, Medline (Ovid), Embase (Ovid), and VetMed (CAB direct), 8,102 publications were identified with a comprehensive search strategy. A further 757 grey literature articles were identified (CORE). Of these, 4,214 duplicates were excluded. Following article screening, 2,677 articles were removed during the title and abstract stage and 697 were excluded after full text screening. The full texts for 68 articles were not available and unobtainable through library resources, so were not carried through to the second stage of the screening process. An additional 76 articles were accepted through stage two screening from F-C and B-C searching and alternative databases, including PubMed, BASE, and PubAg (Perrett *et al*., 2021). During critical appraisal, 237 articles were removed from the dataset, resulting in 285 original articles. The ROSES flow diagram utilised to track the progression and quantity of articles at each level of the review is presented in figure 6 (Haddaway *et al*., 2017).

3.3.2 Narrative synthesis

During narrative synthesis, 55 articles were removed containing data on cooled semen, concentration, and PMOT analysed via microscopy. The final dataset consisted of 230 original articles from publication dates 1991 to 2021. Citations and the reference list are provided as supplementary information (Appendix K1, pages 308-309; Appendix K2, pages 310-324). The meta-regression analysis was based on 231 estimates of fresh stallion sperm PMOT, analysed through objective CASA systems. Given differences observed in CASA settings, VAP and STR threshold values were included in statistical analyses. Year of semen collection utilised within the analysis ranged from 1984 to 2019. The number of studies contributing to each year are presented in table 7. Stallions ranged from 2 to 30 years in age. Breeds analysed included warmbloods (n=64), mixed breeds (n=73), miniature and pony types (n=20), hotbloods (n=5), and coldbloods (n=2). There was a higher percentage of stallions ‘unselected for fertility’ (n=152) than selected (n=79). The majority of articles included were associated with western populations (n=192) compared to non-western populations (n=32). For the northern hemisphere (total studies n=198), articles from stallions in the breeding (n=62), non-breeding (n=18), and year-round seasons (n=13) were

included. For the southern hemisphere (total studies n=26), articles from stallions in the breeding (n=9), non-breeding (n=2), and year-round seasons (n=1) were included.

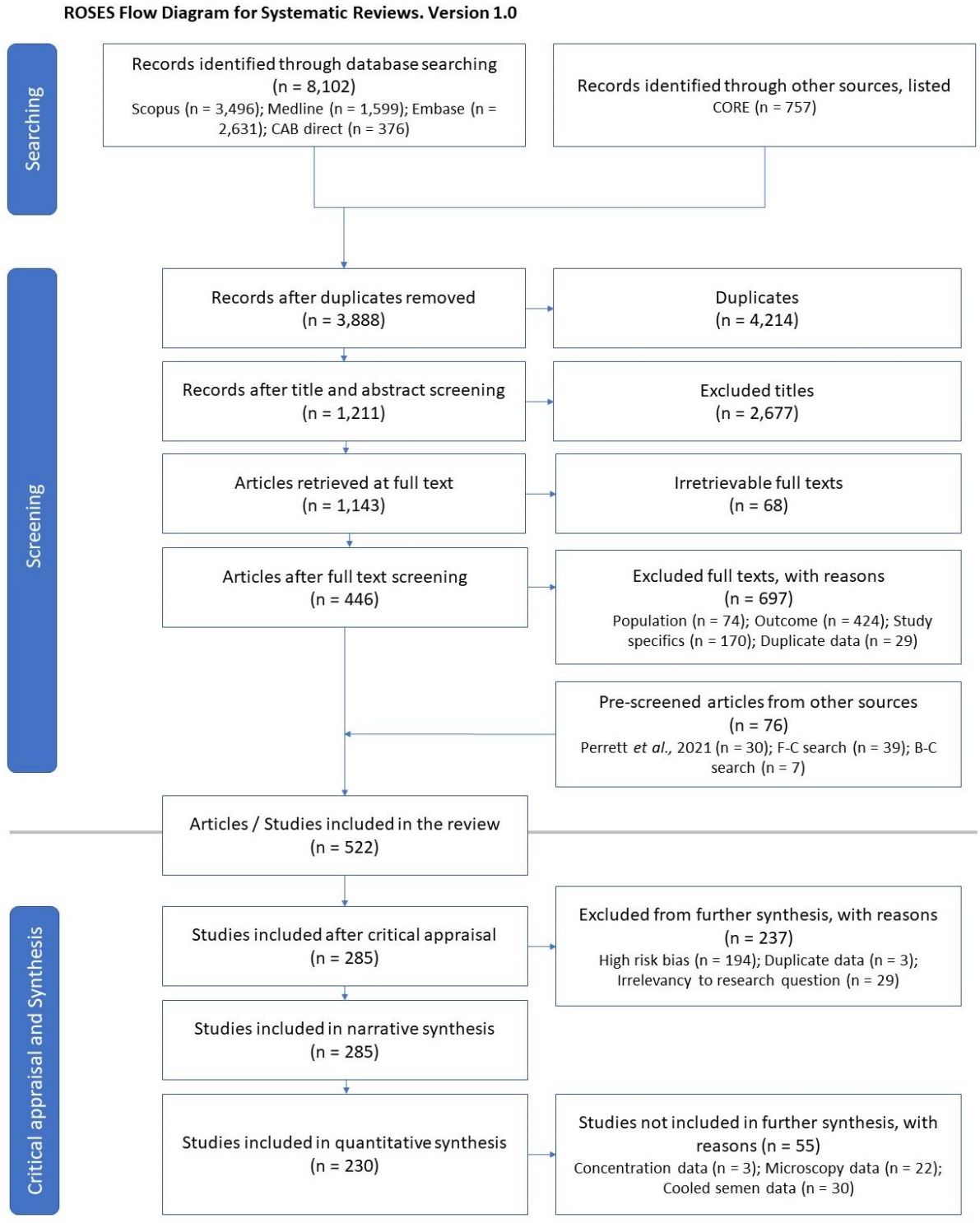


Figure 6: The ROSES Flow diagram for systematic reviews (Version 1.0) used to track the identification, screening, critical appraisal, and synthesis of articles (Haddaway et al., 2017). n=: number of articles; F-C: forward citation search; B-C: backward citation search.

Table 7: Number of articles included in the analysis for each year of semen collection.

Year of collection	**1984**	**1985-1986**	**1987**	**1988**	**1989**	**1990**	**1991**
Article number	1	0	1	1	2	1	3
Year of collection	**1992**	**1993**	**1994**	**1995**	**1996**	**1997**	**1998**
Article number	1	2	2	1	0	2	2
Year of collection	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**
Article number	7	3	4	4	4	1	4
Year of collection	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**
Article number	6	14	8	15	13	18	16
Year of collection	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**
Article number	18	19	21	13	16	6	2

3.3.3 Regression models

The residual diagnostic plots for the simple linear regression analysis indicated that the model fit the test assumptions (VSN International, 2020). The simple linear regression model indicates that PMOT declined significantly over the study period (*b* -0.340; *p* 0.026; figure 7). PMOT declined by a value of 8.47% (1984: 59.11 ± 3.93%, 2019: 50.64 ± 1.77%), a yearly and total decrease of 0.42% and 14.33%, respectively.

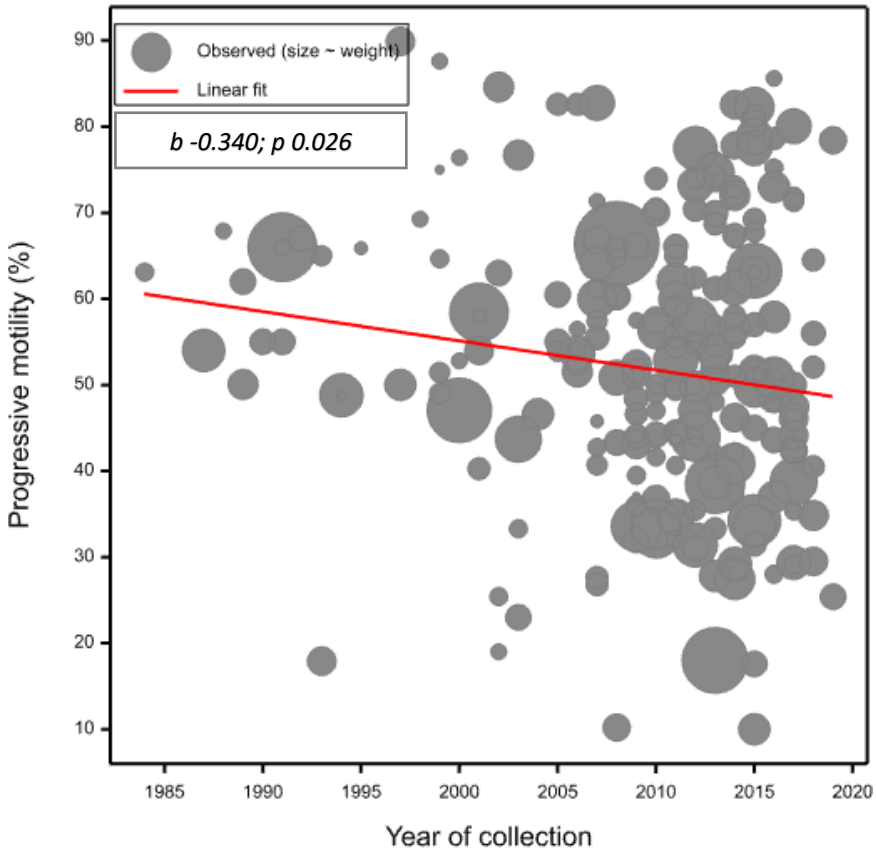


Figure 7: Trends in sperm progressive motility (PMOT; %) as predicted from the simple linear regression model weighted by stallion sample size. Data point size corresponds to the sample size; red line denotes the slope of regression. Graph produced on Genstat.

The residual diagnostic plots for the meta-regression indicated that the model fit the test assumptions (VSN International, 2020). Within the full-refined meta-regression model, accounting for other covariates, PMOT declined significantly (*b* -0.603; *p*<0.001; figure 8). Utilising the Cohen's d method (Nieminen, 2022; Cohen, 1998), there was a large strength of association for the refined meta-regression model. Year of collection (*p* 0.022), location (*p* 0.006), hemisphere, stallion breed, and CASA model (*p*<0.001) were significant within the final refined meta-regression model. Whilst accounting for other covariates, dropping year had a significant effect on the model (*p*<0.001). PMOT declined by a value of 21.10% (1984: 63.59 ± 5.06%; 2019: 42.49 ± 3.69%), a yearly and overall decline of 0.89% and 31.89%, respectively. The refined model accounted for 47% of variance. Predicted PMOT mean (± SEM) values from the final refined meta-regression model are presented in table 8.

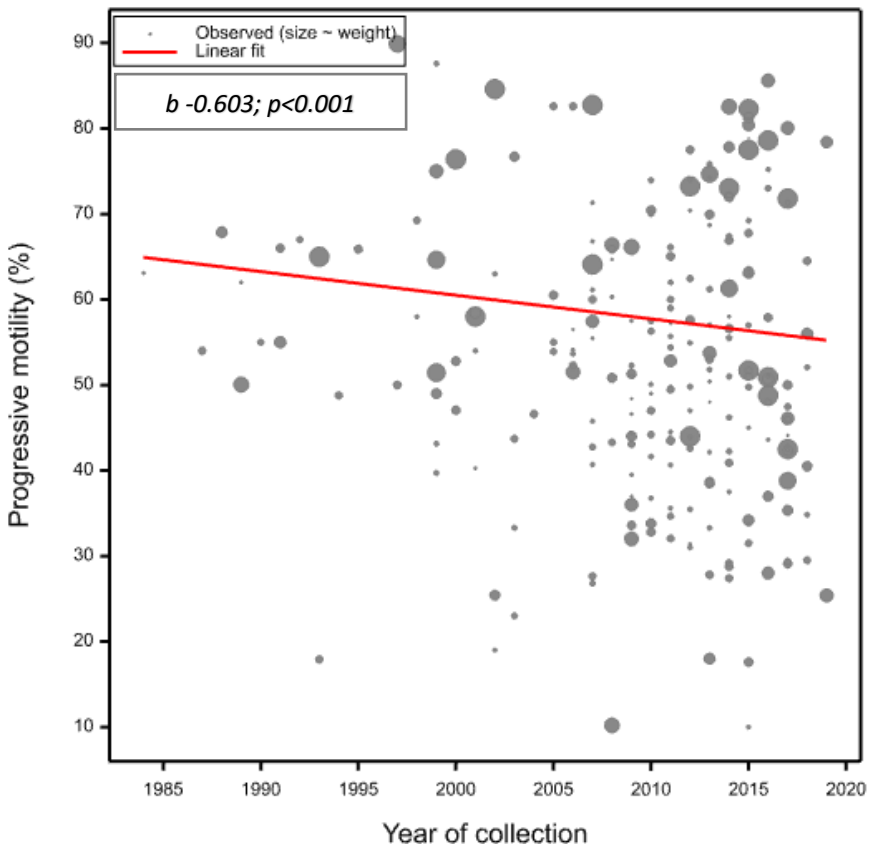


Figure 8: Trends in sperm progressive motility (PMOT; %) between 1984 and 2020 as predicted from the full-refined meta-regression model weighted by study SEM. Data point size corresponds to the sample size; red line denotes the slope of regression. Graph produced on Genstat.

Table 8: Mean (± SEM) progressive motility (PMOT; %) predicted from the full meta-regression.

	PMOT (%)					
Year	**1984**	**1985**	**1986**	**1987**	**1988**	**1989**
Mean ± SEM	63.59 ± 5.06	62.98 ± 4.95	62.38 ± 4.85	61.78 ± 4.75	61.17 ± 4.65	60.57 ± 4.55
Year	**1990**	**1991**	**1992**	**1993**	**1994**	**1995**
Mean ± SEM	59.97 ± 4.46	59.37 ± 4.37	58.76 ± 4.28	58.16 ± 4.20	57.56 ± 4.11	56.95 ± 4.04
Year	**1996**	**1997**	**1998**	**1999**	**2000**	**2001**
Mean ± SEM	56.35 ± 3.96	55.75 ± 3.89	55.15 ± 3.83	54.54 ± 3.76	53.94 ± 3.71	53.34 ± 3.66
Year	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**
Mean ± SEM	52.74 ± 3.61	52.13 ± 3.57	51.53 ± 3.53	50.93 ± 3.50	50.32 ± 3.47	49.72 ± 3.45
Year	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**
Mean ± SEM	49.12 ± 3.44	48.52 ± 3.43	47.91 ± 3.43	47.31 ± 3.44	46.71 ± 3.45	46.10 ± 3.46
Year	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**
Mean ± SEM	45.50 ± 3.49	44.90 ± 3.51	44.30 ± 3.55	43.69 ± 3.59	43.09 ± 3.63	42.49 ± 3.68

3.3.4 Sensitivity analyses

Multiple analyses were completed to assess the sensitivity of the results and determine the influence of covariates, missing data, and study period. Cubic and quadratic functions added to year of collection were insignificant for the meta-regression model (p 0.284; p 0.698), so a linear relationship was accepted. The decline and slope of regression did not change appreciably when restricting the analysis to articles with sample sizes of more than 10 stallions (year of collection p 0.003; b -0.609; p<0.001; n=73) (Borenstein *et al*., 2009). Restricting the meta-regression analysis to articles with full datasets only (n=22) led to a steeper decline in PMOT (year of collection p<0.001; regression: b -1.656; p<0.001). In restricting the analysis to collection years 2000 to 2019 (n=204), PMOT declined significantly (year of collection p 0.058; regression: b -0.474; p<0.001). Whilst variability in PMOT between year of collection was insignificant (p 0.058), the overall decline was significant (p<0.001). In stallions unselected for fertility potential, PMOT declined significantly between 1984 and 2019 (year of collection p<0.001; n=152; b -0.654; p<0.001). PMOT declined by a value of 22.89%, an overall and yearly percentage decline of 36.52% and 1.01%, respectively. A breakdown of trends and regression slopes reported for each sensitivity analyses are reported in table 9.

A significant decline was determined in western (year of collection *p* 0.122; n=191; *b* -0.457; *p*<0.001) and non-western (year of collection *p* 0.4730; n=32; *b* -0.710; *p*<0.001) populations. Whilst year of collection was insignificant (*p*>0.05) in both models, overall PMOT declines were significant (*p*<0.001). In western populations, the predicted overall and yearly declines from 1984 to 2019 were 25.92% and 0.72%, respectively. Predicted means for non-western populations are presented from 1995 to 2017, with an overall and yearly decline of 10.65% and 0.46%, respectively. For a more accurate comparison between western and non-western populations within an equivalent time scale, western means and trends were predicted in relation to the respective dates reported in the non-western analysis (1984 to 2019). From 1984 to 2019, the predicted overall and yearly declines for western populations were 17.74% and 0.77%, respectively, a steeper decline compared to non-western populations.

Table 9: Predicted means and meta-regression outputs. Single asterisk (*): number of articles included in each analysis. Double asterisk (**): means predicted from the fitted model. Full-refined (meta-regression) model; >10 stallions: articles with >10 stallions; complete dataset: studies with complete datasets; date restricted: studies from 2000 to 2019; F-Unselected: Fertility unselected.

Analysis group	n=*	First year	First year PMOT**	Final year	Final year PMOT**	Overall change	Yearly change	Slope (95% CI)
Full-refined model	230	1984	63.59%	2019	42.49%	-31.89%	-0.89%	-0.603
>10 stallions	73	1989	65.98%	2018	36.16%	-24.75%	-0.73%	-0.609
Complete dataset	22	2000	92.48%	2018	34.50%	-45.20%	-2.38%	-1.656
Date restricted	204	2000	51.20%	2019	42.20%	-17.59%	-0.88%	-0.474
F-Unselected	152	1984	62.68%	2019	39.79%	-36.52%	-1.01%	-0.654
Western	191	1984	61.73%	2019	45.73%	-25.92%	-0.72%	-0.447
Non-western	32	1995	57.01%	2017	50.94%	-10.65%	-0.46%	-0.710

One article contributed two individual values to the dataset, whilst the remainder only contributed one (230 original articles; 231 data points). The effect and potential skewing as a result of the inclusion of two values from a single population of stallions was determined. When consecutively removing one of the two included values given by a single article, followed by the whole article (dataset of 229 data points from 229 articles), the slope of the regression was not altered appreciably (*b* -0.590, *p*<0.001; *b* -0.600, *p*<0.001; and *b* -0.580, *p*<0.001, respectively). Whilst the threshold value settings enabling CASA to

define progressively motile spermatozoa were not significant within the meta-regression model, the values were plotted with PMOT trends for graphical interpretation. Whilst VAP and STR presented slight adverse trends, it is unlikely these threshold values were responsible for the decline in PMOT reported in the male equine population (figure 9).

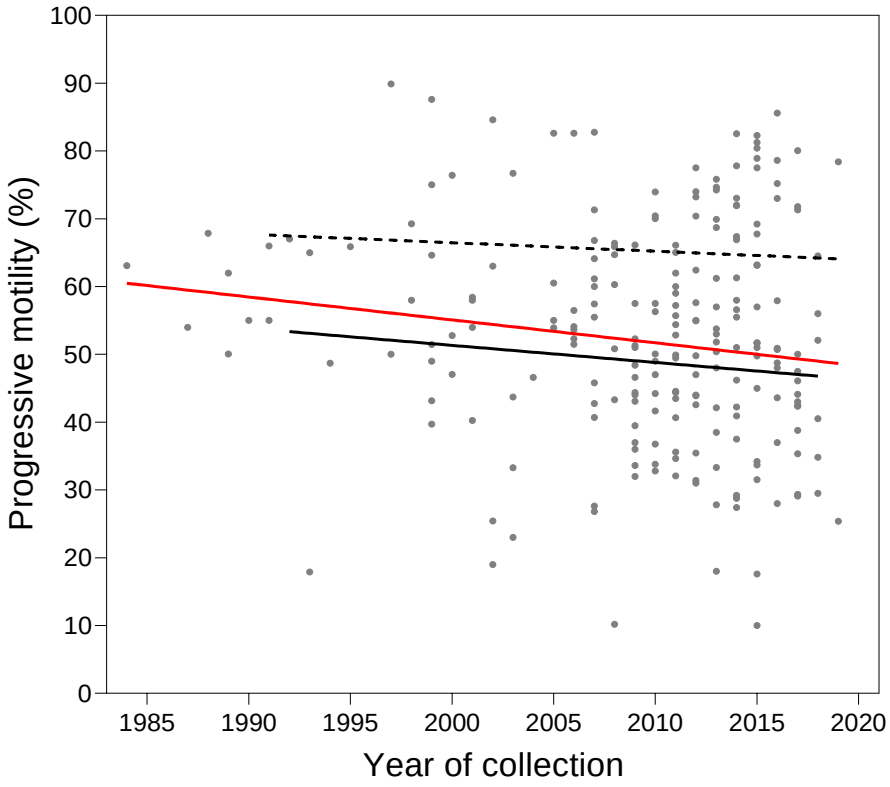


Figure 9: Simple linear regression of PMOT, and CASA technology (VAP and STR) thresholds. n=231; 1984-2019; b -0.340; p<0.05. Simple linear regression line from the statistical output for PMOT (red filled line). Simple linear regression lines for STR (black dotted line) and VAP (black filled line) are for graphical purposes only. Graph produced on GraphPad.

3.4 DISCUSSION

The key output from this study indicates that PMOT in global domestic equine populations has declined by 31.89% between 1984 and 2019, whilst accounting for a set of predetermined covariates. Consistency in trends through multiple sensitivity analyses supports the declines reported in the refined meta-regression model. Declines were consistent within the population of stallions unselected for fertility, supporting the applicability of the results to the wider equine population (Levine *et al*., 2017). Geographical variability in declines existed between western and non-western equine populations, as reported in previous research in alternative species such as humans (Levine *et al*., 2017).

3.4.1 Primary research findings

Results reported within this systematic review and meta-regression analysis consistently report a significant decline over the last four decades in PMOT within global domesticated equine populations, a significant finding for the breeding industry. Adverse trends in PMOT indicate that a higher proportion of stallions are now at risk of testicular dysfunction (Turner, 2019), a serious economic (Shakeel *et al*., 2022), health and welfare concern for the breeding industry. Declining PMOT adds significant weight to the debate surrounding adverse fertility trends and supports the use of the equine model as a comparative species for human populations. The initiation of the equine model as a novel sentinel for the assessment of human-based reproductive trends is a significant outcome of this study.

The rigorous conceptualisation, collection, assessment, synthesis, and analysis of data promotes the quality of the given results. Controlling for a set of predetermined covariates resulted in a steeper slope of regression and a more substantial decline in PMOT when compared to the simple linear regression. Trends within the meta-regression model were determined through the calculation of predicted means accounting for significant covariates, providing a more accurate representation of temporal trends (Thompson and Higgins, 2002). In weighting the meta-regression model by study SEM, the limitation of heterogeneity in article reliability was addressed (Baker and Jackson, 2010), which adds to the quality of the results presented.

Addressing a significant concern raised in previous evidence syntheses (Pacey, 2013), the current analysis did not include historical datasets. Previous research in humans included articles dating back to 1930-1940 (Swan, Elkin and Fenster, 2000; Carlsen *et al*., 1992). Even the current gold standard evidence synthesis in human semen quality trends utilised data from 1973 (Levine *et al*., 2017). Due to the variability in historical semen analysis methods of sperm motility, previous studies chose not to report trends for motility parameters specifically. The current study, analysing recent trends, provides a more accurate evaluation of current reproductive statuses without the limitation of including historical datasets, contributing to the significance of the results. The current research, therefore, acts as a novel study on trends in PMOT through systematic methods across species. As such, this research contributes valuable data to both the equine breeding industry and the general reproductive biology sectors, to further understanding of semen quality trends and male fertility statuses.

To further evaluate trends relative to the 21st century, a date-restricted analysis was carried out. Isolating trends to the past two decades continued to show a significant decline in PMOT. However, the decline was not as prominent when compared to the full-refined meta-regression model. The difference in trends specific to the 21st century could be a result of PMOT levelling off within this population of stallions. Differences in date-restricted trends highlights the importance of the ability to update evidence syntheses (CEE, 2018) to ensure that results are relative to current populations. Whilst CASA is an objective method of analysing PMOT, the progression of technology across the study period must also be considered. The first fully automated CASA system was available in 1985, whilst the design and advancements of systems utilised within the current research occurred throughout the 1990s (Amann and Waberski, 2014). Whilst CASA model was included within all regression analyses, resulting in predicted means accounting for this covariate, trends within the 21st century may be less affected by CASA system developments compared to the refined model. Despite slight adjustments in the regression slope and change in PMOT for the date-restricted analyses, adverse trends were consistent through sensitivity testing, providing a robust indication of declining PMOT.

An important aspect of analysing semen quality trends is the ability to generate data that is applicable to wider populations. Equine industry practices associated with breeding stallions provide a sampling population that is more randomised, unlike human-based research in semen quality trends, which is critiqued for basing results on specific cohorts (Fisch, 2008). Data for the current study were taken from stallions presenting for breeding soundness examinations and collections for ART. Both breeding practices have become routine within the equine sector (Kowalczyk, Czerniawska-Piatkowska and Kuczaj, 2019), and therefore the selection of stallions sampled are likely to be reflective of the greater equine population.

To further test this prediction, stallions categorised as unselected for fertility potential were analysed separately, given that unselected individuals are more representative of wider populations (Levine *et al*., 2017). The predefined covariate was insignificant in the meta-regression models, which could support the prediction that the sample is more randomised. Within the unselected group, PMOT declined substantially, similar to that reported when the analysis was restricted to articles containing complete datasets only. Selection bias is a pitfall of evidence syntheses (Merzenich, Zeeb and Blettner, 2010). Within the current research, each article was rigorously checked for quality by two independent reviewers to mitigate concerns of introducing bias into the dataset (CEE, 2018). Selection bias was based on both population selection and the selectivity of sperm with defined threshold values to ensure that the results were representative of the wider equine population. Together, this would suggest that the equine species holds high importance as a comparative model for trends in reproductive parameters.

Where possible, missing data for covariates were calculated or predicted. However, values were included as missing when the prediction of data were improper. When the model was restricted to articles with complete datasets only, the slope of regression changed substantially, resulting in a steeper decline in PMOT from 2000 to 2018. Whilst the predicted means for the complete model may be more accurate, given that data for every covariate was accounted for, only 22 articles provided full datasets, limiting the conclusions that can be taken from this analysis. The consistency in trends reporting a 1-2% yearly decline in the full-refined model, analysis of articles with complete datasets, and

unselected fertility group provides a robust indication of declining PMOT from 1984 to 2019.

3.4.2 Wider implications of adverse and suboptimal PMOT levels

Adverse trends reported in sperm PMOT within this equine population are of serious concern for the global equine breeding sector. Given that stallion reproductive function is fundamental in the ability to produce successful progeny, these results are also concerning for the sustainability and economy of the wider equine industry. PMOT is significantly correlated with fertilisation rate *in vitro* (Simon and Lewis, 2011). In cooled equine semen, the threshold PMOT value for average and high embryo recovery rates has been reported to stand at 45% (Love *et al*., 2015). Predicted means for the 2019 PMOT readings reported within the current study were below the 45% threshold for high embryo recovery rates. In fresh semen, fertility levels are reported to drop when PMOT values fall below 40% (Colenbrander, Gadella and Stout, 2003). Whilst PMOT in the final year of analysis did not drop below this threshold for the full meta-regression analysis, low values were reported for the group of stallions unselected for fertility, a concerning finding given that this method of analysis is reported to be more applicable to wider populations.

Suboptimal PMOT values are likely to put greater strain on sires with desirable genetic traits, which may result in compromised health and welfare concerns associated with industry breeding practices (Campbell and Sandøe, 2015). Trends do not present any signs of plateau, which is a cause of concern regarding the fertilising capabilities of future individuals and the sustainability of the equine population. A beneficial aspect of utilising publicly available datasets is the ability for research groups to build upon previous reviews as seen within human-based studies (Levine *et al*., 2017; Swan, Elkin and Fenster, 2000; Carlsen *et al*., 1992). The update of this systematic review will enable continual tracking of trends in PMOT over an extended period of time to maintain applicability to the current breeding statuses of stallions in the equine population. An interesting finding within the current analysis was the geospatial differences associated with adverse trends in PMOT.

3.4.3 Geographic variability in trends and associated aetiologies

Results from this present study indicate that declines are more significant in western compared to non-western populations of stallions. The hypothesis of the geographic sensitivity of semen quality trends in the current equine population is supported by previous meta-analyses in humans. Whilst human-based evidence syntheses focus on trends in sperm concentration and total sperm count instead of motility parameters (Swan, Elkin and Fenster, 2000, 1997; Carlsen *et al*., 1992), geospatial variations are reported (Levine *et al*., 2017). The equivalent categorisation method between the current study and previous human-based research (Levine *et al*., 2017) promotes cross-species comparisons, with results indicating that declines in both equine and human semen quality are more significant in western compared to non-western populations. However, whilst this method of geographic categorisation is accepted in previous evidence syntheses, further variations may exist on national or regional levels.

Retrospective studies in humans build on the limitations associated with broad methods of categorisation, and support the hypothesis of geographic-sensitive adverse trends in sperm quality. Significant differences existed in total motile progressive sperm count between regions of Africa, Asia, the USA, Australia, and Europe (Feferkorn *et al*., 2022). In areas of the USA, significant geographic-sensitive declines were reported in human sperm motility (Chang *et al*., 2018). Authors attributed the geographic sensitivity of declines to anthropogenic environmental factors. Additional factors that may impact geospatial differences in human populations include genetic factors (Sharpe, 2010), socioeconomic contexts, nutritional differences, lifestyle, and other unknown aetiologies (Kumar and Singh, 2015). Many of these human-specific factors do not impact reproductive trends in animal species. In the dog sentinel model, research indicates comparable declines in sperm motility (Lea, Byers, *et al*., 2016) to the equine population reported in the current study. Adverse trends in sperm motility in two species that are not exposed to human-specific pressures support the hypothesis of an underlying environmental aetiology. Given that the equine population is not affected by the alternative aetiologies associated with declining sperm motility in humans, this species also provides a valuable comparative model to determine the true effects of environmental toxicology factors within future research.

3.4.4 Aetiologies associated with declining equine PMOT

Perturbed sperm quality across species has been linked to exposure to anthropogenic environmental chemicals (ECs) (Skakkebaek *et al*., 2015), although a current link specific to the equine model has not yet been made. Increasing industrialisation, human consumption, urbanisation, and pressure on agricultural production systems has led to the synthesis and global dissemination of ECs (Liu, Cheng and Li, 2021; Guvvala *et al*., 2020), with an affinity and high toxicity to the reproductive system. Given the use of chemicals in manufacturing, agricultural and pharmaceutical industries, EC contamination of the environment and biological matrices within humans and animals are elevated in areas surrounding high industrial and agricultural productivity.

In China, an area driven by rapid urbanisation and industrialisation, a 3.3% reduction in fertility rate was associated with every 10 $\mu g/m^3$ increment of ambient fine particle pollution (Xue and Zhu, 2018), which contains a range of ECs. Whilst there was not a significant difference in human motility between rural and industrialised areas, alternative kinetic parameters, including curvilinear velocity, linear velocity, and average path velocity, were significantly lower in the latter locations (Zhou *et al*., 2014). Geographic differences were reported in sperm motility in human populations from the USA, with associations between poor sperm quality and elevated urinary concentrations of alachlor and diazinon, two commercial pesticides (Swan, 2006). Given the association of ECs, urbanisation, and perturbations in reproductive parameters, it is plausible that discrepancies in trends reported within the current evidence synthesis are linked to geographical variations in contaminant exposure. Further research is required to elucidate potential aetiologies responsible for significant adverse trends in stallion PMOT, including the evaluation of EC contamination specific to the equine model. Whilst the analysis of an associative link between EC exposure and the adverse trends in PMOT reported here warrants further investigation, additional species-specific factors may also be responsible for declining trends.

3.4.5 Addressing study limitations and the importance of retrospective research

High inbreeding coefficients are associated with poor semen quality in Friesians, Shetland ponies, Colombian Creole horses, and some warmbloods with restricted gene pools (Dini

et al., 2020; Restrepo and Rojano, 2018; Ducro *et al*., 2014; Van Eldik *et al*., 2006). More specifically, inbreeding has been linked to perturbed sperm motion characteristics in horses (Pirosanto *et al.*, 2021, 2019). The impact of inbreeding depression on adverse PMOT trends reported within the current study cannot be overlooked. However, in Thoroughbreds, a breed that is at high risk of inbreeding depression (McGivney *et al*., 2020), whole genome sequencing of a cohort of 150 individuals suggested no association of inbreeding with stallion fertility potential (Castaneda *et al*., 2021). Such research would suggest that there is not a definitive link between inbreeding and sperm quality aberrations. In addition, given that adverse trends in sperm motility characteristics are reported across species (Tiegs *et al.*, 2019; Lea, Byers, *et al*., 2016), it is likely that a common aetiology exists other than species-specific factors alone. In addition, whilst breed is not a direct measure of inbreeding, it was included within the statistical model of the current study, so trends in PMOT were predicted accounting for this variable. Considering the method of breed categorisation, stallions were grouped using recognised methods (Ebel *et al*., 2020). However, differences in sperm quality trends may be on a more specific level than those in the categorised breed types reported here.

An additional factor that should be considered when interpreting trends is the method of semen analysis. Whilst defined as an objective analysis method, CASA systems can impact the readings of sperm motion characteristics, so it is recommended that settings are accounted for when comparing data from multiple laboratories (Hu *et al*., 2013). Comparing two commercial CASA settings using 75% and 50% STR thresholds and 50 µM/s and 30 µM/s VAP thresholds, respectively, total and progressive motility were significantly different (74.6 ± 12.4% versus 69.4 ± 13.5% and 27.4 ± 12.2% versus 40.9 ± 11.7%, respectively) (Whitesell *et al*., 2020). VAP is associated with sperm motion velocity, whilst STR refers to linearity (Lu, Huang and Lü, 2014). VAP and STR were not significant within the meta-regression, although when graphically plotted with the simple linear regression output for PMOT, both settings presented slight declining trends. Given that the slope of CASA settings defining PMOT over time were negligible, results suggest that trends are a result of alternative factors. Whilst the significance of CASA model, VAP, and STR were analysed within the statistical model, many articles did not report the settings assumed. Standardisation of CASA parameter settings specific to the horse would benefit the consistency and interpretation of results in motility and kinetic parameter measurements,

enabling further comparative research in stallion sperm quality. In addition, transparent and consistent reporting of CASA models and settings utilised for the analysis of equine sperm motion and kinetic characteristics in research articles is required for the accurate interpretation of results.

Missing metadata for covariates of interest were dealt with on an individual variable basis. Where possible, predictions were made utilising accepted methods. However, this was not possible for all covariates with reported effects on sperm PMOT. There was no plausible method for calculating abstinence period or age, given the method of reporting in included articles. Therefore, neither of these covariates were included within the model, which is a limitation of this evidence synthesis (Fisch, 2008). In addition, for studies that failed to provide year of semen collection, this variable was calculated from publication date, an accepted method used in previous evidence syntheses (Levine *et al*., 2017). However, whilst this method was a necessary approach for the current study, it has also been critiqued for reduced accuracy in relation to semen collection year and is therefore considered as a limitation (Boulicault *et al*., 2021). Utilising retrospective datasets providing accurate data on timings for individual collections would mitigate this limitation, highlighting the need for further research in single equine populations.

Within the search strategy of the current evidence synthesis, an English-only approach was assumed, which may have introduced a level of language bias to the systematic review. Whilst further international collaborations may mitigate this level of bias, this was an unavoidable limitation associated with the current study. Despite the selected databases providing a high percentage of the articles utilised in the analysis, citation searching resulted in a significant number of original eligible articles. From these results, it is recommended that further evidence syntheses in equine reproductive topics include citation searching within protocols to maximise article returns. However, further research determining the relevance and impact of specific academic databases in relation to equine reproductive systematic reviews is required to promote the development of rigorous and comprehensive evidence syntheses.

3.4.6 Concluding statement

In this comprehensive evidence synthesis, PMOT declined significantly in the global population of domesticated stallions between 1984 and 2019. Consistency in declines across multiple sensitivity analyses adds to the rigour of the results presented. The hypothesis for this study was therefore accepted, and the null hypothesis was rejected. Adverse trends in equine PMOT implies that an increasing proportion of stallions are at risk of perturbed reproductive health and function, with serious implications for stallion breeding, economic statuses, and the health and welfare of breeding stock. The novel equine model has contributed significant data to the debate surrounding declining fertility in human populations, proving an invaluable comparative model for further research in the reproductive biology sector. Further collaborative interdisciplinary research enabling cross-species comparisons could further elucidate to the aetiologies underlying adverse trends across species. However, given the methodological limitations of evidence syntheses discussed, further research assessing reproductive trends in controlled populations is warranted to further understanding of current equine reproductive statuses.

CHAPTER 4

TEMPORAL TRENDS IN SEMEN QUALITY FROM A POPULATION OF STALLIONS AT A SINGLE BREEDING FACILITY IN THE UK (2001-2020).

4.1 INTRODUCTION

4.1.1 Temporal trends in reproductive parameters

Reproductive function, including male factor fertility, is fundamental for the sustainability of a population. Male reproductive aberrations that reduce fertility could diminish the ability to sustain desired heritable stallion traits within the equine gene pool, with practical and economic implications for the equine industry. Exploring trends in reproductive parameters, including semen quality is a useful tool used to further understanding of the past and current fertility statuses of a species. The current research base analysing stallion-specific trends is limited and unrepresentative of the 21st century (Multigner *et al*., 2000, 1999). In humans, there is increasing concern that sperm concentration and sperm count have declined over the last 70 years (Levine *et al*., 2022, 2017; Swan, Elkin and Fenster, 2000; Johnson *et al*., 2015; Carlsen *et al*., 1992), although methodological approaches in evidence syntheses are subjected to high levels of critique (Boulicault *et al*., 2021; Pacey, 2013; Fisch, 2008). Research examining trends in semen quality from single populations with consistent laboratory methods (Sumner *et al*., 2020) and the inclusion of specific participant data reduces many limitations associated with evidence syntheses. As such, retrospective studies provide a fundamental approach in the analysis of reproductive trends.

Epidemiological studies in humans indicate that sperm quality parameters have declined over the past few decades (Tiegs *et al*., 2019; Rolland *et al*., 2013). In southern India, sperm motility of infertility patients declined significantly between 1993 and 2005 (Adiga *et al*., 2008). In a population of fertile sperm donors from France, PMOT declined significantly between 1976 and 2009. Standardisation of microscopy training was reported, with consistency in semen analysis methods across the study period adding to the quality of the results (Splingart *et al*., 2012). Retrospective research in the dog sentinel model indicates a 30% decline in sperm motility between 1988 and 2014 (Lea, Byers, *et al*., 2016). The collection of data from a single laboratory adds to the rigour of these findings, supporting the debate surrounding declining semen quality in humans. The current retrospective research base lacks the analysis of temporal trends in fresh sperm motility parameters within any domesticated herbivorous species, including equine populations.

Previous research in France analysing secular trends in Breton Draught stallions (1981 to 1996) reported a yearly 1.8% decline in semen volume, although no change was observed in sperm count, and there was a yearly 2.8% increase in sperm concentration (Multigner *et al*., 1999). Equivalent findings have been reported in Anglo-Arab Thoroughbred stallions between 1985 and 1995 (Multigner *et al.*, 2000). An increase in concentration and decrease in volume could be due to their inverse relationship, given that sperm count remained consistent. Subsequent research has reported mean seminal volumes below that of the AI referencing ranges (60-120 ml) in the UK (Wilson and Flesner, 2017; Multigner *et al*., 1999), which raises concern over the reproductive health and function of this population. Semen quality is impacted by a range of factors, which need to be accounted for within statistical models determining trends in reproductive parameters.

A number of stallion factors, including age (Darr *et al*., 2017), breed, inbreeding (Pirosanto *et al*., 2019; Wilson and Flesner, 2017), genetics (Gottschalk *et al.*, 2016), discipline, and exercise intensity (Wilson *et al.*, 2019) are reported to impact stallion semen quality. Additional factors, include season (Aurich, 2016), testicular heat stress (Albrizio *et al*., 2020), abstinence period (Sieme, Katila and Klug, 2004), and nutrition (Brinsko *et al*., 2005). The failure to account for such factors, where possible, may lead to a misinterpretation of temporal trends in stallion semen quality. Therefore, further research building on the limitations of the current study determining trends in equine semen quality (Multigner *et al*., 1999) is warranted. In addition, seminal volume is not a direct measure of testicular health (World Health Organization, 2021), with alternative semen parameters reported to be better predictors of testicular function.

Sperm concentration, count, and normal morphology declined by 13.1%, 16.5%, and 46.2%, respectively, between 1970 and 1985 in a population of Holstein bulls. However, in the same population post-thaw motility increased (Wahl and Reif, 2009). Analysing post-thaw samples has limited applicability as a predictor of testicular health, given that values are reflective of freeze-thaw protocols. Research analysing trends in motion characteristics in fresh semen samples is therefore warranted, including in the equine model. Such an analyses could contribute vital data on stallion reproductive trends and further discussions of semen quality declines in general herbivorous species. Equines may also provide an

important herbivorous model that could be utilised in parallel with the carnivorous canine and livestock species to support or oppose trends reported within humans.

Chapter 3 of this thesis (Temporal trends in sperm progressive motility in the novel equine model (1984-2019): a systematic review and meta-regression) initiates the novel equine model as a comparative species for human reproductive trends, which is further investigated in this chapter. The outputs from chapter 3 indicate that sperm PMOT in the global equine population has significantly declined within the past four decades, with means falling below recommended industry thresholds (Love *et al*., 2015). The research presented here builds upon methodological limitations associated with evidence syntheses that are reported in previous literature (Pacey, 2013), by analysing trends in four key semen quality parameters from a single equine population. As such, this retrospective cohort study explores male equine reproductive trends through controlled methodological approaches, contributing understanding on the status of equine reproductive function.

The current study aimed to determine temporal trends in equine semen quality within a single population of breeding stallions from the UK. To address this aim, the research was focused around three main objectives:

1. To collect and analyse retrospective data on semen quality to assess trends in a single equine population at a breeding facility in the UK.
2. To determine time trends in stallion semen quality whilst accounting for alternative variables including stallion age, breed, and abstinence period.
3. To further investigate the suitability of the equine model as a comparative species for human reproductive research.

It was hypothesised that semen quality, including total motility, concentration, volume, and total sperm output, is declining in an equine population from a single breeding facility in the UK. The null hypothesis was that no trends are observed in semen quality parameters from an equine population from a single UK-based breeding facility.

4.2 MATERIALS AND METHODS

An expansion and justifications of the methodology and methods assumed within this study are presented in chapter 2 of this thesis (research methodology and methods; pages 40-67). The research was approved by the Hartpury University Ethics Committee. A data management plan was adhered to throughout the research process to ensure the responsible handling of data, implemented to protect the industry statuses of stallions involved in the study.

4.2.1 Semen collection and analysis

Retrospective data on fresh stallion semen quality was collected from a single breeding facility in the UK. The collection facility was a Defra, and APHA approved center, meeting national regulations associated with equine semen collections. Personnel with standardised training carried out collections at the breeding facility. Stallions were teased with a mare, and the penis was washed with lukewarm water and dried. Semen collection was carried out using an AV with a teaser mare, phantom, or via ground collection, dependent on stallion preference. Whilst there was variation in technicians, training for stallion handling and semen collection was standardised across the study period.

Following successful collection, semen was taken to the laboratory at the same facility for immediate analysis. Parameters of interest included total motility (TMOT; %), concentration (million per ml; $x10^6$/ml) and semen volume (ml). A 10 µl sample of raw semen was analysed subjectively for TMOT readings (microscope; x100 magnification). Volume was calculated by weighing the samples (1 g = 1 ml) (Whigham *et al*., 2014). Sperm concentration was measured using a SpermaCue (2001 – 2011 collections), or a NucleoCounter SP-100 (2012 – 2020 collections), following manufacturer guidelines. One out of two managerial personnel at the facility carried out semen analysis training for technicians, acting as a method of standardization, which contributed to the consistency of methods. Total sperm output (TSO) was calculated by multiplying the semen volume by the sperm concentration of each ejaculate (World Health Organization, 2021; Hering *et al*., 2014). Data on 11,722 samples, from 1,041 stallions of mixed ages and breeds were obtained between the years 2001 and 2020.

4.2.2 Categorisation of variables

Stallions were allocated a numerical code and an ejaculate number as separate variables to account for multiple collections per stallion. All individual stallions reported as a combination of two or more breeds were categorised as crossbred. If an individual had multiple disciplines, it was categorised as multidisciplinary. Samples displaying haematospermia or urospermia were excluded from the dataset, given the detrimental impacts blood and urine have on sperm quality (Ellerbrock *et al*., 2016; Turner *et al*., 2016). In the full dataset, breeds of stallions included; warmbloods (n=6,270), hotbloods (n=1,649), coldbloods (n=442), pony-types (n=1,212), crossbreeds (n=582), and unknown (n=1,267) breeds as defined in appendix M (page 327). Season of collection was categorised as spring (n=3,602), summer (n=2,146), autumn (n=3,044), and winter (n=2,629). Discipline categories included; jumping (n=2,702), flatwork (n=1,782), endurance (n=1,160), breeding (n=348), harness (n=123), American (n=57), and unknown (n=5,250) as defined in appendix M (page 327). Country of birth included; Europe (n=5,241), the Americas (n=352), other (n=71; New Zealand, United Arab Emirates, Russia) and unknown (n=5,758).

4.2.3 Statistical analysis

Data were analysed on GenStat 17th edition (VSN International, 2022b; VSN International Ltd, Hempstead, UK) and graphically interpreted on Genstat and GraphPad Prism 9 (GraphPad Prism, 2022; GraphPad Prism version 9.0, GraphPad Software, California, CA, USA). Whilst normality is assumed in large datasets such as in the current study, extreme outliers, defined as values greater than three times that of the interquartile range, were removed to produce an approximately normal distribution (Curran-Everett and Benos, 2004). For motility, concentration, volume, and TSO, following the removal of outliers, 10,686, 5,185, 11,122, and 5,152 samples were analysed from 984, 524, 1,030, and 523 stallions, respectively.

Data were analysed using a linear mixed model (REML), allowing for the accountability of multiple variables as fixed effects and stallion and ejaculate sample size as random effects. Through an iterative process, dropping and removing terms from the fixed model, significance of variables were evaluated. Variable significance and the AIC value were used to determine the final refined REML model (Verbyla, 2019). Regardless of significance, year

of collection was included within the final refined model for all analyses. As the independent variable, year of collection was fitted last in the fixed effect terms on the statistical input. An expansion of the statistical model assumed, as well as justification behind the selection of the approach, are provided in chapter 2 of this thesis (research methodology and methods; pages 40-67).

For the analyses of all parameters, stallion and ejaculate code were included as random effects. For fixed effects within the REML model, significance was investigated. If $p<0.05$ and the AIC value did not change appreciably by the variables removal, then the parameter was included in the model. Due to the iterative process where values were added and removed from the model to determine significance, some variables were only significant in one direction. If a variable was significant in either the direction of being added or removed from the model, it was included within the final analysis. Variables included for each parameter and significance within the statistical model are presented in table 10.

Table 10: Variables included and associated significance (p) in the fixed effects REML model for TMOT (total motility), concentration, volume, and TSO (total sperm output). Stallion and sample code were included as random effects for all analyses.

Parameter	Fixed model
TMOT	Year of collection ($p<0.001$); abstinence period ($p<0.001$); age ($p<0.001$); extender ($p<0.001$); breed ($p<0.001$); season of collection ($p<0.001$); country of birth (term added: $p<0.001$; term removed: p 0.344)
Concentration	Year of collection ($p<0.001$); abstinence period ($p<0.001$); age ($p<0.001$); breed ($p<0.001$); discipline (term added: $p<0.001$; term removed: p 0.036); season of collection (term added: p 0.031; term removed: p 0.101)
Volume	Year of collection ($p<0.001$); abstinence period ($p<0.001$); age ($p<0.001$); season of collection ($p<0.001$); country of birth (term added: $p<0.001$; term removed: p 0.081)
TSO	Year of collection ($p<0.001$); abstinence period ($p<0.001$); age ($p<0.001$); discipline (term added: p 0.003; term removed: p 0.006); season of collection (term removed: p 0.096; term removed: p 0.019)

Temporal trends in stallion sperm TMOT, concentration, volume, and TSO were evaluated whilst accounting for significant covariates. Each dependent parameter (TMOT,

concentration, volume, and TSO) was treated as an isolated variable within the analyses. As true values, the mean (± SEM) was predicted for each year of collection, accounting for covariates included in the statistical model. The overall decline per year was calculated by dividing the overall decline by the number of collection years, an approach used in previous research (Levine *et al.*, 2017).

Whilst the significance of age was analysed within the REML model and predicted means were calculated accounting for this variable, age-restricted time trends were also assessed. Means plots for each dependent parameter against age were produced. Graphs showed age-based trends in semen quality for each parameter, which were visually interpreted. Based on the variability or trend observed, stallions were grouped as reproductively prime or senescent on an individual parameter basis. For the age range at which the semen quality parameter remained consistent, stallions were grouped as reproductively prime. For those presenting more variability, or a decline in the semen quality parameter based on increasing age, stallions were subsequently defined as reproductively senescent. Isolated REML analyses were then utilised to determine differences in time trends between age groups. A p value of <0.05 was considered statistically significant, whilst a p value of <0.001 was highly significant, and a 95% confidence interval was assumed for all analyses.

4.3 RESULTS

The number of stallions and samples included for each year of collection are provided in the supplementary information (Appendix O; page 332). The models fit the assumptions of the linear mixed (REML) statistical test, as depicted by the residual diagnostic plots (VSN International, 2022a).

4.3.1 Total Motility (TMOT; REML)

Analysis of TMOT included 10,686 samples from 984 stallions. Through the analysis of variable significance within the REML model, year of collection (p<0.001), abstinence period (p<0.001), age (p<0.001), extender (p<0.001), breed (p<0.001), and season of collection (p<0.001) were included. Country of birth was accounted for in the model, given that it was significant in one direction (added: p<0.001; removed: p 0.344). Differences in directional significance could be a result of an unequal spread of country of birth categories within the groups of alternative variables. Predicted mean (± SEM) values calculated from the model are presented in table 11. Overall, TMOT declined by a value of 4.81%, a yearly and overall decline of 0.50% and 10.10%, respectively (2001: 47.64 ± 2.98%; 2020: 42.83 ± 2.17%; figure 10).

Table 11: Predicted (Pr.) total motility (TMOT; %) values for each year of semen collection. Mean (Pr.) ± SEM; predicted values from the final REML model.

Total Motility (%)					
Year of collection	**2001**	**2002**	**2003**	**2004**	**2005**
Mean (Pr.) ± SEM	47.64 ± 2.98	48.84 ± 2.63	49.29 ± 4.95	51.54 ± 2.75	47.76 ± 2.23
Year of collection	**2006**	**2007**	**2008**	**2009**	**2010**
Mean (Pr.) ± SEM	51.49 ± 2.18	51.95 ± 2.13	54.57 ± 2.11	58.14 ± 2.09	55.79 ± 2.17
Year of collection	**2011**	**2012**	**2013**	**2014**	**2015**
Mean (Pr.) ± SEM	52.01 ± 2.05	50.36 ± 2.03	47.25 ± 2.02	45.17 ± 1.98	41.55 ± 1.97
Year of collection	**2016**	**2017**	**2018**	**2019**	**2020**
Mean (Pr.) ± SEM	42.02 ± 1.96	41.98 ± 1.93	37.25 ± 1.93	37.43 ± 1.92	42.83 ± 2.17

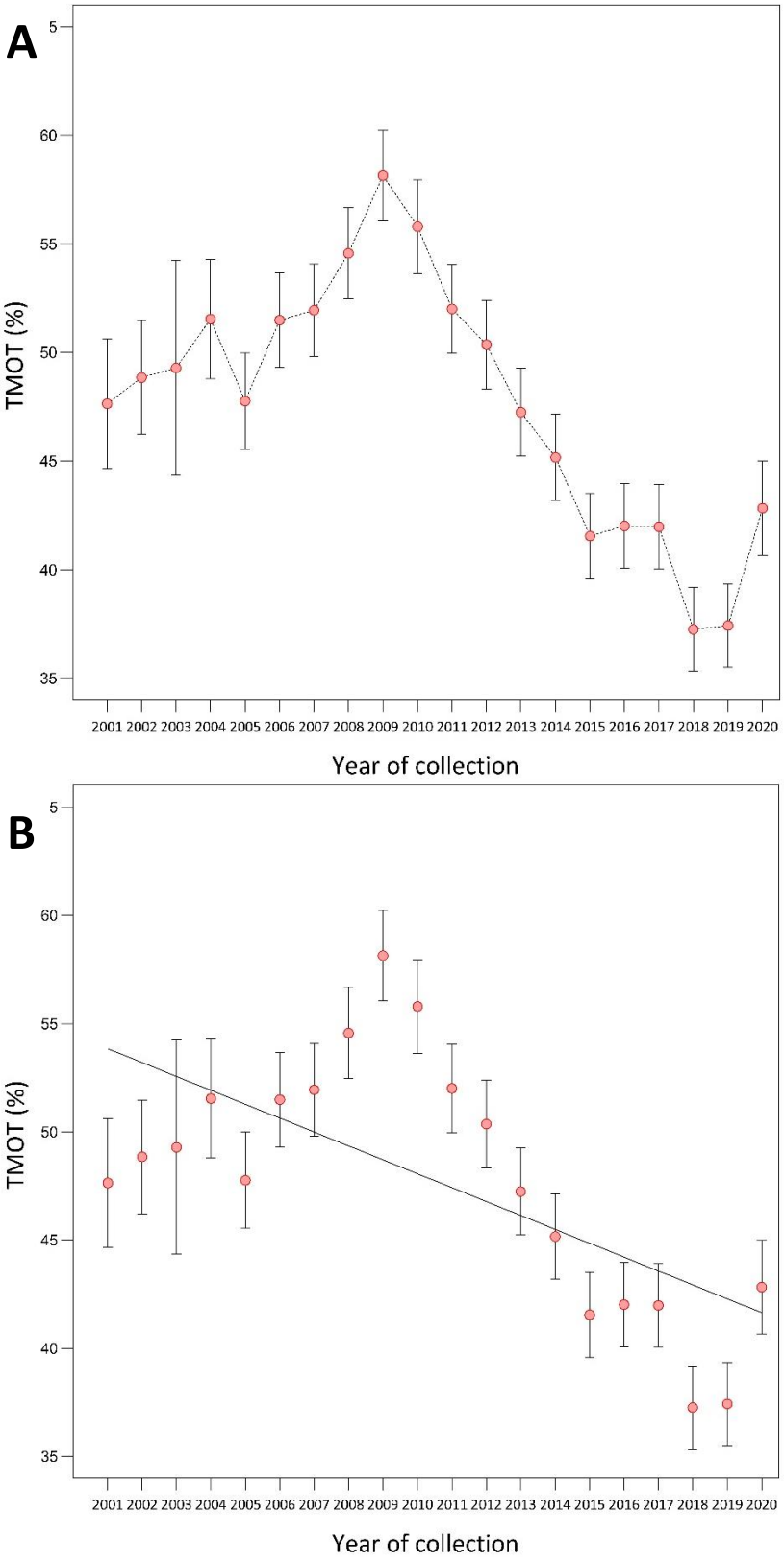


Figure 10: Time trends in mean (± SEM) TMOT (total motility; %) between 2001 and 2020. [A] Statistical REML model output of predicted mean TMOT, accounting for other significant covariates. [B] A regression slope (simple linear) of predicted means plotted for visual purposes. Graphs produced on GraphPad.

4.3.2 Concentration (REML)

The dataset for concentration consisted of 5,185 collections from 524 stallions following extreme outlier removal (n=45 collections; values >725 x10^6/ml). Year of collection (p<0.001), abstinence period (p<0.001), age (p<0.001), breed (p<0.001), and discipline (p 0.036) were all significant within the model. Season of collection was significant when added (p 0.031), although non-significant when removed from the model (p 0.101). The mean (± SEM) predicted sperm concentration values for each year of collection are presented in table 12. Predicted trends in concentration indicated an increase of 352 x10^6/ml between 2002 and 2020 (figure 11; 2002: 221 ± 77 x10^6/ml; 2020: 573 ± 77 x10^6/ml). From 2002 to 2011, concentration was analysed via a SpermaCue and remained consistent. Subsequent samples were analysed via a NucleoCounter (2012 to 2020) and showed a higher degree of variability in concentration.

Table 12: Predicted (Pr.) mean (± SEM) concentration (x10^6/ml) values for each year of semen collection. Mean (Pr.) ± SEM; predicted values from the final REML model. Single asterisk (*): data unavailable for the given year of collection.

Concentration (x10^6/ml)					
Year of collection	**2001**	**2002**	**2003**	**2004**	**2005**
Mean (Pr.) ± SEM	*	221 ± 77	258 ± 26	240 ± 26	238 ± 24
Year of collection	**2006**	**2007**	**2008**	**2009**	**2010**
Mean (Pr.) ± SEM	273 ± 23	276 ± 22	280 ± 23	323 ± 22	327 ± 23
Year of collection	**2011**	**2012**	**2013**	**2014**	**2015**
Mean (Pr.) ± SEM	300 ± 23	286 ± 24	265 ± 50	137 ± 72	246 ± 55
Year of collection	**2016**	**2017**	**2018**	**2019**	**2020**
Mean (Pr.) ± SEM	111 ± 87	142 ± 58	*	586 ± 75	573 ± 77

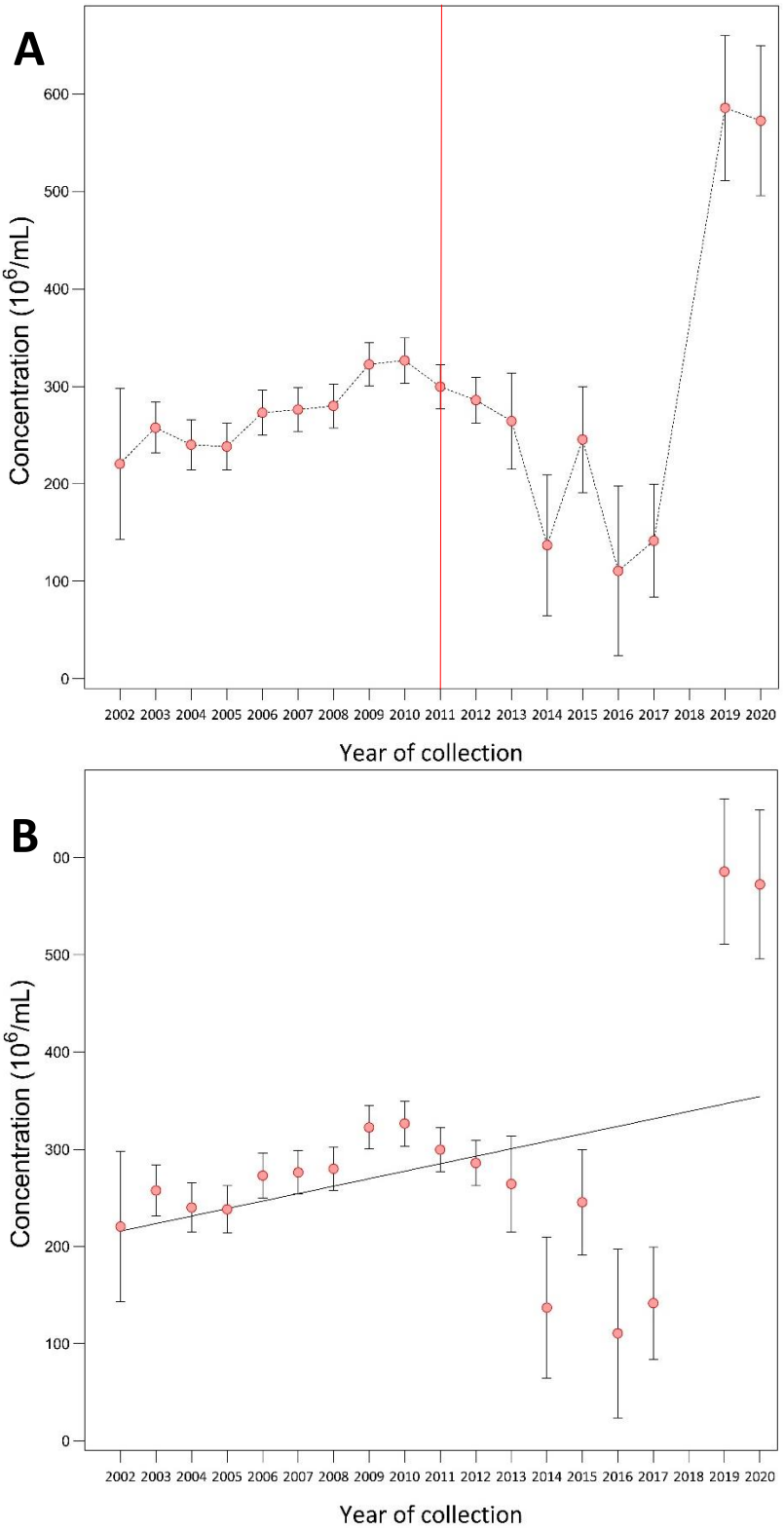


Figure 11: Time trends in mean (± SEM) concentration between 2002 and 2020 (x10^6/ml). [A] Statistical REML model output of predicted mean sperm concentration, accounting for other significant covariates. The red line at 2011 represents the shift in analysis method (SpermaCue to NuceloCounter). [B] A simple linear regression slope of the predicted means plotted for visual purposes. Graphs produced on GraphPad.

4.3.3 Volume (REML)

Following the removal of significant outliers (n=143 collections; volume of >120 ml), the dataset for semen volume included 11,122 samples from 1,030 stallions. Year of collection (p<0.001), abstinence period (p<0.001), age (p<0.001), and season of collection (p<0.001) were significant. Country of birth was a significant term when added (p<0.001), although non-significant when removed (p 0.081). Predicted means (± SEM) for each year of collection are presented in table 13. Means (± SEM) predicted from this model indicated that semen volume increased by 0.05 ml between 2001 and 2020 (2001: 38.98 ml; 2020: 39.03 ml; figure 12) although trends fluctuated between these dates.

Table 13: Predicted (Pr.) mean (± SEM) volume (ml) values for each year of semen collection. Mean (Pr.) ± SEM; predicted values from the final REML model.

Volume (ml)					
Year of collection	**2001**	**2002**	**2003**	**2004**	**2005**
Mean (Pr.) ± SEM	38.98 ± 5.04	43.17 ± 3.57	40.68 ± 3.47	35.17 ± 3.16	36.93 ± 3.14
Year of collection	**2006**	**2007**	**2008**	**2009**	**2010**
Mean (Pr.) ± SEM	35.43 ± 3.08	39.08 ± 3.00	38.68 ± 3.01	33.21 ± 2.98	33.62 ± 3.17
Year of collection	**2011**	**2012**	**2013**	**2014**	**2015**
Mean (Pr.) ± SEM	39.36 ± 2.93	44.33 ± 2.90	46.10 ± 2.89	44.17 ± 2.83	50.96 ± 2.82
Year of collection	**2016**	**2017**	**2018**	**2019**	**2020**
Mean (Pr.) ± SEM	42.56 ± 2.80	45.57 ± 2.74	46.32 ± 2.75	44.06 ± 2.74	39.03 ± 3.36

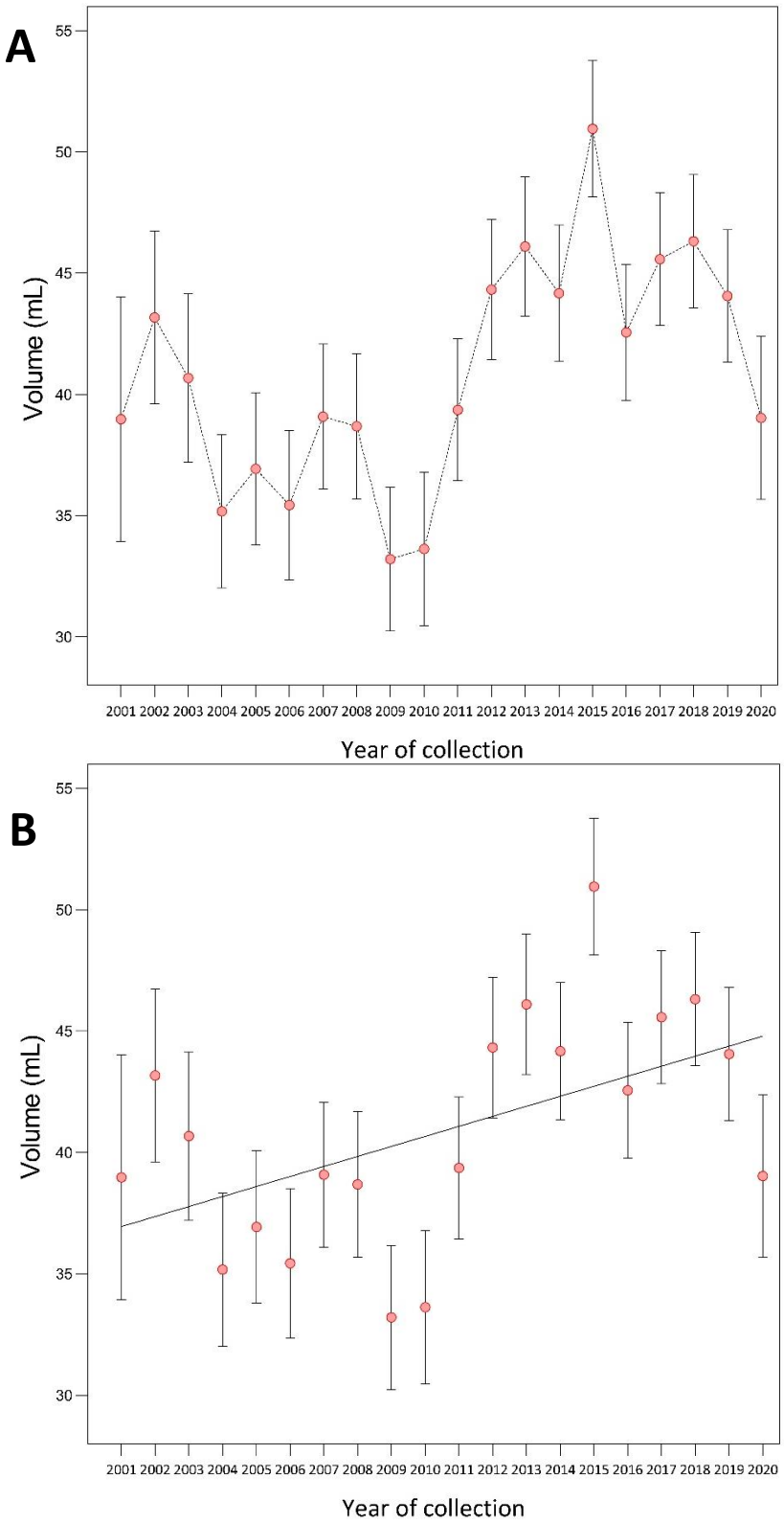


Figure 12: Time trends in mean (± SEM) semen volume (ml) between 2001 and 2020. [A] Statistical REML model output of predicted mean volume, accounting for other significant covariates. [B] A regression slope (simple linear) of the predicted means plotted for visual purposes. Graphs produced on GraphPad.

4.3.4 Total sperm output (TSO; REML)

After the removal of extreme outliers (n=63 collections; >2,100 x10^6 sperm), the dataset consisted of 5,152 samples from 523 stallions. Year of collection (p<0.001), abstinence period (p<0.001), age (p<0.001), and discipline (added: p 0.003; removed: p 0.006) were significant within the model. Season of collection was non-significant when added (p 0.0960) and significant when removed (p 0.019). Predicted TSO declined by a value of 2,908 x10^6 sperm from 2002 to 2016, a yearly and overall decline of 2.63% and 39.42% respectively (2002: 7,377 ± 2,110 x10^6; 2016: 4,469 ± 2,295 x10^6). No data were available for the years of 2017 and 2018, but between 2019 (n=6 samples, n=2 stallions) and 2020 (n=6 samples, n=2 stallions), TSO increased to 12,859 ± 2,331 x10^6 sperm, resulting in an overall increase (5,482 x10^6) over the study period (figure 13). Predicted TSO means (± SEM) for each year of semen collection are presented in table 14.

Table 14: Predicted (Pr.) mean (± SEM) total sperm output (TSO; x10^6) values for each year of semen collection. Mean (Pr.) ± SEM; predicted values from the final REML model. Single asterisk (*): data unavailable for the given year of collection.

Total Sperm Output (x10^6)					
Year of collection	**2001**	**2002**	**2003**	**2004**	**2005**
Mean (Pr.) ± SEM	*	7377 ± 2110	8765 ± 752	8046 ± 718	7778 ± 683
Year of collection	**2006**	**2007**	**2008**	**2009**	**2010**
Mean (Pr.) ± SEM	8638 ± 650	9913 ± 630	8962 ± 635	8583 ± 632	8393 ± 8393
Year of collection	**2011**	**2012**	**2013**	**2014**	**2015**
Mean (Pr.) ± SEM	8293 ± 642	9172 ± 671	9269 ± 1551	5182 ± 1902	5965 ± 1654
Year of collection	**2016**	**2017**	**2018**	**2019**	**2020**
Mean (Pr.) ± SEM	4469 ± 2295	*	*	16354 ± 2158	12859 ± 2331

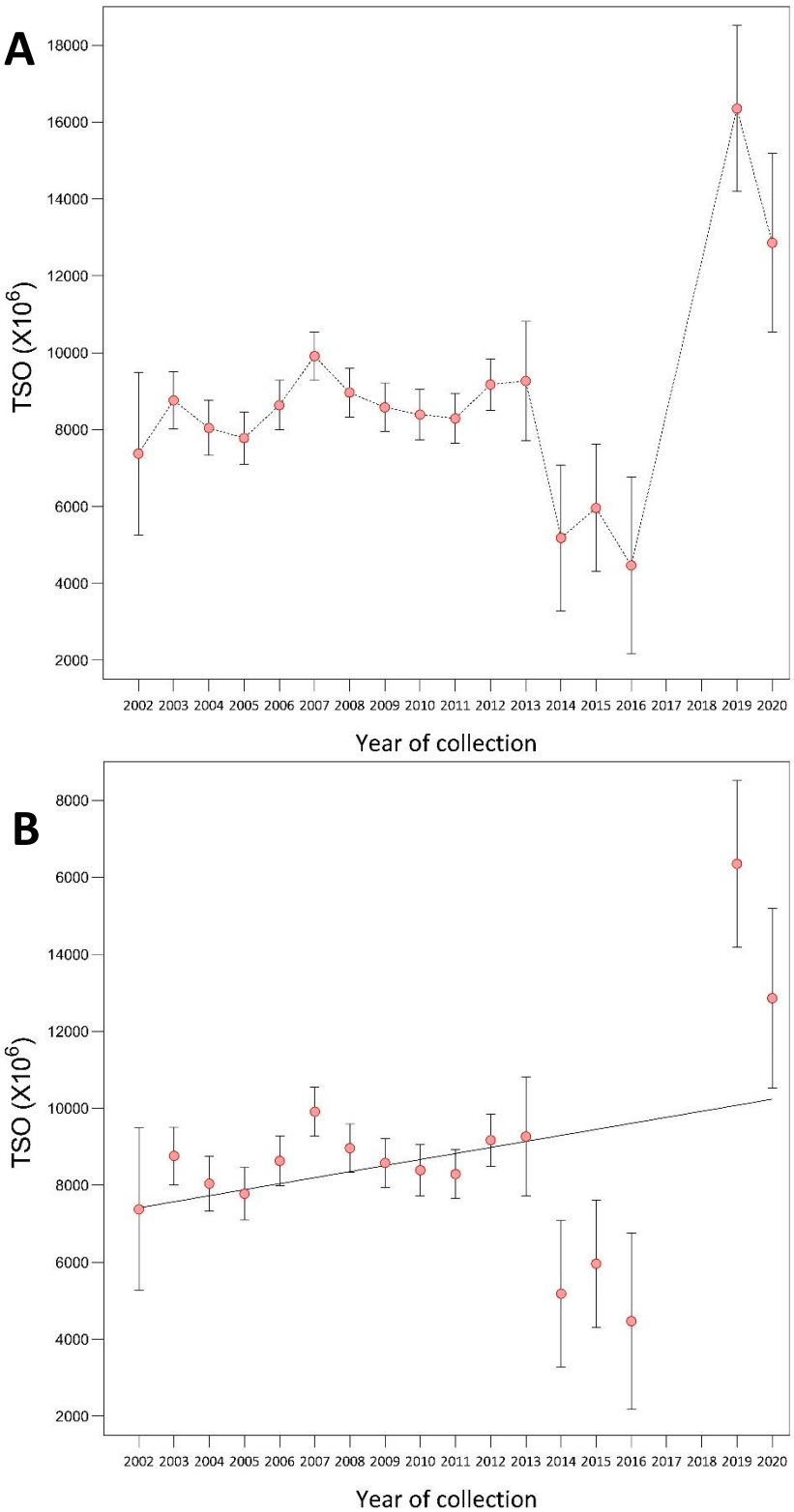


Figure 13: Time trends in mean (± SEM) TSO (Total sperm output; x10^6 sperm) between 2002 and 2020. [A] Statistical REML model output of predicted mean TSO, accounting for other significant covariates. [B] A regression slope (simple linear) of the predicted means plotted for visual purposes. Graphs produced on GraphPad.

4.3.5 Age-restricted trends

Following the analysis of time trends in TMOT, concentration, semen volume, and TSO, age-restricted trends were determined for each parameter, followed by age-restricted time trends. The aim was to assess the sensitivity of results to ensure that trends were not a result of aging stallions across the study period. Prime and senescent age groups were defined based on the variability of each individual parameter. The age range for each category is presented on an individual parameter basis (Table 15).

Table 15: Definitions of reproductively prime and senescent aged stallions used for the assessment of age-restricted time trends for each semen quality parameter.

Parameter	Prime age (years)	Senescent age (years)
TMOT	2 - 17	18 - 31
Concentration	2 - 16	17 - 30
Volume	2 - 25	26 - 31
TSO	2 - 17	18 - 30

4.3.6 Age-restricted trends in TMOT

Means plots were created for age, with TMOT remaining consistent between the ages of 2 to 17 years and declining between the ages of 18 and 31 years (figure 14A). Whilst age was accounted for as a fixed effect within the model, to determine temporal trends in direct relation to age, stallions were grouped as reproductively prime (2 to 17 years; n=8,374 samples; n=735 stallions) or senescent (18 to 31 years; n=1,211 samples; n=79 stallions). Both age and age group had significant interactions with year of collection in the model (separate analyses carried out for age and age group in isolation; $p<0.001$).

Between 2009 and 2020, TMOT in both age groups declined simultaneously (figure 14B). In prime-aged stallions, TMOT declined by a value of 9.43% from 2001 to 2020, a yearly and overall decline of 0.83% and 16.60% respectively (2001: 56.81 ± 3.22%; 2020: 47.38 ± 2.57%). In the reproductively senescent age group, an overall increase by a value of 8.15% in TMOT was detected between 2002 and 2020 (2002: 36.11 ± 6.08%; 2020: 44.62 ± 2.27%). Focussing on the years where TMOT followed the same trend in both age groups (2009 to 2019), TMOT declined by a value of 22.91% (yearly decline: 3.42%; overall decline: 37.61%)

and 29.94% (yearly decline: 4.45%; overall decline: 48.91%) in reproductively prime and senescent stallions, respectively. Means predicted from the statistical model for prime and senescent stallions are presented in table 16.

Table 16: Predicted mean total motility (TMOT; %) values for prime and senescent age groups for each year of semen collection. Values are predicted from the REML model. Prime and senescent age groups were defined as 2 – 17 and 18 – 31 years, respectively. Single asterisk (*): data unavailable for the given year of collection.

Total Motility (%)					
Year of collection	**2001**	**2002**	**2003**	**2004**	**2005**
Predicted mean (prime)	56.81	53.26	51.39	57.85	54.82
Predicted mean (senescent)	*	36.11	*	*	56.66
Year of collection	**2006**	**2007**	**2008**	**2009**	**2010**
Predicted mean (prime)	57.38	57.99	60.92	65.37	62.79
Predicted mean (senescent)	68.38	58.76	62.18	61.21	57.43
Year of collection	**2011**	**2012**	**2013**	**2014**	**2015**
Predicted mean (prime)	57.05	55.52	51.60	49.75	46.23
Predicted mean (senescent)	55.24	51.81	49.72	46.79	40.99
Year of collection	**2016**	**2017**	**2018**	**2019**	**2020**
Predicted mean (prime)	46.73	45.59	41.16	42.46	47.38
Predicted mean (senescent)	36.68	41.42	34.42	31.27	44.62

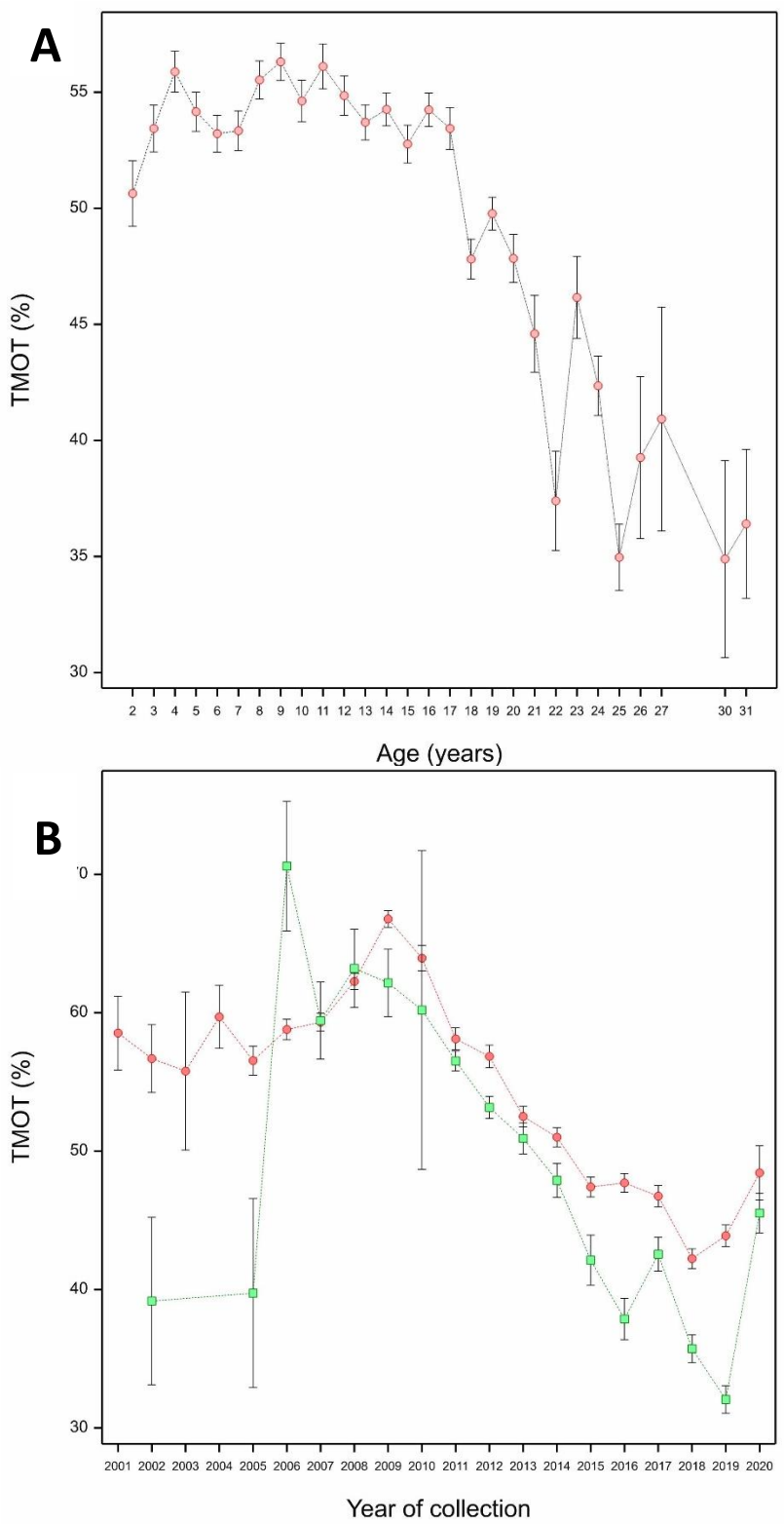


Figure 14: Age (years) and age-restricted time trends in mean (± SEM) TMOT (total motility; %). [A] Age-based trends in TMOT and; [B] time trends in age-grouped stallions (prime, age 2 - 17 years, red circle; senescent, age 18 - 31 years, green square). Each point represents the mean with the SEM extending from each data point. Graphs produced through the statistical output on GenStat.

4.3.7 Age-restricted trends in sperm concentration

Considering age-trends, concentration remained consistent for stallions between 2 and 16 years (categorised as reproductively prime; n=3,702 collections, n=327 stallions), with more variability in older stallions (17 to 30 years; categorised as reproductively senescent; figure 15A). Age and year of collection had significant interactions in the model (p<0.001). When a separate variable was included, categorising stallion age as prime or senescent, there was no significant interaction with year of collection (p 0.151). In prime-aged stallions, concentration declined between 2002 and 2017 (2002: 200 ± 75 x10^6/ml; 2017: 146 ± 55 x10^6/ml; Table 17). Stallion and collection sample size was limited from 2013 onwards (Appendix O; page 332), with two stallions contributing seven samples to the year 2019 and two stallions providing four samples to 2020. Data were only available for senescent stallions between 2003 and 2012, remaining consistent over this time (figure 15B).

Table 17: Predicted mean sperm concentration (x10^6/ml) values for prime and senescent age groups for each year of semen collection. Values are predicted from the REML model. Prime and senescent age groups were defined as 2 – 16 and 17 – 30 years, respectively. Single asterisk (*): data unavailable for the given year of collection.

	Concentration (x10^6/ml)					
Year of collection	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**
Predicted mean (prime)	200	255	249	240	270	276
Predicted mean (senescent)	249	285	285	278	302	332
Year of collection	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**
Predicted mean (prime)	277	299	313	249	235	241
Predicted mean (senescent)	340	346	366	330	315	*
Year of collection	**2014**	**2015**	**2016**	**2017**	**2019**	**2020**
Predicted mean (prime)	97	190	117	146	484	596
Predicted mean (senescent)	281	292	*	*	694	307

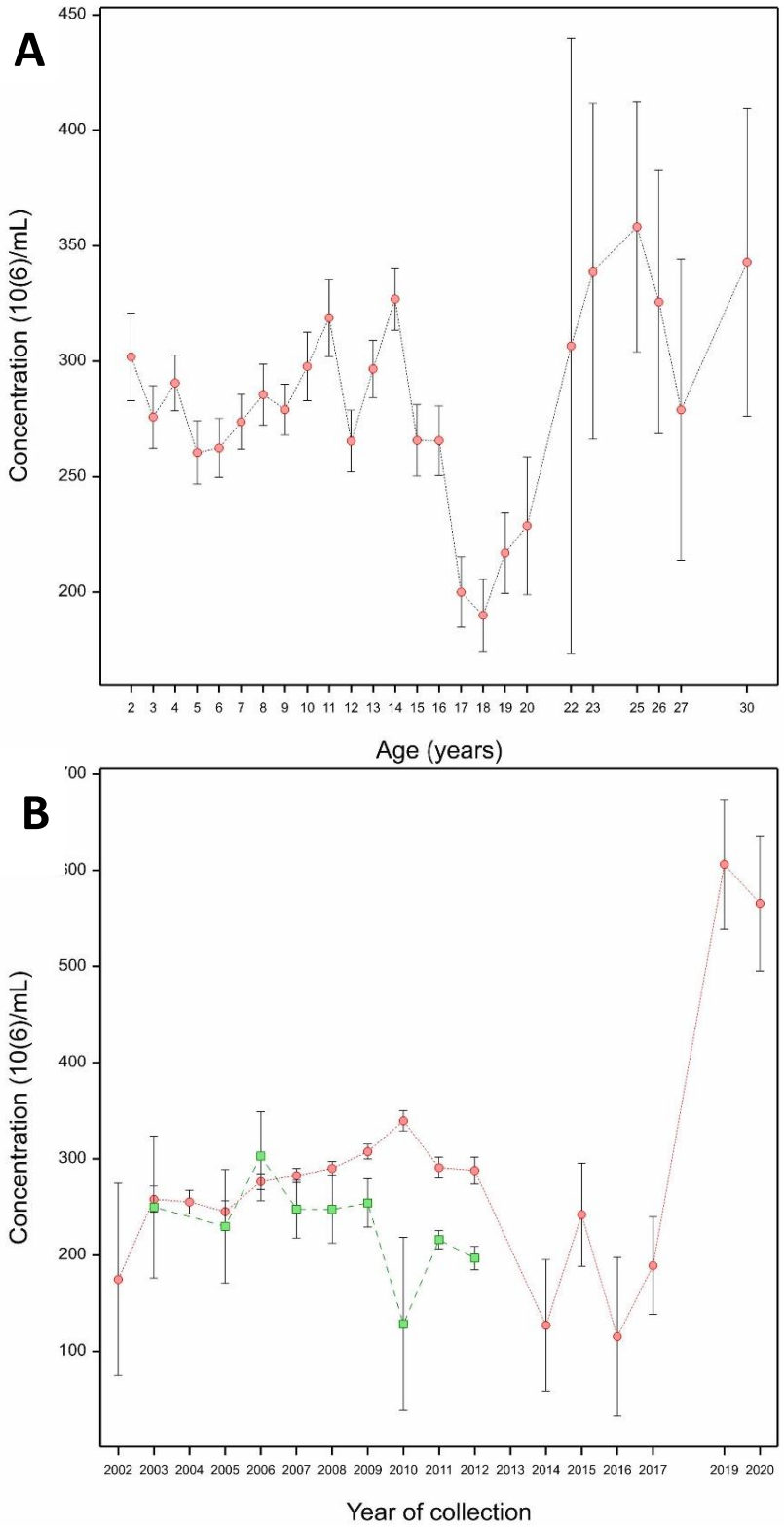


Figure 15: Age (years) and age-restricted trends in mean (± SEM) concentration (x10^6/ml). [A] Age-based trends in concentration and; [B] time trends in age-grouped stallions (prime, ages 2 - 16 years, red circle; senescent, ages 17 - 30 years, green square). Each point represents the mean with the SEM extending from each data point. Graphs produced through the statistical output on GenStat.

4.3.8 Age-restricted trends in volume

Volume declined by 9.68 ml between the ages of 2 and 31 years (2 years: 32.90 ± 3.26 ml; 31 years: 23.22 ± 7.00 ml; figure 16A). Volume remained consistent in stallions aged between 2 and 25 years (subsequently categorised as reproductively prime age), with more variability in stallions aged 26 to 31 years (senescent age group). There was a significant interaction between age and grouped-age and year of collection whilst accounting for all other variables within the refined REML model (p<0.001). Semen volume increased by 6.64 ml between 2001 (38.15 ± 5.66 ml) and 2020 (44.79 ± 3.30 ml) in reproductively prime aged stallions (2 to 25 years; n=9,757 collections; n=818 stallions). Values in the senescent group (26 to 31 years; n=79 samples, n=7 stallions) fluctuated noticeably (Table 18; figure 16B).

Table 18: Predicted mean volume (ml) values for prime and senescent age groups for each year of semen collection. Values are predicted from the REML model. Prime and senescent age groups were defined as 2 – 17 and 18 – 31 years, respectively. Single asterisk (*): data unavailable for the given year of collection.

	Volume (ml)				
Year of collection	**2001**	**2002**	**2003**	**2004**	**2005**
Predicted mean (prime)	38.15	44.52	44.04	36.45	39.40
Predicted mean (senescent)	*	50.71	*	*	13.31
Year of collection	**2006**	**2007**	**2008**	**2009**	**2010**
Predicted mean (prime)	37.12	39.86	40.50	37.67	35.07
Predicted mean (senescent)	*	39.22	53.34	*	*
Year of collection	**2011**	**2012**	**2013**	**2014**	**2015**
Predicted mean (prime)	46.31	47.43	49.07	45.22	53.86
Predicted mean (senescent)	24.75	69.72	*	*	*
Year of collection	**2016**	**2017**	**2018**	**2019**	**2020**
Predicted mean (prime)	45.10	48.43	49.16	45.45	44.79
Predicted mean (senescent)	*	39.84	*	*	7.45

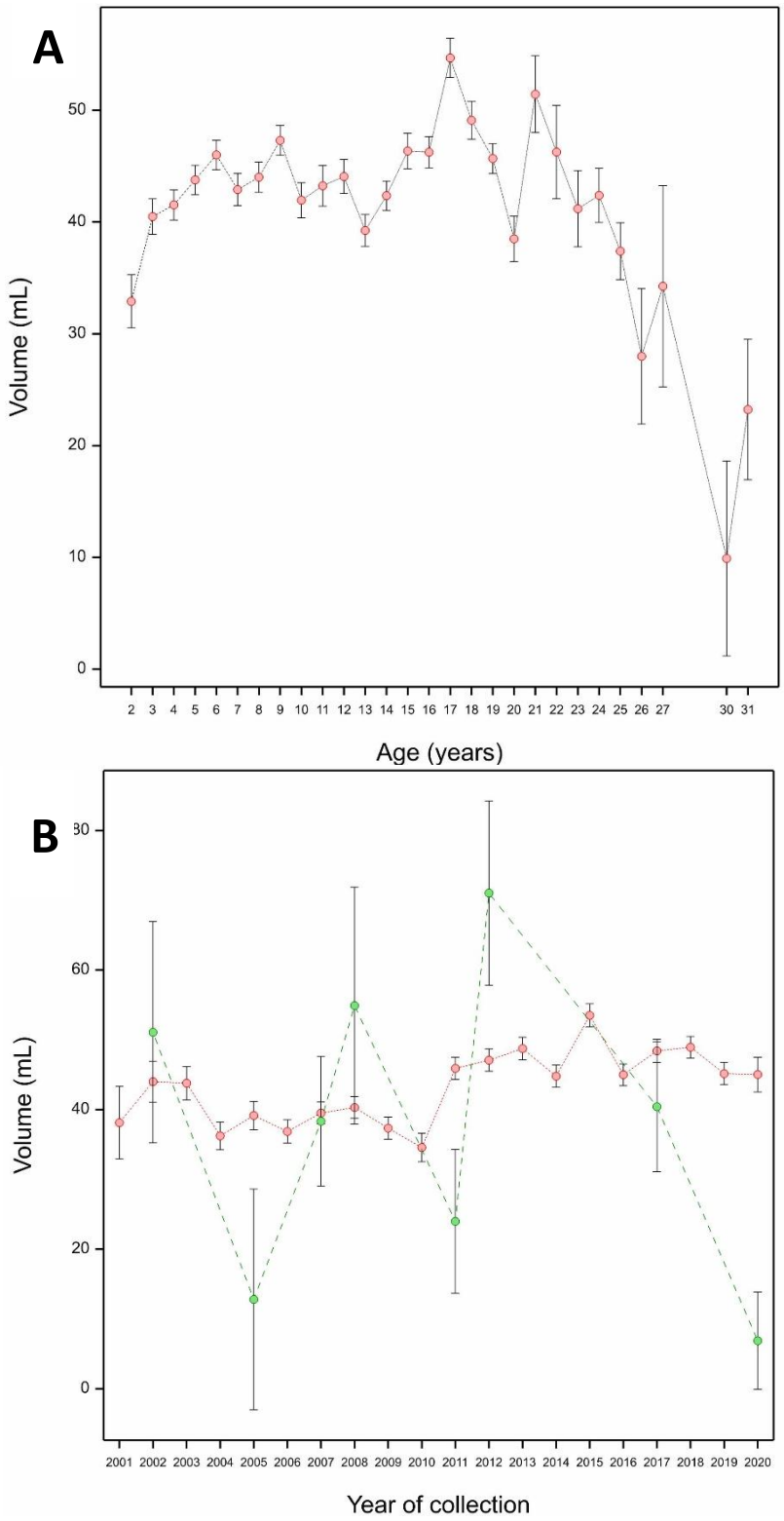


Figure 16: Age (years) and age-restricted time trends in semen volume (ml). [A] Age-based trends in semen volume and; [B] time trends in age-grouped stallions (prime, ages 2 - 25 years, red circle; senescent, ages 26 - 31 years, green square). Each point represents the mean with the SEM extending from each data point. Graphs produced through the statistical output on GenStat.

4.3.9 Age-restricted trends in TSO

Analysing age trends in TSO indicated that mean values did not change appreciably from 2 to 17 years, with a decline and more variability for stallions older than 18 years (figure 17A). There was a significant interaction between age, grouped age, and year of collection (p<0.001). In reproductively prime-aged stallions, TSO remained consistent from 2002 to 2012, declining by 1,409 x10^6 sperm per sample between 2002 and 2016, a yearly and overall decline of 1.48% and 22.24%, respectively (2002: 6,335 ± 2,908 x10^6; 2016: 4,929 ± 2,292 x10^6). In the same population, TSO increased by 7,775 x10^6 sperm per sample between 2016 and 2020 (2017, 2018: no data; 2019: n=6 samples, n=2 stallions; 2020: n=4 samples, n=2 stallions; 12,704 ± 2,315 x10^6 sperm). In reproductively senescent stallions (n=1,198 samples; n=76 stallions), data were only available from 2003 to 2014. TSO remained consistent between 2003 and 2014 (2003: 8,458 ± 1,554; 2014: 8,768 ± 3,290 x10^6; figure 17B). Differences in predicted TSO means between prime and senescent stallions for each year of collection are reported in table 19.

Table 19: Predicted mean total sperm output (TSO; x10^6) values for prime and senescent age groups for each year of semen collection. Values were predicted from the REML model. Prime and senescent age groups defined as 2 – 17 and 18 – 30 years, respectively. Single asterisk (*): data unavailable for the given year of collection.

Total Sperm Output (x10^6)						
Year of collection	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**
Predicted mean (prime)	6335	9674	8607	8467	9494	10275
Predicted mean (senescent)	*	8458	9792	7013	8349	10303
Year of collection	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**
Predicted mean (prime)	9986	9098	9621	9249	9420	*
Predicted mean (senescent)	9250	10269	9999	7064	8514	*
Year of collection	**2014**	**2015**	**2016**	**2017**	**2019**	**2020**
Predicted mean (prime)	3929	6229	4926	*	17045	12704
Predicted mean (senescent)	8768	*	*	*	*	*

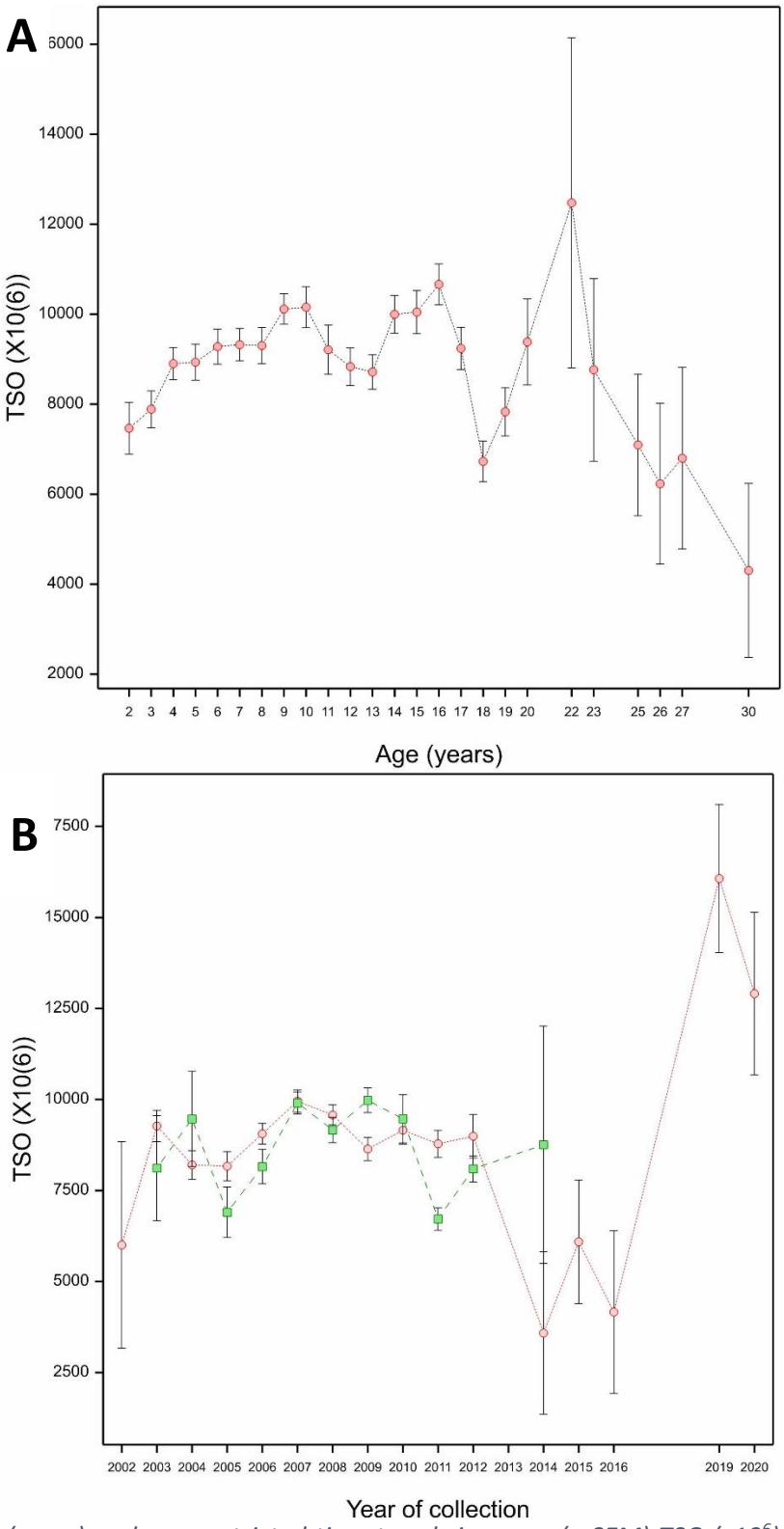


Figure 17: Age (years) and age-restricted time trends in mean (± SEM) TSO (x10^6). [A] Age based trends in TSO and; [B] time trends in age-grouped stallions (prime, ages 2 - 17 years, red circle; senescent, ages 18 - 30 years, green square). Each point represents the mean with the SEM extending from each data point. Graphs produced through the statistical output on GenStat.

4.4 DISCUSSION

Novel outputs from this retrospective study provide a comprehensive understanding of trends in fundamental semen quality parameters specific to the UK-based male equine population in the 21st century. Trends were consistent with the inclusion of a set of predetermined variables, providing a robust indication of the reproductive status of this population of stallions. Results contribute significant data towards the equine breeding sector and supplement the debate on adverse trends in human populations, supporting the utilisation of the equine species as a novel comparative model.

4.4.1 Trends and consequences of suboptimal sperm TMOT

The novel results presented within the current retrospective analysis report temporal changes in key semen quality parameters in an extensive UK-based equine population between 2001 and 2020. This is the first retrospective study to present results on TMOT trends specific to a UK-based equine population. In addition, to the authors knowledge, this is the first study to analyse TMOT trends in fresh semen samples in general domesticated herbivorous populations through any methodological approach, adding to the novelty and significance of the results. Results collected from a single, controlled equine population, with consistency in semen collection and analysis methods and the ability to account for a set of predetermined variables, provide a robust indication of the current reproductive statuses of this equine population. Accounting for the interaction of age and year of collection, TMOT declined comparably to the combined age-group analyses, confirming that adverse trends are unlikely a result of aging stallions. Such results are indicative of the involvement of alternative aetiological factors in the adverse trends presented.

Total motility is a fundamental parameter for the assessment of the fertilising capabilities of semen samples in humans (World Health Organization, 2021) and forms part of the stallion breeding soundness examination (Varner, 2016). Out of other motion and kinetic parameters, TMOT is reported to be the most correlated with equine per-cycle pregnancy rate (Love, 2011). Within the current study, TMOT declined from 47.64 ± 2.98% in 2001 to 42.83 ± 2.17% in 2020. In cooled semen, the threshold values of TMOT for embryo recovery rate are >65% (Love *et al*., 2015), which is significantly higher than the values reported in

the current study. Stallions with high and low fertility have been defined as those with fresh TMOT values of 73 ± 11% and 63 ± 17%, respectively (Jasko *et al*., 1992). All predicted TMOT values within the current study were below the threshold for both high and low fertility individuals, raising concern over the fertilising capabilities of the current equine population. Poor semen quality can have significant implications for the economic value of sires within the breeding industry (Darr *et al*., 2017) and the ability to maintain desirable heritable traits in the gene pool. The low TMOT values within the population of the current study, and declining trends, raise substantial concern over the reproductive health and breeding potential of stallions in the UK. Poor reproductive function could have significant industry implications, influencing the economic status of breeding stock and placing excess stress on stallions due to increased collections and coverings required for conception. However, when considering the method of analysis of motility within the current study, the parameter was assessed utilising subjective microscopy. Whilst standardisation in training for semen analysis was carried out across the study period, subjective analysis methods could introduce a level of variability into the readings provided, a limitation of the current study. In addition, advancements in semen collection and treatment methods, such as the use of specific semen extenders, may have further impacted the results presented. Advancements in semen collection and analysis methods is an inherent limitation of analysing semen quality trends across time.

Previous research in alternative species supports the findings presented within the current study (Lea, Byers, *et al*., 2016). In human populations, motility declined by 1.37% (Wang *et al*., 2017) and 0.66% (Mínguez-Alarcón *et al*., 2018) in China. Comparing the rate of decline in the current study to previous literature, TMOT declined by 0.50% per year in this equine population, whilst a yearly decline of 1.2% was reported in a UK population of dogs between 1988 and 2014 (Lea, Byers, *et al*., 2016). Research indicates that semen quality declines are more prominent in carnivorous and omnivorous species compared to herbivorous populations. Variability in trends between herbivorous and carnivorous species could indicate that dietary factors are associated with the magnitude of adverse motility trends (Kljajic *et al*., 2021). Considering the interplay between environmental contaminants and meat consumption, the species-sensitive inconsistencies between carnivores and herbivores may also be a result of differing levels of EC exposure.

In areas of the USA, significant geographic-sensitive declines were reported in human sperm motility between 2007 and 2017 (Chang *et al*., 2018). In a group of sub-fertile couples from France, total motile sperm count declined between 2002 and 2017 (Tiegs *et al.*, 2019). However, the lack of randomisation in the individuals sampled within this study reduces the applicability of these results to the wider population (Fisch, 2008). The selection of individuals within a population on which to base reproductive trends is fundamental in the applicability of results. Within the equine industry, the use of ART is common practice (Campbell and Sandøe, 2015), regardless of stallion fertility. Therefore, it is likely that adverse trends in TMOT are more applicable to the wider equine population. Adverse trends in motility across three species, including the novel equine model presented here, the dog sentinel (Lea, Byers, *et al*., 2016) and human populations (Wang *et al*., 2017; Geoffroy-Siraudin *et al.*, 2012) add significant weight to the debate surrounding declining sperm quality. These results support the initiation of utilising the equine species as a comparative model for reproductive health. In developing the novel equine model as a comparative species for reproductive trends, this research also initiates the utilisation of this species in the debate surrounding adverse trends in concentration and sperm count.

4.4.2 Revisiting the debate on declining sperm concentration and TSO

The current study analysed trends in sperm concentration and TSO to contribute data to the sperm count debate, and to analyse equine testicular function, given the association of these parameters with reproductive and overall health (Latif *et al*., 2017). To the authors knowledge, this is the first study to assess temporal trends in equine sperm concentration and TSO specific to the 21st century, adding to the novelty of the results presented. Both sperm concentration and TSO increased between 2002 and 2020, suggesting that the UK-based equine population is not currently at risk of the declines reported in humans (Mínguez-Alarcón *et al*., 2018; Wang *et al*., 2017) and bulls (Wahl and Reif, 2009). However, limited stallion and ejaculate sample sizes (Appendix O; page 332) contributing data to the collection years of 2012 to 2020 may have influenced the variability in concentration. Whilst a true overall increase in TSO cannot be eliminated, this trend was led by a limited number of stallions between 2019 and 2020. The age-restricted analysis indicated that between 2002 and 2012, TSO did not change appreciably in the prime stallions and that this age group led the decline up to the year 2014.

Sperm concentration and count are important parameters for conception (Zinaman *et al*., 2000). In cooled semen, concentration and TSO thresholds for embryo recovery rate were reported at >31.8 $x10^6$/ml and >1,140 $x10^6$ sperm, respectively (Love *et al*., 2015). Considering the threshold values for embryo recovery rate in cooled semen samples (Love *et al*., 2015), the mean concentration did not drop below the lower bound for these given values. Highly fertile stallions were defined as those with concentration and TSO values of 182 ± 111 $x10^6$/ml, and 9,400 ± 6,000 $x10^6$, respectively, whilst stallions with low fertility had values of 165 ± 126 $x10^6$/ml, and 8,700 ± 6,000 $x10^6$, respectively (Jasko *et al*., 1992). Results in concentration and TSO are reassuring, indicating that this population of stallions are within the recommended threshold values for sufficient fertility potential. However, a lack of definitive and industry-accepted threshold values for sperm concentration, TSO, and the other parameters analysed within the current study make it difficult to fully interpret the consequences of the values presented here.

The findings in TSO are supported by previous research in stallions. A previous study in Breton Draught stallions between 1981 and 1996 reported no trends in sperm output (Multigner *et al.*, 1999). However, the data included in the study by Multigner *et al.* (1999) failed to undergo such rigorous statistical analysis compared to the current study. The rigorous statistical approach harnessed here provides a more accurate representation of trends compared to previous research. In addition, the research associated with the current study observed trends across a large range of breeds and is therefore, more applicable to the general equine population instead of being breed-specific (Multigner *et al.*, 1999).

Considering an alternative herbivorous model, in a population of Holstein dairy bulls, a significant decline of 13.1% and 16.5% were reported in daily sperm concentration and TSO, respectively (Wahl and Reif, 2009). Adverse trends reported in dairy bulls could be associated with environmental and breed differences. Perturbed reproductive health has been reported in dairy bulls due to intensive selective breeding programmes designed to increase milk yield (Lucy, 2001). Given that the study of Holstein bulls only included a single breed, the effects of inbreeding may have contributed towards declines. However, some equine breeds are also at risk of the effects of inbreeding on semen quality, given the

industry selection pressures on performance and conformation (Dini *et al*., 2020). The current dataset included five breed categories based on 71 individual breeds (Appendix M; page 327), so it is possible that the inclusion of stallions with a reduced risk of inbreeding equalised the trends. However, further research analysing semen quality trends in stallions accounting for differential inbreeding coefficients is required in order to determine to what extent this factor is influencing the trends presented. The inclusion of a significant number of horse breeds is important for the applicability of the research to wider equine populations. However, for the statistical model, such inclusion required a broad method of categorisation, which could be considered as a potential limitation of this research. Differences in semen quality trends within breeds are likely to exist on a more specific level than the categorisation method presented for these analyses. Differences in semen quality trends within breeds are likely to exist on a more specific level than the categorisation method presented for this analyses. Given that dairy bulls are livestock species, increased exposure to agrochemicals, with suspected reprotoxic properties (Pallotta *et al*., 2019), could also be associated with declines reported in this population (Wahl and Reif, 2009). However, other research is indicative of no long-term trends in sperm output in dairy bulls from a centre in the Netherlands between 1977 and 1996 (Van Os *et al*., 1997) and in Spain between 1990 and 2007 (Karoui *et al*., 2011). However, neither of these studies are applicable to trends in the 21st century, which limits the comparability of the results with current research.

Considering the trends in concentration and TSO, both followed a similar pattern. It is worth noting that the shift in analysis methods from a SpermaCue to NucleoCounter during 2011 had the potential to impact the output of trends observed in both parameters, and is therefore considered a limitation of this study. The NucleoCounter is a fluorescence-based automated cell counter instrument, which requires no calibration and the accurate, precise, and sensitive analysis of semen concentration (Brito *et al*., 2016). The SpermaCue is a photometric device used to determine sperm concentration. In bulls, there was no significant difference in sperm concentration measurement between photometric devices and other instruments (Atiq *et al*., 2011). However, when looking at the exact instruments utilised within the current study, research in stallions suggests that concentration readings by a SpermaCue are less accurate than those read by a Nucelocounter (Comerford, 2009). Given the findings from published literature, it can be proposed that accuracy in analysis

method is likely to impact the variability in concentration readings post-2012, highlighting a limitation of analysing semen quality trends (Pacey, 2013). Given the shift in analysis method for concentration and TSO, interpretations of trends must be split at 2011 to avoid misassumptions of trends based on the potential effects of inconsistencies between devices. Focussing on trends in concentration between 2002 and 2011, where the results are produced from consistent analysis methods, concentration and TSO did not change appreciably over this period.

4.4.3 Trends in semen volume, and association of suboptimal levels with reproductive health

Within the current study, semen volume increased by 0.05 ml between 2001 and 2020, although there were evident fluctuations in trends across the study period. Within the age-restricted analyses, volume in the prime age group continued to increase over the study period, with more variability reported in the older age group. The consistency in method of analysis for volume across the study period adds to the quality of the results. The trends in semen volume presented here are reassuring, indicating that over the past two decades, this parameter has improved. However, the AI referencing range in the equine industry is recommended at 60 to 120 ml (Wilson and Flesner, 2017). All predicted means for volume fell below the lower bracket of this threshold value, raising concern over the reproductive health of the equine population studied. Suboptimal semen volumes have also been reported previously in equine studies (Wilson and Flesner, 2017; Multigner *et al*., 1999), which could indicate that reproductive aberrations resulting in low volume exist in the wider equine population.

Whilst volume is not a direct measure of testicular function, in humans, low volume can be an indication of androgen deficiency, obstruction to the ejaculatory duct, or poor development of the seminal vesicles (World Health Organization, 2021), all reflective of poor reproductive health. Considering the involvement of androgens in seminal volume, ECs, otherwise known as endocrine disrupting chemicals (EDCs), are reported to act through endocrinology pathways, including androgen receptors (Amir *et al*., 2021). It is therefore possible that exposure to ECs could be associated with the low ejaculate volume reported in this equine population. To date, understanding surrounding EC exposure and associated reprotoxicity specific to stallions remains unknown. Whilst the results for

concentration and TSO do not seem concerning, aetiologies, incuding those of environmental origin that may be involved in declining TMOT trends, and suboptimal semen levels warrant further investigation.

4.4.4 Age-restricted trends in semen quality

Age and abstinence period were accounted for and significant within the statistical model for all parameters. The failure to include such variables is a primary source of critique in human-based evidence syntheses in reproductive trends, given the involvement of age and abstinence with semen quality (Fisch, 2008). The ability to account for both variables within this research adds to the quality of the results and interpretations of data. Further analysis of age-restricted trends indicated that TMOT, volume, and TSO declined with increasing age, whilst concentration remained consistent, followed by an increase after the age of 22 years. However, small sample sizes of stallions and samples contributing to ages 22 to 30 years for concentration values limit the interpretations that can be taken from this result. Results presented within this research indicate a more extended period of fertility in stallions compared to previous research. Periods of juvenility and senescence are associated with reduced semen quality, with the prime age suggested to fall between 3 and 10 years of age (Darr *et al*., 2017). Within the current study, the prime age for breeding stallions was supposedly broader than that reported in previous research (Darr *et al*., 2017), indicating that 2 to 17 years is optimal for semen quality parameters. Given that the age-restricted analyses confirmed that trends were not a result of aging stallions, an alternative factor is likely to be associated with declines in TMOT and suboptimal semen values.

4.4.5 Concluding statement

This comprehensive retrospective cohort study provides fundamental data on temporal trends in semen quality specific to a controlled UK-based equine population in the 21st century. Results indicate that TMOT has declined between 2001 and 2020, whilst concentration, volume, and TSO have increased over the same time period. Semen quality readings for TMOT and volume are reported to fall below the recommended industry threshold values for successful fertilisation, a concerning finding given the high economic importance of stallion fertility. However, trends and readings for concentration and TSO are reassuring, indicating that not all parameters involved in stallion fertility prediction are

at risk of equivalent declines. Results also contribute data to the debate surrounding adverse trends in semen quality across species, with the equid acting as a valuable model to be furthered within the reproductive sciences sector. Given the adverse trends reported in two motility characteristics in the current study and the previous chapter of this thesis, further research analysing the potential involvement of underlying environmental aetiologies is paramount.

CHAPTER 5

ANTHROPOGENIC ENVIRONMENTAL CHEMICALS IN THE NOVEL EQUINE MODEL: TESTICULAR CONTAMINATION AND ROUTES OF ORAL EXPOSURE

5.1 INTRODUCTION

Chapters 3 and 4 of this thesis addressed the aims associated with determining trends in sperm quality parameters within the equine population, initiating the species as a novel comparative model for reproductive trends in humans. The results from the first two studies of this thesis report adverse trends in two sperm motion characteristics in the global and UK-specific equine populations, respectively. PMOT declined by 31.89% between 1984 and 2019, with noted geospatial variability, as analysed through a systematic review and meta-regression analysis. In a single equine population from a breeding facility within the UK, TMOT declined by 10.10% between 2001 and 2020, although three alternative parameters presented an inclining trend over the same time. Whilst adverse trends were parameter sensitive, consistency in the results of two primary sperm motion characteristics across both equine populations analysed is indicative of a common underlying aetiology. The current study sought to initiate investigations into the potential involvement of anthropogenic ECs with reproductive aberrations specific to the equine model by analysing testicular accumulation and potential routes of exposure.

5.1.1 An environmental aetiology associated with reproductive aberrations

Exposure to anthropogenic ECs is associated with aberrations and adverse trends in the reproductive health and function of male populations in alternative species (Skakkebaek *et al*., 2015). Historical and modern urbanisation, industrialisation, economic growth, and agricultural production are responsible for the increased synthesis and utilisation of chemicals due to their beneficial properties within many anthropogenic materials and manufacturing processes (Guvvala *et al*., 2020). Given the uses of chemicals, geographical differences in environmental levels are related to industrial sources of deposition (Pozo *et al*., 2012). However, long-range transport of persistent ECs results in the widespread spatial distribution of contaminants (Rauert *et al.*, 2018), leading to their ubiquitous presence. More than 140,000 new chemicals have been produced since the 1950s (Landrigan *et al*., 2018), 800 to 1,000 of which have known or suspected endocrine disrupting properties (Godfray *et al*., 2019) with the potential of perturbing reproductive function.

Categories of contaminants include, but are not isolated to, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and Di

(2-ethylhexyl) phthalate (DEHP) (Antignac *et al*., 2016; Mobegi, Nyambaka and Nawiri, 2016). PAHs are produced through the incomplete combustion of organic matter that occurs through burning wood, coal, and petroleum for extensive industrial and domestic processes (Samanta, Singh and Jain, 2002). The phthalate DEHP is a common plasticiser, integrated into plastic to promote the material's malleability and durability (Zhao *et al.*, 2020). Original uses of PBDEs included flame-retardant coatings, whilst PCBs were used in dielectric fluids for capacitors and transformers (Melymuk *et al*., 2022; Kurt-Karakus *et al*., 2019). The synthesis of PBDEs and PCBs was restricted under the Stockholm Convention, given associated toxicity and environmental biopersistence. Despite legislative restrictions, PCBs and PBDEs persist within the current environmental landscape (Lu *et al*., 2021). The extensive and continuous uses of ECs has resulted in the atmospheric deposition of contaminants on a global scale. A complex mixture of ECs now contaminate the environment, referred to as the 'environmental pollutome' (Sumner *et al*., 2020).

Herbivorous species of terrestrial and agricultural ecosystems are exposed to contaminants through the ingestion of contaminated foodstuff and water, inhalation of aerosolised particles, and percutaneous absorption (Rhind *et al*., 2010). Chemical exposure is a recognised health and welfare concern in humans and companion animals, livestock, and wildlife species (Williams *et al*., 2021; Sumner *et al*., 2020; Desforges *et al*., 2018; Paul *et al.*, 2005). Despite research indicating that EC exposure is associated with overall health risks in the general equine population (Mullen *et al*., 2020; Novello *et al*., 2020), there is limited data analysing the interaction of ECs with stallion reproductive function specifically.

5.1.2 Equine-specific EC contamination of biological samples

From an agrochemical perspective, ɣ-HCH and DDT have been detected in the milk of Konik Polski mares in Poland (Pietrzak-Fiećko *et al*., 2009). The transfer of contaminants from the milk to the suckling foal is likely to perturb reproductive development and future fertility, as reported in alternative species (Higuchi *et al*., 2003). From a female perspective, the plasticiser, DEHP has been detected in the follicular fluid of mare ovaries at a mean concentration of 7.85 ± 2.45 nM (Marzano *et al*., 2019), indicative of EC bioaccumulation within the reproductive organs. Further analysis of the testis is required, given specific anatomical differences between the internal positioning of the ovaries and external

positioning of the testes, which may result in differing levels of bioaccumulation. Contaminants with persistent properties are otherwise referred to as persistent organic pollutants (POPs) in literature (Yavuz *et al*., 2022; Myhre *et al.*, 2021; Lundin *et al*., 2016). POPs, including PAHs, PBDEs, and PCBs have been detected in horse hair (Yavuz *et al*., 2022; Te *et al*., 2020), manure (Rey-Salgueiro *et al*., 2008), meat (Cimenci *et al*., 2013), and fat (Naert and Van Peteghem, 2007) samples, confirming equine specific exposure. Assessing overall bodily burdens in species is an essential tool within the current research landscape, given the lack of knowledge associated with stallion testicular contamination. However, furthering understanding of levels present within the testis is required to initiate a health risk assessment of contaminant exposure in relation to stallion reproductive function.

5.1.3 Testicular contamination and routes of exposure

Research in other species is indicative of EC bioaccumulation within the testis (Ramesh *et al*., 2001). PBDEs and PCBs have been detected in canine testicular tissue and ejaculate (Lea, Byers, *et al*., 2016). Testicular accumulation is concerning given that structures and mechanistic processes are a primary target of EC induced perturbations. *In vitro* and epidemiological studies within a variety of species have linked EC exposure to a number of testicular and reproductive aberrations. These include perturbed semen quality parameters and physiological malformations, including testicular cancer, cryptorchidism, and hypospadias, together referred to as testicular dysgenesis syndrome (TDS; Skakkebaek *et al*., 2015).

In herbivorous species, research focuses on seminal fluids, with limited understanding of testicular burdens. PCBs are present within the seminal fluid of bulls, goats, boars, and rams (Kamarianos *et al.*, 2003), raising concern over the effects of contaminants on spermatozoa. Whilst such results indicate that grazing species with comparable environments to horses are exposed to ECs, seminal fluid contamination is applicable to concentrations associated with acute sperm exposure during ejaculation. Analysis of testicular samples is therefore required to develop an understanding of EC levels associated with exposure during the process of spermatogenesis. Determining contamination of the male equine gonads could form a foundation for further research determining the potential toxicity of ECs on stallion testicular function and fertility. This is of utmost importance for

the breeding and wider equine industry, given the association of ECs with reproductive aberrations reported in a range of other species (Sumner *et al*., 2021; Williams *et al*., 2021; Skakkebaek *et al*., 2015; Paul *et al*., 2005). Furthering this area could also initiate the utilisation of the equine model as a novel biomonitor species of EC contamination from a reproductive perspective and as a predictor of terrestrial ecosystem environmental health.

5.1.4 Pasture and feedstuff contamination as routes of oral exposure

Within terrestrial environments, contaminants are present in the soil, water, air, and vegetation (Schulz *et al*., 2005). Considering potential exposure routes to horses specifically, pastures established in contaminated soils are of significant concern (Madejón, Domínguez and Murillo, 2012, 2009). Soil is a primary sink for pollutants (Delannoy *et al*., 2015), acting as a potential exposure source to grazing herbivores, including equines. Horses have incisors on both the mandibular and maxillary jaw, enabling grazing in close proximity to the ground (Jurjanz *et al*., 2021). Grazing patterns in horses are likely to result in the direct consumption of contaminated soil when pasture levels are low. Horses are reported to ingest approximately 600g of dry soil per day (Jurjanz *et al*., 2021). A secondary route of oral exposure in grazing species is the consumption of plant materials contaminated with ECs from soils and the air (Wang *et al*., 2007). Phytoremediation, the ability of grasses to assist in the extraction and biodegradation of ECs from contaminated soils, is an area of environmental interest (Gao and Zhu, 2004). Given the extensive root systems of legumes, including alfalfa, species are recognised for assisting in phytoremediation and, together with rhizospheric microbiomes, the bioremediation of contaminated soils (Bourceret *et al*., 2018). Whilst this is an important process for soil health, the ingestion of pastures grown on contaminated soils could act as an oral exposure route in grazing species such as horses.

Due to the digestible energy content of alfalfa, it is a recommended commercial feedstuff for breeding stock, including stallions and mares (DeBoer *et al*., 2018). Currently, a link between the bioremediating properties of alfalfa and its utilisation as a feedstuff remains unexplored. Alfalfa and concentrate feed materials are processed and packaged for commercial distribution within the equine industry. Contamination of feedstuff may occur during crop growth and management, and in the processing and packaging of

commercialised products (Yavuz *et al*., 2022). Many EC's have the ability to migrate from product matrices into feed contents (Wania, 2003). Migration of a range of ECs has been reported in plastic packaging, resulting in the contamination of food materials designed for human consumption (González-Castro *et al.*, 2011). Chemical leaching and contamination of equine feed sources specifically remain unknown, with potentially significant implications for equine management and feeding practices. Given the ubiquitous nature of ECs and their extensive reprotoxic abilities, further research analysing testicular accumulation rates and exposure routes specific to the equine model is warranted.

The aim of research associated with the current chapter was to investigate testicular contamination levels specific to the equine species and determine routes of oral exposure. To address this research aim, the study was separated into four objectives:

1. To analyse stallion (*Equus caballus*) testis, using the Thoroughbred as a model, for four categories of anthropogenic ECs; Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP.
2. To analyse commercialised equine feedstuff materials and grazing pastures (soil and grass) for Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP as potential routes of oral exposure.
3. To analyse geographical differences in Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP between testicular, soil and grass samples from two regions in the UK (Cambridgeshire and Gloucestershire).
4. To evaluate the suitability of the equine species as a novel comparative model for reproductive EC contamination and environmental health in terrestrial ecosystems.

For this research objective, it was hypothesised that anthropogenic ECs including, PCBs, PBDEs, PAHs, and DEHP, accumulate in stallion testis and exposure includes the ingestion of contaminated pastures and feedstuffs. The null hypothesis was that anthropogenic ECs, including PCBs, PBDEs, PAHs, and DEHP, do not accumulate in stallion testicular tissue, and the equine species are not exposed to contaminants through the ingestion of contaminated pastures or feedstuffs.

5.2 MATERIALS AND METHODS

Justifications of the methodology and methods employed for this study are presented in chapter 2 of this thesis (research methodology and methods; pages 40-67). Hartpury University Ethics Committee approved the research associated with this study. Signed consent was obtained from all stallion owners, veterinarians, and property owners prior to sample collection, and anonymity was maintained throughout the research process, as outlined by the data management plan (Appendix A; pages 246-263).

5.2.1 Sample collection: Thoroughbred (*Equus caballus)* testicular tissue

Six clinically healthy Thoroughbreds (*Equus caballus)* from the racing sector were included in this study. The Thoroughbred was used as a model with applicability to the wider equine population. Age at castration ranged from 19 months to 44 months (31.50 ± 9.65 months). Testes with no abnormalities were collected as surplus tissue from routine castrations completed by qualified veterinarians. Castrations were carried out in November 2020 at two UK locations (Cambridgeshire, n=3; Gloucestershire, n=3). The principal investigator (IH) carried out the subsequent stages of sample treatment. Immediately post castration, the tunica vaginalis and epididymis were removed, and the right and left testis were weighed separately. Each testis was bisected down the axial plane. A 4mm disc was cut, from which a biopsy (biopsy punch; Stiefel; 4mm) was taken from the centre of the parenchyma. The biopsy was immediately submerged in Allprotect Tissue Reagent (Qiagen; Cat. No./ID: 76405) following manufacturer guidelines (Qiagen, 2011). Remaining tissue was wrapped in tin foil and transported to the laboratory on ice. A second 4mm disc was cut and treated with Bouin's fixative (Sigma-Aldrich; HT10132) for histological observation. Due to COVID-19 restrictions resulting in limited laboratory availability, histology treatment methods were inconsistent, resulting in the inability to continue with this element. Remaining tissue was wrapped in tin foil and frozen at -80°C until chemical analysis. Testicular samples in Allprotect Tissue Reagent were archived at -20°C.

5.2.2 Sample collection: soil and grass collection

Soil and grass were collected in April 2021 from Thoroughbred breeding and racing facilities in Gloucestershire (n=2) and Newmarket, Cambridgeshire (n=3). Convenience sampling was used, determined by participant willingness to engage in the study. Nine soil and grass

samples from each plot were taken using a 'W' transect for the spatial distribution of sampling sites (The Soil Carbon Project, 2021). Shears were used to take cuttings at ground level, just above the roots. Once the grass cutting was collected, the roots were cleared, and a soil core was taken with a stainless steel probe (VOSAREA; 2.8 cm diameter by 10 cm depth). Grass (n=2) and soil samples (n=2) were pooled separately from the Gloucestershire and Cambridgeshire areas, respectively, and stored in glass jars. Samples were transported to the laboratory on ice and frozen at -20°C until chemical analysis.

5.2.3 Sample collection: baled haylage and concentrate feed collection

Samples from a small commercially plastic-wrapped bale (n=1) and a large farm baled plastic-wrapped haylage (n=1) were collected for chemical analysis. Samples were taken from the external and internal portions of the bale to provide a representative sample and stored in tin foil at -20°C until analysis. Four concentrate feeds, including a forage-based alfalfa with a high oil content, a breeding cube fed to stallions and mares, a racehorse-specific cube, and a pellet for weaning foals, were also analysed. The specific feed constituents are presented in the supplementary information (Appendix P; page 333). Feed from internal and external portions of the bag was taken and stored in glass jars at -20°C.

5.2.4 Chemical analysis of tissue, pasture, and feedstuff samples

Chemical analysis was undertaken by an ISO-17025 accredited laboratory (James Hutton Institute) utilising standardised extraction protocols and gas chromatography-mass spectrometry (GC-MS) analysis. An extension of this analytical method is presented in previous literature (Rhind *et al*., 2010; Rhind *et al*., 2005). All samples were analysed for anthropogenic ECs including; Polychlorinated biphenyl (PCB) congeners (28, 52, 101, 118, 138, 153, 180), Polybrominated diphenyl ether (PBDE) congeners (28, 47, 99, 100, 153, 154, 183), Di (2-ethylhexyl) phthalate (DEHP) and Polycyclic aromatic hydrocarbons (PAHs). PAH congeners included; Naphthalene, Acenaphthalene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo[a]anthracene, Chrysene, Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[a]pyrene, Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene and Benzo[ghl]perylene. A summary of chemical congeners analysed is included in the supplementary information (Appendix N; pages 328-331).

5.2.5 Statistical analysis of sample contamination levels

Statistical analysis and graphical outputs for this research objective were performed on GraphPad Prism 9 (GraphPad Prism, 2022; GraphPad Prism version 9.0, GraphPad Software, California, CA, USA). Mean concentrations (± SD) were calculated for grouped chemicals in individual samples, followed by individual chemical congeners in grouped samples of respective sample types. A Shapiro-Wilk test and graphical interpretation of a Normal QQ Plot and a histogram were used to determine the distribution and normality of the data. Data exhibiting a skewed distribution were normalised utilising the Logarithmic (Log) transformation. An extension of the statistical methods utilised within this research are presented in chapter 2 (research methodology and methods; pages 40-67). Following confirmation of normal distribution, subsequent analyses included parametric statistical tests.

Data were first analysed to determine differences in Σ7PCBs, Σ7PBDEs, and Σ16PAHs in individual testis, feedstuff, soil, and grass samples. A one-way analysis of variance (one-way ANOVA) and Tukey's post hoc determined statistical differences between stallion testis concentrations and different feedstuff materials. An independent t-test was used to analyse differences in Σ7PCB, Σ7PBDE, and Σ16PAH concentrations between isolated soil and grass samples from Cambridgeshire and Gloucestershire regions. Geographical differences in testicular concentrations of Σ7PCBs, Σ7PBDEs and Σ16PAHs, and DEHP between Cambridgeshire and Gloucestershire were analysed with an independent t-test. Results for t-tests are presented as (mean difference, t(df); t value (degrees of freedom); probability). Data were then analysed to determine differences between specific congeners of chemical categories in grouped testis and feedstuffs, soils, and grass. A one-way ANOVA with Tukey's post hoc was used to determine differences in PCB, PBDE, and PAH congeners in pooled sample categories. Results for one-way ANOVAs are presented as (F (DFn, DFd); F value (degrees of freedom numerator, degrees of freedom denominator); probability). *P* values of <0.05 and <0.001 were considered statistically significant and highly significant, respectively, and a confidence interval of 95% was assumed.

5.3 RESULTS

5.3.1 Testicular contamination

Mean (± SD) testicular Σ7PCBs, Σ7PBDEs and Σ16PAHs ranged from 0.04 ± 0.05 µg/kg to 0.15 ± 0.10 µg/kg (figure 18A), 0.04 ± 0.05 µg/kg to 0.20 ± 0.33 µg/kg (figure 18B) and, 2.83 ± 3.39 µg/kg to 39.90 ± 99.04 µg/kg (figure 18C), respectively (Table 20). DEHP ranged from 436.68 to 1,176.19 µg/kg (figure 18D). Total testicular Σ7PCBs, Σ7PBDEs, Σ16PAHs and DEHP means were calculated at 0.08 ± 0.07 µg/kg, 0.14 ± 0.19 µg/kg, 31.35 ± 97.70 µg/kg and 739.60 ± 317.50 µg/kg, respectively. Mean Σ7PCB concentrations were highest for testis one (0.15 ± 0.10 µg/kg). Concentrations of Σ7PBDEs, Σ16PAHs and DEHP were highest for testis four (0.28 ± 0.33 µg/kg; 82.16 ± 196.92 µg/kg; 1,176.19 µg/kg). Testis two had the lowest levels of Σ7PBDEs (0.04 ± 0.05 µg/kg), Σ16PAHs (2.83 ± 3.39 µg/kg) and DEHP (436.54 µg/kg). Testis five had the lowest Σ7PCB concentration (0.04 ± 0.05 µg/kg). No significant differences were detected amongst mean concentrations of Σ7PCBs (1.85(5,32); *p* 0.132), Σ7PBDEs (0.58(5,23); *p* 0.713) or Σ16PAHs (0.35(5,73); *p* 0.862).

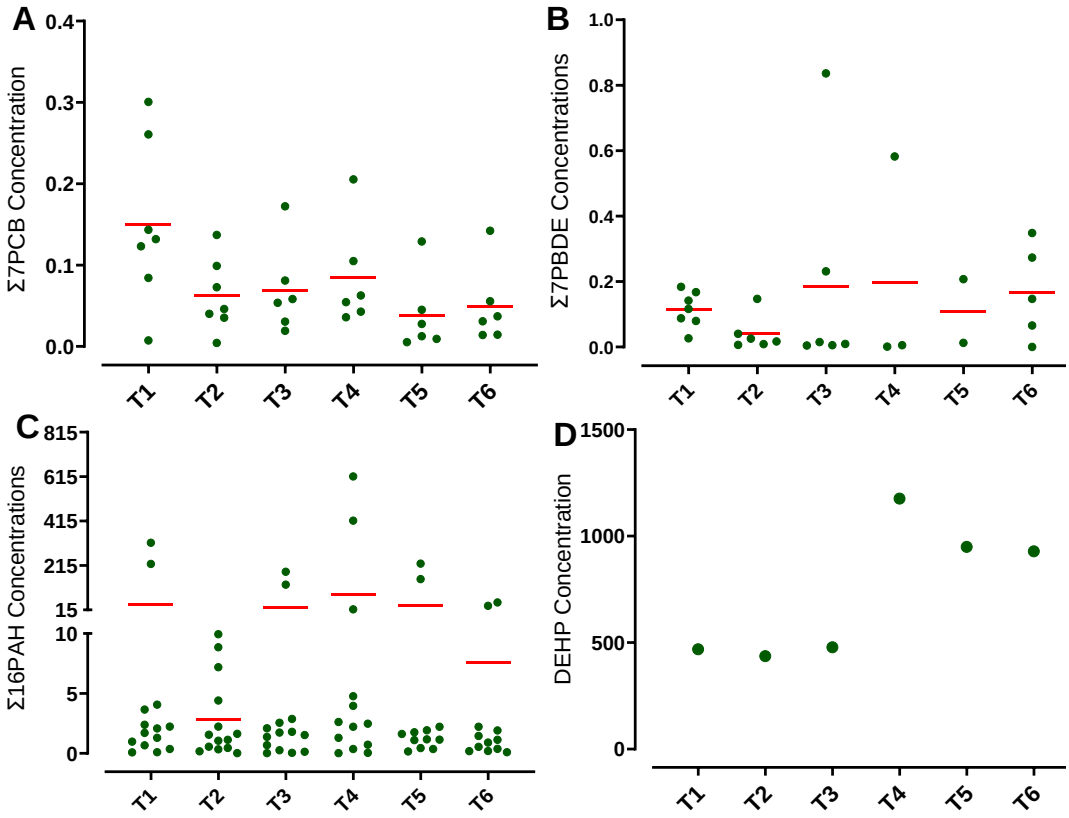


Figure 18: [A] Σ7PCBs, [B] Σ7PBDEs, [C] Σ16PAHs and [D] DEHP testis concentrations (µg/kg DW). T1-T6: Testis 1-Testis 6. Each point represents a congener value. Red lines denote mean values of the Σ-congeners in each chemical category ([A] Σ7PCBs; [B] Σ7PBDEs; [C] Σ16PAHs; [D] DEHP).

5.3.2 Geographic differences in testicular concentrations

Raw data and mean (± SD) values for testicular contamination levels between the two sampling regions are presented in table 20. Mean Σ7PCBs, Σ7PBDEs and Σ16PAHs in the Gloucestershire and Cambridgeshire regions were detected at 0.10 ± 0.08 µg/kg and 0.06 ± 0.05 µg/kg (figure 19A), 0.10 ± 0.08 µg/kg and 0.06 ± 0.05 µg/kg (figure 19B), and 22.11 ± 65.64 µg/kg and 41.84 ± 123.62 µg/kg (figure 19C), respectively. No significant differences were detected in mean testicular concentrations of Σ7PCBs (1.69(36); *p* 0.0990), Σ7PBDEs (0.24(27); *p* 0.816), Σ16PAHs (0.52(77); *p* 0.605) between the Gloucestershire and Cambridgeshire regions, respectively. Testicular concentrations of DEHP were significantly higher in the samples from Gloucestershire compared to Cambridgeshire samples (6.94(4); *p* 0.002), with mean concentrations of 1,018.11 ± 112.10 µg/kg and 461.04 ± 17.73 µg/kg, respectively (figure 19D).

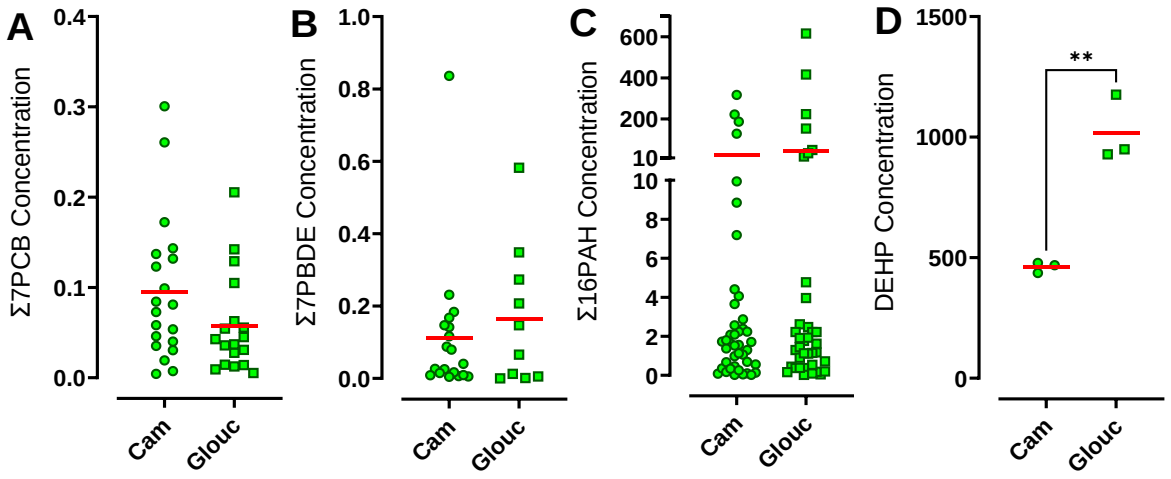


*Figure 19: Geographical differences in testicular concentrations (µg/kg DW) of [A] Σ7PCBs, [B] Σ7PBDEs, [C] Σ16PAHs, and [D] DEHP between Cambridgeshire (Cam) and Gloucestershire (Glouc) regions. Each point represents a value for each congener (Cam: green circle; Glouc: green square). The red line represents the mean of the total congeners for the respective chemical classification. Asterisks represent differences between means of statistical significance (t-test); **p<0.005.*

Table 20: Raw data and mean testicular concentrations (µg/kg DW) of Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP. Single asterisk (*) denotes mean (± SD) burdens of Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP in each testis. Double asterisk (**) denotes mean (± SD) levels of chemical congeners in all testicular samples (n=6). ND: not detected; <LOD: below limit of detection; LOD values are presented in appendix Q (page 334).

Chemical congener	Cambridgeshire			Gloucestershire			Summary
	Testis1	Testis2	Testis3	Testis4	Testis5	Testis6	*Mean ± S.D. **
PCB 28	0.26	0.14	0.17	0.21	0.13	0.14	0.17 ± 0.05
PCB 52	0.01	<LOD	<LOD	<LOD	<LOD	<LOD	0.01 ± 0.00
PCB 101	0.08	0.04	0.03	0.04	0.03	0.03	0.04 ± 0.02
PCB 118	0.30	0.10	0.06	0.04	0.01	0.01	0.09 ± 0.11
PCB 138	0.12	0.04	0.02	0.05	0.01	0.06	0.05 ± 0.04
PCB 153	0.13	0.07	0.08	0.10	0.04	0.04	0.08 ± 0.04
PCB 180	0.14	0.05	0.05	0.06	0.01	0.01	0.06 ± 0.05
*Mean ΣPCB * ± SD **	0.15 ± 0.10	0.06 ± 0.04	0.07 ± 0.06	0.08 ± 0.06	0.04 ± 0.05	0.05 ± 0.05	
PBDE 28	0.12	0.01	0.02	<LOD	<LOD	<LOD	0.05 ± 0.06
PBDE 47	0.18	0.15	0.84	0.58	0.21	0.35	0.38 ± 0.27
PBDE 99	0.14	0.03	0.23	0.01	<LOD	0.27	0.14 ± 0.12
PBDE 100	0.09	<LOD	<LOD	<LOD	<LOD	0.15	0.12 ± 0.04
PBDE 153	0.17	0.02	0.01	<LOD	<LOD	0.07	0.05 ± 0.07
PBDE 154	0.08	0.01	0.01	<LOD	<LOD	<LOD	0.03 ± 0.04
PBDE 183	0.03	0.04	0.00	<LOD	0.01	<LOD	0.02 ± 0.02
*Mean ΣPBDE * ± SD**	0.11 ± 0.05	0.04 ± 0.05	0.18 ± 0.33	0.20 ± 0.33	0.11 ± 0.14	0.17 ± 0.14	
Naphthalene	ND	ND	ND	ND	ND	ND	ND
Acenaphthalene	0.10	0.56	0.05	ND	ND	ND	0.24 ± 0.28
Acenaphthene	0.08	1.06	0.14	0.05	0.44	0.18	0.32 ± 0.39
Fluorene	2.39	0.46	2.09	2.62	2.22	2.22	2.00 ± 0.78
Phenanthrene	2.08	1.63	1.73	2.22	1.76	1.91	1.89 ± 0.23
Anthracene	ND	ND	ND	ND	ND	ND	ND
Fluoranthene	1.71	1.13	1.38	1.30	1.17	0.90	1.27 ± 0.27
Pyrene	0.36	8.85	0.69	0.37	0.37	0.38	1.83 ± 3.44
Benzo[a]anthracene	316.94	9.94	186.78	615.84	223.53	48.62	233.61 ± 218.90
Chrysene	222.07	7.19	128.42	416.53	153.62	33.18	160.17 ± 148.41
Benzo[b]fluoranthene	2.24	0.17	2.87	17.22	1.62	1.45	4.26 ± 6.41
Benzo[k]fluoranthene	0.98	0.02	1.80	4.77	1.15	0.54	1.54 ± 1.69
Benzo[a]pyrene	4.06	1.56	2.56	2.48	1.93	1.13	2.29 ± 1.02
Indeno[1,2,3-cd]pyrene	3.65	2.24	1.53	3.96	1.11	0.20	2.11 ± 1.47
Dibenzo[a,h]anthracene	1.30	0.34	0.26	0.72	0.16	0.09	0.48 ± 0.46
Benzo[ghl]perylene	0.67	4.41	0.02	0.02	ND	ND	1.28 ± 2.11
*Mean ΣPAH * ± SD**	39.90 ± 99.04	2.83 ± 3.39	23.59 ± 57.92	82.16 ± 196.92	32.42 ± 74.45	7.57 ± 15.93	
DEHP	468.68	436.54	477.90	1176.19	949.54	928.60	739.60 ± 317.50

5.3.3 Soil samples

Raw data and mean (± SD) values for chemical burdens in soil are presented in table 21. In the soil samples analysed from Cambridgeshire and Gloucestershire, mean Σ7PCBs were 0.09 ± 0.06 µg/kg and 0.07 ± 0.01 µg/kg, respectively (figure 20A). Concentrations of Σ7PBDEs in Cambridgeshire and Gloucestershire soil samples were 0.05 ± 0.31 µg/kg and 0.01 ± 0.29 µg/kg, respectively (figure 20B). Σ16PAHs in Cambridgeshire soil were 76.12 ± 70.30 µg/kg and 34.50 ± 31.57 µg/kg in Gloucestershire (figure 20C). Raw values for DEHP in Cambridgeshire and Gloucestershire were 108.00 µg/kg and 206.30 µg/kg, respectively (figure 20D). No significant differences were detected in Σ7PCBs (-0.02; 0.53(12); *p* 0.607) or Σ7PBDEs (-0.03; 1.69(9); *p* 0.125) between the two sampling locations. There was a significant difference in Σ16PAH concentrations between Gloucestershire (34.50 ± 31.57 µg/kg) and Cambridgeshire (76.17 ± 70.03 µg/kg) areas (-41.68; 2.17(30); *p* 0.038).

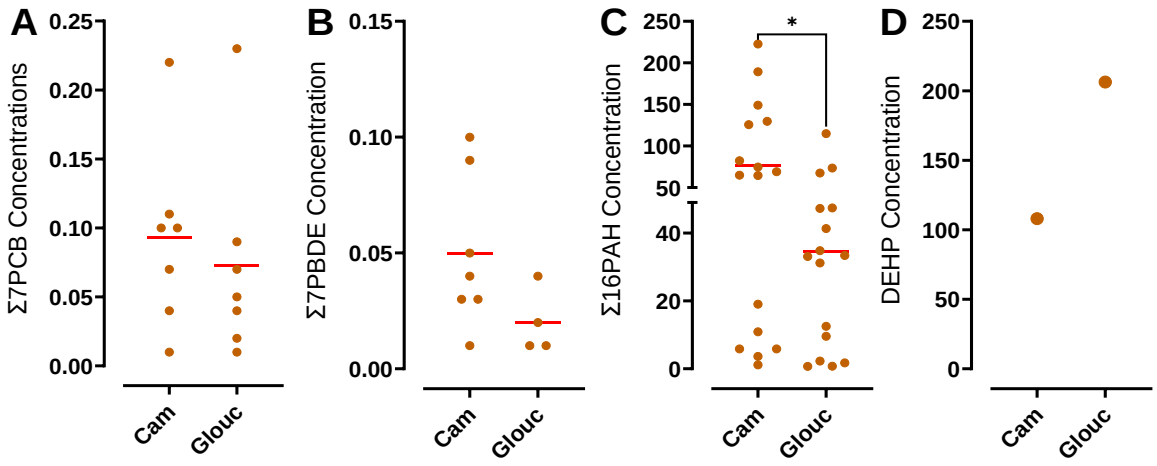


*Figure 20: Soil contamination (µg/kg DW) of [A] Σ7PCBs, [B] Σ7PBDEs, [C] Σ16PAHs, and [D] DEHP. Cam: Cambridgeshire; Glouc: Gloucestershire. Each point represents a value for each congener. The red line represents the mean of the total congeners within each chemical category ([A], Σ7PCBs; [B], Σ7PBDEs; [C], Σ16PAHs; [D], DEHP). Asterisks represent differences between means of statistical significance (t-test); * p<0.05.*

5.3.4 Grass samples

Contamination levels of all congeners within the grass samples analysed are presented in table 21. Mean Σ7PCBs in grass collected from the Cambridgeshire and Gloucestershire sampling regions were 0.64 ± 0.31 µg/kg and 0.28 ± 0.05 µg/kg, respectively (figure 21A). For Σ7PBDEs, mean concentrations were 0.28 ± 0.14 µg/kg in Cambridgeshire and 0.06 ± 0.05 µg/kg in Gloucestershire (figure 21B). Mean Σ16PAHs detected in grass samples from Cambridgeshire and Gloucestershire were 18.52 ± 23.63 µg/kg and 37.87 ± 61.32 µg/kg, respectively (figure 21C). DEHP concentrations in Cambridgeshire were 4373.77 µg/kg, and 713.32 µg/kg in Gloucestershire grass samples (figure 21D). For grass sampled at either location, no significant differences in Σ7PCBs (-0.35; 1.93(11); *p* 0.080), or Σ16PAHs (19.35; 1.11(29); *p* 0.277) were detected. There was a significant difference in Σ7PBDE concentrations between Gloucestershire (0.06 ± 0.05 µg/kg) and Cambridgeshire (0.28 ± 0.14 µg/kg) regions (-0.22; 3.83(11); *p* 0.003).

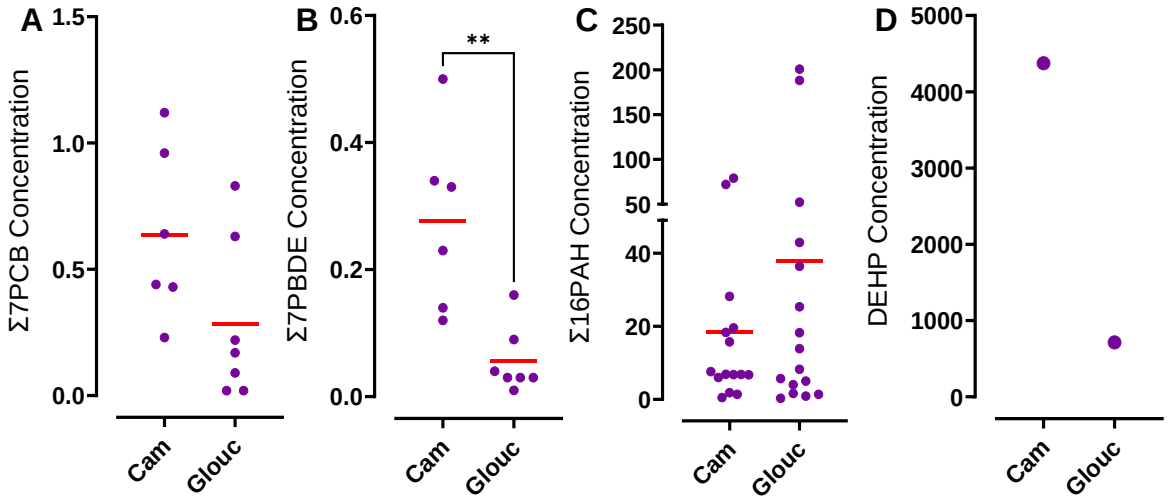


Figure 21: Levels (µg/kg DW) of [A] Σ7PCBs, [B] Σ7PBDEs, [C] Σ16PAHs, and [D] DEHP in grass. *Cam: Cambridgeshire; Glouc: Gloucestershire. Each point represents a value for each congener. The red line represents the mean of congeners within each chemical category ([A] Σ7PCBs; [B] Σ7PBDEs; [C] Σ16PAHs; [D] DEHP). Asterisks represent differences between means (t-test); ** p<0.005.*

Table 21: Raw data and mean concentrations (µg/kg DW) of Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP in soil and grass. Single asterisk (*): mean (±SD) Σ7PCB, Σ7PBDE, Σ16PAH, and DEHP burdens in soil (n=2) and grass (n=2). Double asterisk (**): mean (± SD) concentrations of each chemical congener in soil and grass. Cam: Cambridgeshire; Glouc: Gloucestershire. ND: not detected; <LOD: below limit of detection; specific LOD values are presented in appendix Q (page 334).

Chemical congener	Soil		Summary	Grass		Summary
	Cam	Glouc	*Mean± SD **	Cam	Glouc	*Mean± S.D. **
PCB 28	0.22	0.23	0.23 ± 0.00	0.64	0.83	0.74 ± 0.09
PCB 52	0.01	0.01	0.01 ± 0.00	<LOD	0.02	0.02 ± 0.00
PCB 101	0.04	0.02	0.03 ± 0.01	0.23	0.09	0.16 ± 0.07
PCB 118	0.11	0.05	0.08 ± 0.03	0.96	0.22	0.59 ± 0.37
PCB 138	0.10	0.07	0.09 ± 0.01	0.43	0.02	0.23 ± 0.20
PCB 153	0.10	0.09	0.10 ± 0.01	0.44	0.17	0.31 ± 0.13
PCB 180	0.07	0.04	0.06 ± 0.01	1.12	0.63	0.87 ± 0.25
*Mean ΣPCB **	0.09	0.07		0.64	0.28	
*± SD **	± 0.06	± 0.07		± 0.31	± 0.29	
PBDE 28	0.04	<LOD	0.02 ± 0.02	0.23	0.03	0.13 ± 0.10
PBDE 47	0.09	0.02	0.06 ± 0.03	0.50	0.16	0.33 ± 0.17
PBDE 99	0.10	0.04	0.07 ± 0.03	0.34	0.09	0.21 ± 0.13
PBDE 100	0.03	<LOD	0.03 ± 0.00	0.12	0.03	0.07 ± 0.04
PBDE 153	0.05	0.01	0.03 ± 0.02	0.33	0.04	0.19 ± 0.15
PBDE 154	0.03	0.01	0.02 ± 0.01	0.14	0.03	0.09 ± 0.06
PBDE 183	0.01	<LOD	0.01 ± 0.00	<LOD	0.01	0.01 ± 0.00
*Mean ΣPBDE **	0.05	0.01		0.28	0.06	
*± SD**	± 0.03	± 0.01		± 0.14	± 0.05	
Naphthalene	10.91	9.59	10.25 ± 0.66	ND	5.04	5.04 ± 0.00
Acenaphthalene	1.13	0.69	0.91 ± 0.22	1.84	0.89	1.36 ± 0.47
Acenaphthene	3.66	0.78	2.22 ± 1.44	1.40	1.40	1.40 ± 0.00
Fluorene	5.84	2.30	4.07 ± 1.77	6.86	13.87	10.36 ± 3.50
Phenanthrene	129.79	33.11	81.45 ± 48.34	72.18	200.99	136.59 ± 64.40
Anthracene	5.87	1.75	3.81 ± 2.06	0.51	0.31	0.41 ± 0.10
Fluoranthene	222.62	73.45	148.04 ± 74.58	79.15	188.36	133.76 ± 54.60
Pyrene	125.68	34.79	80.23 ± 45.44	19.59	18.26	18.93 ± 0.66
Benzo[a]anthracene	82.39	31.20	56.80 ± 25.60	6.77	5.71	6.24 ± 0.53
Chrysene	149.18	67.49	108.34 ± 40.84	28.18	52.37	40.27 ± 12.10
Benzo[b]fluoranthene	189.35	114.91	152.13 ± 37.22	18.37	42.95	30.66 ± 12.29
Benzo[k]fluoranthene	64.55	33.38	48.96 ± 15.58	15.77	25.38	20.58 ± 4.80
Benzo[a]pyrene	74.78	41.34	58.06 ± 16.72	6.02	1.66	3.84 ± 2.18
Indeno[1,2,3-cd]pyrene	69.11	47.26	58.18 ± 10.93	6.75	8.25	7.50 ± 0.75
Dibenzo[a,h]anthracene	19.04	12.52	15.78 ± 3.26	7.60	36.43	22.01 ± 14.41
Benzo[ghl]perylene	64.85	47.39	56.12 ± 8.73	6.85	4.06	5.45 ± 1.40
*Mean ΣPAH **	76.17	34.50		18.52	37.87	
*± SD**	± 70.03	± 31.57		± 23.63	± 61.32	
DEHP	108.00	206.30	157.20 ± 69.51	4373.77	713.32	2,544.00 ± 2,588.00

5.3.5 Feedstuff materials: haylage and commercial concentrate feeds

Mean (± SD) concentrations for feedstuffs are presented in table 22. Mean Σ7PCBs ranged from 0.05 ± 0.05 µg/kg (haylage (B); bailed) to 0.15 ± 0.10 µg/kg (haylage (CB); commercially bailed; figure 22A). Mean Σ7PBDEs were detected at the lowest concentrations in haylage (B), and highest in race cubes, ranging from 0.01 ± 0.01 µg/kg to 0.23 ± 0.12 µg/kg (figure 22B). Mean Σ16PAHs ranged from 0.74 ± 0.73 µg/kg (foal cubes) and 7.54 ± 13.07 µg/kg (haylage (CB); figure 22C). DEHP concentrations ranged from 19.39 µg/kg (foal cubes) to 447.42 µg/kg (haylage (CB); figure 22D). There were no significant differences in Σ7PCB concentrations between feeds (1.79(5,34); *p* 0.142). Significant differences were detected between Σ7PBDE (4.61(5,18); *p* 0.007) and Σ16PAH (2.64(5,79); *p* 0.029) concentrations. Significant differences for Σ7PBDEs existed between haylage (CB) and race cubes (*p* 0.036), and haylage (B) and race cubes (*p* 0.025), and for Σ16PAHs differences were between haylage (CB) and foal cubes (*p* 0.034), and foal cubes and stud cubes (*p* 0.040). There were no significant differences between concentrations and any other feed types.

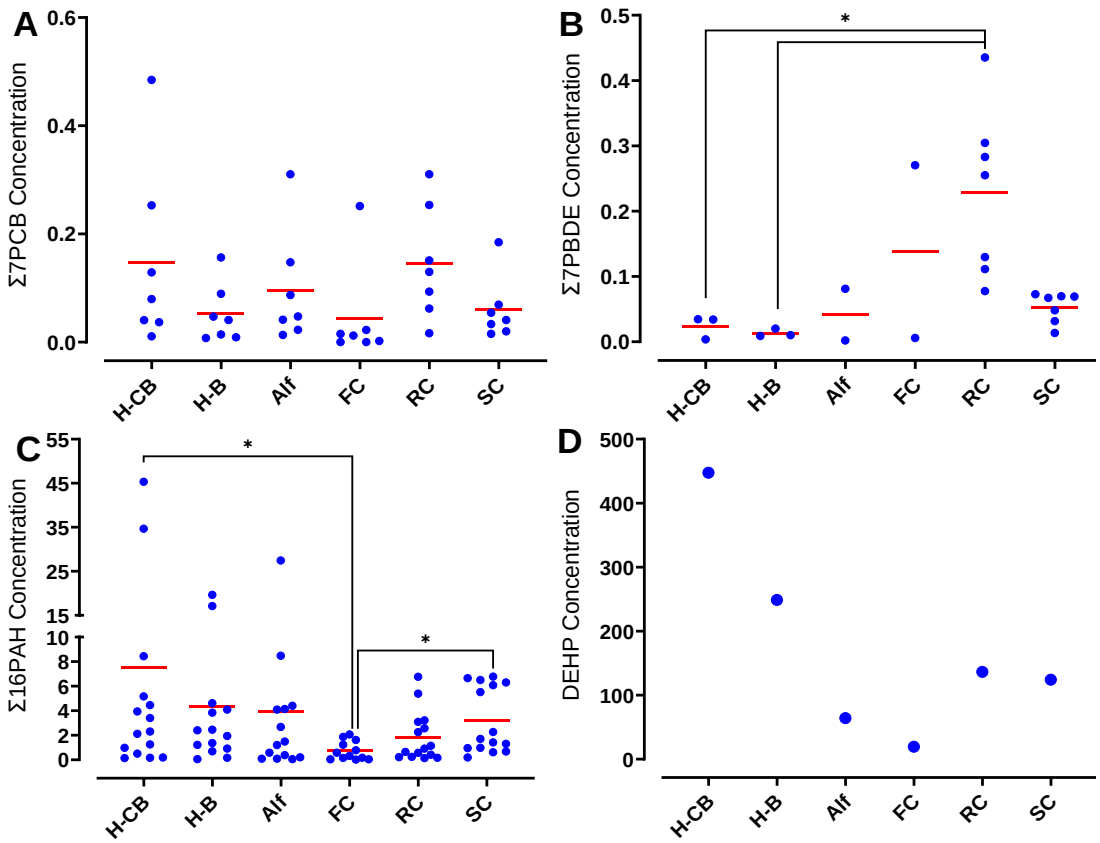


*Figure 22: [A] Σ7PCB, [B] Σ7PBDE, [C] Σ16PAH and [D] DEHP levels (µg/kg DW) in feedstuff. H-CB: commercially baled haylage; H-B: baled haylage; Alf: alfalfa; FC: foal cubes; RC: race cubes; SC: stud cubes. Each point: congener value. Red line: mean value. Asterisks: Tukey's post hoc; * p<0.05.*

Table 22: Raw data and mean concentrations (µg/kg DW) of Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP in feedstuffs. Haylage (CB), commercially baled haylage; Haylage (B), baled haylage. Single asterisk (*) denotes mean (± SD) Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP in individual feed types (n=6). Double asterisk (**) denotes mean (± SD) concentrations of individual congeners in grouped feedstuffs (n=6). <LOD; below limit of detection; specific LOD values are presented in appendix Q (page 334).

Chemical congener	Feed type						Summary
	Haylage (CB)	Haylage (B)	Alfalfa	Foal cubes	Race cubes	Stud cubes	*Mean ± S.D* **
PCB 28	0.25	0.09	0.31	0.25	0.31	0.18	0.23 ± 0.08
PCB 52	0.01	0.01	0.01	0.02	0.02	0.02	0.01 ± 0.00
PCB 101	0.04	0.01	0.02	0.02	0.06	0.02	0.03 ± 0.02
PCB 118	0.13	0.04	0.05	<LOD	0.25	0.07	0.11 ± 0.09
PCB 138	0.04	0.01	0.04	<LOD	0.13	0.03	0.05 ± 0.05
PCB 153	0.08	0.05	0.09	0.01	0.09	0.04	0.06 ± 0.03
PCB 180	0.48	0.16	0.15	<LOD	0.15	0.05	0.17 ± 0.17
*Mean ΣPCB **	0.15	0.05	0.10	0.06	0.15	0.06	
*± SD **	± 0.16	± 0.05	± 0.10	± 0.10	± 0.10	± 0.05	
PBDE 28	<LOD	<LOD	<LOD	<LOD	0.25	0.07	0.16 ± 0.09
PBDE 47	<LOD	0.02	0.08	0.27	0.44	0.07	0.18 ± 0.16
PBDE 99	<LOD	<LOD	<LOD	<LOD	0.30	0.07	0.19 ± 0.12
PBDE 100	0.03	0.01	<LOD	<LOD	0.13	0.05	0.06 ± 0.04
PBDE 153	<LOD	<LOD	<LOD	<LOD	0.28	0.07	0.18 ± 0.11
PBDE 154	<LOD	<LOD	<LOD	<LOD	0.11	0.03	0.07 ± 0.04
PBDE 183	0.03	0.01	<LOD	0.01	0.08	0.01	0.03 ± 0.03
*Mean ΣPBDE **	0.03	0.01	0.08	0.14	0.23	0.05	
*± SD**	± 0.00	± 0.01	± 0.00	± 0.13	± 0.12	± 0.02	
Naphthalene	2.31	<LOD	<LOD	<LOD	0.64	6.51	3.15 ± 2.47
Acenaphthalene	0.20	0.17	0.09	0.05	0.56	1.42	0.41 ± 0.48
Acenaphthene	0.17	0.91	0.39	<LOD	0.22	0.68	0.47 ± 0.28
Fluorene	8.45	0.69	2.69	0.17	2.57	5.51	3.35 ± 2.85
Phenanthrene	5.17	4.10	8.49	2.07	3.22	6.65	4.95 ± 2.14
Anthracene	0.50	<LOD	<LOD	<LOD	<LOD	<LOD	0.50 ± 0.00
Fluoranthene	3.94	4.61	4.10	1.86	3.09	6.30	3.98 ± 1.36
Pyrene	0.14	2.41	1.50	1.23	0.92	2.27	1.41 ± 0.78
Benzo[a]anthracene	<LOD	1.22	0.21	0.58	0.24	0.96	0.64 ± 0.40
Chrysene	4.46	3.85	4.41	0.78	2.26	6.78	3.76 ± 1.88
Benzo[b]fluoranthene	45.33	2.46	1.20	0.05	0.41	0.99	8.41 ± 16.53
Benzo[k]fluoranthene	34.68	17.11	27.46	1.62	6.76	6.08	15.62 ± 12.05
Benzo[a]pyrene	2.12	19.66	0.09	0.02	5.40	1.31	4.77 ± 6.90
Indeno[1,2,3-cd]pyrene	0.98	1.95	0.58	<LOD	1.14	0.62	1.05 ± 0.49
Dibenzo[a,h]anthracene	3.41	1.37	4.14	0.16	0.15	1.71	1.82 ± 1.51
Benzo[ghl]perylene	1.26	0.06	0.06	0.32	0.17	0.19	0.34 ± 0.42
*Mean ΣPAH **	7.54	4.33	3.96	0.74	1.85	3.20	
*± SD**	± 13.07	± 5.92	± 6.93	± 0.73	± 1.97	± 2.59	
DEHP	447.42	248.92	64.12	19.39	136.52	124.32	173.40 ± 155.10

5.3.6 Polychlorinated biphenyls (PCBs)

Testicular PCBs ranged from 0.01 ± 0.00 µg/kg (PCB 52) to 0.17 ± 0.05 µg/kg (PCB 28). There were significant differences between mean concentrations of PCBs in the testes analysed (5.08(6,31); *p* 0.001). Multiple comparisons specified that the differences lay between PCB 28, and PCB 52 (*p* 0.0003) and PCB 138 (*p* 0.043); PCB52 and PCB 153 (*p* 0.011) (figure 23).

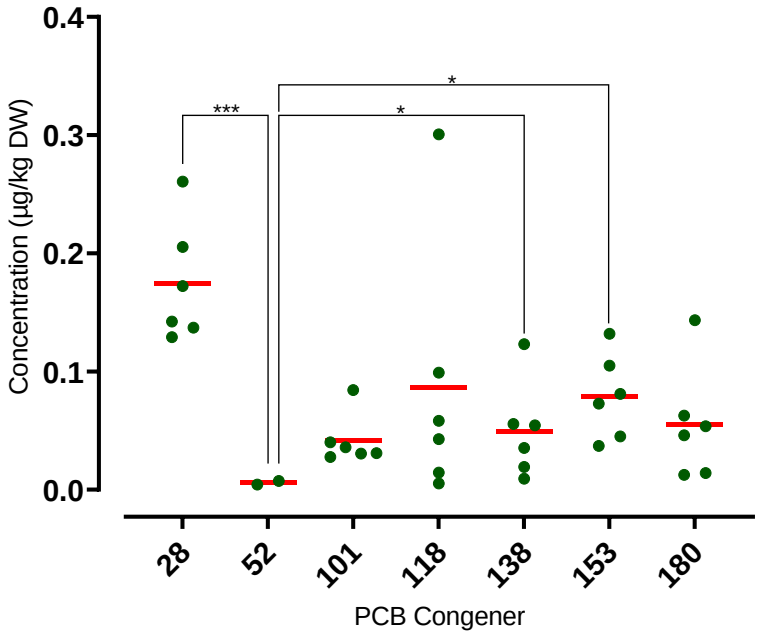


*Figure 23: Individual PCB congener concentrations (µg/kg DW) in grouped testicular samples. PCB congeners (28, 52, 101, 118, 138, 153, 180). Each point represents the concentration of each sample. The red line represents the mean. Asterisks represent pairwise comparisons; *p<0.05; ***p<0.001.*

All PCB congeners were detected in soil samples from the Cambridgeshire and Gloucestershire areas (figure 24A). PCB 28 was detected at the highest level (0.23 ± 0.00 µg/kg), whilst PCB 52 was detected at the lowest concentration (0.01 ± 0.00 µg/kg). All PCB congeners were higher in grass samples than in the respective soil samples analysed. PCB 52 was the only congener in grass that was below limit of detection (Cambridgeshire sample; figure 24B). As reported in soil, PCB 52 was the lowest congener detected in grass (0.02 ± 0.00 µg/kg). The highest congener was PCB 180 with a mean concentration of 0.87 ± 0.25 µg/kg across the two sampling areas. There were significant differences between PCB congeners in pooled soil samples (22.74(6,7); *p*<0.0001). Significant differences existed between PCB 28 and all other congeners (PCB 28 - PCB 52: *p* 0.0002; - PCB 101: *p* 0.0004; - PCB 118: *p* 0.0023; - PCB 138: *p* 0.0028; - PCB 153: *p* 0.0044; - PCB 180: *p* 0.0008), and, PCB

52 and PCB 153 (*p* 0.042). In pooled grass, there were no significant differences between congeners of PCBs (2.68(6,7); *p* 0.112).

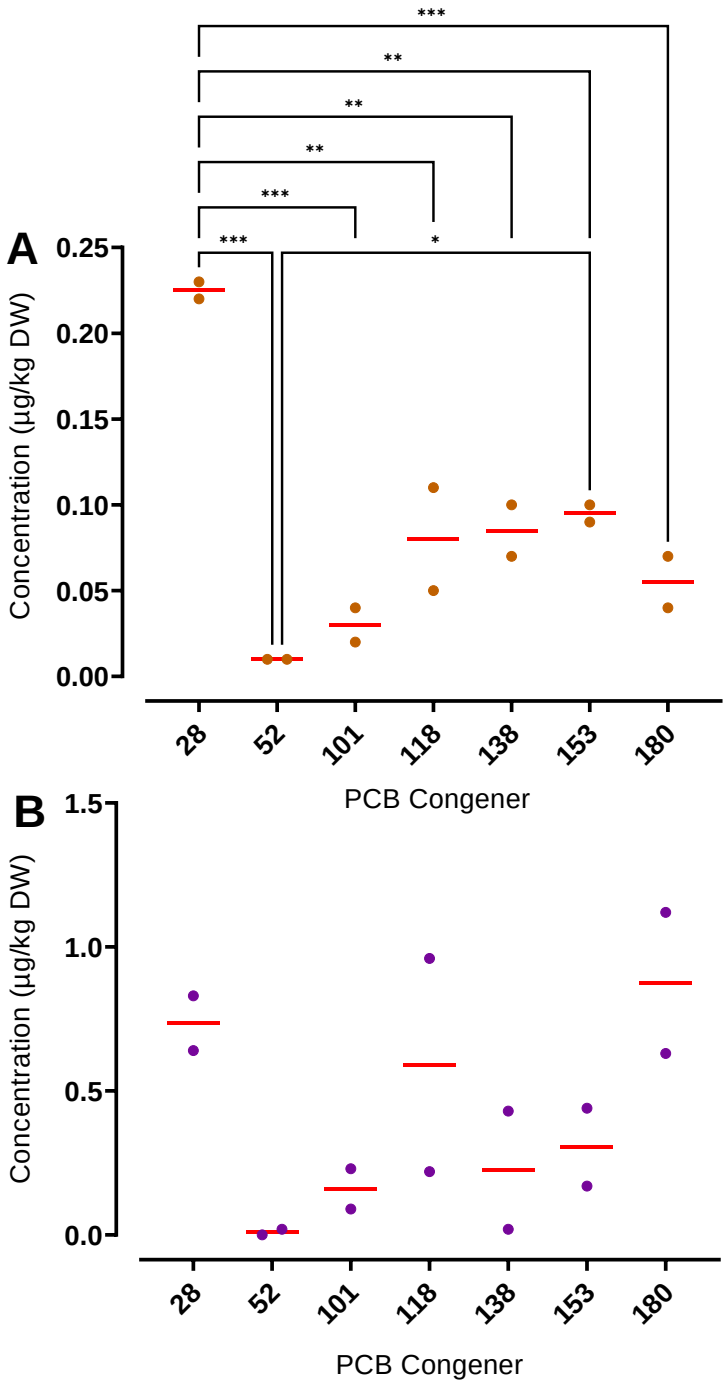


*Figure 24: Individual PCB congener concentrations (µg/kg DW) for grouped [A] soil and [B] grass. PCB congeners (28, 52, 101, 118, 138, 153, 180). Each point represents a single sample value. Red line represents the mean. Asterisks represent Tukey's post hoc; *p<0.05; **p<0.005; ***p<0.001.*

In the grouped feedstuffs analysed, PCB 28 was the congener with the highest mean concentration (0.23 ± 0.08 µg/kg), whilst PCB 52 was reported at the lowest mean level

(0.001 ± 0.00 µg/kg). All PCB congeners were detected in both haylages and stud cubes, race cubes, and alfalfa. The foal creep pellets were contaminated with PCB congeners 28, 52, 101, and 153, although congeners 118, 138, and 180 were reported at levels below the LOD. There were significant differences amongst the means of PCB congeners in grouped feedstuffs (6.87(6,33); p<0.0001; figure 25). The Tukey's post hoc specified that differences lay between PCB 28, and PCB 52 (p 0.0005), PCB 101 (p 0.001), PCB 118 (p 0.047), PCB 138 (p 0.008) and PCB 153 (p 0.009). Significant differences also existed between PCB 52 and PCB 180 (p 0.007) and PCB 101 and PCB 180 (p 0.016) for grouped feedstuff materials.

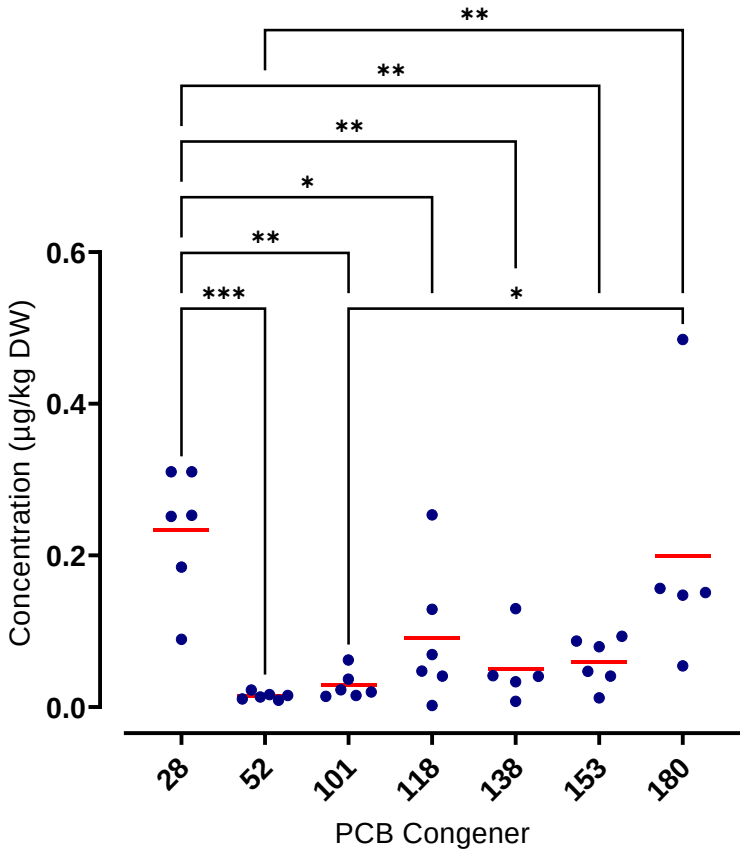


*Figure 25: Individual PCB congener concentrations (µg/kg DW) in grouped feedstuff materials. PCB congeners (28, 52, 101, 118, 138, 153, 180). Each point represents a sample value. The red line represents the mean. Asterisks represent Tukey's post hoc; * p<0.05;** p<0.005; ***p<0.001.*

5.3.7 Polybrominated diphenyl ethers (PBDEs)

In testicular samples, PBDE 47 was the highest congener, with a mean concentration of 0.38 ± 0.27 µg/kg, whilst PBDE 183 was the lowest (0.02 ± 0.02 µg/kg). There were significant differences between mean PBDE congener concentrations in testicular samples

(4.22(6,22); *p* 0.006; figure 26), between PBDE 28 and PBDE 47 (*p* 0.044) and PBDE 47 and PBDE 153 (*p* 0.015), PBDE 154 (*p* 0.033) and PBDE 183 (*p* 0.006).

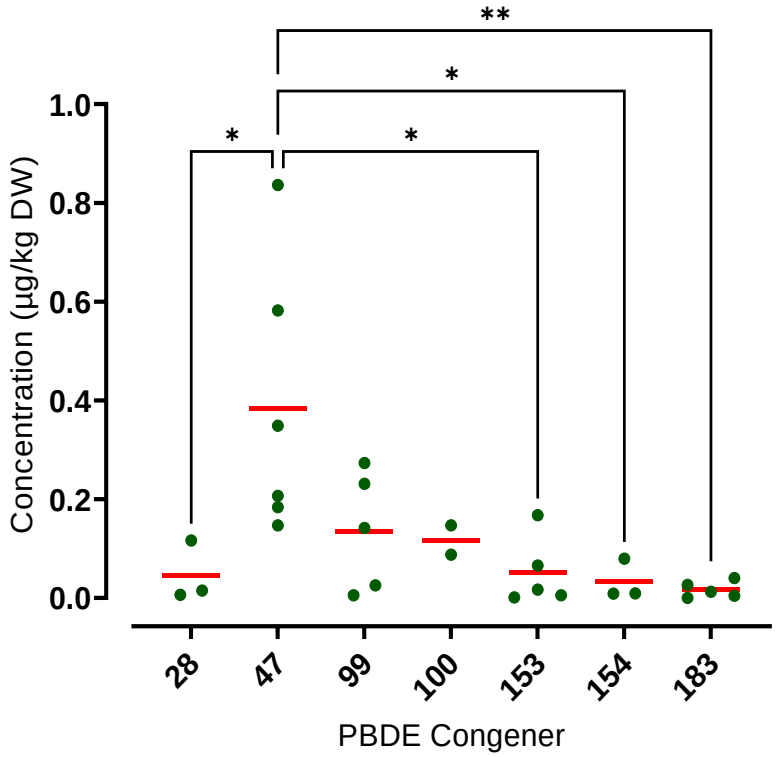


*Figure 26: Individual PBDE congener concentrations (µg/kg DW) in grouped testicular samples. PBDE congeners (28, 47, 99, 100, 153, 154 and 183). Each point represents a sample concentration value. The red line represents the mean. Asterisks represent Tukey's post hoc; * p<0.05; ***p<0.001.*

All PBDE congeners were detected in Cambridgeshire soil and Gloucestershire grass samples. In soil and grass samples, PBDE 47 was the highest congener with mean concentrations of 0.06 ± 0.03 µg/kg and 0.33 ± 0.17 µg/kg, respectively, whilst PBDE 183 was the lowest in both materials (0.01 ± 0.00 µg/kg). There were no significant differences in PBDE congeners in the pooled soil (0.54(6, 4); *p* 0.763; figure 27A) or grass (0.71(6,6); *p* 0.654; figure 27B) samples analysed.

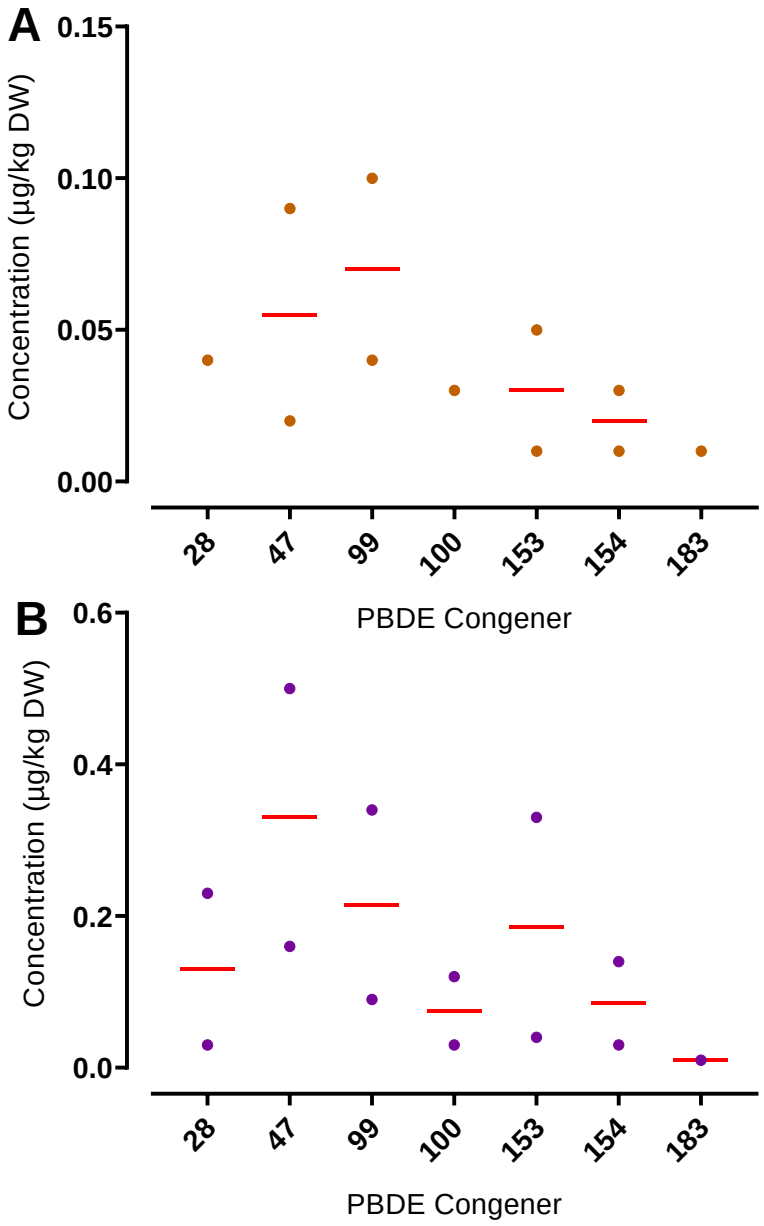


*Figure 27: Individual PBDE congener concentrations (µg/kg DW) for grouped [A] soil and [B] grass. PBDE congeners (28, 47, 99, 100, 153, 154 and 183). Each point represents a sample value. The red line represents the mean. Asterisks represent pairwise comparisons; *p<0.05; **p<0.005; ***p<0.001.*

In feedstuff materials, PBDE 99 had the highest mean concentration (0.19 ± 0.12 µg/kg). PBDE 183 was reported at the lowest concentration in feedstuffs, with a mean value of 0.03 ± 0.03 µg/kg. With respect to PBDE contamination in feedstuff materials, all congeners were detected in the stud cubes, and race cubes analysed. No significant differences were detected between any PBDE congeners in grouped feedstuff materials (1.15(6,17); *p* 0.379; figure 28).

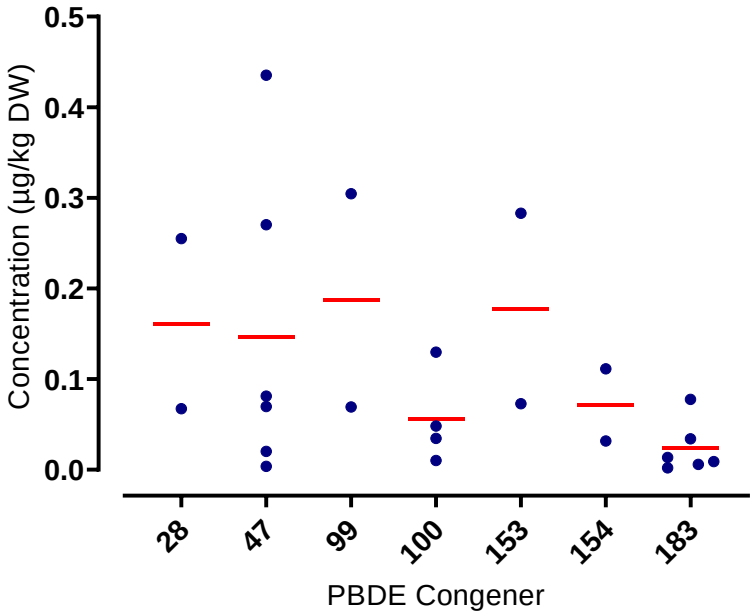


*Figure 28: Individual PBDE congener concentrations (µg/kg DW) in grouped feedstuff materials. PBDE congeners (28, 47, 99, 100, 153, 154 and 183). Each point represents a sample value. The red line represents the mean. Asterisks: Tukey's post hoc; * p<0.05;** p<0.005; ***p<0.001.*

5.3.8 Polyaromatic hydrocarbons (PAHs)

Benzo[a]anthracene was detected at the highest testicular concentration, with a mean of 233.61 ± 218.90 µg/kg. Naphthalene and Anthracene were not detected in any testes analysed. Acenaphthalene was detected at the lowest concentration in the testicular samples analysed (0.24 ± 0.28 µg/kg). There were significant differences amongst PAH congeners in the testis analysed (5.77(13,65); p<0.0001; figure 29). Significant differences existed between Pyrene-, Benzo[a]anthracene (p<0.0001), and Chrysene (p 0.025); Fluoranthene-, Benzo[a]anthracene (p<0.0001), and Chrysene (p 0.024); Phenanthrene-, Chrysene (p 0.025), and Benzo[a]anthracene (p<0.0001); Fluorene-, Chrysene (p 0.025), and Benzo[a]anthracene (p<0.0001); Acenaphthene-, Chrysene (p 0.022), and Benzo[a]anthracene (p<0.0001); Acenaphthalene- and Chrysene (p 0.002). Significant differences were detected between Benzo[a]anthracene and all other PAH congeners (p<0.001), apart from Chrysene (p 0.896). Significant differences were detected between Chrysene and all other PAH congeners (p<0.05), apart from Benzo[a]anthracene (p 0.896) and Benzo[ghl]perylene (p 0.070).

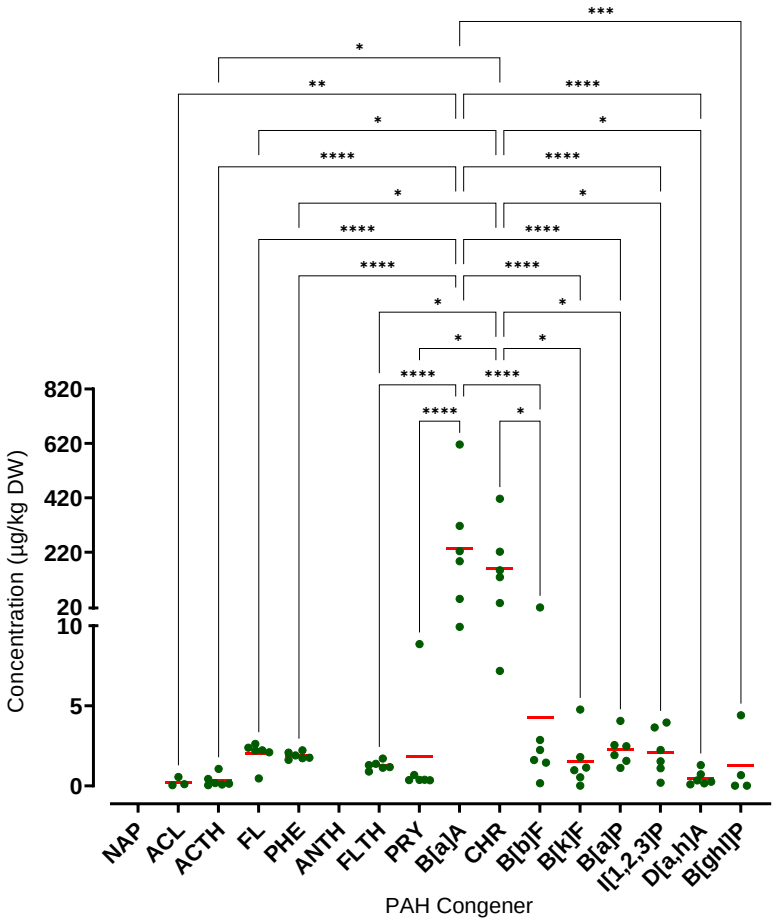


*Figure 29: PAH congener concentrations (µg/kg DW) in grouped testicular samples. Each point represents a sample value. Red line represents the mean. Asterisks: Tukey's post hoc; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Naphthalene (NAP), Acenaphthalene (ACL), Acenaphthene (ACTH), Fluorene (FL), Phenanthrene (PHE), Anthracene (ANTH), Fluoranthene (FLTH), Pyrene (PY), Benzo[a]anthracene (B[a]A), Chrysene (CHR), Benzo[b]fluoranthene (B[b]F), Benzo[k]fluoranthene (B[k]F), Benzo[a]pyrene (B[a]P), Indeno[1,2,3-cd]pyrene (I[1,2,3cd]P), Dibenzo[a,h]anthracene (D[a,h]A) and Benzo[ghI]perylene (B[ghI]P).*

In soil samples, Benzo[k]fluoranthene was the highest congener, with a mean concentration of 152.13 ± 37.22 µg/kg. Phenanthrene was the highest in the corresponding grass samples (136.59 ± 64.40 µg/kg). In soil and grass, Acenaphthalene and Anthracene were detected at the lowest concentrations of 0.91 ± 0.22 µg/kg and 0.41 ± 0.10 µg/kg, respectively. There were significant differences between PAH congeners in soil samples (2.72(15,16); *p* 0.028; figure 30A). Through further statistical analysis (Tukey's post hoc), the driver behind differences for PAH soil analysis was not specified, and so these interactions remain unknown, a potential outcome of post hoc analyses (Chen *et al.*, 2018). Significant differences were detected between PAH congeners in pooled grass samples

(3.65(15,15); *p* 0.008; figure 30B). Differences lay between Acenaphthalene-, Phenanthrene (*p* 0.036), and Fluoranthene (*p* 0.043); Acenaphthene-, Phenanthrene (*p* 0.037), and Fluoranthene (*p* 0.043); Phenanthrene-, Anthracene (*p* 0.035), Benzo[a]anthracene (*p* 0.048), Benzo[a]pyrene (*p* 0.042), and Benzo[ghl]perylene (*p* 0.046); Anthracene and Fluoranthene (*p* 0.040), and Fluoranthene and Benzo[a]pyrene (*p* 0.049).

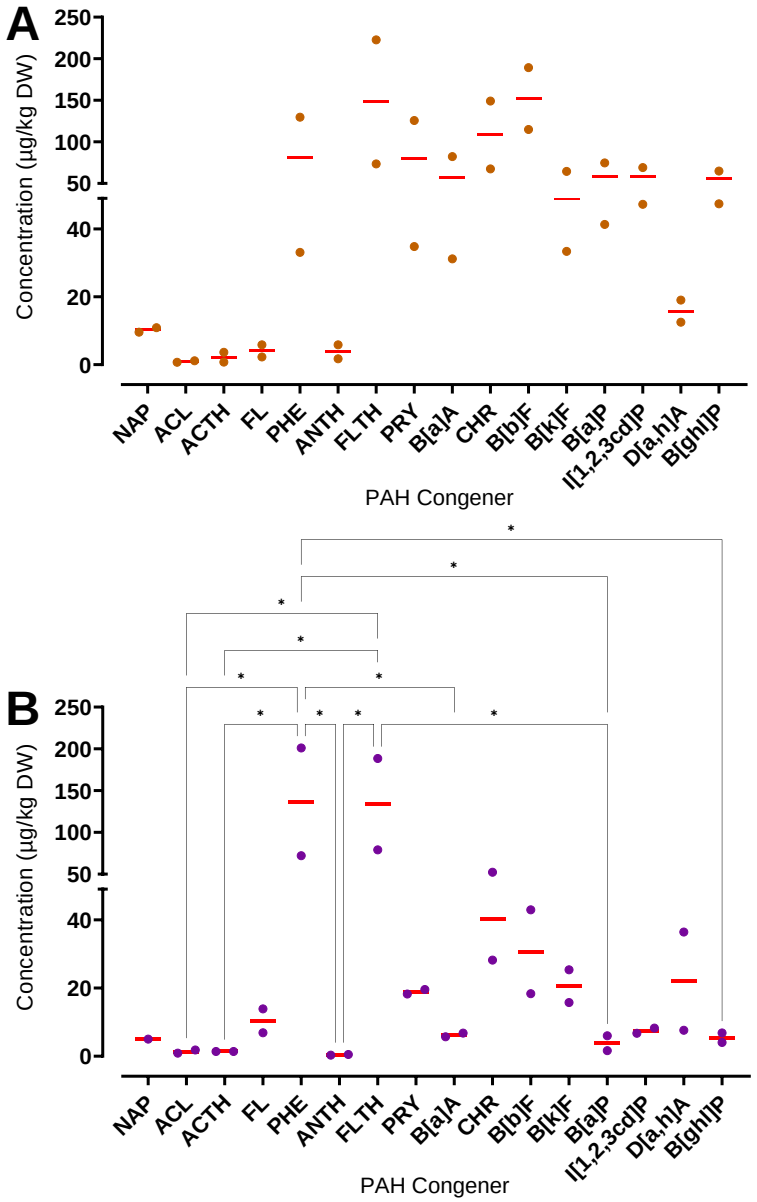


*Figure 30: PAH congener concentrations (µg/kg DW) in grouped [A] soil and [B] grass. Each point represents a sample value. The red line represents the mean. Asterisks: Tukey's post hoc; *<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Naphthalene (NAP), Acenaphthalene (ACL), Acenaphthene (ACTH), Fluorene (FL), Phenanthrene (PHE), Anthracene (ANTH), Fluoranthene (FLTH), Pyrene (PY), Benzo[a]anthracene (B[a]A), Chrysene (CHR), Benzo[b]fluoranthene (B[b]F), Benzo[k]fluoranthene (B[k]F), Benzo[a]pyrene (B[a]P), Indeno[1,2,3-cd]pyrene (I[1,2,3cd]P), Dibenzo[a,h]anthracene (D[a,h]A) and Benzo[ghl]perylene (B[ghI]P).*

Benzo[k]fluoranthene was the highest congener in the feedstuffs analysed, detected at 15.62 ± 12.05 µg/kg, whilst the lowest was Benzo[ghl]perylene at a mean concentration of 0.34 ± 0.42 µg/kg. Significant differences were determined amongst mean concentrations of PAH congeners in feedstuffs (4.52(15,64); p<0.0001; figure 31). Differences were detected between Acenaphthalene-, Phenanthrene (p 0.002), and Fluoranthene (p 0.044); Acenaphthalene and Benzo[k]fluoranthene (p 0.040); Acenaphthene and Phenanthrene (p 0.005); Fluoranthene and Benzo[ghl]perylene (p 0.036); Benzo[k]fluoranthene and Benzo[ghl]perylene (p 0.03); and Phenanthrene-, Pyrene (p 0.048), Benzo[a]anthracene (p 0.009), Benzo[b]fluoranthene (p 0.026), Indeno[1,2,3-cd]pyrene (p 0.029), and Benzo[ghl]perylene (p 0.002).

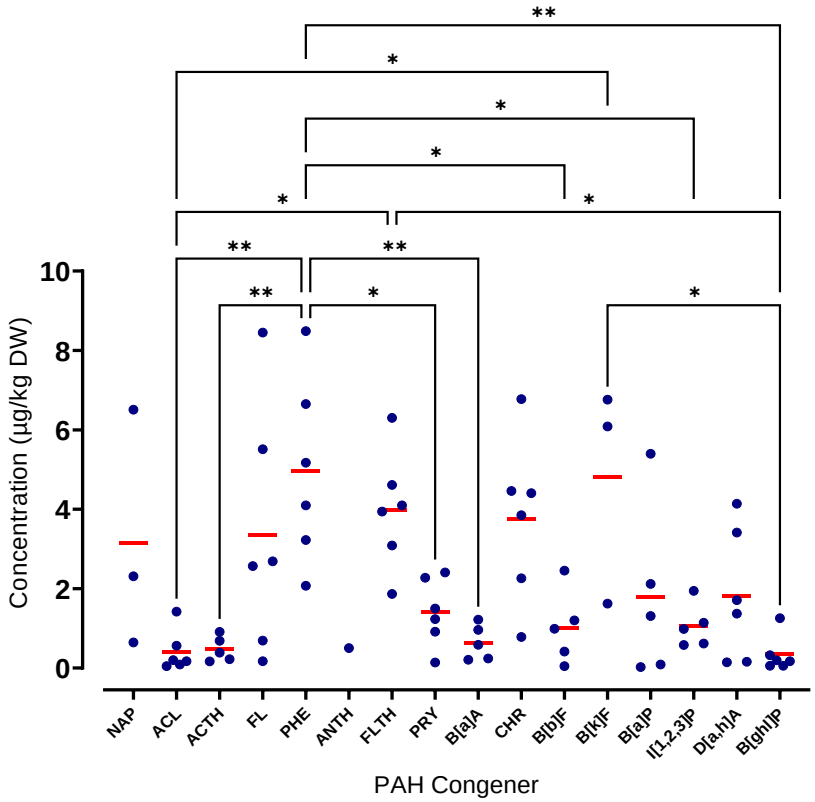


*Figure 31: Individual PAH congener concentrations (µg/kg DW) in grouped feedstuffs. Each point represents a sample value. The red line represents the mean. Asterisks represent pairwise comparisons; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Naphthalene (NAP), Acenaphthalene (ACL), Acenaphthene (ACTH), Fluorene (FL), Phenanthrene (PHE), Anthracene (ANTH), Fluoranthene (FLTH), Pyrene (PY), Benzo[a]anthracene (B[a]A), Chrysene (CHR), Benzo[b]fluoranthene (B[b]F), Benzo[k]fluoranthene (B[k]F), Benzo[a]pyrene (B[a]P), Indeno[1,2,3-cd]pyrene (I[1,2,3cd]P), Dibenzo[a,h]anthracene (D[a,h]A) and Benzo[ghl]perylene (B[ghI]P).*

5.3.9 Di (2-ethylhexyl) phthalate (DEHP)

Mean DEHP concentrations in testicular (figure 32A), soil (figure 32C), grass (figure 32D), and grouped feedstuff (figure 32B) samples analysed were reported at 739.60 ± 317.50 µg/kg, 173.40 ± 155.10 µg/kg, 2,544.00 ± 2,588.00 µg/kg, and 157.20 ± 69.51 µg/kg, respectively. DEHP was the only congener that was not statistically analysed, given its sample size.

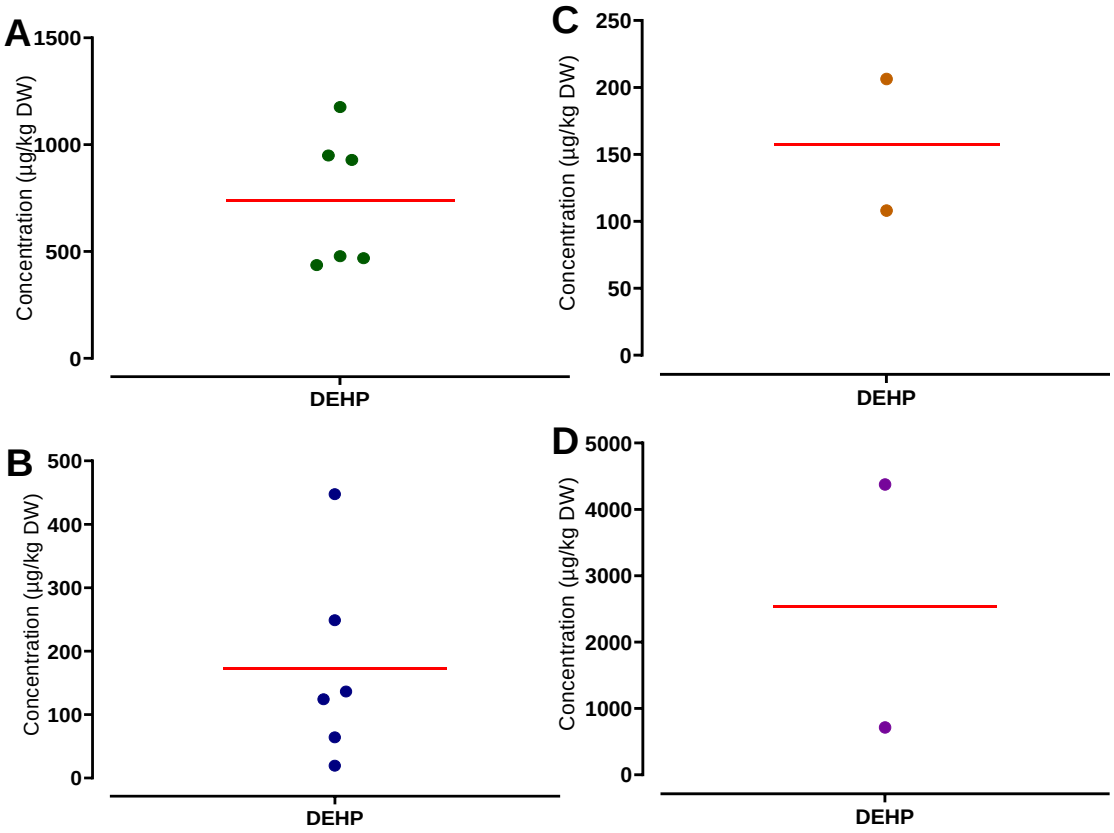


Figure 32: Individual DEHP concentrations (µg/kg DW) in grouped [A] testicular, [B] feedstuff, [C] soil, and [D] grass samples. Each point represents a sample value. The red line represents the mean.

5.4 DISCUSSION

A range of anthropogenic ECs accumulate in equine testicular tissue, as determined within the current study. This research confirms the contamination of equine pasture, haylage, forage-based, and concentrate feed materials, indicating that the ingestion of contaminated feedstuffs is a key equine-specific exposure route. The research contributes significant data to be utilised within environmental toxicology research with regards to reproductive aberrations and analysing the potential link between ECs and adverse trends reported in equine sperm motion characteristics (chapter 3, pages 68-93; chapter 4, pages 94-126). Results of Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP in biological and environmental samples also provide data on the status of EC contamination within terrestrial ecosystems, indicating the value of the novel equine model as a biomonitor species.

5.4.1 Testicular EC accumulation and associated reproductive aberrations

The novel findings from the current study indicate that PCBs, PBDEs, PAHs, and DEHP accumulate within equine testicular tissue. To date, testicular contamination of ECs, including that of anthropogenic origin, have not been quantified in the herbivorous equine model, with the results presented here contributing valuable data to the environmental toxicology and reproductive biology sectors. Previous research on EC contamination in alternative species has focussed on seminal plasma (Lea, Byers, *et al*., 2016), hair (Peng *et al*., 2021; Yang *et al*., 2020), breastmilk (Antignac *et al*., 2016), blood (Manz *et al*., 2022), and amniotic fluid (Dusza *et al*., 2022; Barmpas *et al*., 2019) accumulation. The current study therefore provides a novel understanding of bioaccumulation specific to the testis of an herbivorous population, adding to the significance of the results. Additionally, there is limited research associated with EC contamination of general biological tissue samples with regards to the equine species. In confirming testicular accumulation, this research initiates the utilisation of the novel equine model as a biomonitor species regarding environmental toxicology from a reproductive perspective. GC-MS is an accepted, sensitive and accurate method for EC analysis in tissue (Lea, Byers, *et al.*, 2016) and herbage materials (Wang *et al.*, 2007), contributing towards the quality of the results obtained.

The testes are responsible for two fundamental functions, producing spermatozoa and synthesising hormones (Weinbauer *et al*., 2010). Testicular accumulation of a complex EC

mixture raises concern over perturbed stallion reproductive health and function, given the reprotoxic nature of contaminants detected (Lea, Byers, *et al*., 2016). The individual congener, Benzo[a]anthracene was consistently detected in all stallion testes analysed, and was found at the highest concentration compared to other PAHs. In humans, Benzo[a]anthracene is significantly correlated with reduced semen quality (Song *et al*., 2013). Chrysene was also detected at elevated concentrations in all testes analysed. Benzo[a]anthracene and Chrysene, both high molecular weight (HMW) PAHs, are associated with aberrations in sperm morphology (Chen *et al*., 2021). Whilst there were significant differences in testicular contamination of specific congeners, Σ14PAHs were detected at varying concentrations, indicating that horses are exposed to complex chemical mixtures. Σ16PAH exposure is associated with increased oxidative stress markers in human sperm, although Benzo[a]anthracene was reported to be a primary oxidative driver (Jeng *et al*., 2022). Given the reprotoxic association of PAH mixtures and the primary role of Benzo[a]anthracene, the detection of such congeners in equine testis is of great concern for stallion testicular health and function.

DEHP was detected in all testicular samples analysed, at a mean concentration higher than that reported in any other congener. DEHP exposure is associated with increased sperm cell apoptosis (Sun *et al.*, 2018) and collapse of vimentin filaments, the intermediate structural proteins of the S-Cs and important structural components involved in holding spermatogenic cells within the seminiferous epithelium (Erkekoglu and Zeybek, 2012). Given the fundamental role of S-Cs in supporting spermatogenesis (Walker and Cheng, 2005), DEHP exposure is likely to result in the alteration of key sperm quality parameters and reduce the fertilising capabilities of the sperm, as reported within *in vitro* studies (Sumner *et al*., 2019). In immature rat testis, DEHP compromised the integrity of the blood-testis-barrier (BTB) through oxidative pathways resulting in autophagy (Yi *et al*., 2018). Given that the testes are particularly sensitive to reactive oxygen species (ROS) (Yi *et al*., 2018), oxidative stress is considered to be a primary mechanism of DEHP-associated toxicity (Zhou *et al*., 2013). The PAH congeners benzo(a)pyrene and pyrene, both of which were detected in all equine testis analysed, are reported to disrupt the BTB through cellular apoptosis (Zhang *et al*., 2022), which could also be associated with ROS production (Azad, Chen and Gibson, 2009). Alterations to the integrity of the BTB is likely to result in the impairment of the microenvironment required to sustain spermatogenesis and

subsequently, lead to aberrations in sperm quality. Whilst four key categories of environmental chemicals were analysed, the contaminant profile of the equine testis are likely to be more complex than reported here, given the significant number of ECs present within the environment. This is of high concern for equine testicular function, given the effects of complex chemical mixtures on the health of the testis in alternative species.

5.4.2 Geographical variability in contaminant exposure and canine comparissons

Contamination of DEHP was significantly higher in testicular samples collected from Gloucestershire compared to Cambridgeshire regions. Whilst research analysing geospatial differences of EC levels in biological tissues is limited, research in the dog sentinel indicates that variability exists in testicular concentrations of DEHP, PBDEs, and PCBs. Differences in EC contamination existed on both an international level, between Scandinavia and the UK, and at a regional level between areas of the UK specifically (Sumner *et al*., 2021). Geospatial differences in environmental contamination could be a result of the variability of DEHP between the two sampling regions. Whilst geographical differences in DEHP within soil and grass samples were not statistically analysed, DEHP was higher in Gloucestershire soil and grass samples compared to those taken from Cambridgeshire. It is therefore likely that differential levels of DEHP in the testis from the two regions was in relation to levels of environmental contamination and exposure. Elevated levels of DEHP contamination raises significant concern over the health of the environment and levels available for uptake by all animals and humans existing in these regions. However, the limited sample size restricts the ability to draw a definitive conclusion regarding the geospatial variability of DEHP within the current analysis. In addition, whilst soil and grass samples were taken from the general Gloucestershire and Cambridgeshire regions, they were not sampled from the precise locations where stallion testis were collected. Therefore, it is difficult to make a full comparison between the concentrations of environmental ECs that the specific stallions were exposed to.

When compared to previous findings in the dog sentinel model, mean DEHP concentrations in both canine and equine species were higher than that of PCBs and PBDEs (Lea, Byers, *et al*., 2016). Such research would indicate that currently, DEHP exposure in animals is of high concern given elevated concentrations detected with the testis and reported reprotoxicity

in alternative species. It is not plausible to compare specific testicular concentrations of ECs between the horse and dog, given that the current study provides contamination levels on a dry weight basis, whereas research in the dog is on a wet weight basis (Lea, Byers, *et al*., 2016). In order to utilise the concentrations presented within the current study for further research, the water content of equine testicular tissue would need to be determined and the wet weight concentration calculated. Whilst DEHP is a key congener associated with equine-specific exposure that requires further investigation, tissue analysis confirmed the testicular accumulation of two alternative categories of ECs; PBDEs and PCBs.

The synthesis of PBDEs and PCBs, as well as alternative persistent organic pollutants (POPs), was regulated under the United Nations Environment Programme at the Stockholm Convention in 2001 (United Nations Environment Programme, 2001). Despite legislative actions, the current study confirms that both EC classifications continue to pose a health risk within the current environment, indicative of their biopersistence. The congeners PBDE 47 and PCB 28 had the highest concentrations out of their respective chemical classifications, although the differences were not significant. In dog testis, PBDE 47 and PCB 28 were also reported at the highest mean concentrations compared to other PCB congeners (Lea, Byers, *et al*., 2016). PBDE 47 ($C_{12}H_6Br_4O$) and PCB 28 ($C_{12}H_7Cl_3$) are low in molecular weight compared to the alternative congeners within their respective chemical categories, which may enable a more effective diffusion across the BTB, resulting in higher levels of accumulation. The physiochemical properties of specific congeners can assist in explaining the contaminant's affinity for testicular bioaccumulation.

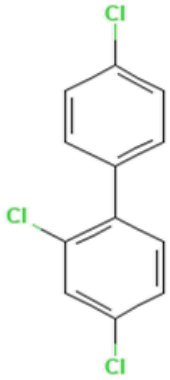
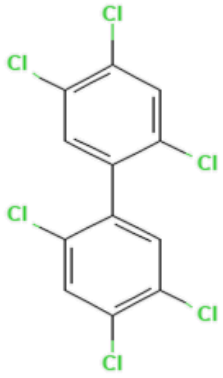
5.4.3 Physiochemical properties

Structurally, PCB 28 has only three chlorine-substituted atoms (Appendix N; pages 328-331). PCB 28 has the lowest molecular weight (LMW) and log P value compared to alternative congeners, reported at 258 g/mol and 5 g/mol, respectively (Bourez *et al*., 2013). The Lipinski Rule states that for the efficient absorption of a molecule, a molecular weight of no greater than 500 g/mol, a logP value of no more than 5, a maximum of 5 hydrogen bond (H-bond) donators and 10 H-bond receivers is required (Lipinski *et al*., 2001). PCB 28 meets the criteria associated with the Lipinski rule, so it is possible that the ease of transport into the testicular tissue could result in a higher level of deposition of this

congener specifically. However, from a molecular weight perspective, considering the alternative congeners measured within this thesis, all PAHs, PCBs, DEHP and PBDE 28, and PBDE 47 have a molecular weight less than 500 g/mol (Appendix N; pages 328-331), which is likely to assist in their affinities for testicular deposition. Many ECs have high lipophilic affinities, depending on the specific chemical structures and components. The log P value is the partition coefficient, a fundamental parameter associated with the uptake of ECs by fat cells, and is subsequently a reading of lipophilicity (Bourez *et al*., 2013). The testis is formed of complex structures with high lipid content (Sun *et al*., 2020), and so the tissue is particularly susceptible to bioacummulation of ECs. Physiochemical properties such as the log P value are therefore reported to affect the ability of ECs to cross the BTB and may impact the affinity of congeners to accumulate within the testis.

In adipocytes, PCB 28 was reported to accumulate more readily compared to PCB 118 and PCB 153, with a respective cellular transfer of 20 ± 5% to 49 ± 5%, 6 ± 2% to 32 ± 3%, and 5 ± 2% to 28 ± 1% depending on adipocyte cell line (Bourez *et al.*, 2013). In comparison to the tri-chlorinated PCB 28, PCB 118 and PCB 153 have five and six chlorine substituents, respectively (Table 23; Appendix N; pages 328-331). Whilst both congeners still meet the Lipinski rule criteria, it is possible that their higher molecular weights may partially restrict diffusion into the testicular tissue. The order of highest mean concentrations within the testes analysed as part of this research was PCB 28 > PCB 118 > PCB 153, which supports previous research in adipocytes and the Lipinski rule.

Table 23: Low and high levels of chlorination in two example PCB congeners (PCB 28 and PCB 153). Full chemical structures and classifications for PCBs and other EC congeners analysed within this research are presented in appendix N, pages 328 to 331 (PubChem, 2022).

Low chlorinated congener	High chlorinated congener
PCB 28 ($C_{12}H_7Cl_3$) 	PCB 153 ($C_{12}H_4Cl_6$) 

Interestingly, one of the LMW PCB congeners, PCB 52, was present in the testis at the lowest mean concentration. The physiochemical properties of this congener would suggest a higher affinity to testicular accumulation. However, levels present within the soil, grass, and feedstuff materials were also low, so it is likely that exposure to this congener is minimal. In grass silage intended for bovine consumption, PCB 52 was abundant, although low concentrations were found within the cattle ingesting the feedstuff (Driesen *et al.*, 2022). A high metabolic clearing potential of low-chlorinated congeners such as PCB 52 (four substitute chlorine atoms) may be associated with a reduced uptake (Mclachlan, 1993). However, differences between rumination, the digestive process in cattle, and hindgut fermentation processes occurring in equine digestion may result in alternative metabolism and excretion of contaminants. Despite that Naphthalene and Anthracene were not detected in any testicular samples analysed, concentrations were present in pastures and feedstuffs, which could suggest the effective metabolism and excretion of congeners. Further research determining the metabolism, accumulation, and excretion of ECs is required specific to the equine model to provide a holistic understanding of the health risks posed by contaminant exposure.

When comparing the concentrations of specific chemical groups within the current study, DEHP presented the highest levels of contamination, followed by PAHs, PBDEs, and PCBs. In humpback dolphins, PCBs predominantly accumulated within the blubber, whilst PAH

accumulation in the brain and adipose tissue were approximately equal (Sun *et al*., 2020). A higher proportion of PAHs also accumulated within the testicular tissue compared to PCBs. Within the testis, blood, and blubber, mean ΣPAH concentrations were 511 ± 98 ng g^{-1}, 2060 ± 940 ng g^{-1}, and 2569 ± 233 ng g^{-1}, respectively. In comparison, ΣPCB concentrations in the testis, blood and adipose tissue of the dolphins were 60 ± 34 ng g^{-1}, 32 ± 10 ng g^{-1}, and 1717 ± 108 ng g^{-1}, respectively (Sun *et al*., 2020). Whilst differences in PCB and PAH concentrations are likely to be a result of specific environmental and exposure levels, the proportional differences of accumulation in biological matrices with differing lipid contents seem to be a fundamental component. It was outside the scope of this research to analyse EC burdens within stallion blood. However, testicular Σ7PCBs had lower total means when compared to Σ16PAHs. Given that testicular accumulation of ECs was confirmed within the current study, it is likely that other equine organs are also exposed to elevated contamination levels, as reported in humpback dolphins (Sun *et al*., 2020). This could be of significant concern for the overall health and wellbeing of the equine population, given the broad-spectrum toxicity of ECs on a number of organs and biological processes reported in other species.

5.4.4 EC exposure, brain function, and hypothalamic-pituitary-gonadal axis

Contaminants, including PAHs, were reported to readily accumulate within the brain tissue of humpback dolphins (Sun *et al*., 2020). Contaminant accumulation within the brain is reported to alter brain and cognitive function through the modulation of genetic pathways (Myhre *et al*., 2021). The hypothalamus is a structure in the brain and a fundamental component of the hypothalamic-pituitary-gonadal (HPG) axis, the controlling mechanism of the reproductive system (Plunk and Richards, 2020). The gonadotropin-releasing hormone (GnRH) is the main reproductive hormone secreted by the hypothalamus that initiates the anterior pituitary gland to synthesise follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Walker and Cheng, 2005). FSH and LH act directly on testicular structures and are essential in gonadal function (Zirkin and Papadopoulos, 2018). Given the endocrine disrupting effects of the ECs analysed within the current study and likely distribution around the equine body, accumulation within the brain could result in the disruption of the HPG axis and the endocrinology pathways associated with testicular function. Reproductive aberrations resulting from EC exposure could therefore exist

through the direct effects of ECs on testicular structures and through secondary pathways, interrupting brain function and hormonal cascades associated with the HPG.

Accumulation of ECs within the brain may also impact reproductive behaviours in seasonal breeders, as suggested in previous research analysing the effects of POPs on arctic foxes (Sonne *et al.*, 2017). Maintaining manageable reproductive behaviours in stallions is fundamental in breeding regime risk prevention and optimising animal health and welfare standards (de Oliveira and Aurich, 2021). Neuroendocrinology interactions are associated with the regulation of reproductive behaviours in stallions (Veronesi *et al.*, 2010) and with overall reproductive performance (Rua *et al*., 2015). Therefore, accumulation in the stallion brain of the endocrine disrupting ECs detected in the testis within the current study may impact the reproductive behaviours and abilities of stallions, with serious breeding behavioural implications, reproductive efficiency, health and welfare consequences for the equine breeding sector. Determining the potential accumulation of ECs within equine brain tissue and effects on stallion behaviours is therefore warranted to provide a holistic understanding of the interactions of contaminants with stallion reproduction. Multiple factors responsible for variable biological concentrations of EC congeners indicate the complexity of contaminant exposure, although it is clear that physiochemical properties and environmental levels are fundamental counterparts of this narrative.

5.4.5 Equine-specific exposure to PCBs, PBDEs, PAHs, and DEHP

In equine management feedstuffs, including forage-based and concentrate feeds, the order of overall mean concentrations was: DEHP > Σ16PAHs > Σ7PBDEs > Σ7PCBs. Considering EC contamination of environmental pasture, overall concentrations in soil and grass, respectively were detected in DEHP > Σ16PAHs > Σ7PCBs > Σ7PBDEs. Despite levels of PCBs and PBDEs reported at levels above the limit of detection, concentrations below that of PAHs and DEHP may be a result of regulations surrounding their synthesis (United Nations Environment Programme, 2001). Whilst there are some discrepancies in the order of Σ7PBDEs and Σ7PCBs, testicular accumulation of ECs is reflective of environmental levels, indicating that contaminated feedstuffs and pastures are fundamental exposure routes in the equine model. DEHP was detected at elevated levels in soil and grass samples. Given that DEHP is a common plasticiser, it is possible that accumulation within pasture materials

is a result of MPP and EC deposition from sewage sludge land application in surrounding agricultural areas. In wastewater used in the processing of sewage sludge fertiliser, DEHP was reported at higher concentrations when compared to alternative pollutants (Cai *et al*., 2008). In addition, leaching from plastic films present within the environment, including those packaging haylages, may be associated with elevated DEHP concentrations. Soil acts as a primary sink to pollutants, an accepted hypothesis due to contaminant insolubility and subsequent adsorption onto organic matter (Tang *et al*., 2005). Soil may act as an exposure route through the direct ingestion of contaminated soils, inhalation of airborne particles, and dermal contact (Tarafdar and Sinha, 2018; Madejón, Domínguez and Murillo, 2012).

With the exception of Anthracene and Dibenzo[a,h]anthracene, HMW PAHs (>4 benzene rings) contaminated the soil at higher concentrations compared to LMW PAHs. HMW PAHs exist in a solid phase, which may result in higher levels of soil deposition and elevated contamination levels. In addition, within the rhizosphere, 4-6 ring PAH congeners are more resistant to degradation (Meng, Qiao and Arp, 2011). The uptake of PAHs by plants, as well as their ability to biodegrade EC contaminants, is associated with the chemical's molecular size (Meng, Qiao and Arp, 2011). Therefore, as seen within the current research study, HMW PAHs have a higher affinity for soil accumulation compared to LMW PAHs. Whilst this may reduce the uptake of ECs into grass species, a horse is reported to ingest around 600g of soil per day (Jurjanz *et al*., 2021). The consumption of contaminated soils could therefore act as an exposure route to PAHs within the population of horses analysed here.

The elevated hydrophobicity of PAHs enables strong sorption to the organic matter components of soil (Un Nisa and Rashid, 2015). In three agricultural soil types, DEHP rapidly adsorbed onto the material, which is likely a result of its hydrophobic nature (Murillo-Torres *et al*., 2012). The process of making the sequestered ECs bioavailable includes the desorption of molecules during digestion (Delannoy *et al*., 2015), which is impacted by the organic carbon content in soils (Shen *et al*., 2019). It was beyond the scope of this research to analyse carbon content in soil samples, although further research into equine exposure through contaminated soil would benefit from the inclusion of these factors. Such research would develop an understanding of the bioavailability of contaminants sequestered to soil within the equine gastro-intestinal tract. A risk analysis of soil PAHs found that absorption

through dermal contact was the main route of exposure, followed by oral pathways, whilst inhalation was negligible in humans (Tarafdar and Sinha, 2018). Soil-to-skin contact within horses could therefore result in the percutaneous absorption of contaminants detected within samples analysed within the current study.

The adsorbtion of ECs onto the organic content of soil may also impact their bioavailability to root structures of grass species and subsequent uptake into the leaves. The results from this research indicate that grasses have higher levels of contamination compared to their respective soil samples. Highly chlorinated congeners, including PCBs, bond strongly to soil organic matter, reducing the uptake into plants through root structures (Terzaghi *et al.*, 2018). Instead, such congeners are readily absorbed from the surrounding air (Terzaghi *et al.*, 2018). This could explain the higher concentrations present in grass compared to respective soil samples. However, within the current study, soil and grass were pooled with respective samples, which limits the conclusions that can be made regarding the absorption of contaminants into vegetation. Elevated concentrations of ECs in grass samples could result in a direct exposure route to grazing horses, with a secondary level of exposure likely to exist through the processing of herbage materials for haylages and commercialised feedstuffs.

5.4.6 Feedstuff material contamination

This study presents novel preliminary data on the contamination of commercial equine feedstuffs with anthropogenic ECs. All feeds analysed were contaminated with Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP, indicating that the ingestion of feedstuffs is a key exposure route in stallions. Ingestion of feeds was also considered the main exposure route in cattle when compared to air and water (Mclachlan, 1993). In the current study, stud cubes had a significantly higher level compared to foal cubes, which could be partially explained by the feedstuff content (Appendix P; page 333) and manufacturing processes. This suggests that foal cubes have relatively low levels of contamination. However, given that only one bag of foal cubes was sampled, further analysis, including more feed samples, is required before this material can be defined as low risk. The sample sizes used here are considered as a study limitation, although results provide a preliminary understanding of the ECs present within a broad range of equine-specific materials to be furthered within future research.

Stud cubes analysed are designed for breeding stallions, broodmares, and lactating mares. Elevated levels of contamination within this feed could impact a stallion at multiple stages throughout its life. Exposure is likely to occur through the maternal unit and directly through the consumption of this feed. Contamination of feedstuff could occur during forage growth, feed processing, and chemical leaching from product packaging. Commercially baled haylage had the highest concentration of DEHP and PAHs. Given that haylage is packaged in plastic, it is possible that leaching of DEHP from the wrapping into the feedstuff leads to a higher level of contamination (Taylor and Sapozhnikova, 2022). Legislations outlined by the European Food Safety Agency include a tolerable daily intake and EU-specific migration limits. For DEHP, the specific migration limit is 1.5 mg/kg of packaging material (Fang, Wang and Lynch, 2017). No such regulations concerning EC contamination or migration have been outlined for the production of equine feeds. Further research determining tolerable daily intake levels and migration limits is required to inform practice and implement policy change to reduce equine exposure and reduce the environmental deposition of ECs resulting from the equine manufacturing sector.

An additional feed analysed within the current study was alfalfa, processed into a commercially available forage-based feedstuff. Alfalfa was contaminated with Σ7PCBs, Σ3PBDEs, Σ16PAHs, and DEHP, although concentrations were not significantly higher than those present in other feed types. Given the lipophilic nature of many ECs (Lehmann *et al*., 2015), elevated contamination levels were expected compared to alternative feeds analysed, due to the high oil content (Appendix P; page 333). In addition, alfalfa is an effective plant species at remediating contaminated soils, given the affinity of ECs to accumulate within plant structures (Bourceret *et al*., 2018). It was therefore hypothesised that alfalfa-based feeds with high oil contents would have elevated EC levels, although this was not the case within this research. During the growth of alfalfa, the rhizospheric microbiome is reported to assist with the biodegradation of ECs (Fatima *et al*., 2015). Therefore, whilst chemicals may accumulate within the plant, effective biodegradation could result in a reduction in parent compounds within the leaves. This could partially explain the lower levels of contamination in alfalfa-based feeds. Further research is required to confirm the contamination of differing processing and storage techniques of feedstuffs, given the limitation of the low sample size utilised for these materials within the current study. Such data could contribute towards informing regulations for the safe

production of equine feedstuffs and reduce the environmental impact of the sector from the perspective of EC contamination.

5.4.7 Concluding statement

This novel research indicates that equine testes are susceptible to the accumulation of PCBs, PBDEs, PAHs, and DEHP. Exposure to a complex mixture of ECs is likely to result in perturbed reproductive health and function as reported in alternative species, although equine-specific toxicity remains unknown. This raises concern over the reproductive abilities of both current and future breeding stallions in the equine population. The results presented also initiate the use of the novel equine model as a biomonitor species of environmental health of terrestrial ecosystems in relation to EC contamination. This research focused on the oral exposure route, with results indicative of exposure through the ingestion of contaminated pastures and feeds. Data provided forms an essential foundation for further research concerning the effects of ECs on equine reproductive health and function.

CHAPTER 6

GENERAL DISCUSSION

6.1 Summary of research and outcomes

Chapter 1 of this thesis, a general literature review (pages 1-39), explored reproductive trends and associated environmental aetiologies across species. As a result of the analysis of literature, key gaps of knowledge were highlighted, including the understanding of temporal trends in equine semen quality and the potential aetiological association of anthropogenic ECs and stallion reproductive aberrations. Therefore, the research carried out as part of this doctorate analysed temporal trends in equine semen quality parameters and initiated investigations into plausible environmental aetiologies (figure 33). To address this, the doctorate was structured around three objectives.

Objective A (chapter 3, pages 68-93) assessed temporal trends in sperm PMOT in global domesticated equine populations through systematic review and meta-regression analysis. Results indicated a significant decline in PMOT of 31.89% between 1984 and 2019, so the research hypothesis was accepted, and the null hypothesis rejected. Trends did not change appreciably regardless of sensitivity analyses imposed. Geospatial variability in trends between western and non-western populations were detected. Whilst trends indicated that declines existed in the global population, a key gap of knowledge that emerged was determining whether such trends existed in controlled equine populations.

Objective B (chapter 4, pages 94-126) built upon the limitations of evidence synthesis methodologies, determining trends in semen quality from a controlled population of stallions at a single breeding facility in the UK. TMOT declined by 10.10% between 2001 and 2020, whilst overall concentration, volume, and TSO increased over a similar time. Age-restricted trends confirmed that declines in TMOT were not a result of aging individuals, indicating an alternative aetiology. Given that adverse trends were not consistent across all semen quality parameters, the research hypothesis was partially accepted on the grounds of declining sperm TMOT. The potential aetiological involvement of anthropogenic ECs and reproductive aberrations had not been explored in the equine model, thus the following study of this thesis addressed this gap in knowledge.

Objective C (chapter 5, pages 127-165) provided a putative understanding of the risk of exposure to ECs with suspected reprotoxicity specific to the equine population.

Equine testis, pastures, and feedstuffs were analysed utilising accepted and sensitive methods. All samples were contaminated with a complex mixture of PCBs, PBDEs, PAHs, and DEHP, confirming equine-specific testicular accumulation and plausible exposure routes. The research hypothesis for objective C was accepted, indicating the importance of further research analysing the associative link between declining sperm motility and EC exposure.

Evidence of declining sperm motion characteristics in both the global and UK-isolated equine populations are significant findings reported in this thesis. The combination of two comprehensive datasets utilising supportive methods, designed to mitigate limitations associated with previous research approaches, adds weight and credibility to the results. Data provide an essential understanding of the reproductive status of stallions in a changing environment, which could contribute to defining methods for the preservation of equine reproductive health and function. Adverse trends reported in sperm motion characteristics and values below industry standards raises concern over the reproductive health of the equine population, with poor semen quality likely to have serious consequences for the economic profiles of stallions within the breeding sector. Results also add significant contributions to the debate surrounding declining reproductive trends in humans, companion, and livestock animals, indicative of a potential common environmental aetiology, however this remains to be explored further. In confirming adverse sperm motility trends, the research initiates the utilisation of the novel equine model as a comparative species for assessing reproductive trends in human populations, holding global and cross-species significance.

For the first time, novel outputs from this research indicate elevated testicular burdens of ECs specific to the equine population. In confirming accumulation of anthropogenic ECs within equine biological tissue, this research initiated the use of the species as a novel biomonitor for the environmental health of terrestrial ecosystems, which has significant global implications. The presence of potentially reprotoxic PCBs, PBDEs, PAHs, and DEHP within equine testicular tissue raises concern over the reproductive health and function of stallions. Contamination of the testis, the reproductive organ responsible for sperm production, may contribute to the adverse trends and geographic variability of semen

quality reported in the first two objectives of this thesis. This research provides strong evidence that exposure to contaminated pastures and feedstuff materials is a fundamental equine-specific exposure route. Data concerning environmental contamination levels are also an indicator of current EC accumulation rates within the soil and grass of terrestrial ecosystems.

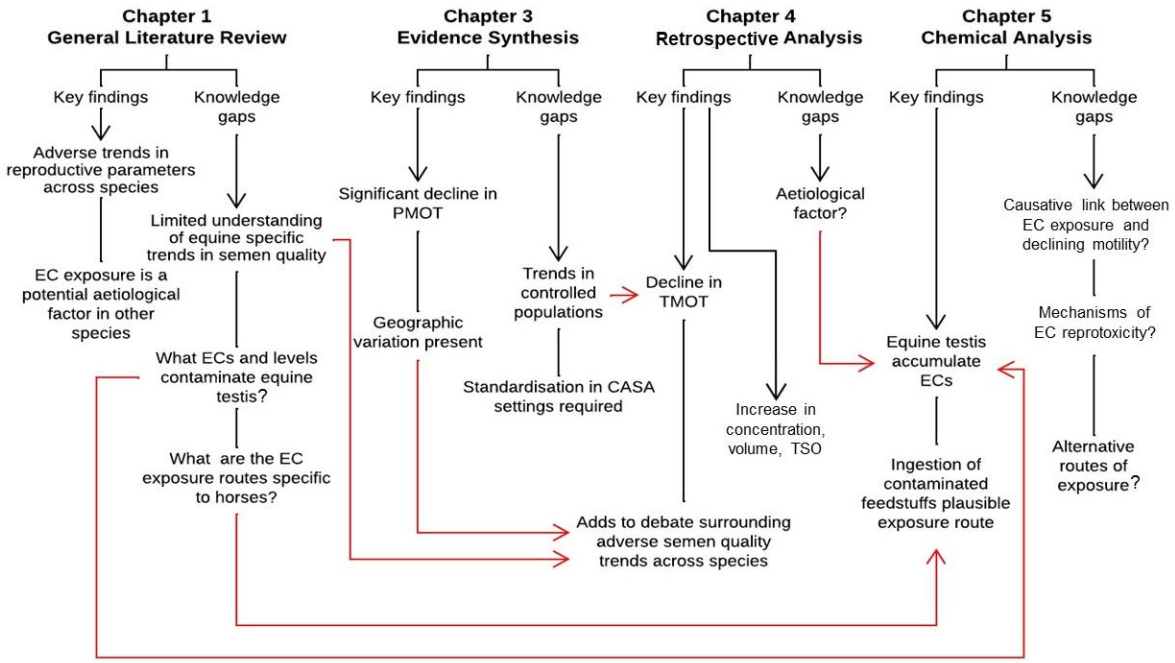


Figure 33: Key findings and knowledge gaps highlighted in each chapter. Chapter 1: A General Literature Review (pages 1-39). Chapter 3: Evidence Synthesis; Temporal trends in sperm progressive motility in the novel equine model (1984-2019): a systematic review and meta-regression (pages 68-93). Chapter 4: Retrospective Analysis; Temporal trends in semen quality from a population of stallions at a single breeding facility in the UK (2001-2020) (pages 94-126). Chapter 5: Chemical Analysis; Anthropogenic environmental chemicals in the novel equine model: testicular contamination and routes of oral exposure (pages 127-165).

6.2 Temporal trends in motility parameters within equine and wider species

Through systematic review and meta-regression analysis, a significant decline of 31.89% (1984 to 2019) in sperm PMOT across global equine populations was reported here. The analysis of a comprehensive retrospective dataset from a single controlled population of breeding stallions in the UK supported these findings. The novel results are the first to confirm adverse trends in sperm motion characteristics relevant to the current equine population. The research initiates the equine as a model to further understanding of reproductive statuses across species, including in human reproductive biology. This

development is imperative for providing a holistic understanding of fertility statuses in a changing environment and working towards a One Health approach. Adverse trends in motion characteristics are likely to have detrimental effects on the economic status of breeding stallions, given the high commercial value associated with stallion semen sales and natural coverings in the equine industry (Juddmonte Farm, 2022; Stallion AI Services, 2022). In addition, reduced fertility potential is likely to result in additional costs associated with managing stallions with poor semen quality, such as the need for an increased number of collections, coverings, and inseminations required to achieve a successful pregnancy. Sperm quality aberrations are also an indicator of poor testicular health and function (Jacobsen *et al*., 2000), with the declines in motility reported here likely to hold serious health and welfare consequences for the male equine species. A reduction in the fertilising capacity of stallions raises concern over the sustainability of current and future equine populations, given the significant importance of stallion reproductive function for the successful conception of progeny.

As current human-based evidence syntheses focus on sperm count and concentration (Levine *et al*., 2017; Johnson *et al*., 2015; Swan, Elkin and Fenster, 2000; Carlsen *et al*., 1992), the novel equine study presented here advances research by assessing motility characteristics through systematic review and meta-regression analysis. However, this study presented an issue associated with the lack of standardisation of CASA settings utilised within the equine sector. Further industry recommendations are required to standardise the threshold values for settings of CASA technology used to define sperm motility parameters. This will promote further cross-comparisons of motility between differing laboratories. However, the influence of CASA models and settings were analysed within the current evidence synthesis where possible. No significant effects of model or settings were reported, indicating that the influence of such factors on the trends presented here is negligible. The yearly trends in motility parameters for chapters 3 and 4 are comparable, reporting a 0.89% and 0.50% decline from 1984 to 2019 and 2001 to 2020, respectively. For the retrospective analysis, age-restricted trends confirmed that TMOT declines were not a result of aging stallions, indicative of the involvement of other aetiological factors. Typically, reports in other species have reported a decline in motility, however, considering the involvement of alternative aetiological factors, these data are often using samples that have been freeze-thawed (Wahl and Reif, 2009), which is not an

accurate method to assess true trends. When determining trends, it is paramount that efforts employed prevent the effects of semen preservation and treatment methods.

Given that motility for the current research project utilises fresh semen analysis values, the results are a more accurate predictor of testicular health. Previous research on fresh semen characteristics in horses (Multigner *et al.*, 2000, 1999) and bulls (Snoj *et al*., 2013; Setchell, 1997) failed to include sperm motility in the analyses. Therefore, this research provides novel data on temporal trends in motion characteristics across general domesticated herbivorous species. Due to declines in the current equine study, and previous research indicating the deteriorating quality of alternative semen parameters in bulls (Wahl and Reif, 2009), a reanalysis of trends in fresh sperm motion characteristics within livestock species is recommended. This is required to truly determine the status of fertility potential, given that reproduction is one of the most significant economic factors for production animals (Velasco and Ruiz, 2020).

In relation to alternative established sentinel species, trends in the dog sentinel model indicate comparable trends to those reported in equines, supporting the hypothesis of declining sperm motility (Lea, Byers, *et al.*, 2016). The results of this study are also supported by trends in retrospective human-based research, which suggest geographic-sensitive declines in sperm motion characteristics (Chang *et al*., 2018; Centola *et al.*, 2016; Comhaire, Mahmoud and Schoonjans, 2007; Irvine *et al*., 1996; Auger *et al.*, 1995). Adverse trends in sperm motility across horses, dogs, and humans, are unlikely to be a result of primarily species-specific factors, indicative of a common underlying environmental aetiology. However, there are species-specific differences that must be considered when interpreting trends reported.

Inbreeding is a phenomenon reported in specific equine breeds (McGivney *et al*., 2020; Ducro *et al*., 2014; Van Eldik *et al*., 2006), and is associated with impaired reproductive performance (Pirosanto *et al.*, 2021, 2019). Therefore, it is important to consider the effects of inbreeding when speculating on the aetiological factors involved in declines reported in the current research. A negative correlation between TMOT, PMOT, and inbreeding has been reported in ungulate species (Gomendio, Cassinello and Roldan, 2000). However, in

Utrecht, between 2003 and 2018, no adverse trends were reported in the semen quality of stallion breeds associated with inbreeding (Dini *et al*., 2020). Between 1987 and 2002, consistent trends in sperm quality were reported in Friesian stallions from Utrecht, a breed subjected to high levels of inbreeding (Boer, 2007). Whilst results from other research could indicate that the declines in sperm motility reported in the current equine studies are not primarily a result of inbreeding, the influence of this factor cannot be discredited completely. It is therefore essential that the equine industry and breeding organisations implement selective breeding strategies associated with reproductive health and fertility potential. It is in the interest of the breeder to select sires with sufficient semen quality in order to maintain high health and welfare standards, the monetary value of the stallion's semen, and prevent additional labour required to manage poor fertility in stallions.

6.3 Industry defined motility thresholds and implications of results

A significant correlation between sperm motion characteristics and equine fertility has been reported (Jasko *et al.*, 1992). Therefore, given the overall decline in sperm motility presented here, it stands to hypothesise that equine fertility potential has also declined over time. Significant correlations have been reported between TMOT and PMOT with equine first cycle, seasonal, and overall pregnancy rates (Love, 2011), indicating the importance of this parameter for successful breeding outcomes. Dutch Warmblood studbooks have threshold values for stallion PMOT of 50% (Aurich *et al*., 2020; Parlevliet, Kemp and Colenbrander, 1994), with values reported in this thesis suggesting populations are falling below this bracket. Alternative research suggests that compromised fertility is expected when PMOT values drop below 40% (Colenbrander, Gadella and Stout, 2003). There is no universally accepted threshold level for semen quality parameters. The lack of standardisation in industry values defining the fertility of an ejaculate makes it difficult to fully interpret the true status of current equine reproductive function. The formation of standardised industry thresholds would promote further comparative analysis of stallion semen quality and hold clinical relevance by providing vital data for breeding management practices. Pregnancy rates of 77.0 ± 10.4% have been achieved by stallions with PMOT values of 41.1 ± 19.9% (Neild *et al.*, 2000). Using these referencing values for PMOT, findings within this thesis showcase that current motion characteristics are at an acceptable level for maintaining fertility and successful pregnancy outcomes. However, trends in PMOT show no sign of plateau, indicating that lower values are a likely outcome

in future generations. Further declines and suboptimal PMOT values may result in an increased proportion of stallions presenting compromised fertility, with serious complications in sustaining the equine population.

If PMOT was to decline past threshold values, the use of ART is an established means of supporting stallion breeding within the equine sector (Colenbrander, Gadella and Stout, 2003). Cooled-stored semen is a common procedure to enable greater flexibility in AI protocols in relation to timing, transportation, and location of insemination (De Oliveira, De Oliveira Viu and Gambarini, 2015). To obtain high embryo recovery rates with cooled equine semen *in vitro*, threshold PMOT and TMOT values were reported at 45% and 65%, respectively (Love *et al.*, 2015). Both motility parameters reported within the current studies fall below these threshold values, indicating that semen samples would not be sufficient in obtaining high embryo recovery rates, an indicator of reduced fertility potential. Given the detrimental effects associated with cooling semen on sperm motility (Neuhauser, Säcker and Handler, 2017; Haadem *et al*., 2015), it is likely that a higher threshold would be required for fresh samples to achieve this percentage following cooling protocols. Semen samples from stallions with suboptimal fresh motility values reported within the current global and UK-restricted studies are, therefore, unlikely to be used for semen cooling practices. The reduced ability to use this breeding method could have serious economic and ergonomic consequences for the national and international trade market of equine semen.

The impact of the findings directly relate to the racing industry, for which racing levy boards, in 2022, illustrate that research strategies should be for 'male and female reproductive success, stallion sub-fertility and fertility regulation'. Other regulations preventing the use of ART in breeding management protocols for registered Thoroughbreds (Rogers *et al*., 2009), means that this breed relies on natural covering. Therefore, registered Thoroughbreds may be more at risk of the results proposed within the current research, given the inability to use assistive techniques for stallions with low fertility. Given the significant monetary and industry values of high-quality stallions within the racing sector (Juddmonte Farm, 2022), significant economic consequences could occur

if the maintenance of desirable heritable traits in the gene pool are lost due to the decline in sperm PMOT of valuable sires (Darr *et al.*, 2017).

Whilst research indicates that motility is associated with reproductive potential and infertility in stallions, it does not hold absolute power for the prediction of fertilisation (Varner, 2016; Colenbrander, Gadella and Stout, 2003). Poor sperm quality parameters, including motility, are associated with reduced overall wellbeing (Eisenberg *et al*., 2014). Semen quality assessment acts as a predictor of reproductive health and function (Colenbrander, Gadella and Stout, 2003) and is a suggested universal health marker (Latif *et al*., 2017). Therefore, from a welfare perspective, it is paramount that efforts are made to optimise equine health, including that of the reproductive system. Within humans, a significant association was reported between poor sperm motility, hospitalisation cases, and mortality in a population of 4,712 men (Latif *et al*., 2017). Therefore, the declines and low sperm motility values reported within the current studies could be an indicator of reproductive and overall health aberrations, which is a welfare concern for the equine population. Compromised health and welfare in stallions is likely to have significant ethical implications for the equine industry. Ensuring that horses have high welfare standards is the responsibility of those who breed, train, care, and own them. Trends in semen quality and values below threshold levels reported in this thesis, could be associated with reduced stallion reproductive health, and have welfare implications regarding the need for increased collections and coverings to achieve successful pregnancies. It is therefore the responsibility of the equine breeding sector to implement practices to optimise semen quality, including integrating fertility into selective breeding programmes, and investing in further research determining the effects of contaminants on equine reproductive health and function. This is essential for the welfare of the equine population, and to support and optimise the social licence of industry practices. The analysis of TMOT is a fundamental measurement taken for breeding soundness examinations, which is carried out to determine the seminal quality and health of stallions (Varner, 2016). Additional parameters associated with this examination include sperm concentration, TSO, and volume, all of which were analysed within the current retrospective study.

6.4 The declining sperm count debate, trends in concentration, volume, and TSO

Whilst semen volume increased over the study period, values were reported below industry accepted AI referencing ranges (Wilson and Flesner, 2017). Given the suboptimal semen volumes reported within the current retrospective study, the assessment of potential aetiological factors involved in results is warranted. Seminal fluids are synthesised by the accessory glands, and so volume is not a direct indicator of testicular health. However, the secretions of the accessory glands are associated with sperm function (Soriano-Úbeda *et al*., 2021; Samanta *et al*., 2018) and with pregnancy outcomes (Robertson and Sharkey, 2016). The production of the seminal fluid from the accessory glands is an androgen-mediated process (Multigner *et al*., 2000). Many ECs act through androgenic pathways, exerting aberrations on semen quality parameters (Kharlyngdoh, Pradhan and Olsson, 2018). In adult rhesus monkeys, exposure to Aroclor 1242, a commercially available PCB mixture, resulted in significant structural and morphological alterations to the accessory glands (Ahmad *et al.*, 2003). In rats, Aroclor 1242 resulted in the decreased weight of accessory glands (Gray *et al*., 1993). Glandular aberrations induced by contaminant exposure are likely to result in impaired functionality, including the production of seminal fluids. Given the presence of complex EC mixtures within equine testes, as reported within the current study, it is possible that exposure to endocrine disrupting contaminants is associated with low stallion semen volumes. However, considering the trends reported in the UK-restricted equine population, semen volume increased, which contradicts the hypothesis of EC toxicity regarding semen quality parameters including those associated with the accessory glands.

The analysis of temporal trends within the current study included sperm concentration and TSO, parameters associated with reproductive health and function (Ferlin *et al.*, 2021), including ART efficiency in humans (Zacà *et al*., 2020). Results presented for these two parameters are supportive of the sperm count biovariability hypothesis (Boulicault *et al*., 2021). The results indicate that TSO has either increased or remained consistent dependent on age group, a reassuring result suggesting that equine populations are currently not at risk of reducing sperm count parameters. Adverse trends in sperm concentration and TSO have been a controversial topic since the first evidence synthesis in humans was published three decades ago (Carlsen *et al.*, 1992). It is clear that the debate surrounding declining sperm counts is still a current topic. Whilst concentration and TSO did not decline within

the current UK-based equine population, these findings do not devalue the equine model as a comparative species for human trends. Therefore, the novel equine model continues to hold importance in resolving the discrepancies presented in human populations. In addition, research suggests that the analysis of trends should not be limited to sperm concentration and instead include additional parameters as a better biomarker of testicular health and function (Jouannet *et al*., 2001). Results on four semen parameters reported in the current retrospective study, which are also utilised within human fertility evaluations (World Health Organization, 2021), are therefore invaluable for cross-species comparative analyses. In addition, given that the semen quality parameters analysed here form a fundamental part of stallion breeding soundness examinations (Varner, 2016), the results contribute vital data on semen quality with clinical relevance for the equine breeding sector.

6.5 Geographic variability, reproductive trends, and EC distribution

Geospatial differences in reproductive trends are reported to exist on global and regional levels and form a fundamental part of the debate surrounding declining semen quality. Research carried out in the current study supported the hypothesis that adverse trends are geographically sensitive (Merzenich, Zeeb and Blettner, 2010). Declines in PMOT were more prominent in stallions from western versus non-western populations. Definitions of western and non-western categories were equivalent to those utilised in previous evidence syntheses in humans, which support the current findings, reporting that declines were more significant in westernised groups (Levine *et al*., 2017). Whilst the current equine and human-based evidence syntheses report data on different sperm quality parameters, the comparable results of geospatial differences in trends from western and non-western populations are indicative of a common environmental aetiology. However, given that an English language restriction was placed on the search strategy, it is also possible that language restrictions based on geographic origin of the articles included could have impacted the geospatial differences reported both here and in previous literature (Levine *et al*., 2017). An additional factor that must be considered in humans is dietary differences.

Westernised populations rely on a high percentage of processed meats as a nutritional source (Gaskins *et al*., 2012), with high saturated fat diets associated with adverse trends in reproductive health and function (Attaman *et al*., 2012). Therefore, sperm quality declines in humans from western regions may be associated with lifestyle and dietary differences (Sharpe, 2010). Whilst there is variation in the equine diet, with the introduction of more cereal-based materials, their main nutritional source is forage (Raspa *et al*., 2022). Given the equine's herbivorous diet, it is unlikely that changes or differences in foodstuffs is primarily responsible for adverse trends in sperm motility reported here, thereby supporting the argument of an underlying environmental aetiology.

The method of categorising populations as western and non-western provides a means for cross-species comparisons and a broad understanding of geospatial variability in trends. However, reproductive differences may also exist on national or regional levels. In canine populations from the UK, whilst geospatial differences in declining reproductive parameters were not analysed (Lea, Byers, *et al.*, 2016), regional differences in testicular contamination levels of DEHP, PCB, and PBDE were associated with geospatial variability in testicular pathologies (Sumner *et al*., 2021). The inability to histologically analyse the testicular samples within the current study prevents the ability to compare testicular pathologies in relation to the geospatial variability in contamination levels, which were significant for DEHP between the Cambridgeshire and Gloucestershire regions. Analysing pathologies in relation to testicular accumulation could begin to elucidate to whether contamination of ECs is associated with perturbed testicular health and function, contributing vital data to the equine breeding sector and the wider industry.

Previous research in humans is supportive of the hypothesis of geospatial variability in reproductive trends. In the USA, there was significant variation in human sperm motility and concentration depending on collection area, with a conclusion that parameters were lower for areas of high agricultural demand compared to urbanised locations (Swan *et al*., 2003). In Spain, men in areas of high industrialisation had a higher prevalence of oligospermia (López-Teijón, Elbaile and Alvarez, 2008). Whilst this is only an association and not causation, and focussing on low sperm count rather than motility, the reduction in

equine sperm quality over time presented in this thesis could be due to increased industrialisation and subsequent exposure to EC contaminants.

6.6 Environmental chemical exposure

Declines and geospatial variability in semen quality trends are associated with differential exposure to ECs, as reported in humans (Skakkebaek *et al*., 2015; Huang *et al*., 2014). Testicular structures are particularly sensitive to the toxicity of ECs (Paul *et al*., 2017), including those that were found within the testis of the current equine population. Whilst it was outside the scope of this research to assess a potential aetiological link between adverse sperm motility trends and testicular ECs, previous research in alternative species is indicative of an association. PCBs at environmentally relevant doses are reported to compromise both sperm TMOT and PMOT in pigs (Campagna *et al*., 2009). Sperm motility was reduced in dog samples treated individually with environmentally relevant concentrations of PCB 153 and DEHP (Lea, Byers, *et al*., 2016). Rats exposed to PBDE 47 through maternal transfer *in utero* and during lactation presented perturbed sperm quality, which was mainly reflected in poor motility (Li *et al*., 2021). Given the reported reprotoxicty of PBDEs, PCBs, and DEHP, and the contaminants found present within the stallion testis analysed here, it is plausible that ECs are associated with declining motility trends reported in the current equine populations.

Whilst research analysing toxicity of single chemicals provides an important understanding of isolated contaminant effects, real-life exposure in the equine model is to a complex mixture of ECs, as identified within the current research. Depending on specific chemical combinations, interactions resulting in toxicological effects differ (Fiandanese *et al*., 2016). Contaminant mixtures can exert their toxicity through synergism, antagonism, independent action, dose-addition, interaction, or potentiation (Hernández *et al*., 2013), indicating the complexity of exposure to a broad range of ECs.

A commercial mixture of PCBs was reported to reduce sperm motility in humans (Jiang *et al*., 2017). It is possible that chemicals in specific categories with similar structures, could act through comparable mechanisms. Given that equine testicular accumulation is associated with a range of contaminants, it would therefore be more applicable for

research to focus on the analysis of contaminant mixtures of a variety of EC categories. Gonadal concentrations of DEHP and PCB 153 in combination reduced sperm motility in humans and the dog sentinel model, with significant interactions reported between the two contaminants (Sumner *et al*., 2019). Whilst *in vitro* research is essential in understanding isolated effects of ECs and mechanisms of toxicity, methodologies are associated with their own limitations. Results are based on analysing the effects of acute exposure to ECs, despite real-life exposure occurring through chronic means (Krzastek *et al*., 2020). Considering results from *in vitro* and *in vivo* research, alongside epidemiological observational studies, is therefore essential in providing a holistic understanding of the health risks associated with EC exposure.

When analysing trends in bovine sperm quality in relation to year of birth, pesticide use had a significant correlation with semen quality parameters (Snoj *et al*., 2013). Given that horses exist in comparable environments to livestock species, it is possible that a similar association of *in utero* EC exposure and semen quality exists, although further exploration of this potential link is required. A negative correlation was reported between PAH contamination and sperm motility in men from the USA (Hammoud *et al*., 2010). Seminal PBDE levels were negatively associated with motility, sperm count, and semen volume (Yu *et al.*, 2019), indicative of the reprotoxic effects of this chemical category. High urinary concentrations of DEHP metabolites were associated with reduced sperm motility and concentration values (Huang *et al*., 2014). DEHP, PAHs, and PBDEs were detected in the equine testis analysed in the current study, raising concern over their effects on stallion testicular function. It is therefore a plausible interpretation that adverse trends and suboptimal motion characteristic values reported in the current equine populations are at least partially associated with EC exposure. However, whilst analysing urinary concentrations is a useful tool as a predictor of overall bodily burdens and a biological sample with low ethical considerations, it has limited applicability to the levels exposing developing spermatozoa. The analysis of testicular tissue, as carried out within chapter 5 of this thesis, provides a more accurate understanding of the reproductive perturbations associated with testicular EC accumulation.

As the organ of spermatogenesis, testicular health is fundamental in sustaining the microenvironment to support spermatogenesis (Yan Cheng and Mruk, 2012), sperm quality, and the ability of the gamete to transfer valuable genetic material to offspring during fertilisation (figure 34). Effects of ECs on motility may result from a range of mechanisms, including endocrine disruption, alterations in gene expression, and epigenetic pathways, as reported in alternative species (Elmetwally *et al.*, 2019; Baccarelli, 2011; Scott, Mason and Sharpe, 2009). The effects of ECs are also reported to have transgenerational implications (Goldsby, Wolstenholme and Rissman, 2017), which raises concern regarding the breeding potential of future equine generations. Whilst Bisphenols were not investigated in this thesis, in mice, prenatal exposure to Bisphenols resulted in a reduction in sperm motility and concentration and was linked to spermatogenic aberrations in F3 generations (Shi *et al*., 2019). In rats, exposure to DEET and permethrin mixtures, chemicals commonly used in commercial equine insect sprays (Baker *et al*., 2015), results in testicular aberrations in F1 and F3 generations (Manikkam *et al*., 2012). A current link between the use of equine insecticides and stallion reproductive function has not been explored, with serious implications for equine management practices within the industry.

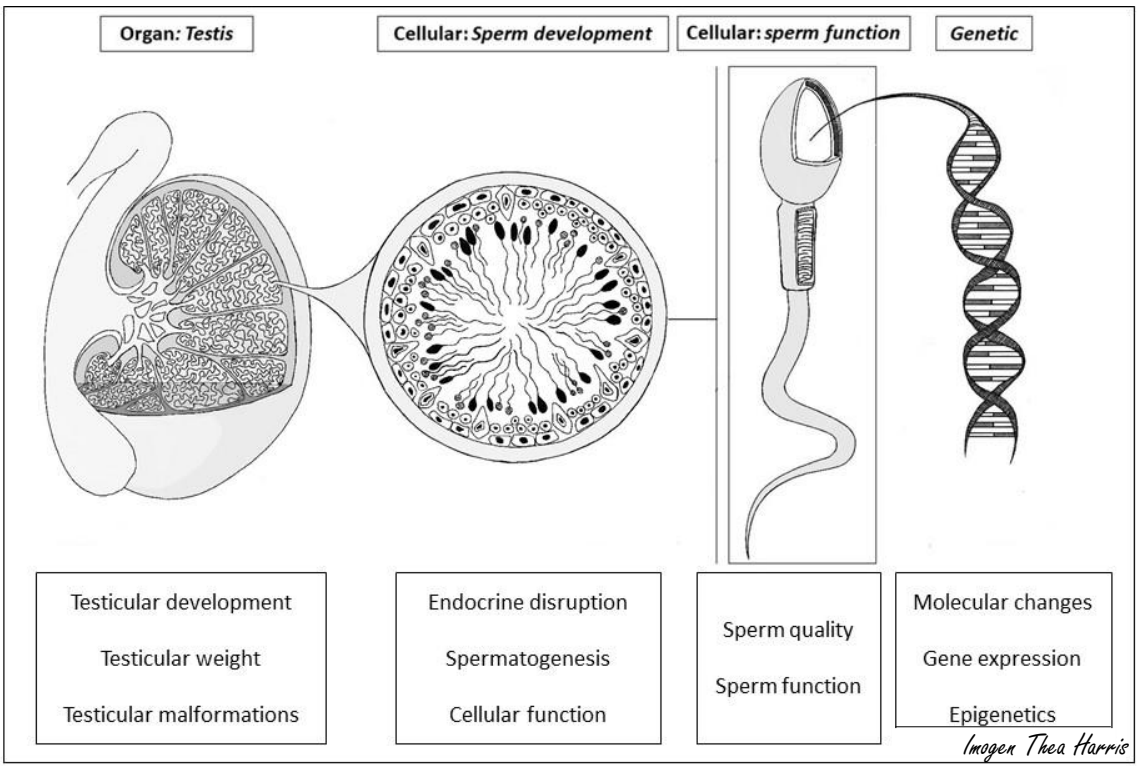


Figure 34: Loci at which ECs induce reproductive aberrations in male individuals (authors own).

Exposure to mixtures of ECs through sewage sludge treated pastures has also been reported to have cross-generational effects on testicular development in sheep (Paul *et al.*, 2005). If transgenerational effects of ECs exist in horses, testicular levels present within the equine species are likely to result in reproductive aberrations in future generations of offspring with significant health and welfare implications. *In utero* exposure is clearly fundamental in the onset of contaminant-associated reproductive aberrations. However, exposure in the paternal lineage is also reported to have detrimental effects on sperm quality across multiple generations (Maurice *et al*., 2021; Lessard *et al*., 2019). The transgenerational effects of ECs on male reproductive health and function of the equine species could therefore result from maternal *in utero* and paternal exposure. Transgenerational reproductive aberrations are likely to result in a long-term decline in the economic values of breeding stock, a serious concern for the sustainability and trade market of the equine industry. Given the transgenerational effects of ECs on testicular function and sperm quality, it is essential to explore the mechanisms by which contaminants exert their toxicity.

Sperm motility may be impaired by the ECs present within equine testis through interaction with the Ca^{2+} signalling of CatSper channels and cAMP pathways (Warner *et al.*, 2019; Tavares *et al.*, 2013). The two mechanisms are associated with the regulatory function of mammalian sperm motility (Pereira *et al*., 2017). In stallions, it has been suggested that both calcium/calmodulin and cAMP pathways regulate sperm motility (Lasko *et al.*, 2012). In L-Cs, the common herbicide Atrazine is reported to result in an increase in cAMP through the inhibition of enzymatic pathways (Karmaus and Zacharewski, 2015). Organochlorine insecticide exposure also impairs cAMP signalling pathways, thereby reducing steroidogenesis (Njembele, Bailey and Tremblay, 2014). A reduction in steroidogenesis of the L-Cs, impacting T_4 biosynthesis (Zirkin and Papadopoulos, 2018), may result in testicular perturbations, including impaired sperm function.

ECs are also reported to act by interrupting Ca^{2+} signalling of the CatSper pathway, an important mechanism in the hyperactivation of sperm (Marquez and Suarez, 2004). In humans, individual and pesticide mixtures are reported to induce Ca^{2+} signalling via the

CatSper channel *in vitro* (Birch *et al*., 2022). However, whilst CATSPER1 mRNA and proteins have been localised to equine sperm, researchers suggested that within the equine, the association of motility hyperactivation and the influx of Ca^{2+} was weak (Loux *et al*., 2013). In stallions, *in vitro* fertilisation is an unsuccessful ART, given the reduced capacitation ability of equine sperm (Lange-consiglio *et al*., 2011). Researchers have suggested that difficulties associated with equine *in vitro* fertilisation may be linked to the inability of sperm to achieve hyperactivated motility in these conditions (Loux *et al*., 2013), an issue that is not reported in humans. Such research indicates that there are species-specific variabilities in the control mechanisms of sperm hyperactivation (Hinrichs and Loux, 2012), although, to date, this physiological difference remains unknown.

Mycotoxins with oestrogenic properties are reported to inhibit equine sperm motility characteristics whilst stimulating kinetic parameters associated with hyperactivated motility (Filannino *et al*., 2011), demonstrating the susceptibility of equine sperm to chemicals with oestrogenic properties. Whilst oestrogen-related pathways are reported to be key mechanisms of EC action in alternative species, the stallion has particularly high levels of testicular oestrogens (Hess, 2003). Therefore, when considering equine specific contaminant modes of action associated with oestrogenic mechanisms, it may be of interest to focus the attention of further research on alternative pathways. A deeper understanding of the physiological differences in hyperactivation between horses and humans is required prior to the use of the equine species as a comparative model for this specific topic. However, this does not discredit the use of the novel equine model for other aspects of reproductive research discussed within this thesis. Whilst the direct link between declining sperm motility and the involvement of ECs has not yet been elucidated, both mechanistic pathways described above could be key in the declines surrounding sperm motility characteristics reported here. Given the extensive reprotoxic actions of ECs outlined, limiting exposure through the determination of specific routes is fundamental in reducing associated health risks.

6.6.1 Exposure to environmental chemicals

Key oral-based EC exposure routes specific to the stallion have now been determined within chapter 5 of this thesis. Considering that DEHP is a common plasticiser, elevated environmental levels in terrestrial ecosystems, as found within this thesis, may be related

to MPPs, given the inability of DEHP to covalently bond to polymer matrices (Fiandanese *et al*., 2016). It was outside the scope of this research to analyse MPPs in environmental samples, although previous research indicates high levels of accumulation. In European agricultural land, top-level soils are reported to be contaminated with approximately 607 plastic particles per kg of soil, a large proportion of which is a result of plastic films and coatings, including those from the agricultural sectors (Lind, 2020). Chemical leaching from MPPs is likely to result in elevated concentrations of ECs, including DEHP, within terrestrial environments.

PAHs were the second highest chemicals detected within soil samples in the current study. In previous literature, the proportion of HMW PAHs in soil was significantly higher than LMW PAHs, accounting for 80.8% and 75.8% in industrial and rural sites, respectively. Researchers suggested that pyrogenic and coal burning were likely sources responsible for soil contamination (Xu *et al*., 2021). With reference to burning organic matter, global warming has resulted in the increased risk of forest fires, which are associated with the elevated deposition of PAHs into terrestrial ecosystems (Campos and Abrantes, 2021). Changing climate conditions may therefore result in an increase in the presence of environmental PAHs and increased exposure to a range of animals, including horses. Given that PAH levels detected within the equine testis analysed here are already elevated, further exposure in horses surrounding areas susceptible to forest fires is therefore of significant concern, with potential risk for further health aberrations and reproductive declines. Increased environmental deposition of PAHs is also likely to pose a further environmental and human health risk. Such research indicates the importance of a holistic One Health approach in reducing the risks posed by the environmental deposition of contaminants.

Whilst there are many exposure routes applicable to the horse that could have deleterious impacts within the body, complex EC mixtures present in the soil of pastures designed for equine grazing may act as a primary oral exposure route to horses through direct ingestion when grass levels are low (Jurjanz *et al.*, 2021). Previous research suggests that a state of solubilisation must be met to enable the absorption of ECs into the blood system (Delannoy *et al*., 2015). High organic carbon content in soils and dust reduces the bioavailability of

PCBs exposed to digestive fluids (Shen *et al*., 2019), which could limit absorption and subsequent distribution to testicular tissues. However, in goats exposed to ECs through soil, the bioavailability for PCB 180 was 73%, whilst for PCBs 118, 138, and 153, relative bioavailability ranged from 36-50%. Researchers suggested that soil ingestion may act as a key exposure route in ruminants (Feidt *et al*., 2013).

Given the presence of contaminants in soil samples analysed here, it is likely that ingestion acts as a key exposure route in grazing horses, leading to testicular accumulation, as confirmed within the current study. However, due to physiological differences between ruminants and hindgut fermenters, further research is required to assess soil ingestion as an exposure route in horses. During dry months, airborne soil dust with adsorbed PAHs and other ECs may result in an additional exposure route through the inhalation of particles. Absorption of inhaled PAHs occurs mainly through the bronchial epithelium and is influenced by the molecular weight and adsorption to other particles (Castano-Vinyals *et al*., 2004). Therefore, whilst soil was analysed as a potential oral exposure route, the current study has also initiated investigations into exposure through alternative means, with significant implications for grazing management in the equine industry.

Soil-to-skin contact in horses is also a route of exposure that warrants further investigation in the equine model, given the ability of ECs to cross the dermis. In humans, soil residue on clothing was reported as a potential key exposure route to PAHs and PBDEs through dermal contact (Easter, Lander and Huston, 2016). Out of other phthalates, DEHP was reported to have the maximum skin uptake in a mouse model (Pan *et al*., 2014). This could be a key exposure route to DEHP in equines, given that testicular bioaccumulation in the current study was particularly high. In a monkey model, a commercial mixture of PCBs present within soil were able to cross over the dermis, with soil organic content modulating the rate of percutaneous absorption (Mayes *et al*., 2002). Given that research highlights percutaneous absorption as a key exposure route in alternative species, and equine behaviours resulting in skin-to-mud contact (Pan *et al*., 2014), further research assessing this exposure risk specific to the equine model is warranted. Research could contribute vital data to the equine industry in order to promote effective pasture management and horse turnout protocols in relation to contaminant exposure. Once in the bloodstream, PAHs are

distributed around the body to tissue structures, including the testis (Castano-Vinyals *et al.*, 2004), as confirmed within the current study.

Whilst not analysed in the current study, chemicals utilised in fly deterrent sprays that are commercially available within the equine industry (Baker *et al*., 2015), may also result in equine exposure through dermal contact, although this route has not yet been explored in horses specifically. It is plausible that dermal exposure to chemicals such as DEET may result in testicular perturbations and could be partially associated with adverse motility trends reported here, given the link between exposure and transgenerational testicular aberrations in murine models (Manikkam *et al*., 2012). It is therefore concerning that the equine industry continues to promote the use of such chemicals for widespread commercial use. Raising awareness of the effects of chemicals used within the equine industry that are reported to impair reproductive health in alternative species (Guvvala *et al*., 2020), is paramount in preventing excess EC deposition, exposure, and associated health risks. The application of insect deterring sprays is also likely to result in exposure to humans and the environment. The ability of such chemicals to impact animal, human, and environmental health further supports the need for a holistic, One Health approach to reduce the risks associated with EC contaminants. The equine industry must contribute to this approach by reducing contaminant outputs through policy change and implementing informed equine management procedures to reduce EC deposition.

Poor management of equine pastures, leading to soil erosion, is associated with the environmental displacement of pollutants (Westendorf *et al*., 2012). This could result in further exposure to grazing horses. Whilst previous research has explored soil contamination heavily (Wang *et al*., 2007), this thesis confirms exposure in equine pasturelands specifically, a novel output of the research. Horse owners and carers must be educated surrounding the additional risks associated with poor pasture management and increased contaminant exposure in order to lead industry change. The results also provide data on the accumulation of ECs within grasses, holding relevance for the trophic accumulation of ECs within terrestrial ecosystems. Results indicate the current contamination of EC burdens in the nutritional sources for production animals designed for

human consumption. The ingestion of contaminated meats is therefore likely to result in the biomagnification of ECs within human populations, posing as an additional health risk.

Grass species are reported to remediate soils contaminated with PAHs (Un Nisa and Rashid, 2015), DEHP (Ma *et al*., 2012) PCBs (Zeeb *et al*., 2007), and PBDEs (Lu and Zhang, 2014). However, a link between the phytoremediation properties of grasses and the consumption of materials by horses requires evaluation. Equine diets require fibrous feeds for healthy digestive function, and therefore, grasses and baled forages form a main part of their diet (Müller, 2018). PAHs (Un Nisa and Rashid, 2015) and PCBs (Zeeb *et al*., 2007) accumulated more readily in roots compared to shoots. Given that elevated levels were reported in equine pastures, consumption of root portions of grasses when pasture levels are low could result in a higher degree of exposure than reported within the current study. Biodegradation of contaminants by grasses is enhanced by microbial populations within the soil (Khan *et al*., 2013). Considering pasture management regimes in the equine industry, growing grasses that have a reduced affinity for the uptake of contaminants may be a means of reducing EC contamination in grazing herbivores. Alternatively, given the importance of soil health in sustaining life on earth (Fierer, Wood and Bueno De Mesquita, 2021), a more sustainable approach could include producing effective protocols to degrade soil contaminants with grasses and bacterial populations, resulting in a method that simultaneously remediates soil without sacrificing the safety of forage feedstuff production (Liu *et al*., 2022). From the current published research base, it is suggested that microbial soil populations could be fundamental in this process (Liu *et al*., 2022; Khan *et al.*, 2013).

6.6.2 Processed and packaged equine feedstuff materials

Cut grasses and other herbage materials are processed to produce concentrate and forage-based feedstuffs for commercial distribution, a common practice in the equine sector (Robles et al., 2017). It is thus concerning that concentrate feeds analysed here were contaminated with a range of ECs. Contamination of feeds designed for breeding stock raises significant concern regarding equine bodily burdens, which was confirmed in the current study, and potential reprotoxicity. Research in cattle is supportive of this finding, suggesting that hard feeds are a primary route of EC exposure (Mclachlan, 1993). Given that feed materials are reported to be a key route of EC exposure to animals, including

those designed for human consumption, it is concerning that research and industry practices have not sought to reduce this as a contamination route. Producing an evidence based understanding to inform equine management practices in relation to the potential welfare-threat of EC contaminants, could strengthen social licence in the equine sector and improve welfare within the future. Whilst acute effects of ECs on testicular function and sperm quality have been reported (Jiang *et al*., 2017), prenatal and early life development are stages at which individuals are particularly sensitive to the toxicity of ECs (Rhind, 2005), often referencing the 'foetal origins of disease' (Barker and Osmond, 1986).

Given that the recommended breeding stock for stud cubes is stallions and broodmares and the ability of ECs to be transferred to the foetus (Vizcaino *et al.*, 2014), EC exposure to developing male individuals could occur indirectly through the maternal unit. Exposure to individuals during these key windows of development is likely to be reflected in adult reproductive health and function (Rhind, Rae and Brooks, 2003). Considering the postpartum period, the ECs present within feedstuffs given to lactating mares may also be transferred to suckling offspring, which, as reported in other species, often suffer from higher burdens compared to the mother (Polischuk, Norstrom and Ramsay, 2002). In calves, concentrations of PCBs and PCDDs were double that of their respective mothers, indicating the multigenerational exposure of ECs (Driesen *et al*., 2022). In murine and ovine models, exposure to contaminants during foetal and postnatal development results in multi- and transgenerational reproductive aberrations in male offspring, including reduced sperm quality (Sanabria *et al*., 2016; Paul *et al*., 2005). If such effects of ECs exist in equines, future generations are likely to be at risk of the same semen quality declines reported in the current study. It is imperative that industries, including equine sectors, implement regulations and policy changes to reduce the synthesis and deposition of contaminants, to protect the health of animals, humans, and the environment on a global scale.

An additional process of contaminant environmental deposition associated with the equine industry is the harvesting of equine feedstuffs. Haylages were reported to have the highest contamination levels of PAHs compared to other feedstuffs analysed within the current study, which could indicate that specific processes associated with harvesting result in the deposition of elevated EC levels into the feed. Haylage is harvested by agricultural

machinery run on petroleum gas or diesel (Hanna and Schweitzer, 2015). Incomplete combustion of organic fuel materials is a key source of environmental deposition of PAHs (Pant and Harrison, 2013). It is therefore likely that harvesting of herbages using fuel-run machinery results in the elevated contamination of haylages, as reported within the current study. Whilst exhaust emissions have long been associated with particulate matter, and atmospheric deposition of PAHs, a current area of interest is the wear patterns of tyres and the resuspension of contaminants into the atmosphere (Vicente *et al*., 2022). Whilst this research is associated with wear between tyres and roads, it is possible that friction resulting from tractor tyres and soils could result in a similar distribution of PAHs into forages being harvested for hays, haylages, and other animal feeds.

Haylages analysed within the current study were also contaminated with the highest concentrations of DEHP when compared to other feedstuff materials. Haylage is typically plastic wrapped, which may result in a release of plasticising chemicals into the feedstuff contents. The elevated concentrations of DEHP in plastic baled haylages is likely a result of leaching from the polymer wrappings. However, given that hay was not analysed within the current study, contamination of this feedstuff material cannot be disregarded. Considering plastics as a source and vector of DEHP and other ECs, the presence of MPPs in equine environments may result in elevated deposition and exposure to contaminants. Hays are bound by polypropylene twines and fed in nets made of polyesters and polypropylenes (Lind, 2020). Such feeding management regimes may result in MPP, and EC plasticiser feed contamination and act as a subsequent route of exposure to the ECs detected in horses within the current study. MPPs are detected in equine manure samples (Lind, 2020), likely to result in EC exposure throughout the digestive tract during desorption of chemicals from MPPs. The use of these materials within the equine industry, and presence in equine manure is also likely to be associated with the redistribution of contaminants and plastics into the environment. Interlinks between equine and environmental health support the need for industry change formulated around the One Health approach.

Considering alternative packaging materials, whilst paper bags are now commonly used for concentrate feeds, the development of alternative materials for wrapping hays, haylages,

and chaff-based feeds is warranted. Reducing plastic packaging for feeds is required to limit MPP and EC contamination associated with the equine feed industry, which is of utmost importance for equine and environmental health. However, steps must be taken to ensure that alternative materials reduce the environmental and health risks associated with contaminants. Considering the practicalities of paper feed packaging, materials must be sturdy enough to maintain the quality of the feed contents. Perfluorinated chemicals are often utilised as water-resistant materials, including for the treatment of paper and cardboard packaging (Surma *et al*., 2015), which could result in an additional means of environmental and feed contamination. It is recommended that a holistic approach is developed within the equine industry to produce alternative packaging and sustainable feed distribution methods whilst maintaining the quality and preservation of feedstuffs.

Considering the widespread use of plastic packaged equine supplements, this sector of the equine feeding industry requires attention. In Ireland, 98% and 86% of professional and non-professional horse owners were reported to include supplements within their feeding regimes, with up to 70% feeding more than one (Murray, Hanna and Hastie, 2018). In human consumer industries, refill schemes, and innovative packaging designs are used to reduce the deposition of plastics resulting in the synthesis, use, and disposal of this material (Okada *et al.*, 2021). Such methods could be introduced into the equine sector to reduce the requirement for the synthesis of virgin plastics and raw materials, limiting contaminant and MPP deposition and the industry's environmental impact. Development of regulations associated with maximum migration limits and animal tolerable daily intakes, as seen within human food supply chains (Fang, Wang and Lynch, 2017), are also required to limit contaminant exposure through chemical leaching of packaging. The equine sector is a lucrative, global industry and holds a responsibility to reduce its environmental impact, including minimising the synthesis and deposition of EC contaminants.

6.7 The novel equine research model

For the first time, the research associated with the current project initiates the equine model as a sentinel species from a reproductive perspective and biomonitor of terrestrial ecosystem environmental health. The worth of the equine species in relation to environmental monitoring programmes has been suggested in previous research.

However, focus has been on using hair to predict EC contamination levels (Yavuz *et al*., 2022; Madejón, Domínguez and Murillo, 2009). Whilst the analysis of hair samples provides an essential, non-invasive material, it holds limited applicability when considering monitoring reproductive EC contamination and toxicity. Contamination of hair samples is associated with endogenous routes from the blood to hair follicles and exogenous exposure routes related to the transportation of chemicals from air and dust (Zheng *et al*., 2011). Given the fundamental differences between hair and tissue, the analysis of equine testis, as determined within the current research, provides an indicator of accumulation levels specific to the male reproductive organ. Castrations are carried out as routine procedure across the UK to facilitate domestication and ease of training (Price *et al*., 2005). Routine semen collections for breeding soundness examinations and ART are also common practice in the equine industry (Colenbrander, Gadella and Stout, 2003). Such materials, together with databases and published metadata on reproductive parameters, emphasise the importance of the horse as a holistic model for reproductive and environmental toxicology research. This thesis takes advantage of these data and sample sources to develop the novel equine species as a holistic research model for the analysis of reproductive and environmental health. From the research presented within this thesis, it is recommended that broad populations of horse breeds are used when determining reproductive trends and EC contamination levels in relation to differing management practices, to further develop the equine species as a novel comparative model.

6.8 Further research following the completion of this doctorate

Whilst it was outside the scope of this research to form a statistical associative link between testicular burdens of ECs and adverse sperm motility trends, elevated testicular levels are a likely aetiological factor, the mechanisms for which warrant further investigation. The collection and analysis of stallion testicular tissue could be used to develop a cross-sectional study to enable the determination of EC burdens in relation to testicular and seminiferous tubule structure, endocrinology, genomics, epigenetics, and epididymal sperm quality parameters. Longitudinal research could analyse seminal concentrations in relation to sperm motion characteristics and alternative semen quality parameters (figure 35). Both research methodologies could contribute to the understanding of EC effects on stallion fertility and potential mechanisms resulting in declining motility trends reported within the current studies. This research provides mean values of chemicals specific to the

equine model that could be used for further controlled *in vitro* research to determine the effects of exposure and mechanistic actions of ECs. Given the declines in equine sperm motility reported here, focussing on mechanisms associated with this parameter could elucidate to a potential associative link with EC exposure. However, the water content of the equine testis needs to be specified, and the wet weight chemical concentration calculated from the dry weights provided within this thesis to be utilised for *in vitro* studies. In addition, to fully interpret the effects ECs may have on equine reproductive potential in relation to impaired semen quality, the equine industry must establish standardised semen quality threshold values to define stallion fertility.

The assessment of EC toxicodynamics within the equine testis will elucidate to the potential interactions of contaminants with the testicular target to develop an understanding of the biological effects that may result from exposure. Further analysis of species-specific internal EC exposure and contaminant toxicokinetics is required to increase understanding of contaminant distribution, metabolism, and excretion, to initiate investigations into overall equine health risks associated with exposure (figure 35). Given the presence of ECs within the testis, it is likely that alternative organs are exposed to similar mixtures of contaminants. It is therefore recommended that further research considers the aetiological involvement of contaminants with other endocrinology-mediated mechanisms in relation to overall equine health and welfare.

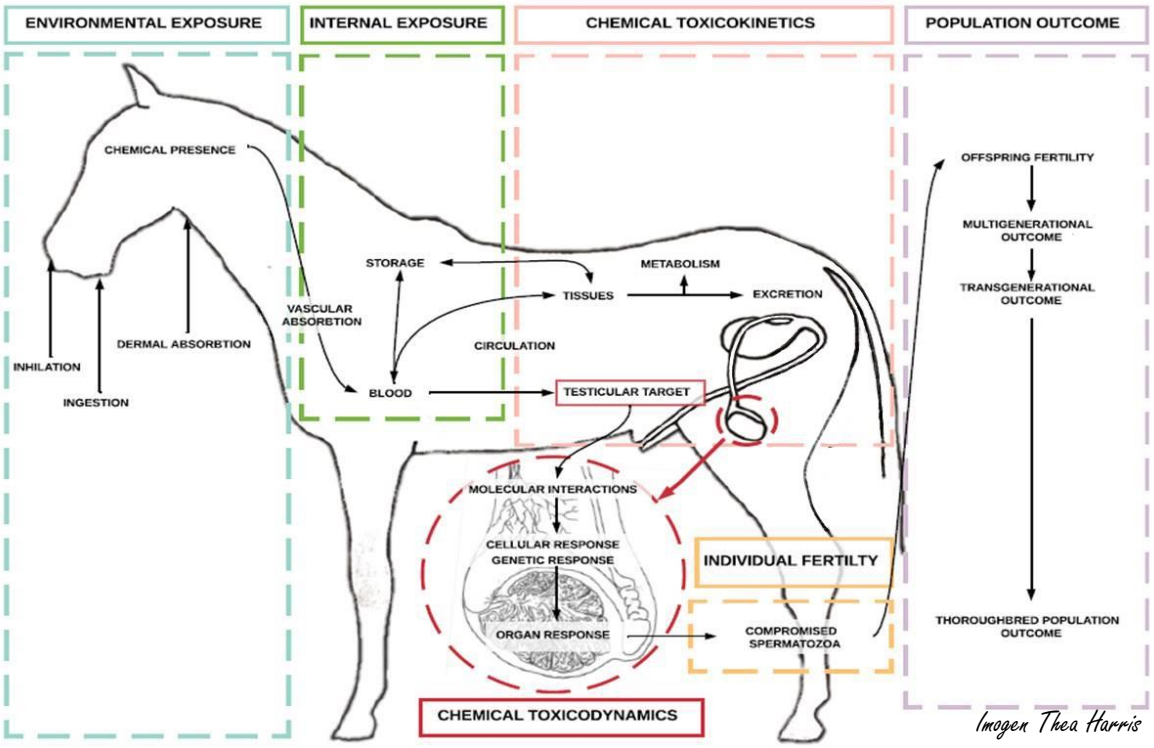


Figure 35: Potential environmental and internal exposure routes, EC toxicodynamics, and outcomes specific to the equine population. This diagram indicates the key areas addressed within this thesis, including routes of oral ingestion and accumulation in equine testicular tissue. The diagram also highlights the areas that require further analysis to provide a holistic understanding of contaminant exposure specific to the male equine population (authors own).

Given that research in alternative species indicates that EC exposure during the *in utero* and postnatal periods are essential in the manifestation of reproductive aberrations (Priya *et al*., 2018; Higuchi *et al*., 2003), further analysis associated with contaminants, pregnancy, and postnatal development are required. This is essential in understanding the effects ECs may have on equine reproductive development and subsequent fertility potential in adult individuals. Chemically analysing samples such as placental tissue, amniotic fluid, and mare's milk could begin to elucidate to the maternal transfer of chemicals from the dam.

Considering the ingestion of contaminated feedstuffs, determining pasture management and feed processing protocols that are responsible for elevated EC contamination is essential to reduce this as a primary exposure route. Sampling populations of horses with differing management protocols may enable further elucidation to specific practices associated with variable levels of contamination. Collaborative, interdisciplinary projects with environmental toxicologists, agricultural sectors, feed companies, and policy makers

are required to implement informed legislation surrounding the production, storage, and distribution of equine feedstuffs in relation to EC contamination. Further analysis and development of alternative feed packaging and holistic approaches to the commercial distribution of equine feeds are required to reduce the environmental impact of the equine industry. Such practices could include refill schemes, which is an established method utilised within wider industries.

Further research is also required to determine alternative routes of exposure through inhalation of aerosolised particles and the absorption of ECs through the dermis. Analysing contaminant presence and the potential percutaneous transfer of chemicals used to treat equine rugs is essential to determine the risks associated with this exposure route. An additional area requiring further attention is the assessment of the percutaneous absorption and inhalation of aerosolised particles of chemicals used in equine insect deterring sprays, bedding materials, dust and soil, which may act as a risk for both horses and stable staff and result in the redistribution of contaminants into terrestrial environments.

6.9 The One Health research approach and accountability of the equine sector

In order to provide a holistic understanding of the effects of ECs on species and environments in terrestrial ecosystems, a detailed analysis of species from a variety of taxa is required (Rhind, 2009). The equine species forms a part of this narrative, with current results of reproductive trends and EC contamination initiating investigations into equine-human, equine-animal, and equine-environment interactions in support of the One Health approach (figure 36). The research carried out as part of this doctorate indicates that the equine population is exposed to a range of anthropogenic ECs at elevated levels, raising concern over the reproductive health and function of this species. ECs pose interconnected health risks to the environment, animal, and human health. Therefore, the impact of ECs on the health and welfare of companion, livestock and wildlife animals, humans, and the environment should not be deliberated in isolation but as one unit within an ecosystem. Horses exist in close environments to humans (Yavuz *et al*., 2022), have the potential to monitor environmental contamination (Madejón, Domínguez and Murillo, 2009), produce manure for arable land fertiliser (Hadin and Eriksson, 2016), form part of the human food

chain in some cultures (Jurjanz *et al*., 2021), and can have negative impacts on the surrounding environment (Westendorf *et al*., 2012). The broad-spectrum implications of ECs on the environment, humans, and animals indicate that a shared research approach under the One Health framework is required to provide a holistic understanding of contaminants and produce informed industry and policy changes to reduce the risks associated with exposure (figure 36).

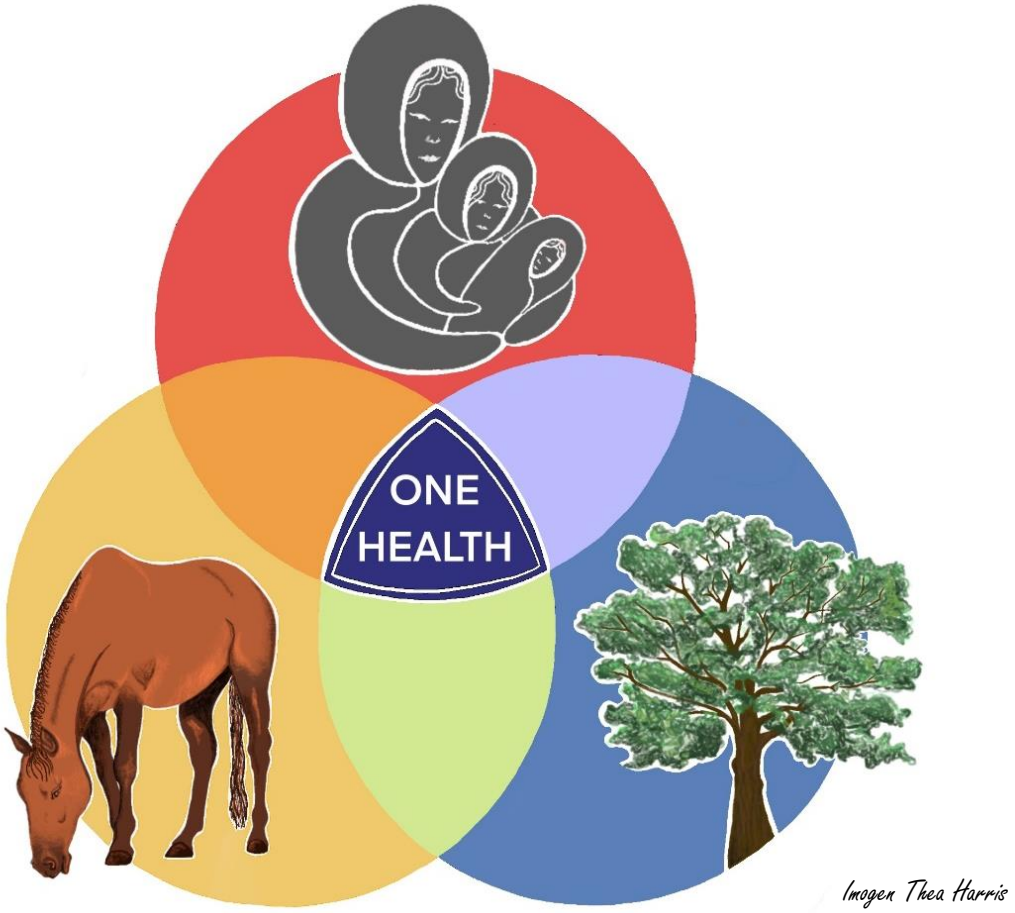


Figure 36: One health diagram consisting of human-animal (equine), animal-environment, and human-environment interactions (authors own).

The current study confirmed that the male equine population is exposed to elevated levels of a complex mixture of ECs, as reported in humans (Peng *et al*., 2021), wildlife (Haraguchi, Hisamichi and Endo, 2009), livestock (Kamarianos *et al*., 2003) and domestic (Lea, Byers, *et al.*, 2016) animal species. Such chemical exposure is reported to result in reproductive aberrations across species, although the true effects on equine testicular health and function remain unknown. Integrating comparative anatomy and physiology, including that of the reproductive system, is important in contributing towards the One Health approach

(Bhattacharjee *et al*., 2022). Such research will support a holistic understanding of the interactions of ECs on reproductive health and function. Currently, the environmental impact of equine operations is focused on the output of phosphorous, nitrogen, and methane (Hadin and Eriksson, 2016), which now needs to be expanded to include EC contamination. Conservation of equine environments is important for the sustainability of the equine industry and to minimise the sector's impact on surrounding ecosystems (Westendorf *et al*., 2012). Research needs to focus on defining industry practices to minimise the output, exposure, and subsequent health implications of EC contamination. A global, multidisciplinary research approach is fundamental to bring knowledge together and inform procedures and regulations to limit the environmental deposition, exposure and toxicological effects of contaminants (Sonne *et al*., 2022). Researchers, industry personnel and policy makers within the equine sector must work together to optimise the environmental efficiency of management and commercial practices. As with other environmental policies, whilst change on an individual consumer basis is important, pressure must be placed on equine organisations, businesses, and policy makers to make fundamental changes to production lines in order to reduce the overall environmental impact of the equine industry.

6.10 Conclusions

The work that has been carried out within this thesis indicates that equine sperm motility has declined in global and UK-restricted domesticated equine populations. The research confirms the accumulation of four anthropogenic EC categories within equine testicular tissue and indicates that exposure includes the ingestion of contaminated pasture and feedstuff materials. The analysis of a potential associative link between EC exposure and adverse reproductive trends in the equine model requires further investigation, although the research presented here initiates the discussion of this topic and supports the need for this further work. Temporal trends in stallion reproductive parameters and the contamination profile of the equine testis warrant further monitoring given the trends in semen quality and presence of ECs reported within this thesis.

These results provide valuable data for the equine breeding sector, which holds responsibility in preserving the fertility of the equine population through managing and

reducing EC exposure. The equine sector also holds responsibility for putting legislation and protocols in place to reduce its environmental impact, including the production and deposition of ECs, both for the benefit of the equine population and wider species and environments. Research presented provides reference values of testicular EC contamination, opening a window of opportunity for further toxicology research in the equine model using *in vitro* methods. The development of the novel equine model within this research adds weight to the debate surrounding adverse semen quality trends across species. This raises considerable concern regarding the reproductive potential of species on a global scale, which requires immediate attention in a changing environment. Results support the hypothesis of utilising the equine model as an alternative comparative and biomonitor species for reproductive biology and environmental research in terrestrial ecosystems. It is recommended that the equine species be utilised within further environmental toxicology research in support of the One Health approach to provide a holistic understanding of reproductive perturbations in an environment led by anthropogenic change.

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APPENDICES

APPENDIX A: Ethics and data management

A1 Ethics application approval

A2 Data management plan (UWE)

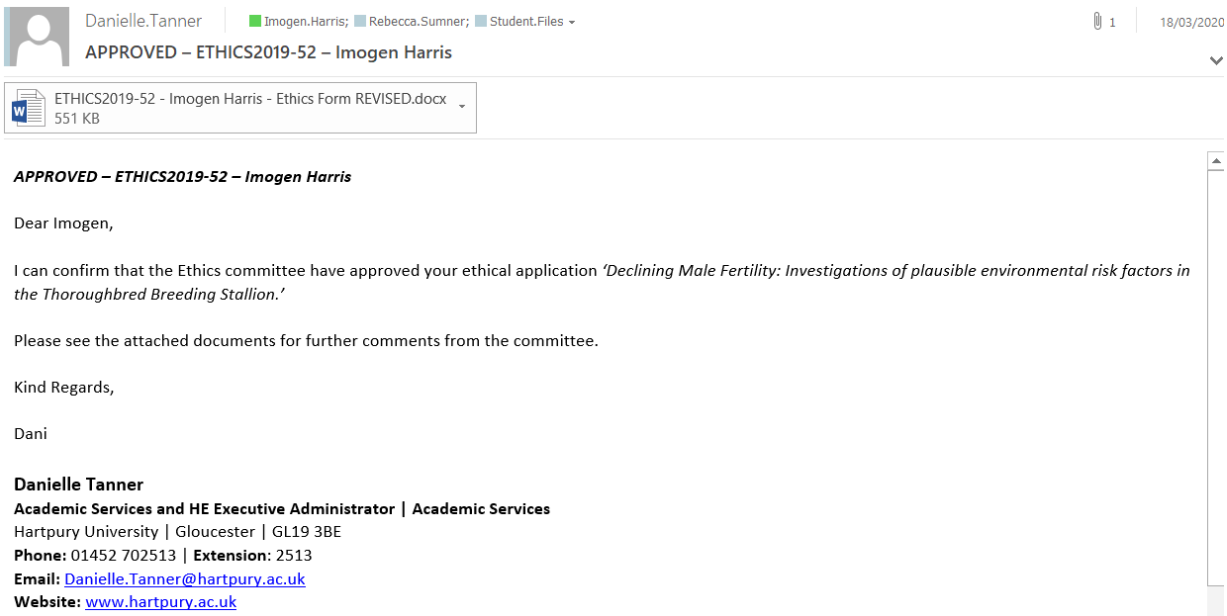
A3 Site permission form

A4 Veterinarian information sheet, consent form and data sheet

A5 Stallion owner information sheet, consent form and data sheet

A6 Yard owner information sheet, consent form and data sheet

Appendix A1; Ethics application approval



Danielle.Tanner Imogen.Harris; Rebecca.Sumner; Student.Files 1 18/03/2020

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APPROVED – ETHICS2019-52 – Imogen Harris

Dear Imogen,

I can confirm that the Ethics committee have approved your ethical application *'Declining Male Fertility: Investigations of plausible environmental risk factors in the Thoroughbred Breeding Stallion.'*

Please see the attached documents for further comments from the committee.

Kind Regards,

Dani

Danielle Tanner
Academic Services and HE Executive Administrator | Academic Services
Hartpury University | Gloucester | GL19 3BE
Phone: 01452 702513 | **Extension**: 2513
Email: Danielle.Tanner@hartpury.ac.uk
Website: www.hartpury.ac.uk

**Following the acceptance of the ethics application, the title was changed to 'Male reproductive trends in the novel equine model: investigations into anthropogenic environmental chemical exposure'.*

Appendix A2; Data management plan (UWE)

UWE Project manager name:	Dr Kathryn Nankervis
Student name, where applicable:	Imogen Thea Harris
Faculty:	Hartpury Equine, Hartpury University and College
Project Title:	Male reproductive trends in the novel equine model: investigations into anthropogenic environmental chemical exposure.

Research Data Management Plan version number:	Version 3
Date:	04/09/2022

If you have the following reference numbers, please enter them below.	
PASS code:	N/A
UREC / FREC / AWEC application numbers:	N/A Ethical acceptance has been granted by the Hartpury University Ethics Committee (ETHICS2019-52).
HTSC registration number:	N/A
GM registration number:	N/A

What data will you collect, create or use? Give a brief description. See Note 1
The first objective of this research project is a systematic review and meta-analysis, by which published data, from publicly available databases, was collated electronically. Meta-data collected, included semen quality parameters (progressive motility) and factors associated with semen quality. Supporting data were obtained from a singular UK based stud farm, collated from electronic files on past stallion semen collections. Stallion AI Services is the industry partner that granted access to retrospective data from electronic databases. An agreement of collaboration was signed prior to the completion of data collection, outlining the collection, use and publication of associated data. Data including stallion age, breed, discipline and location of birth and semen quality data including total motility, volume and concentration, were recorded electronically. 230 articles were included in the systematic review. Data collection at Stallion AI Services resulted in an unrefined dataset of >10,000 ejaculates. For objective C, testicular samples were collected from routine castrations carried out by qualified veterinarians. Soil, grass and haylage were collected from collaborating Thoroughbred racing and breeding yards. Feeds were bought direct from commercial distributers. Samples were analysed for a range of environmental chemicals, for which quantitative data were recorded electronically. Prior to the collection of samples, an information sheet, consent form and data sheet was provided to the stallion or yard owners and attending veterinarians where appropriate. Signed consent was required before sample collection. All consent forms and questionnaires were collected in a hardcopy format, and digitalised once returned to Hartpury University. All electronic data were stored on OneDrive on a password protected Hartpury University staff laptop.
Please classify your data here as public, restricted or confidential. See Note 2
Confidential.

How will you collect, create or access the data? See Note 3

All data were collected by myself as hardcopies that were digitalised directly onto password protected electronic files. Data for the retrospective study were from past semen collections, already maintained electronically by the stud yard for breeding management purposes. Subsequent biological samples were collected by qualified veterinarians and chemical analysis data via GC-MS were obtained by a collaborating laboratory. All samples were anonymised with a random numerical code prior to analysis. Consent forms and questionnaires that were associated with the collection of biological samples were collected in a hard copy format and subsequently digitalised.

Data were obtained from third party veterinary practices and a stud farm. Owners and veterinarians provided full informed consent, complying with the General Data Protection Act (2018). Collaborating industry partners included veterinarians at Three Counties Equine Hospital and Rossdales Veterinary Surgeons. Stallions, owners and organisations maintained full anonymity and are allocated a randomised numerical code when data were collected to maintain this. This is a condition that was stipulated when obtaining informed consent for their collaboration. A secured, password protected file, for which only the researcher (myself) had access to, was created containing explanations of codes used.

How will the data be stored and backed up at all stages during its life course? See Note 4

All data were collected and stored on excel spreadsheets. Data were processed via NVivo, GenStat, and graphically interpreted on GraphPad Prism 8. All data collected, stored and processed were secured on a password protected Hartpury staff laptop and placed within password protected online resources (Hartpury OneDrive). Sensitive data had additional password protection on the file itself. Data were backed up on an external hard-drive at the end of each month, of which was also password protected and maintained in a locked desk in the secured postgraduate research office at Hartpury University or in a locked draw in a personal working space when working from home. The researcher (myself) was responsible for the collection, processing and backing up of all data. During disposal of data, a method of disk wipe shall be used to prevent further use by anyone other than the researcher. The IT department at Hartpury University shall be contacted to ensure that the method of disk wipe is in line with specific regulations. Hard copies were disposed of in confidential waste bins onsite at Hartpury University.

How will the data be documented, described and maintained? See Note 5

Data were documented with suitable headings including name of collector, date of collection and data contents to prevent manipulation. When raw data were subject to statistical analysis, a new file was created to ensure a record was kept of the data's progression. Excel, NVivo, Genstat, GraphPad Prism 8 and ImageJ were used to obtain, process, maintain and store data for this project.

How will your data be processed? See Note 6

Only myself and supervisory team (Dr Kathryn Nankervis, Dr Rebecca Sumner and Dr Alison Pyatt) had access to the password or data sets. To ensure only those with a legitimate right had access to the data, it was not shared or sent to any other parties. Data were stored on a single OneDrive and all editing was carried out on that account and device to prevent it being shared. Regarding the digitalisation of hardcopy files of data collected offsite, paper copies were transported back to Hartpury University by the researcher for digitalisation. During transportation, hardcopies were concealed and not left unattended.

Does the Data Protection Act (2018) apply to your research? See Note 7

Full-informed owner consent was obtained complying with the General Data Protection Act (2018) before data collection begun. Although human participants themselves were not involved in the collection of data,

owners of stallions used for sample collection, veterinarians completing associated procedures and organisations and yard owners (dependent on the research objective) provided informed consent.
Export controls and other legislation and regulation. See Note 8
N/A
What Intellectual Property will be created or used in this research? See Note 9
In the retrospective study, the stud farm owns the rights to the data. A collaboration agreement was signed before data collection. For objective C, the researcher and supervisor shall maintain full rights for the data collected.
What are your plans for long-term preservation and data sharing, where appropriate, and data disposal? See Note 10
If required, data will be disposed of using disk wipe technologies. The IT department at Hartpury University shall be contacted to ensure that university regulations are met regarding the disposal of electronic data. During publication of research and included data, an embargo period of 1 year is standard policy.
Who is responsible for enacting the different elements of the research data management plan? See Note 11
Dr Kathryn Nankervis and I (Imogen Harris) were responsible for ensuring the elements of this data management plan were maintained throughout the research process.
What resources are needed to deliver the plan, and are these available? See Note 12
A password protected Hartpury University Laptop and OneDrive account were provided. An encrypted external hard-drive was purchased by the researcher for the storage and backup of data.

Appendix A3; Site permission form

Name: Imogen Harris
Email: imogen.harris@hartpury.ac.uk

Site permission form:

To whom it may concern,

I _____, in my capacity as manager/owner of _____ grant permission for Imogen Harris to collect retrospective data from databases on previous semen collection processes at my establishment on _____.

I understand that all stallions/stallion owners will maintain fully anonymity for the purposes of this research and future presentations and publications. I understand what the project involves and will facilitate work, which will be presented as a PhD thesis, with aims to publish in an appropriate journal. I also understand that a paper and electronic copy of such data will be kept at Hartpury University on a personal, password protected device, with access restricted to the researcher (Imogen Harris).

Yours sincerely,

Signature of manager/owner_____

Location/facility_____

Please print name/s: _____

Date: _____

Appendix A4; Veterinarian information sheet

Date: November 2020

Testicular sample collection – veterinarian information sheet

To whom it may concern,

Thank you for allowing us access to Thoroughbred testis as part of routine castrations.

Environmental chemicals including those used in plastics and fertilisers, are globally disseminated and are reported to have detrimental effects on semen quality in multiple species including humans, yet research in stallions is minimal.

This research aims to assess the environmental chemical burden in stallion testis and gene expression in reference to equine fertility potential, to assess potential impacts on stallion fertility. By doing this, we hope to be able to advise breeders on management factors to reduce chemical exposure and optimise stallion fertility.

Our research sees us collecting a pair of testis from castration procedures completed by a veterinarian surgeon, freezing one to undertake chemical analysis and treating the other with All Protect Tissue reagent to preserve the testis for RNA and protein samples.

The horse will not undergo any additional procedures other than the routine castration carried out by the attending veterinarian. Treatment of the testes post castration shall be carried out by the researcher (Imogen Harris). Testicular tissue will be disposed of safely by Novus Environmental, complying with Hartpury University regulations.

It is intended that results shall be published, and full anonymity shall be retained throughout this. If you wish to withdraw the data you have provided, please contact me directly via the contact details provided below. Please provide the random stallion code that will have been given to you during data collection. It is possible for data to be withdrawn up until June 2020.

If you are still happy to provide such samples, we would like to ask you to complete the consent form and data sheet and return it at your earliest convenience. If you have any further queries, please do not hesitate to get in touch with either myself or director of studies, the contact details for which are provided below.

Yours sincerely,
Imogen Harris
PhD candidate, Hartpury University
Imogen.harris@hartpury.ac.uk, 07792125604

Director of study: Dr Alison Pyatt, Alison.Pyatt@hartpury.ac.uk, 01452 702100

This research project has received ethical approval from the Hartpury University, Research Ethics Committee

Attending veterinarian consent form – testicular sample collection

Individual Consent

I can confirm that I have read the information sheet and fully understand the use of testicular samples within this research.

I provide full consent for the use of stallion testicular samples for chemical analysis and the *in vitro* studies associated with this PhD project and understand it is intended that results shall be published.

I understand that no horse will undergo any additional procedures other than the routine castration procedure carried out by the attending veterinarian.

I understand that full anonymity and confidentiality shall be retained throughout, including future publications and that all data shall be treated with respect and stored in a secured location.

(PRINT NAME)	hereby give consent for the use of testis from castrations to be obtained and utilised for the research purposes of this PhD project.
Signature of attending veterinarian:	Date
Signature of Investigator:	Date
PRINT NAME OF INVESTIGATORS: **Imogen Harris, Alison Pyatt, Rebecca Sumner**	

Contact details:

Researcher: Imogen Harris, Imogen.harris@hartpury.ac.uk, 07792125604
Director of studies: Dr Alison Pyatt, Alison.Pyatt@hartpury.ac.uk, 01452 702100

This research project has received ethical approval from the Hartpury University, Research Ethics Committee

Protocols and data required from attending veterinarian

To be filled out by the attending veterinarian.

Animal details

Animal weight:_____ Animal height:_____

Body score condition (Please refer to the chart attached) :_____

Castration procedure preparation

Sedation**:**_____

Dose administered (ml):_____

Anaesthetic:_____

Dose administered (ml):_____

Testicular appearance

Do the testis appear healthy (please provide reasoning if not)?

Body Condition Score Chart (Henneke *et al.*, 1983).

Body Condition Score Chart

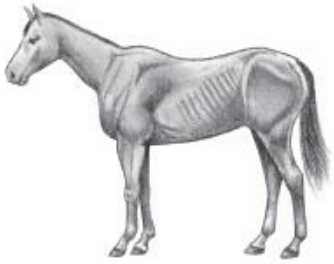
Areas of emphasis for body condition scoring: thickening of the neck, fat covering the withers, fat deposits along backbone, fat deposits on flanks, fat deposits on inner thighs, fat deposits around tailhead, fat deposits behind shoulders, fat covering ribs, shoulder blends into neck

Kentucky Equine Research®

859-873-1988, www.ker.com

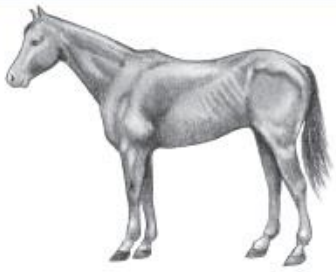
1 Poor

Animal extremely emaciated; spine, ribs, tailhead, points of hip and buttock projecting prominently; bone structure of withers, shoulders, and neck easily noticeable; no fatty tissue can be felt.



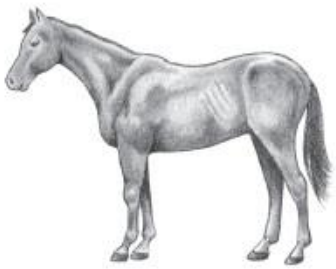
2 Very Thin

Animal emaciated; slight fat covering over base of spine; ribs, tailhead, points of hip and buttock prominent; withers, shoulders, and neck structure faintly discernable.



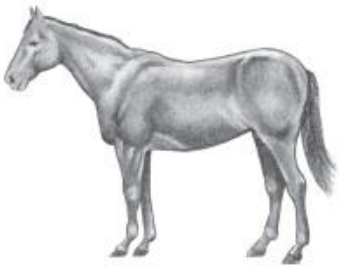
3 Thin

Fat buildup about halfway on spine; slight fat cover over ribs; spine and ribs easily discernable; tailhead prominent, but individual vertebrae cannot be identified visually; points of hip appear rounded but easily discernable; points of buttock not distinguishable; withers, shoulders, and neck accentuated.



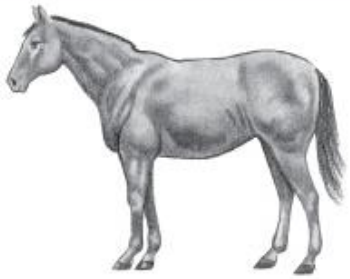
4 Moderately Thin

Slight ridge along back; faint outline of ribs discernable; tailhead prominence depends on conformation, fat can be felt around it; points of hip not discernable; withers, shoulders, and neck not obviously thin.



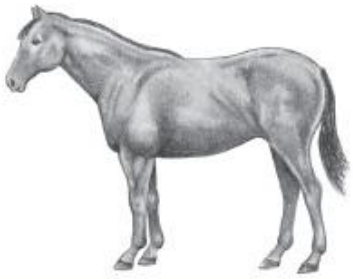
5 Moderate

Back is flat (no crease or ridge); ribs not visually distinguishable but easily felt; fat around tailhead beginning to feel spongy; withers appear rounded over spine; shoulders and neck blend smoothly into body.



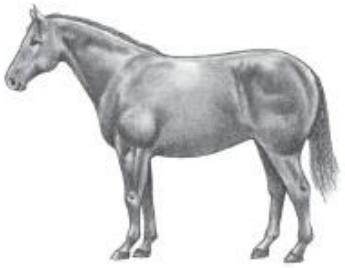
6 Moderately Fleshy

May have slight crease down back; fat over ribs fleshy/spongy; fat around tailhead soft; fat beginning to be deposited along sides of withers, behind shoulders, and along sides of neck.



7 Fleshy

May have crease down back; individual ribs can be felt, but noticeable filling between ribs with fat; fat around tailhead soft; fat deposited along withers, behind shoulders, and along neck.

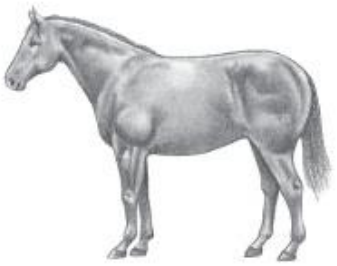


8 Fat

Crease down back; difficult to feel ribs; fat around tailhead very soft; area along withers filled with fat; area behind shoulders filled with fat; noticeable thickening of neck; fat deposited along inner thighs.

9 Extremely Fat

Obvious crease down back; patchy fat appearing.



Kentucky Equine Research, 3910 Delaney Ferry Rd., Versailles, KY 40383, 859-873-1988, www.ker.com

Appendix A5; Stallion owner information sheet, consent form and data sheet

Testicular sample collection – owner consent form

To whom it may concern,

Thank you for allowing us access to Thoroughbred testis as part of routine castrations.

Environmental chemicals including those used in plastics and fertilisers are globally disseminated and are reported to have detrimental effects on semen quality in multiple species including humans, yet research in stallions is minimal.

This research aims to assess the environmental chemical burden in stallion testis and gene expression in reference to equine fertility potential, to assess potential impacts on stallion fertility. By doing this, we hope to be able to advise breeders on management factors to reduce chemical exposure and optimise stallion fertility.

Our research sees us collecting the pair of testis from castration procedures completed by a veterinarian, freezing one to undertake chemical analysis and treating the other with All Protect Tissue reagent to preserve the testis for RNA and protein samples.

The horse will not undergo any additional procedures other than the routine castration carried out by the attending veterinarian. Treatment of the testis post castration shall be carried out by the researcher (Imogen Harris). Testicular tissue will be disposed of safely, complying with Hartpury University regulations.

Additionally, we would be looking to collect data from a short questionnaire for stallion specific details including age. All stallions shall retain full anonymity throughout and allocated random numerical codes to ensure this.

It is intended that results shall be published, and full anonymity shall be retained throughout this. If you wish to withdraw the data you have provided, please contact me directly via the contact details provided below. Please provide the random stallion code that will have been given to you during data collection. It is possible for data to be withdrawn up until June 2020.

If you are still happy to provide such samples, we would like to ask you to complete the consent form and data sheet and return it at your earliest convenience. If you have any further queries, please do not hesitate to get in touch with either myself or director of studies.

Yours sincerely,
Imogen Harris
PhD candidate, Hartpury University
Imogen.harris@hartpury.ac.uk, 07792125604

Director of study: Dr Alison Pyatt, Alison.Pyatt@hartpury.ac.uk, 01452 702100

This research project has received ethical approval from the Hartpury University, Research Ethics Committee

Owner consent form – testicular sample collection

Individual Consent

I can confirm that I have read the information sheet and fully understand the use of testicular samples within this research.

I provide full consent for the use of stallion testicular samples for chemical analysis and the *in vitro* studies associated with this PhD project and understand it is intended that results shall be published.

I understand that no horse will undergo any additional procedures other than the routine castration procedure carried out by the attending veterinarian.

I understand that full anonymity and confidentiality shall be retained throughout, including future publications and that all data shall be treated with respect and stored in a secured location.

If at any point prior to the publication period, I desire to prevent the use of data provided, I understand I have to contact the researcher directly with the details that have been provided.

(PRINT NAME)	hereby give consent for the use of testis from castrations to be obtained and utilised for the research purposes of this PhD project.
Signature of Participant:	Date
Signature of Investigator:	Date
PRINT NAME OF INVESTIGATORS: **Imogen Harris, Alison Pyatt, Rebecca Sumner**	

Contact details:

Researcher: Imogen Harris, Imogen.harris@hartpury.ac.uk, 07792125604
Director of studies: Dr Alison Pyatt, Alison.Pyatt@hartpury.ac.uk, 01452 702100

This research project has received ethical approval from the Hartpury University, Research Ethics Committee

Equine Fertility and Management Data Sheet (to be completed by the owner).

General information

Date of birth of horse: __ / __ / ____ or age if DOB unknown_____

Breed:_____ Country of birth:_____

Intended discipline:_____

Reasoning for castration (routine or other):_____

Does this horse have previous issues of reduced fertility or reproductive problems?

_____

Heritage

Sire and dam of horse (if known):_____

Location

Current address horse is kept at (post code only):_____

Years horse has been kept at this address:_____

Has this horse ever lived abroad (if so, where)?_____

Management factors

Is this horse stabled?_____

What bedding is this horse kept on (straw/shavings)?_____

Does this horse get turn out (if so how often)?_____

Are the pastures treated with any of the following:

Fertiliser:_____ Pesticides:_____

Herbicides:_____ Fungicides:_____

Sewage sludge:_____ Unknown:_____

What hard feed is this horse fed?_____

Is the feed plastic wrapped?_____

Do you provide the horse with hay or haylage?_____

Details of vaccination history:_____

Details of worming history:_____

Appendix A6; Yard owner information sheet, consent form and data sheet

Date: March 2021

Information sheet for: informed consent form

To whom it may concern,

Thank you for your consideration of involvement within this research project. Our research sees us collecting samples of soil, grass and haylage, for chemical analysis.

Sample collection involves removing small cores (with a diameter of no more than 7cm) of soil and taking grass cuttings. Collection methods minimise any associations with disruption to the land, and will pose no risk to horses. If you consent, the collection of the samples shall be carried out by the researcher (Imogen Harris).

Additionally, we would be looking to collect data from a short questionnaire, to investigate management practices of Thoroughbred racing yards.

All details associated with the yard shall retain full anonymity throughout and allocated random numerical codes. If you are happy to provide such samples, we would like to ask you to complete the informed consent form (page 2) and data sheet (pages 3 and 4) and return it (to the email provided) at your earliest convenience. If you have any further queries, please do not hesitate to get in touch.

A note from the researcher:

Given the current circumstances and COVID-19, I would like to reassure you that I shall adhere to all government guidelines during the collection of samples, and if I or anyone I am in contact with falls ill with COVID-19 symptoms or tests positive, I will contact you immediately to rearrange a date.

Yours sincerely,

Imogen Harris – PhD candidate, Hartpury University
Imogen.harris@hartpury.ac.uk, 07792125604

Director of study: Dr Kathryn Nankervis, Kathryn.nankervis@hartpury.ac.uk

This research project has received ethical approval from the Hartpury University, Research Ethics Committee

Informed consent form

Individual Consent

I can confirm that I have read the information sheet and fully understand the collection methods and use of samples within this research.

I provide full consent for the researcher (Imogen Harris) to come on to the property for the purposes of collecting soil, grass and/or haylage samples and understand government guidelines regarding COVID-19 shall be adhered to at all times.

I provide full consent for the analysis of samples for the purposes of this PhD project.

I understand it is intended that results shall be published. I understand that full anonymity and confidentiality shall be retained throughout, including future publications and that all data shall be treated with respect and stored in a secured location.

If at any point prior to the publication period, I desire to prevent the use of data provided, I understand I have to contact the researcher directly with the details that have been provided.

(PRINT NAME)	I hereby give consent for the collection and use of soil, grass and haylage samples to be obtained and utilised for the research purposes of this PhD project and future publication.
Signature of Participant:	Date
Signature of Investigator:	Date
PRINT NAME OF INVESTIGATORS: **Imogen Harris**	

Director of study: Dr Kathryn Nankervis, Kathryn.nankervis@hartpury.ac.uk

Land Management Data Sheet

General information

Please circle the samples you are willing to provide for analysis:

Soil Grass Haylage

Current land address (postcode only):_____

How long have you managed this land for?_____

Pasture treatment and land management

If known, which species or types of grass are present on the pastures being sampled?

_____
_____
_____

How would you describe the soil type in the pastures being sampled?

_____
_____
_____

Are the pastures currently treated with any of the following (Y/N):

Fertiliser:_____ Pesticides:_____

Herbicides:_____ Fungicides:_____

Sewage sludge:_____ Other:_____

Are the pastures used for purposes other than the turnout of animals (including hay, haylage or silage harvest)? If it is, please specify its uses.

_____
_____
_____

Please provide a description of land management (mechanical treatment, resting, rotations) and turnout regimes (turnout frequency, turnout of animals other than horses) utilised for each season:

Autumn (September, October, November):

_____
_____
_____

Winter (December, January, February):

_____
_____
_____

Spring (March, April, May):

_____
_____
_____

Summer (June, July, August):

_____
_____
_____

Exposure to environmental contaminants and the impact on reproductive health

Imogen Harris,[a] Richard Lea,[b] Rebecca Sumner[b]
[a]Hartpury University and Hartpury College, Gloucester, United Kingdom
[b]School of Veterinary Medicine and Science
The University of Nottingham, Sutton Bonington, United Kingdom

Abstract

Reports are illustrating increasing evidence of perturbed reproductive health in a variety of species. Given the rate of change and the widespread occurrence amongst a variety of species, such observations allude to an environmental drive opposed to a natural genetic change. Extensive use and dissemination of plastics that contain anthropogenic organic chemicals is suggested as a plausible etiology of such adverse fertility trends given the drastic increase in global plastic production. Direct industrial emission and chemical migration from plastic product matrices, in which such chemicals originate, leads to environmental deposition. Once ubiquitous within the environment, such environmental chemicals, otherwise known as xenobiotics that are known to modulate endocrine signalling, are consistently available for uptake by humans and animals on a global scale. A variety of species are proposed as sentinel models to further explore the impact of a polluted ecosystem on reproductive health. Pregnant animal exposure to xenobiotics, during the key developmental programming window, is of great concern due to the potential for epigenetic modifications on the developing fetus. This review aims to discuss such concepts and routes of exposure, to highlight areas for further research within the field.

Keywords: Xenobiotics, reproduction, dog, fertility, environment

Introduction

Global plastic production reportedly stands at 320 x 10^6 tons per annum. Around 40% of such plastics are single use and contain a variety of anthropogengic organic chemicals. Direct industrial emission and migration from product matrices, in which such chemicals originate, leads to environmental deposition.[1] Exposure to environmental chemicals (ECs), often endocrine disruptive in nature, have been suggested in the etiology of adverse fertility trends.[2] Common anthropogenic organic chemical classes include; bisphenols, dioxins, phthalate ethers, parabens, polycyclic aromatic hydrocarbons, and perfluorinated compounds (PFCs), with existing overlap between some chemical congeners.[3,4] Such chemicals are typically produced through or utilized in a range of industrial and agricultural processes.[5] These include uses as plasticisers, flame-retardants, solvents, preservatives, additives, coatings, pesticides, herbicides, fungicides, and fertilizers. Given the ability of ECs to leach from products into nearby surroundings, chemicals remain ubiquitous within the environment; present in air, water, soil, and sediment.[6,7] Here, ECs are consistently available for uptake by humans and animals on a global scale, through a variety of means.[8]

With global increases reported in the occurrence of obesity, metabolic syndrome, and associated diseases (e.g., PCOS and diabetes) it has been postulated that this is also linked to exposure to chemicals, giving rise to the term 'metabolism disrupting chemicals'.[9] In humans, reports suggest that the etiology and pathophysiology of metabolic diseases could be due to environmental chemical exposure.[10] Many chemicals that appear to impact on metabolic function, also have endocrine disrupting activity and thus may adversely affect both reproductive function and metabolic disease.[11]

Exposure routes of environmental chemicals

One of the main deposition pathways for EC contamination is via a carnivorous diet. Consumption of contaminated meat, particularly when fat content is high, is considered as a main exposure route in carnivorous and omnivorous species, given the lipophilic nature of many ECs. This leads to biomagnification across trophic levels, with apex predators incurring the highest bodily burdens. Considering 3 aquatic species with high blubber content but differential dietary sources, the herbivorous dugongs has polybrominated diethyl ester biological burdens of 120 ng/g lipid weight.[12] This level, whilst high, is still 8 times less than the apex predator species, the killer whale.[13,14] Similar biomagnification of ECs has been reported in terrestrial ecosystems and agricultural food chains,[15] as part of human consumption.[16]

Dog is a close companion to man and shares the same habitat. For this reason, dog is exposed to the same environmental conditions, including environmental chemicals present in home. For this reason, dog is considered, an ideal sentinel model to investigate human exposure to environmental pol-

lutants.[14] Canine diet (commercially available pet foods) has common environmental chemicals.[17] Since similar chemical types were detected in dog semen and testes (collected from routine neuters), effects of environmental/gonadal contaminants on sperm quality parameters were tested. Deleterious chemical effects were reported on the quality of DNA and sperm motility in dog and human.[18] Food assessed for environmental chemicals contained meat sources from grazing animals (consumed by man who have a meat-based diet).[17]

Pasture contamination through the routine use of sewage sludge fertilizers (biosolids) is therefore of primary concern.[19] Such fertilizers promote the deposition of a broad range of toxic chemicals onto agricultural land, in addition to complex mixtures of microplastics.[20] The ability of chemicals originating from their polymer matrices, to leach into the surrounding environment or digestive tract, if ingested, provides an initial exposure route to biota. Fetuses from pregnant ewes grazed on such pastures, as well as their offspring, exhibit perturbations in in both female and male reproductive development.[17,21,22]

Highly chlorinated congeners, including polychlorinated biphenyls (PCBs), bond strongly to soil organic matter, reducing the uptake into plants through root structures, and instead are readily absorbed from the surrounding air.[8] However, when pasture concentrations are lower, direct consumption of contaminated soil may result in an additional exposure route to grazing species. Around 21,000 tonnes of surface soil are reported to contain PCBs[23] that are known reproductive toxicants.[17,18] Although banned in the 1970's, these ECs are reported to be present within UK soil at an average concentration of 2.52 mg/kg, with the highest concentrations reaching 80.6 mg/kg.[24] As alluded to above, biomagnification within the human can then ensue from a meat-based diet, by consuming animal species that have grazed on such treated lands. Theoretically, this would mean that a plant-based diet would result in lower biomagnification of contaminants.

Unpublished, preliminary data in the horse actually showcases concentrations of certain contaminants to be higher than sentinel models (e.g., dog) fed on meat-based diets (Harris, unpublished data). This is potentially indicative of alternative environmental exposure (e.g., water or plant-based feedstuffs). A common approach to breaking down contaminated materials in soil is a process called bioremediation. Using fungi or bacteria alongside plant material, ECs can be drawn from the soil into plant matter. Reports illustrate the ability of plant 'alfalfa' to remove PCBs from contaminated soil by uptake into roots and leaves.[25] Although this is a beneficial aspect for clearing contaminated soil, due to the digestible energy content of alfalfa, this feedstuff is suggested for pregnant herbivores and breeding stallions.[26] Giving rise to a potential exposure route for herbivorous diets.

A further risk factor is xenobiotic run off into water systems. Although a range of xenobiotics are reported present in water, [27,28] di-ethyl hexyl phthalate (DEHP) is a common plasticizer and a reported carcinogen,[29] known to perturb reproductive health at lower exposure concentrations.[17] This phthalate is present in tap water, bottled water and barrelled water supplies. Concentrations of DEHP were initially greater in tap water, but increased concentrations of DEHP were observed in plastic bottled water that was heated to 60°C, a finding expected due to the ability of chemicals to leach from the product matrices.[30] This increase in chemical pollutants is not restricted to plastic products. It is estimated that with every 1°C increase in environmental temperature, the volatility of polychlorinated bi-phenyls would rise by around 10 - 15%, increasing pollutant mobility and promoting further uptake within the ecosystem.[31] Such change could be an addition to the concept of biomagnification (Figure 1).

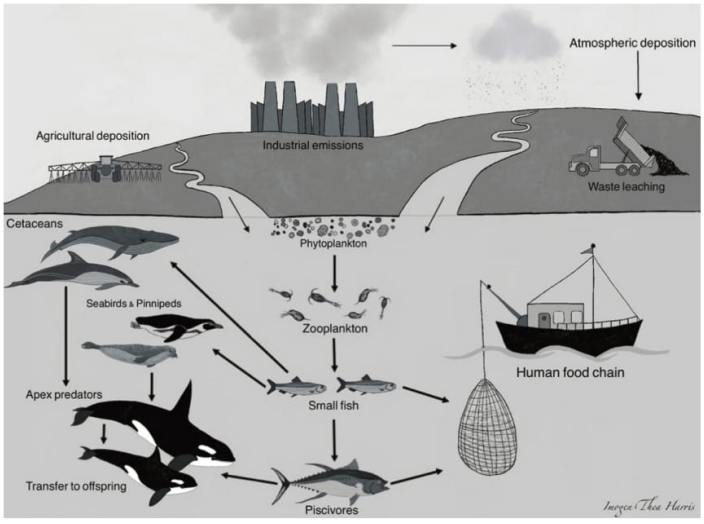


Figure 1. Biomagnification of xenobiotics within the aquatic ecosystem. Industrial processes, agricultural deposition and waste leaching gives rise to biomagnification within the ecosystem, starting from consumption of phytoplankton, magnifying within endangered apex predators. A similar process occurs on land. Figure not to scale. Figure is authors own (I.T.H).

Fetal environment

Environmental chemical exposure is likely to occur throughout life, beginning in utero, continuing postpartum, throughout adolescence, adulthood and gametogenesis. Placenta is a dynamic endocrine organ and has incorporating roles (e.g., homeostasis, fetal growth and sustaining pregnancy).[32] Perturbed placental function can impact fetal development and growth, contributing to chronic health issues in adult life.[33] Mono (2-ethylhexyl) phthalate, the primary metabolite of DEHP, is reported to perturb trophoblast differentiation, thereby acting as an endocrine disruptor to the very early developing placenta.[34] Intrauterine environment is where the fetus is most susceptible to the exposure of external ECs due to endocrine mediated developmental period.[35] It is thus concerning that ECs (e.g., phthalates[36] and bisphenols [BP][37]) have the ability to cross the placental barrier, exposing the developing fetus to a range of toxic chemicals. In an in vivo murine model, BP-A and BP-S altered 13 sets of identical placental genes, causing morphological defects within the midpregnancy placenta that persisted until parturition.[38] Such exposure may lead to the indirect disruption of essential developmental processes of the gonads and reproductive system, leading to chronic reproductive perturbations in an adult life.

Fetal transfer is complex, although placenta works to protect the fetus against exposure to xenobiotics there is the common consensus that ECs have an accumulatory nature towards fetal compartment due to cross talk of signalling pathways.[39] Analogue lipophilicity, polarity and hydrogen-bonding are reported to impact placental transfer efficiency.[37,40] Relatively lower concentrations of bisphenol congeners are actively transported to the fetal compartment.[41] Aryl hydrocarbon receptor is highly expressed within the placenta and a key receptor that works to protect the maternal-fetal interface and placental barrier from xenobiotic exposure. It is hypothesized that pollutants (e.g., bisphenol-A), interfere with the activity of the aryl hydrocarbon receptor, reducing the typical endocrinological function and metabolic activities of the placenta.[42] Due to the physiochemical specificities of chemical metabolites and their inability to be removed from the fetal compartment in their glucurono-conjugated forms, a back-metabolism cycle is initiated by which the bioactive forms are resynthesized. This cycle increases fetal exposure substantially.[43] For BP-S, although the placental transfer in the materno-fetal direction was only 0.4%, this back-metabolism increases fetal exposure to the bioactive form by 87%.[41] Additionally, it has been suggested that amniotic fluid acts as a reservoir by which the fetus is reexposed to BPS through swallowing and dermal adsorption, raising further concern over fetal exposure.[37,41]

ECs are also reported to have reprotoxic effects through endocrinological interactions.[44,45] Depending on specific chemical composition, steroidogenic perturbations are a result of affinities for different receptors. This is an area that certainly needs to be furthered within an appropriate sentinel model. Preliminary data assessing the addition of PCB-153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) and DEHP on LH-induced testosterone secretion in the canine sentinel, to determine the impact of toxicants on endocrine function, did not appear to inhibit endocrine function.[17]

Male reproductive health

There is an increasing body of published evidence indicating that human male fertility and reproductive health has declined over the last 40 - 60 years. Geographically dependant temporal declines in human semen quality are becoming an increasing concern, with meta-analytical studies suggesting an approximate 50% decline in sperm concentration over the past 70 years.[46,47] Results from these meta-analytical studies remain heavily scrutinized, a result of developments in semen analysis methodologies, heterogeneity and the inclusion of historical data sets. Such limitations were suggested to be substantial factors influencing the adverse trends reported, thus questioning the true declines in semen quality.[48] Following the application of stricter inclusion criteria, in addition to completion in accordance with standardized protocols (meta-analysis of observational studies in epidemiology)[49] more recent meta-analyses continue to suggest a decline in fertility.[50] This rate of decline appears to have no plateau, raising substantial concerns for future male fertility.

Temporal declines are specific to the Western world, including Europe, North America, Australia and New Zealand, with trends failing to prevail in South Africa, Asia and South America.[50] Such trends, with distinct geographical variation would suggest the influence of environmental factors, although socio-economic contexts could pose as additional contributors. Parallel trends in sperm quality parameters to that in humans have been reported in a dog sentinel model, with an overall decline of 30% in progressive sperm motility.[17] Geographical variation is also evident within exposure to environmental chemicals. Dog sentinel model showcases how testicular chemical profiles vary regionally, alongside varying testicular pathological profiles. Testis collected from Finland had reduced pathologies compared to testis collected from Denmark and the UK. Such findings provide additional support to the concept that the environment likely influences reproductive function.[51] Many socio-economic interactions do not retain relevance in such species. Additionally, data were collated from a single lab with consistent analytical methods, thereby adding to the weight of evidence exhibited in the human and limiting the criticism over semen quality assessments. Decline in semen quality is proposed to be associated with bioaccumulation from meat-based diets, supported by the fact that a meta-analysis of temporal trends in the herbivorous stallion sperm quality does not appear to be overly altered. [52] However, collecting sperm quality data from a singular lab, preliminary data do actually have similar patterns of declining sperm quality within the herbivorous breeding stallion (Harris, unpublished).

As mentioned, dog, given the association to human lifestyle, is proposed as a useful sentinel model to assess the deleterious impact of environmental chemicals on human reproductive health. Undertaking an updated analysis of temporal trends in canine sperm quality, originally assessing sperm quality over a 26-year time frame,[17] continued to have a decline in sperm motility over time, yet not to as a substantial degree (Figure 2).

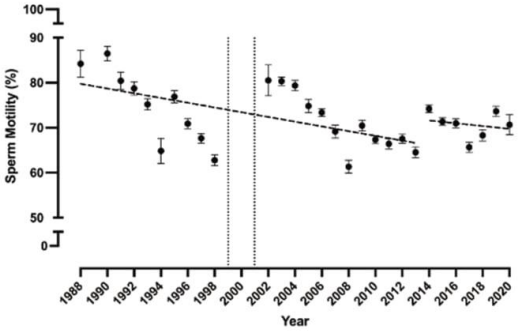


Figure 2. Updated analysis of temporal trends in canine sperm quality over a 32-year timeframe. Percent normal sperm motility. Data expanded utilising part of published data.[17] Scientific report articles are published under a CC BY license allowing for maximum dissemination where users are free to adapt data. Error bars represent ± 1 SEM. Vertical dotted lines represent time point within the programme where dogs with poor semen quality were removed from the programme. The cause of this is unknown. Diagonal lines showing declining sperm quality are plotted for graphical purposes only.

Temporal trends in sperm quality are also linked with global increases in reproductive perturbations; including testicular cancer (TCa) and genitourinary abnormalities, such as cryptorchidism and hypospadias.[2,53] This is evident within both the primate and dog. The reproductive trends present today are collectively termed testicular dysgenesis syndrome.[2] Globally, TCa incidences in humans have increased 2-fold over the past few decades, with most cases apparent in younger generations. Although a variety of factors could give rise to such changes, these trends are suggested to be a result of toxicant exposure at vital periods of sexual development.[54,55] Over a similar time frame, increases in cryptorchidism have been reported in male pups from the same population of stud dogs that exhibited a decrease in sperm motility.[17] Preliminary evidence within the canine additionally suggests an increased incidence of testicular tumours over a 40-year period.[56] Geographical variation in human reproductive perturbations are also heavily reported, with higher incidence rates in industrialised and agricultural areas, indicative of interactions between chemicals utilized within these industries.[19,57]

Female reproductive health

A further sensitive window of exposure is during the complex process of follicle development from the primordial follicle pool to mature preovulatory Graafian follicles. Ovarian somatic cells are specialised, multidisciplinary cells that are paramount for optimum reproductive function and follicle differentiation; with roles in germ cell support steroidogenesis and growth.[58] In canine sentinel model studies, certain environmental chemicals were present at higher concentrations in the dog ovary then in the testis (Van der Mescht, unpublished data). In addition, when coculturing murine ovarian tissue with the chemicals present within the canine ovary, there is an enhanced sensitivity to of the earlier follicle types (primordial and primary follicles). With the follicular population formed during development determining the reproductive lifespan of an individual, ensuring a balance between apoptosis, proliferation and differentiation is crucial. Primordial follicles are those that define the ovarian reserve, therefore modifications to such follicles could be detriment to fertility. Understanding how environmental chemicals affect the molecular and biochemical signalling of the granulosa cell, to support the oocyte, is an area that requires further study.

Key programming window and transgenerational impact of ECs

It has been suggested that EC exposure may manifest long after initial exposure due to epigenetic modifications originating from exposure at the critical window of genitourinary development in the foetus and new-born.[59,60] Modifications include DNA methylation, microRNA or histone alterations.[59] As discussed above, placenta is a dynamic organ that supports fetal development. Studies are beginning to indicate how exposure to phthalates during pregnancy is associated with genome wide modifications of placental DNA methylation, impacting fetal development.[61] Focussing on reproductive development, the bipotential gonad during early pregnancy incorporates many signalling pathways and molecules that instigate, and control, crucial embryological developmental pathways. Utilization of mouse knockout models provides insight into such genetic determinants of the bipotential gonad development. GATA binding protein 4 (Gata4) remains as 1 of the earliest markers crucial for formation and development of the gonadal ridge.[62] Loss of Gata4 gene expression is reported to inhibit formation of gonadal ridge.[62] Further development and maintenance of the gonadal ridge is determined by genes (e.g., Wilms' tumour suppressor 1 gene and steroidogenic factor 1) essential for early gonadal development.[63] Under the influence of WNT signalling, the binary fate decision of gonadal formation is chosen. A recent review discusses how the observed human male reproductive disorders might have a fetal origin, due to an androgen dependant programming window during early gestation.[64] One of the major signalling pathways throughout ovarian differentiation is that of WNT4/RSPO1 signalling.[65] In the absence of the male sex determinant gene, the cascade of genetic pathways to promote female development sees WNT mediated stimulation, like that of the ligand WNT4 induce the expression of downstream effectors,[66] such as follistatin and β-catenin.[67] β-catenin is a pro-ovarian signalling molecule which induces expression of the pivotal female development transcriptional target, FoxL2.[68-70] Should a toxicant impair the expression of male sex determining genes in an XY embryo, then it is plausible for WNT mediated stimulation to ensue, to follow the feminisation pathway.[71]

Epigenetic mechanisms, with adverse effects on reproductive potential, are considered to be transgenerational.[72,73] In killer whale populations, EC concentrations in calves are higher than in their lactating mothers.[74] Comparably, in suckling polar bear cubs, PCB concentrations surpassed that of maternal contamination.[75] Such research demonstrates the accumulatory nature of toxic ECs within the developing neonate. The subsequent exposure of such chemicals to the suckling offspring is likely to perturb reproductive development and future fertility, as reported in other species,[76] having future transgenerational impact. The transgenerational impact of contaminants

has been shown to induce delayed pubertal onset, impaired gametogenesis and impaired steroidogenic gene expression. Furthermore, maternal behaviours have also been shown to be impaired following transgenerational studies of toxicant mixtures, raising concern of not only reproductive health, but also wellbeing of future generations.[77] Within the sheep model, exposure to environmental contaminants has been shown to induce testis transcriptome modifications, which authors report, if not corrected by or during puberty, would likely have adverse outcomes for future generations adult life.[22] Reports discuss how epigenetic mechanisms are the means by which xenobiotics mediate such transgenerational effects.[78]

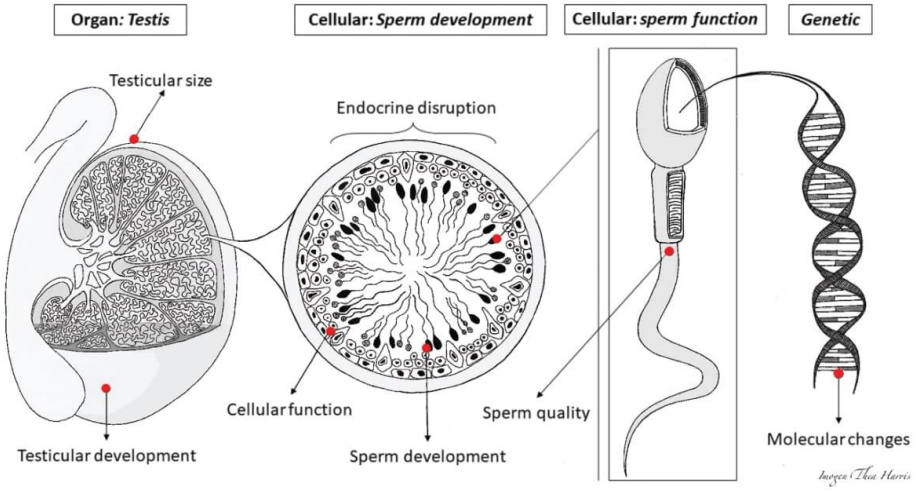


Figure 3. Avenues for xenobiotic perturbations within testicular tissue. As the housing unit of sperm development, testicular health has a significant impact on sperm quality and reproductive success, from pathological perturbations to epigenetic modifications. Figure is authors own (I.T.H).

Conclusion

The sentinel has historically been exploited as a model for bio-monitoring environmental conditions in addition to health responses to toxic and infectious agents in other populations of species, including humans.[79] Common biomonitor species include ants,[1] birds,[80,81] sheep,[82] dogs,[14] and aquatic species (oysters, fish, and killer whales).[83-85] Many socio-economic interactions do not retain relevance in such species, although exception is given to species undergoing artificial reproductive techniques, which may still influence trends in reproductive health. Canids and felines share close environmental conditions with that of their owners, thus representing important models for bio-monitoring human health.[14] Although the precise drivers behind these reproductive temporal trends remain uncertain, there is increasing evidence that anthropogenic environmental change may be a key factor.[86] There is a distinct need to increase public understanding in order to promote a sustainable future. Although microorganisms are reported to be able to degrade toxic environmental compounds,[87] the use of the dog as a sentinel model, or additional sentinel models, would add substantial information in order to further or dispute previous research, and add to the weight of evidence that showcases how xenobiotics are perturbing the environment and future health of individuals. Only with further research can we work to drive change within a polluted environment.

Conflict of interest

There are no conflicts of interest to disclose.

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Appendix C: Systematic analysis of breed, methodological, and geographical impact on equine sperm progressive motility

animals

MDPI

Systematic Review

Systematic Analysis of Breed, Methodological, and Geographical Impact on Equine Sperm Progressive Motility

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Simple Summary: Semen quality is an important indicator of reproductive health and fertility. With adverse temporal trends in human semen quality over the past 50 years paralleled in male animals, there is increasing concern for the causes and implications of perturbed male reproductive health. The evaluation of equine progressive motility (PM), a parameter closely associated with fertility, provides information on the fertilising capacity of equine ejaculate and current reproductive health of the equine stallion. Using systematic analysis, recent trends in equine PM were determined from 696 estimates from 280 individual studies. Temporal trends indicate equine PM has not significantly changed between the years 1990 and 2018. Significant breed, methodological, and geographical variations observed in equine PM may considerably influence actual and reported stallion fertility potential. Information on stallion PM meaningfully contributes to the wider literature on semen quality and provides avenues for future stallion fertility research. This systematic analysis presents the wider challenges associated with semen quality assessment, particularly within the equine sector, and provides recommendations to promote consistency across industry and research.

Abstract: Over the past five decades, there has been increasing evidence to indicate global declines in human semen quality. Parallel adverse trends measured in male animals indicate a potential environmental aetiology. This study evaluated the progressive motility (PM) of stallion ejaculate through a systematic review and meta-analysis. A total of 696 estimates of equine PM from 280 studies, which collected semen samples between the years 1990 and 2018, were collated for meta-analysis. The method of motility analysis, breed, season of collection, and geographical location were extracted. Simple linear regression determined temporal trends in stallion PM. Studies using microscopy estimated PM to be significantly greater compared to computer-automated methods ($p \leq 0.001$). For Arabian breeds, PM was consistently higher than other breeds. Over time, there was a significant decline in PM for studies from Europe ($n = 267$) but a significant increase for studies from North America ($n = 259$). Temporal trends indicate the fertilising capacity of equine ejaculate has remained consistently high in the last three decades. That being so, variations observed suggest methodological, geographical, and individual stallion differences may significantly influence actual and reported stallion fertility potential.

Keywords: equine; progressive motility; temporal trends; semen analysis; breed; seasonality; geographical location

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1. Introduction

Semen quality is an important indicator of male reproductive health and fertility [1,2]. Over the past five decades, there has been increasing concern over declining semen quality across species [3–6]. Reduced sperm count is associated with reduced reproductive potential and the development of reproductive disorders, such as cryptorchidism, hypospadias, and testicular cancer [2,7]. The decline in human male fertility has remained controversial since the first meta-analysis reported a global reduction of 50% in mean sperm density [3]. Aggregation of heterogenous studies and absence of systematic approaches to control bias were identified as limiting the validity of semen quality trends [3,8–10].

Subsequent reanalysis and independent studies support global temporal declines in semen quality, suggesting increasing concern around the causes and implications of reduced male fertility [6,11–15]. A rigorous meta-analysis of global trends in human semen quality reported a 50–60% decline in sperm count in North America, Europe, Australia and New Zealand in the period from 1973 to 2011 [6]. Geographical variations in semen quality, manifested by stronger declines in Westernised regions, suggests sperm count may reflect environmental and lifestyle influences.

Global adverse trends in male reproductive health have been shown in different species. Between 1988 and 2014, progressive motility (PM) declined by 30% in a population of breeding dogs, whilst incidences of cryptorchidism increased [5]. Temporal changes in semen quality have been reported in the Breton Draught and Anglo-Arab Thoroughbreds in France (1981–1996 and 1985–1995, respectively), as well as the Holstein Dairy bull in the USA (1965–1995) [4,16,17]. Semen volume decreased for both stallions and bulls alike. Sperm concentration was found to have increased for stallions, yet decreased for bulls over their respective study periods [4,16]. Stallion sperm production remained unchanged between 1981 and 1996; as such, the increasing trend for sperm concentration may be linked to its inverse relationship with seminal volume [16]. As seminal volume is androgen-dependent, this raises concerns for the cause of declining stallion seminal volume [16,18].

Information on stallion semen quality trends is limited to Breton Draught and Anglo-Arab Thoroughbred stallions in the late 1900s and may not be considered generalisable to stallion populations in the 21st century. The development of assisted reproductive technologies (ARTs) mitigates the effects of male factor infertility, diminishing evolutionary pressure for fertility in stallions [19–21]. Current fertility indexes in the equine sector are influenced by factors extrinsic to the stallion, such as per-cycle conception, pregnancy, and foaling rates [22,23]. There is a need to evaluate global reproductive trends in the breeding stallion to determine the status of stallion fertility. This review initiates an approach to elucidate stallion PM trends during the period 1990 to 2018, and therefore identify factors impacting stallion PM. A meta-analysis assessing equine PM will meaningfully contribute to the wider literature on semen quality trends and provide recommendations for future research to support stallion fertility. This study presents significant methodological, geographical, and breed variations in stallion PM, representing the wider challenges in the equine sector that need to be addressed to understand the considerable variation in semen quality among stallions.

2. Materials and Methods

2.1. Ethics

Ethical approval for this study was granted by the Hartpury University Ethics Committee (Ethics Application Number: ETHICS2020-21-LR).

2.2. Systematic Review

The conduct and reporting of the systematic review adhered to Meta-analysis Of Observational Studies in Epidemiology (MOOSE) guidelines throughout. A comprehensive search of literature was conducted to identify articles that reported data on equine progressive motility. PubMed (access to MEDLINE database; https://pubmed.ncbi.nlm.nih.gov/), PubAg (the U.S Department of Agriculture and National Agricultural Library search

engine https://pubag.nal.usda.gov/), and BASE (Bielefeld Academic search engine; https://www.base-search.net/) were searched in the period from 13 January 2021 to 10 February 2021. Boolean search phrases were used to improve the specificity and sensitivity of the search: (stallion* OR equine* OR colt* OR horse*) AND (semen AND quality OR sperm* OR insemin* NOT human*) NOT horseradish. The 'Mendeley reference management system' was used to manage studies retrieved from the literature search.

2.2.1. Eligibility Criteria

The review considered all studies that reported primary or retrospective data on equine PM, defined by WHO as "sperm moving actively, either in a linear or large circle, regardless of speed" [1]. Studies were included if semen samples were collected from healthy reproductively normal stallions using standard procedures. Microscopy and computer-assisted sperm analysis (CASA) were considered acceptable methods of PM assessment (Table 1). Typically, stallion sperm quality is assessed via assessment of morphology and motility but with greater emphasis placed upon per cycle foaling rates. Although computer-automated methods have increased repeatability due to the ability of computer algorithms to track swimming speed, agreement between CASA and manual microscopy assessment is frequently reported in several species, including for stallion motility [19]. All subgroups within an individual study, which met the eligibility criteria, were included [24,25].

Table 1. Eligibility criteria for the systematic review and meta-analysis.

Inclusion	Exclusion
• Domesticated *Equus caballus* only. • Semen collection via an artificial vagina including Hannover, Colorado, Missouri, French, Botupharma, Avenches, and Roanoke models. • Semen collection utilising a phantom, live mare, or via ground collection. • Semen quality analysis of progressive motility. • Progressive motility assessment via Computer-Assisted Sperm Analysis or microscopy analysis. • Semen samples assessed less than 24 h after collection without the addition of extender (raw), or with the addition of an extended (fresh). Semen samples assessed after cool-storage within a 72-h period (cool).	• Alternative sub-species of the *Equus* genus. • Non-standard methods of semen collection, including epididymal retrieval or stimulation of ejaculation via pharmacological methods or electroejaculation. • Semen quality parameters given for sexed semen samples. • Stallions displaying signs of perturbed reproductive health including anatomical, seminal, and bacterial or poor libido. • Parameters recorded for cryopreserved or thawed semen samples.
• English language documents only. • Peer-reviewed published literature including primary and retrospective data sets and case reports. • Academic grey literature including dissertations and theses, conference presentations, and posters.	• Published in a language other than English and without translation. • Data presented in the format of a review article or opinion article. • Duplicate datasets.

2.2.2. Article Screening

An adapted MOOSE systematic review flow diagram was used to identify and screen articles eligible for inclusion (see Figure 1). Title and abstract screening formed the first stage of the screening process. Studies that indicated stallion PM was a measurable outcome in either the title or abstract were considered eligible. Duplicate datasets were identified and removed at stage one. Unique studies eligible for stage two were exported to NVivo (QSR International version 12) to tabulate justifications for inclusion/exclusion of studies in the stage two screening process. The full text was reviewed and either assigned as eligible for quality assessment or to exclusion with justified reasoning based on predefined categories informed by the eligibility criteria [25]. Articles accepted for stage two screening were used to identify additional articles using citation searches. Backward citation searches were conducted using the reference lists of obtained articles [24]. Forward citation searches were conducted by inputting the title of each record into Google Scholar (https://scholar.google.com) and using the "Cited by" function.

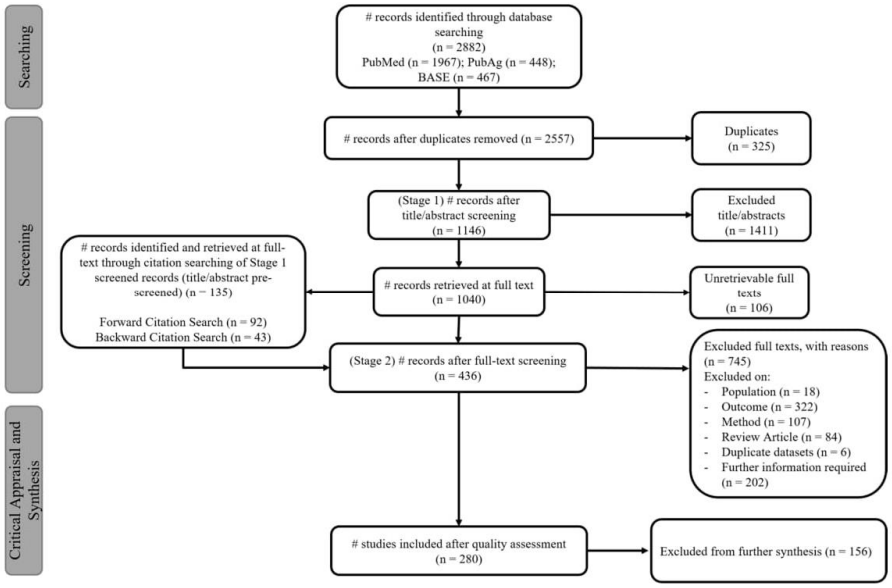

Figure 1. An adapted MOOSE systematic review flow diagram to identify, screen, and critically appraise records for inclusion in the current review [24].

2.3. Quality Assessment

Assessment of study quality was informed by the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Checklist and based on six domains of bias: selection, performance, detection, attrition, reporting, and confounding [25,26]. In response to the signalling questions, a category for high, uncertain, or low risk was assigned for each domain of bias. For each study, an overall judgement was made to accept or reject the study for data extraction based on the risk of bias assigned to each domain [25,26]. Quality assessment was conducted in NVivo to tabulate the risk of bias for each domain [27].

2.4. Data Extraction

Data extraction was performed in MS Excel (Microsoft Office, version 2019). The summary statistics on percentage of the progressively motile sperm (mean, median, standard deviation, standard error) were extracted from 260 peer-reviewed journal articles and 20 grey literature records (Master's theses, doctoral theses, non-peer reviewed articles, and conference proceedings/abstracts). Additional data on the year of collection, season of collection, geographical location, method of PM assessment (CASA or microscopy), and stallion breed were also extracted (see Table 2). If not specified, the year of sample collection was estimated by calculating the average difference from other publications and subtracting this from year of publication, as followed by other literature [6]. The geographical location was determined by the continent of the first author affiliation and/or the continent in which ethical approval was granted [7]. The breed categories were determined based on the predominant breed-types present among all individual studies [28]. For studies in which the population comprised of multiple breeds, the predominant breed type was chosen for assigning the breed category.

Table 2. Categorisation of variables for statistical analysis (geographic location, breed, and season of collection) based on data extracted from individual studies.

Category for Analysis	Data Extracted from Individual Studies	
Geographic Location	**Countries Included**	
Africa	Egypt, South Africa	
Asia	India, Iran, Saudi Arabia, Thailand	
North America	United States, Canada, Mexico	
South America	Argentina, Brazil, Chile, Colombia	
Europe	Austria, Belgium, Czech Republic, Finland, France, Germany, Italy, Netherlands, Poland, Portugal, Spain, Sweden, Switzerland	
UK	United Kingdom	
Other	Australia, New Zealand, Russia, Turkey	
Breed category [1]	**Horse breeds included**	
Andalusian	Lusitano, Peruvian Paso, Lipizzaner, Mangalarga Marchador, Brazilian Jumping, Spanish Purebred, Sorraia, Garrano	
Arabian	Arabian, Anglo-Arab	
Draught	Draught, Polish Coldblood	
Miniature	Miniature, Shetland, Miniature Caspian Pony	
Pony	Brazilian Pony, Pony, Connemara, Welsh Pony	
Quarter Horse	American Quarter Horse; Azteca Horse, Thoroughbred; Trakehner, Manipuri, Standardbred, American Paint Horse, Friesians, Thai Native X, Pantaneiro, Trotter, Maremmano, Finnhorse	
Warmblood	Hanoverian, Holsteiner, Appaloosa, Dutch Warmblood, German Warmblood, Oldenburg Warmblood, Rhinelander, Chilean Purebred, Marwari, Kathiawari, Zanskari, French Saddlebred, Westphalian, Swedish Warmblood, Old Kladruber, Criollo Colombiano, Brandenburg, Belgium Draft, Franches-Montagnes, French Warmblood, Polish Warmblood, Haflinger	
Month of Collection	**Northern Hemisphere**	**Southern Hemisphere**
November-February	Winter	Summer
March-June	Spring	Autumn
July-August	Summer	Winter
September-October	Autumn	Spring

[1] Breed category = breed categories were determined based on the predominant breed types reported among individual studies. For studies in which the population comprised of multiple breed types, the predominant breed type was chosen for assigning the breed category, as such breed categories are not exclusive.

2.5. Statistical Analysis

Data extracted and recorded in the digital spreadsheet were analysed using GraphPad Prism 9 (GraphPad Prism version 9.0, GraphPad Software, California, CA, USA). Statistical analysis was based on 696 unique estimates for PM collected from 280 studies between 1990 and 2018. The Shapiro–Wilk test determined whether parametric or nonparametric statistical tests were used [29]. Simple linear regression was used to predict PM as a function of year of semen collection, and in relation to method of motility analysis (CASA or microscopy), geographical location, and to investigate whether year of semen sample collection predicted PM dependent on preparation method prior to assessment. One-way analysis of variance (ANOVA) or Kruskal–Wallis test investigated the differences in PM between season of collection, geographical location, and breed category. Dunn's multiple comparison post-hoc test analysed differences between stallion breed and geographical location. A Mann–Whitney test was used to compare significant differences in PM between CASA and microscopy methods. A two-way ANOVA determined whether stallion breed affected PM in raw, fresh, and cool semen samples (Table 1). Tukey's multiple comparison post-hoc test analysed differences between seasons and stallion breeds, respectively, for PM separated by raw, fresh, and cool semen samples. Results were considered significant when $p \leq 0.05$.

3. Results

The systematic search of stallion semen quality literature returned 2882 records and an additional 135 records through citation searches. Of these, 325 duplicates were removed, 1411 records were excluded after title/abstract screening, 106 could not be retrieved at full text, 436 were excluded after full-text screening, and 156 were excluded from further synthesis after critical appraisal (Figure 1). The results are based on 696 unique estimates for stallion PM from 280 studies between 1990 and 2018.

3.1. Year of Semen Collection

The mean percentage of PM in the period from 1990 to 2018 was 51.06% ± 0.33 (±SEM). There was no significant change in PM in the period from 1990 to 2018 (Figure 2A; slope = −0.007497, R^2 = 0.00005, $p \geq 0.05$). When PM was separated into raw, fresh, and cooled samples and plotted against the year of semen collection, there was no significant change in PM for all factors (Figure 2B: raw: slope = 0.5337, R^2 = 0.1326, $p \geq 0.05$; fresh: slope = 0.0735, R^2 = 06637, $p \geq 0.05$; cool: slope = 0.1301, R^2 = 0.009240, $p \geq 0.05$).

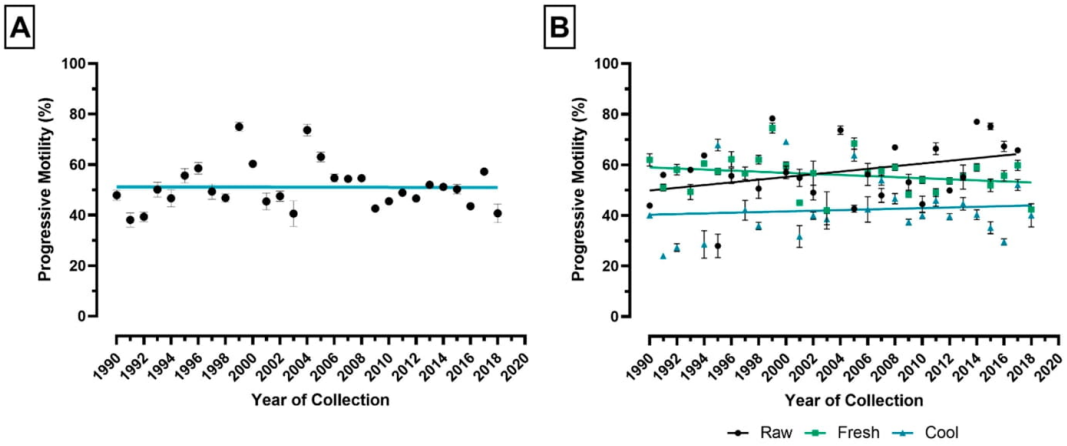

Figure 2. Progressive motility (PM) by year of semen collection in the period from 1990 to 2018. (**A**) PM plotted by year of semen collection. Each point represents the mean (±SEM) PM for a different year. (**B**) PM plotted by year separated by ejaculate type: raw (black), fresh (green), and cool (blue). Each point represents the mean (±SEM) PM for a different year for each ejaculate type. The lines for both figures denotes the best-fit lines. Error bar ± 1 SEM.

3.2. Method of Sperm Motility Assessment

There was a significant difference in PM between the method of motility assessment ($p \leq 0.001$). Figure 3A shows that PM was significantly higher for studies that assessed motility using microscopy (168 data points) compared to those using CASA (538 data points). There was a significant increase in PM for studies using microscopy in the period from 1990 to 2018, but no significant change for CASA systems overtime (Figure 3B: microscopy: slope = 0.4394, R^2 = 0.1675, $p \leq 0.05$; CASA: slope = −0.02396, R^2 = 0.0004617, $p \geq 0.05$).

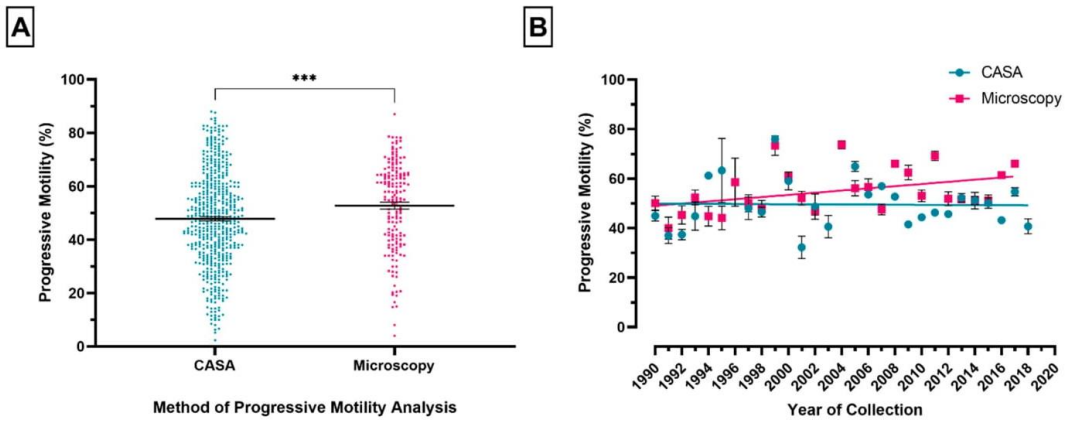

Figure 3. The influence of methods of motility analysis on progressive motility (PM). (**A**)Differences in mean (±SEM) PM between microscopy and CASA. Each point represents the mean PM for each individual study that provided PM estimates assessed by CASA (blue) or microscopy (pink). (**B**) PM plotted by year (1990–2018) separated by motility analysis method: CASA (blue) or microscopy (pink). Each point represents the mean (±SEM) PM for a different year for each method of motility analysis. The lines denote the best-fit lines.*** $p \leq 0.001$. Error bar ± 1 SEM.

3.3. Breed Category

There were significant differences in PM between breed categories ($p \leq 0.0001$; Figure 4A). Figure 4A shows that PM for the Arabian breed category was consistently greater than other breed categories, and significantly greater than Andalusian, Draught, Miniature, Quarter Horse, and Warmblood breed categories ($p \leq 0.0001$; $p \leq 0.01$; $p \leq 0.05$; $p \leq 0.0001$; $p \leq 0.05$, respectively). There were significant intra- and inter-breed differences between raw, fresh, and cool semen samples ($p \leq 0.0001$; $p \leq 0.0001$; $p \leq 0.0001$). Figure 4B shows intra-breed differences for PM were consistently lower for cool semen samples compared to fresh semen samples and significantly for lower for all breed categories except Draught (Andalusian fresh:cool $p \leq 0.001$; Arabian fresh:cool $p \leq 0.05$; Miniature fresh:cool $p \leq 0.0001$; Pony fresh:cool $p \leq 0.001$; Quarter horse fresh:cool $p \leq 0.0001$; Warmblood fresh:cool $p \leq 0.0001$).

3.4. Season of Semen Collection

There was no significant difference in PM between the seasons of collection ($p \geq 0.05$), with a mean PM of 55.13% ± 2.92, 49.79% ± 1.58, 51.41% ± 1.52, and 48.21% ± 1.96 for autumn, winter, spring, and summer, respectively (Figure 5A). There were significant differences in PM between and within seasons for raw, fresh, and cool semen samples ($p \leq 0.0001$, $p \leq 0.05$, $p \leq 0.0001$). Figure 5A shows PM was consistently lower for cool semen samples compared to fresh, and significantly lower in winter, spring, and summer ($p \leq 0.0001$, $p \leq 0.0001$, $p \leq 0.0001$). For cool semen samples, PM was significantly higher in the autumn compared to winter, spring, and summer ($p \leq 0.05$, $p \leq 0.0001$, $p \leq 0.01$).

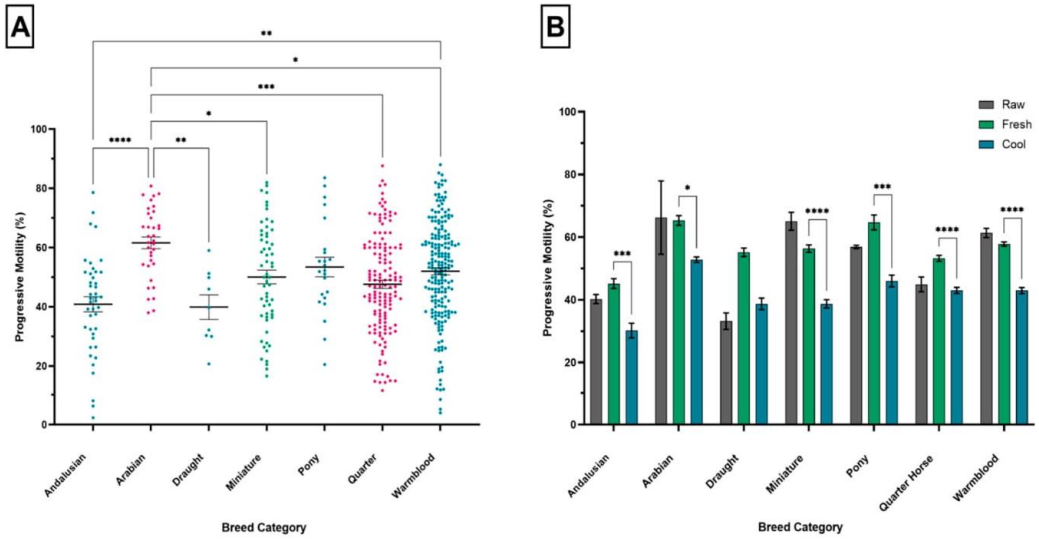

Figure 4. The influence of breed category on progressive motility (PM). (**A**) Differences in mean (±SEM) PM between breed categories. Each point represents the mean PM for individual studies that provided PM estimates for each breed category. (**B**) Effect of ejaculate type on PM within breed categories. Each bar represents the mean (±SEM) PM for raw (grey), fresh (green), and cool (blue) semen samples for each breed category. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Error bar ± 1 SEM.

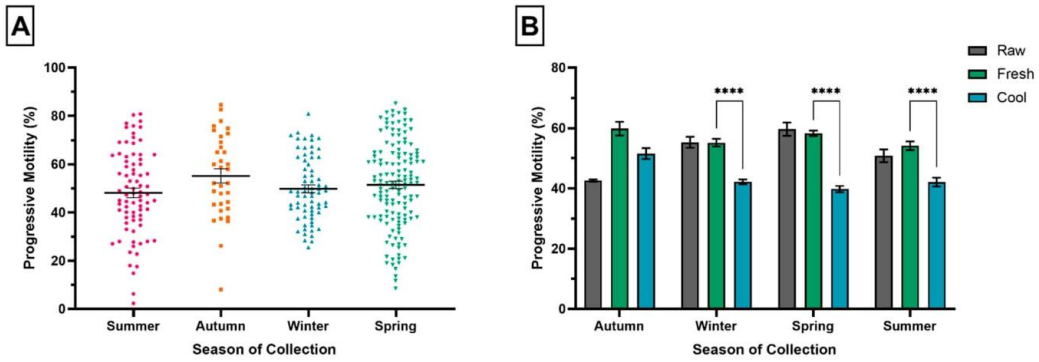

Figure 5. The influence of season of collection on progressive motility (PM). (**A**) Differences in mean (±SEM) PM between seasons of collection. Each point represents the mean PM for individual studies that provided PM estimates for each season. (**B**) Effect of ejaculate type on PM within seasons. Each bar represents the mean (±SEM) PM for raw (grey), fresh (green), and cool (blue) semen samples for each season. **** $p \leq 0.0001$. Error bar ± 1 SEM.

3.5. Geographical Location

There were significant differences in PM between geographical locations ($p \leq 0.0001$). Figure 6A depicts that PM was consistently lower for stallions located in South America, and significantly reduced compared to Africa, Asia, Europe, and North America ($p \leq 0.0001$, $p \leq 0.0001$, $p \leq 0.0001$, and $p \leq 0.0001$). There were significant changes in PM over time for both Europe and North America ($p \leq 0.05$, $p \leq 0.0001$). Figure 6B shows PM significantly decreased in Europe (slope = −0.2073, R^2 = 0.01709) and significantly increased in North America (slope = 0.3737, R^2 = 0.06986) in the period 1990–2018.

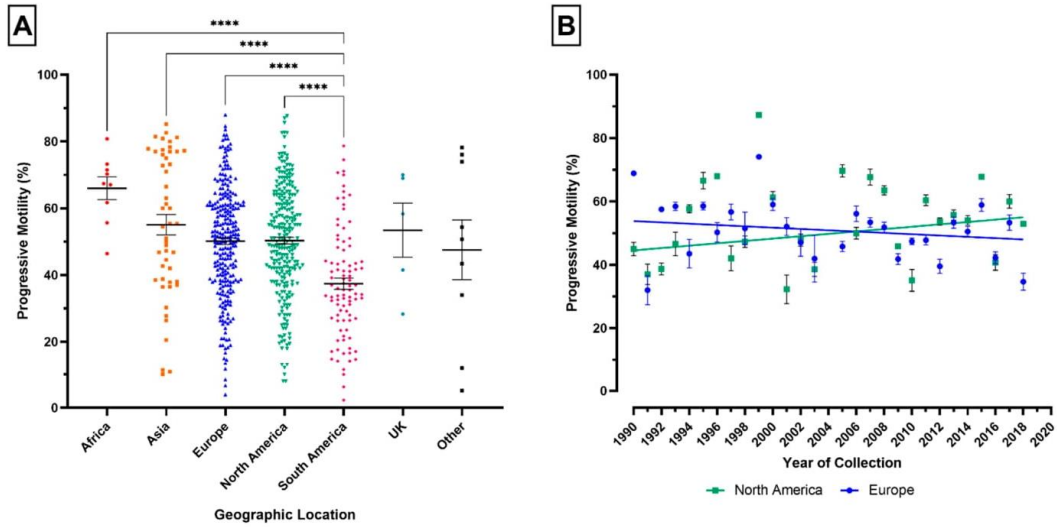

Figure 6. The influence of geographical location on progressive motility (PM). (**A**) Differences in mean (±SEM) PM between geographical locations. Each point represents the mean PM for individual studies that provided PM estimates for each geographical location. (**B**) PM plotted by year (1990–2018) separated by geographical location: North America (green) and Europe (blue). Each point represents the mean (±SEM) PM for a different year for North America and Europe. The lines denote the best-fit lines. **** $p \leq 0.0001$. Error bar ± 1 SEM.

4. Discussion

Our findings represent the status of equine PM across the last three decades, for which research is limited [16]. This present study suggests the fertilising capacity of equine ejaculate has remained consistent, but there is considerable variation in PM.

Variations in stallion PM have been reported to account for only 20% of the total variation in fertility, as such, PM below 40% is likely to compromise stallion fertility [30]. A study utilising equine embryo recovery rates identified that a PM greater than or equal to 45% is the threshold value associated with a change from lesser to higher fertility [31]. Our findings highlight that stallions with no history of perturbed reproductive health have a current PM globally that is above the threshold for high fertility [30,31]. Despite significant seasonal differences between fresh and cool ejaculates, overall seasonal differences were not significant and ejaculate type did not significantly change PM overtime. Since PM is considered an important parameter for fecundity, our results suggest satisfactory reproductive performance can be achieved outside optimal breeding periods, irrespective of whether ejaculates are fresh or cool-stored. Inferences drawn from retrospective analysis are limited due to inherent heterogeneity between individual studies, but our study is the first to examine temporal trends in stallion PM and provides avenues for future stallion fertility research.

It is argued that changes in human laboratory andrology methodologies resulted in systematic errors being interpreted as a decline in semen quality [3,10]. Data presented here show a significant temporal increase in equine PM for studies utilising microscopy, but no significant change in PM over time for studies utilising CASA. The findings presented here highlight the extent to which methodological differences, incorporating visual assessment, contribute to variations in equine PM, manifested by the significant modification of temporal trends.

Graphically, equine PM appears to be normally distributed for studies using CASA, while microscopy has a distinct cluster of studies at a greater motility [29]. Within the literature, boar ejaculates assessed visually reported significantly higher PM (77%) compared

to CASA (39%), consistent with our results [32]. The results of the present study propose CASA to be a more consistent methodology for equine motility assessment and underscore the considerable heterogeneity of microscopy studies due to technical variability [10,25,33]. Evaluation of the canine ejaculate by different technicians has demonstrated high inter-observer variability (30–60%), highlighting that the skills, experience, and training of individual technicians can influence the interpretation of motility [34]. Methodological differences within and between laboratories over time may explain the considerable variation in PM. Evidence of individual stallion and inter-breed variation indicates methodological changes overtime are not solely responsible [35–38].

The significance of breed variations in equine PM is evident, since semen traits have been found to be related to genotype and breed, and are therefore heritable [37,38]. This study provides further evidence that breed could account for the considerable variation in semen quality among stallions, manifested by significant between-breed variation in PM. Breed variations in equine PM reported here are consistent with literature reporting whereby Arabian stallions exhibit greater PM compared to warmblood and light horse breeds [28,39,40]. In mares, reports suggest reproductive genes involved in oocyte maturation, development, and function differ amongst Spanish breeds alongside certain fertility traits [41,42]. Evidence of breed differences in reproductive traits indicates significant differences in equine PM may be a function of genetic factors. Methodological and technical differences between individual studies limits assumptions on the extent breed differences contributed to variations in PM [10,25].

In human reproductive studies, geographical location is a potential confounder for variations in semen quality. Our study included global stallion PM data, as such geographic location was varied and unequally distributed. Therefore, variations should be interpreted with caution. Europe and North America provided similarly distributed datasets to further investigate geographical differences from a temporal aspect. Included studies from Europe assessing stallion PM parallel the declines observed in European human semen quality and for UK stud dogs [5,12,43]. The increase in PM for stallions in North America conflicts with existing human semen quality data, perhaps related to inherent limitations of meta-analyses or an effect of inter-species differences [3,6,44]. To interpret geographical differences in stallion PM, the method of motility analysis could be considered a confounding factor in the present study due to the inclusion of both manual and computer-automated methods within this meta-analysis. Steeper declines in human semen quality and higher incidences of testicular cancer have been observed in Europe compared to other continents [7,45,46]. Taken with the data presented here, it could be inferred that an environmental aetiology may be at least partly responsible for geographical differences in equine PM, presenting an area worthy of future consideration.

Environmental pollution and contaminants are reported for impairing sperm quality and causing reproductive abnormalities [47]. The relationship between geographical variations in semen quality and the potential burden of environmental pollution is contentious, yet sufficient observational and experimental data exist to indicate there is a cause for concern. Research concerning exposure levels of environmental contaminants in the equine is limited; however, environmental pollutants, namely polychlorinated biphenyls, have been detected in horse meat and donkey milk [48,49]. In Pennsylvania (USA), there were increased incidences of dysphagic foals born in an active unconventional gas development, highlighting areas within North America with high environmental chemical burdens [50]. For Europe, a higher proportion of studies of particularly low progressive motility may have originated from contaminated areas that may have confounded the progression lines in the current meta-analysis, similarly for North America. Single-centre studies would provide a more accurate representation of semen quality trends with the potential to identify sources of variation across regions.

The prognostic value of our results as markers for stallion fertility potential cannot be substantiated due to the lack of standardised reference limits for semen parameters in the equine sector [19]. For human sperm quality analysis, the WHO guidelines that define

PM when using manual and computer-automated methods are the same, and these give rise to the recommended categories and sperm swimming speeds for progressive motility assessment. This information can be placed into CASA algorithms to assess sperm motility. Information on the direct CASA algorithms for sperm swimming speed in data obtained for this study was not possible. Development of internationally accepted equine sperm quality reference limits would increase consistency across the equine sector in stallion semen evaluations for the selection of future breeding prospects [51]. Using equine pregnancy rates as comparative markers for stallion sperm quality reference limits, similarly to the WHO reference limits for human sperm quality, the predictive value of semen parameters to assess stallion fertility potential could be improved [1,52]. Determining the source of variation in equine PM is challenging due to the inherent limitations of heterogeneity in meta-analytical studies. Missing data for variables of interest limited the choice of statistical methods. The statistical methods used limit equine PM inferences as they did not account for within and between study variation. Variations in equine PM could be related to discrepancies within the data at breed-level. Grouping breeds is easier when genetic lines are similar, as seen in the livestock industry in which genetic differences arise due to different breeding goals [53]. Genetic lines within dairy cow breeds are similar but are genetically different to beef cattle or dual-purpose breeds [53]. This inconsistency is representative of some of the wider challenges of heterogeneity in retrospective analysis, particularly within the equine sector. There is a need for validation and standardisation of objective methodologies to assess equine motility to replace visual estimation to reduce technical variability within and between laboratories [10,33,51]. An international collaboration utilising standardised objective motility analysis would overcome inherent limitations in methodological differences in retrospective analysis and provide a more robust explanation for variations in equine PM.

5. Conclusions

Monitoring stallion reproductive trends can inform decisions on the selection of future breeding prospects to navigate the potential economic impacts of poor reproductive efficiency. The results from the present study indicate stallion PM has remained unchanged globally across the last three decades. Comparative markers to assess the fertility potential of equine sperm values would increase the predictive value of our results. Significant differences between methods of motility assessment showcase the wide variability in semen evaluation in the equine sector. The present study highlights the need for standardisation across the equine breeding industry to support stallion fertility research. Research should focus on validating objective methodologies to assess equine motility to promote consistency across the equine sector. It is the authors' recommendation that implementation of internationally-accepted semen evaluation methodologies be developed to reduce technical variability, allowing for geographical and breed-level variations to be further explored.

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REPRODUCTION
REVIEW

The dog as a sentinel species for environmental effects on human fertility

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Abstract

Despite the vast body of evidence that environmental toxicants adversely affect reproductive development and function across species, demonstrating true cause and effect in the human remains a challenge. Human meta-analytical data, showing a temporal decline in male sperm quality, are paralleled by a single laboratory study showing a similar 26-year decline in the dog, which shares the same environment. These data are indicative of a common cause. Environmental chemicals (ECs) detected in reproductive tissues and fluids induce similar, short term, adverse effects on human and dog sperm. Both pre- and post-natal stages of early life development are sensitive to chemical exposures and such changes could potentially cause long term effects in the adult. The environmental 'pollutome' (mixtures of ECs) is determined by industrialisation, atmospheric deposition and bioaccumulation and characterises real-life exposure. In Arctic ecosystems, dietary and non-dietary chemical contaminants are detectable in biological tissues and linked with adverse health effects in both dogs and their handlers. In the female, such exposure could contribute to disorders such as ovarian insufficiency, dysregulated follicle development, ovarian cancer, and polycystic ovarian syndrome. In the dog, ovarian chemical concentrations are greater in the testis. In addition, preliminary studies indicate that dietary exposures may influence the sex ratio in the offspring in favour of females. Within this article, we review current knowledge on chemical effects on human reproduction and suggest that the dog, as a sentinel species for such effects, is an essential tool for addressing critical data gaps in this field.

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Sentinels: a historical perspective

In the early twentieth century, coal miners exploited the enhanced sensitivity of the canary as an early warning system to carbon monoxide. As soon as the canary birds fell off their perch, miners had time to respond to ensure their own safety (reviewed Burton 2014). This highly cited example of the use of an animal as a bio-monitor illustrates the utility of a sentinel species for assessing risk to human well-being when exposed to environmental hazards (Bowser & Anderson 2018). Since the use of canaries in coal mines, a range of other species have been proposed as sentinels for infectious agents, food hazards, or toxic substances present in the immediate environment (Rabinowitz *et al.* 2010). Some key examples of these are outlined in Table 1.

While not demonstrating true cause and effect, sentinel species have been used as an index of environmental pollution in soil, air, plants, water, and human habitats. For example, the common earthworm can provide information on the detritus in the soil in addition to the dietary exposure of birds that prey on such species. Another example is that of sheep, providing an index of the exposure from plant material ingested throughout grazing and any soil swallowed in the process. In the aquatic world, sentinel species include oysters, fish, and animals that obtain their food from the aquatic environment, such as seals, dolphins, and killer whales. With respect to the latter, bioaccumulation of PCBs through the food chain has been associated with a decline in species number, placing the highly contaminated killer whale at a high risk of population collapse (Desforges *et al.* 2018) and eliciting severe health consequences (Haraguchi *et al.* 2006, Alava & Gobas 2016, Kurt-Karakus *et al.* 2019).

In the human household, dogs and cats share our environment more than any other species and are exposed to household contaminants similar to that of humans. In the Western world, dogs tend to live and travel with their owners and are thus exposed to not only the same infectious agents, but also to non-infectious environmental factors, such as chemical pollutants, of

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Table 1 Animal models proposed as sentinel models for the study of environmental exposure to endocrine disrupting chemicals.

Proposed model	Specific area of study	Evidence	Reference
Companion animals			
Feline	Low-level chronic exposure to PBDEs	Empirical	Dye *et al.* (2007)
Canine		Empirical	Schilling *et al.* (1988), López-Alonso *et al.* (2007), Sonne *et al.* (2010, 2020), Lea *et al.* (2016*b*)
	Metal concentration in tissue		
	Exposure of chemicals within environment		
Canine/feline	2,3,7,8 Tetrachlorodibenzodioxin	Empirical	Schilling & Stehr-Green (1987)
Production animals			
Bovine/ovine		Empirical	Wahl & Reif (2009), Petro *et al.* (2010), Lea *et al.* (2016*a*)
	Sperm quality		
	Tissue and fluid concentrations of PCB, OCPs, and PBDEs		
Wildlife species			
Primate		Empirical	Mitchell *et al.* (2008)
	Germ cell differentiation		
	Testicular germ cell tumour		
Mink	Mercury and PCBs	Review	Basu *et al.* (2007)
Marine mammals	Anthropogenic toxins	Case Study and review	Bossart (2006), Sonne (2010), Jepson *et al.* (2016), Sonne *et al.* (2019)
General reports			
Domestic animals	Endocrine disruptors	Review	Majdič (2010)
Mammals	Toxic environmental contaminants	Review	O'Brien *et al.* (1993)
Animals	ECs	Review	Van Der Schalie *et al.* (1999)
Companion animals		Review	Schmidt (2009)
	Public health		
	Environmental contaminants		

ECs, Environmental chemicals; OCPs, Organochlorine pesticides; PBDEs, Polybrominated diphenyl ethers; PCBs, Polychlorinated biphenyls.

household or recreational origin. It follows therefore that environmentally linked health conditions in the dog may be mirrored in the owners. Sentinel species typically provide a range of tissues for chemical analysis post-slaughter. In contrast, the routine surgical neutering of hundreds of thousands of dogs and cats provides easy access to surplus reproductive tissues which could be used for this purpose. Furthermore, after routine neutering, the male reproductive tract can be further utilised to provide sperm from the tail of the epididymis (Ponglowhapan *et al.* 2006). Another method by which the dog may be used as a sentinel is through the provision of sperm as part of routine fertility monitoring. This is a procedure tolerated by many species (Hermansson & Forsberg 2006) and provides a further, cost effective, resource for monitoring contaminants and the quality of the ejaculate. For these reasons, our recent work has focussed on the domestic dog as an index of the reproductive health of humans, particularly as it shares the same environment.

Temporal trends in human reproductive and metabolic health

In humans, perturbed reproductive potential is characterised by reports of declining semen quality over several decades, an increase in incidence of testicular cancer, malformations of male babies at birth, precocious female puberty, premature menopause, and ovarian cancer. These temporal changes are not limited to reproductive parameters, since a striking increase in global obesity, other cancers, and metabolic syndrome have occurred over the same period (Borch-Johnsen 2007).

Although the precise drivers behind these temporal trends remain uncertain, ECs have been implicated as playing a role alongside Westernised lifestyles and gene-environment interactions (Ellulu & Jalambo 2017). In support of this hypothesis, many chemicals have been described as endocrine disrupting, obesogens, and metabolic disrupting, raising considerable concern over their effects on animal and human health (Landrigan *et al.* 2018).

Male semen quality and testicular dysgenesis syndrome

There is an increasing body of published evidence to indicate that human male fertility and/or reproductive health has declined over the last 40 to 60 years (Levine *et al.* 2017). Meta-analytical studies suggest an approximate 50% decline in sperm concentration over the past 70 years, averaging a 2% decline per annum (Carlsen *et al.* 1992, Swan *et al.* 2000, Levine *et al.* 2017). Alarmingly, this rate of decline appears to show no 'levelling off' in more recent years and an increasing proportion of men with less than 40 million sperm per mL of ejaculate has been reported (Sharpe 2012).

Other parameters of sperm function, such as motility and morphology, have been less extensively reported. There are reports indicative of a decline in percentage morphologically normal sperm along with a decline in sperm count over a 17-year period limited to France (1989–2005) (Rolland *et al.* 2013). More recently, two separate studies have reported a temporal decline in total motile sperm count at fertility centres in Europe and North America from 2002 to 2017 (Chang *et al.* 2018, Tiegs *et al.* 2019). Such adverse temporal trends have been linked with additional temporal global increases in reproductive perturbations, such as testicular cancer (TCa), and genitourinary abnormalities, such as cryptorchidism and hypospadias.

Collectively, the adverse reproductive trends present today are termed testicular dysgenesis syndrome (TDS) (Skakkebaek *et al.* 2016). Over a 10-year period, the global total incidence rate of TCa has increased by 5.6%, with higher incidences in developed vs undeveloped countries (Park *et al.* 2018). Similarly, subtypes of TCa, including seminoma and nonseminomas, increased annually by 3.26% and 1.15%, respectively, with significant variation between racial and ethnic populations (Ghazarian *et al.* 2018). TCa incidence appears to have increased incidence at a younger age, adding further evidence of a possible environmental aetiology (Pishgar *et al.* 2019).

The human sperm quality debate

Adverse temporal trends in male sperm quality have raised much controversy ever since the concept was first proposed by Carlsen *et al.* (1992) (Pacey 2013). This is predominantly a result of limitations in the heterogeneity of meta-analytical publications, in addition to the inclusion of historical data sets (Carlsen *et al.* 1992, Swan *et al.* 2000). Developments in semen analysis have led to a shift towards the use of haemocytometer techniques as opposed to the Makler Chamber. Indeed, when applying modern technologies with increased precision, sperm count values have been reported to be 1.5–2.7 times lower, thus giving the impression of a decline in sperm quality (Pacey 2013). In subsequent studies, the exclusion of those that did not use a haemocytometer and the utilisation of standardised PRISMA and MOOSE meta-analytical protocols still continued to show a decline in fertility (Levine *et al.* 2017). Furthermore, a decline in total motile sperm count between 2002 and 2017 has recently been reported. This was based on the analysis of data generated from just two laboratories (Tiegs *et al.* 2019).

The dog as a sentinel for human environmental chemical exposure

The dog lives in close proximity to humans, where individuals are exposed to similar environmental factors. Parallel temporal trends of semen quality have been reported in the dog sentinel model, with an overall decline of 30% in progressive sperm motility over a period of 26 years (Lea *et al.* 2016*b*). In contrast to the human meta-analyses, the dog study used semen quality parameters collated from a single laboratory by staff with standardised training, promoting consistent analytical methodologies. Consequently, the confounders that complicate the interpretation of meta-analytical studies in the human do not apply and this in turn adds to the weight of evidence for a decline in semen quality in both species.

Genetic, epigenetic, or lifestyle influences on these reproductive trends cannot be ruled out, particularly with respect to the many potential confounders that account for the noise in the human studies. In addition to changing methodologies, tight underwear has been linked with poor sperm quality and other factors such as smoking, alcohol, physical activity, adiposity, and cell phone usage have also been suggested as possible factors (Mínguez-Alarcón *et al.* 2018, Kaya *et al.* 2020). In the dog study, the list of possible alternative causes for declining sperm quality is smaller, mostly due to the 'non meta-analytical approach' used. In addition, within the population of stud dogs studied, heritability measures for a range of sperm quality parameters, including reduced motility, were low (England *et al.* 2010). Given that there was no influence of breed, body weight, or sire, an environmental aetiology would appear to be the most likely cause.

Over the same period as the adverse trends in dog sperm motility, an increased incidence rate of cryptorchidism in male pups was observed from the same population of stud dogs (Lea *et al.* 2016*b*). Preliminary evidence suggests that the incidence of testicular tumours in the dog has increased over a 40-year period and that the histological characterisation of such tumours is similar to that reported in the human (Grieco *et al.* 2008*a*). In total, these studies suggest that the dog exhibits the same range of reproductive abnormalities as reported in the human (Grieco *et al.* 2008*b*, Ghazarian *et al.* 2018). Such trends demonstrate the importance and worth of the dog as a sentinel species for environmental influences on human reproductive health. However, in contrast to the 'canary in the coalmine scenario', the mechanism underlying these reproductive temporal trends remains uncertain. Although we cannot be certain of a common trigger in both species, there is a clear commonality in the temporal reproductive trends observed and thus the proposal is that the underlying aetiology is environmental and consistent in both the dog and human.

The environmental 'pollutome'

Despite the ongoing debate surrounding reproductive perturbations, the weight of evidence suggests a common

aetiology involving the interaction of a complex group of anthropogenic ECs (Bellingham *et al.* 2009, Cabrera-Rodríguez *et al.* 2019, Sumner *et al.* 2019). There exist more than 14,000 classified environmental pollutants inclusive of, but not limited to, organic chemicals, heavy metal ions, and trace elements (Jeon *et al.* 2015, Daoud *et al.* 2017, Dusanov *et al.* 2018). A multitude of *in vitro* studies have sought to investigate the pathogenesis and mechanisms by which ECs disrupt endocrine and metabolic functionality, resulting in a number of reproductive and non-reproductive perturbations. However, such research is limited in its extrapolation to the human population due to the complexity of chemical mixtures involved in real-life exposure. This further emphasises the importance of alternative models including the utilisation of a sentinel species.

Organic ECs are typically utilised for industrial and agricultural processes due to their properties as plasticisers, pesticides, and solvents (Chen *et al.* 2019). Dependent on the congener, ECs are present in a variety of industrialised resources (Fig. 1). ECs are released into the environment either through direct emission from the production process or migration from products themselves (Wania 2003). The latter is possible due to the inability of many ECs to chemically bond to the matrices in which they originate, leaching into the surrounding environment (Lenoir *et al.* 2016). Present in air, water, soil, and vegetation, ECs are consistently available for uptake by humans and other species on a global scale (Terzaghi *et al.* 2018). In reference to the agricultural industry, the use of EC contaminated sewage sludge for pasture fertilisation and organochlorine pesticides is a primary concern due to studies demonstrating detectable levels of repro-toxic ECs including phthalates and Bisphenol-A (BPA) (Rivera *et al.* 2009, Tran *et al.* 2015).

Industry plays a major role in global pollution. The global distribution of chemicals facilitates the spread and atmospheric deposition of ECs in remote non-industrialised areas of the world, thus EC contamination is ubiquitous. This is particularly true for persistent organic pollutants (POPs) – ECs including PCBs, PBDEs, organochlorine pesticides and dioxins – that are categorised by extended half-lives and high lipophilicity, making them bio-persistent in nature (Wania 2003). Numerous chemical congeners have thus been detected in a range of biota and in ecosystems in areas once considered isolated from anthropogenic influence including ant cuticles from the deep Amazonian rain forest; Amazonian caymen and fish; polar zones including the Arctic ecosystems; and endemic arthropods in the Mariana and Kermadec trenches, the two deepest oceanic trenches (Wania 2003, Lenoir *et al.* 2016, Jamieson *et al.* 2017). Shockingly, EC levels have also been detected in Arctic ecosystems. In coastal areas, the Arctic fox has been identified as a sentinel for contaminant monitoring in humans, particularly in relation to the element mercury from a marine diet. In addition, sled dogs living with their handlers have been identified as sentinels to both dietary and non-dietary environmental contaminants (Harley *et al.* 2016). The concept of the sled dog as a sentinel model to monitor arctic ecosystems has recently been highlighted by Sonne and colleagues (Sonne *et al.* 2020).

Marine ecosystems are the final destination for many persistent ECs. Aquatic species are thus exposed to ECs and these tend to accumulate within the adipose tissue (Jeong *et al.* 2019). Elevated bodily burdens of lipophilic chemicals have been found to increase at each trophic level, resulting in bio-magnification in species with higher blubber content and longer life spans, such as apex predators. Consequently, such apex predators are of great scientific interest for further understanding the effect of chronic EC exposure and substantial levels of contamination. An interesting comparison is that of three aquatic species displaying high blubber content with differing diets, from the herbivorous Dugong dugon (otherwise known as the sea cow), to the finless porpoise (surviving mainly on a fish based diet), and finally the killer whale (with a main dietary source of contaminated seal blubber). The Dugong dugon has reported bodily burdens of PBDE of approximately 120 ng/g lipid weight (lw) (Weijs *et al.* 2019). In Korean coastal line populations, the finless porpoise, an endangered marine mammalian species, is reported to have PBDE concentrations at around 294 ng/g lw (Jeong *et al.*

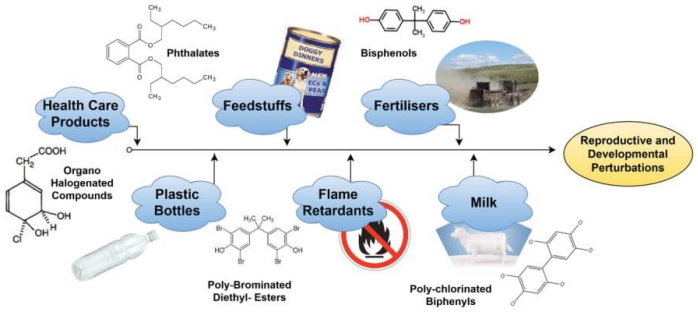

Figure 1 Overview of sources giving rise to environmental chemicals. This figure illustrates sources in which chemicals present within the environmental 'pollutome' could potentially contribute to reproductive and developmental perturbations.

2019). In the male southern Pacific resident killer whale, PBDE levels are reportedly eight times greater than that of the Dugong dugon (Rayne *et al.* 2004). Although geographical variation in the environmental 'pollutome' of each habitat will vary, higher concentrations in killer whale populations are likely due to the consumption of contaminated seal blubber leading to the bio-magnification of these lipophilic ECs. The reported concentrations in each of these species demonstrate the ability of ECs to bio-magnify at each trophic level, but also highlight exposure routes other than the consumption of contaminated meat, raising additional concern of reproductive health in herbivorous species. Given that the three species discussed are endangered populations, concerns of further population decline due to the repro-toxic nature of ECs have been raised.

Due to EC congeners being utilised for specific industrial or agricultural purposes, it follows that the profile of contaminants constituting the 'pollutome' could reflect this variation (Fig. 1). To our knowledge, present research is restricted to geographical variation in EC levels, with limited understanding of contaminant profiles in relation to specific industry processes. Assessing ecotoxicological risks associated with exposure to a multitude of ECs are essential due to their synergistic toxicity (Jeong *et al.* 2019). To asses industrially associated contaminants, ants have been proposed as a valuable bio-monitoring species for environmental contamination and the chemical profiling of differing geographical regions due to the abundance of ant populations on a global scale (Wania 2003). While research in this area has the ability to advance understanding in specific EC profiles and potentially predict the subsequent interactions of ECs within humans, there is a distinct need for a sentinel that closely shares our environment. It is for these reasons that we propose the dog as a sentinel species for better assessing human exposure to ECs.

Geographical differences in chemical exposure

As discussed, EC concentrations present within the environment fluctuate dependening on the geographical location, level of industrialisation, and population density. Given the contribution of industry to pollution, distinct regional variation in reproductive perturbations are reported, with higher incident rates in industrialised areas (Lin *et al.* 2017). Sperm samples collected from sub-fertile men in Middle Eastern and North-African regions have been reported to have lower sperm motility and morphology, alongside increased DNA damage (Elbardisi *et al.* 2018). In the Western world, a temporal decline in sperm quality includes North America, Europe, Australia, and New Zealand (Levine *et al.* 2017). In the same meta-analysis, no such trends were found in South America, Asia, and Africa. Reports had previously shown higher semen quality and a lower incidence of TCa in Finland, compared to other neighbouring countries such as Denmark (Znaor *et al.* 2014, Virtanen & Toppari 2015). More recent studies now indicate that the incidence of TCa in Finland is increasing and that sperm quality is converging to the lower quality levels of Danish men (Rodprasert *et al.* 2019). By contrast sperm quality and TCa rates in Denmark have remained relatively stable (Priskorn *et al.* 2018). While geographical variations in sperm quality and TCa have been reported, data demonstrating a causal relationship with ECs are contentious (Foster *et al.* 2008). In this regard, the dog constitutes an appropriate sentinel model for monitoring geographical differences in seminal or testicular EC concentrations. Figure 2 illustrates that, in the dog, the testicular chemical profiles vary across different areas of the United Kingdom.

Factors influencing tissue profiles of chemicals include diet, obesity, environment, and even genetics. Increased numbers of Westernised fast food chains in these areas have led to a diet consisting of products higher in fatty foods and animal products (Elbardisi *et al.* 2018). Since persistent organic pollutants are lipophilic, it follows that diet may be a key source of exposure and that this, in turn, may impact on reproductive health (Pratt *et al.* 2012). Since the number of studies carried out in more remote areas is limited, it remains uncertain

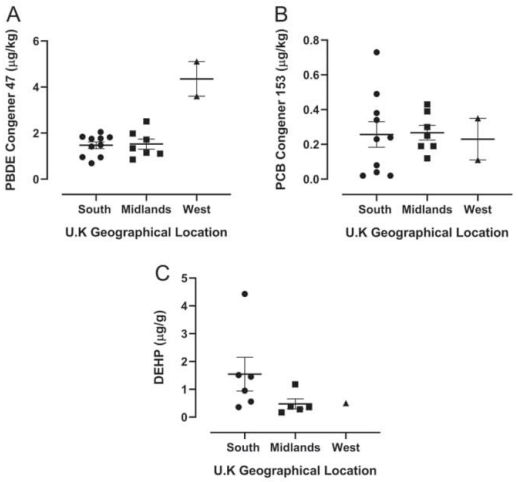

Figure 2 Geographical variation of dog testicular ECs across three regions of the United Kingdom. As shown, PBDE47, a flame retardant, was greater in samples from West England (A). PCB-153 was consistently expressed across England, even though this chemical was banned from the 1970s (B). Finally, DEHP was observed consistently in the majority of samples, other than at a greater concentration in a testicular sample from South of England (C). Data obtained as part of the data published in Lea *et al.* (2016*b*). Scientific report articles are published under a CC BY license allowing for maximum dissemination where users are free to adapt data. Error bars represent ± 1 S.E.M.

if fertility related perturbations are a primary problem of the industrialised world.

Chemical profiling of reproductive tissues and fluids

Returning to the dog sentinel, the availability of tissue from neutering procedures provides an opportunity to determine the profile of chemicals in testis and ovary. A total of 12 chemicals across three chemical types have been detected in both testis and ovary (Lea *et al.* 2016*b*, M.E. Van der Mescht, unpublished observation). These comprise PCBs, PBDEs, and the phthalate DEHP. These chemical types have also been detected in the dog ejaculate and from the limited studies carried out in the human. Such chemicals are likely reflective of what is present in the human. Since the basis of the sentinel paradigm is that ECs have similar effects in both species, two chemicals predominant in testis and seminal fluid, DEHP and PCB153, have been shown to have similar effects on dog and human sperm (Sumner *et al.* 2019). Gonad-relevant concentrations of these chemicals reduced sperm motility and increased DNA fragmentation in both species. This work demonstrates that the development of this research field could be achieved by integrating the use of a sentinel model in combination with *in vitro* experiments.

Real-life exposure to chemical mixtures

Real-life exposure to chemical contaminants constitutes exposure to complex mixtures of chemicals, which inevitably interact, thus influencing the overall biological or toxicological effect. Since the 1950s, it is estimated that more than 140,000 new chemicals have been produced and many of these are released into the air, sea, and soil (Landrigan 2017). Geographic variability will inevitably reflect different degrees of regional industrial activity. Since many chemicals are non-biodegradable and exhibit bioaccumulation and bio-magnification, this constitutes a concern of global magnitude. For these reasons, determining biological responses to real-life mixtures of chemicals presents a challenge to the establishment of a link between environmental contaminants and altered fertility or reproductive function.

Responses to chemicals may be dependent on whether chemicals in a mixture, as well as their respective metabolites, share similar or differing modes of action (Chen *et al.* 2019). Exposure to multiple ECs is reported to have a greater adverse effect compared to exposure to a single contaminant (Woodruff *et al.* 2011). Some *in vitro* studies have utilised commercially available mixtures of chemicals in toxicological *in vivo* studies. For example, in the mouse, the 50-day administration of a commercial mixture of PCBs (Aroclor 1254) by oral lavage every 3 days induced a significant decrease in testicular weight and increased degenerative testicular alterations. Interestingly no changes were seen at lower doses (0.5–50 µg/kg) (Cai *et al.* 2011). Environmental chemicals have been shown to mimic hormones such as prostaglandin and progesterone to activate CatSper channels invoking a Ca^{2+} ion increase. In particular, a metabolite of the pesticide Dichlorodiphenyltrichloroethane (DDT) has been reported to activate CatSper channels on sperm membranes (Schiffer *et al.* 2014), inducing hyper-activation of sperm. While this report demonstrates a potential mechanism of action for ECs to influence sperm quality, not all chemicals investigated invoked effects in this manner. It has been suggested that the observed chemical effects might be specific to human sperm. Some ECs that induced an influx of Ca^{2+} ions in human sperm had no effect in a mouse model (Schiffer *et al.* 2014). In combination, the ECs assessed by Schiffer and colleagues on human sperm evoked greater Ca^{2+} ion responses like that of progesterone *in vivo* (Diamanti-Kandarakis *et al.* 2009). The determination of EC mechanism of action is difficult due to the lack of an appropriate animal model or sentinel.

One approach, more meaningful from a biological perspective, is to use chemical mixtures relevant to everyday exposure to see if chemicals act in synergistic or additive manners. In this regard, the dog has been used to evaluate chemical mixture effects administered in a diet composed of minke whale blubber, contaminated with a cocktail of bio-accumulated chemicals. The feeding of blubber to male sled dog pups elicited direct and trans-generational effects, resulting in significantly reduced testes weight. In addition, the offspring of mothers fed that a total of 20 kg of blubber from 2 months postpartum to time of weaning had reduced testes weight and reduced IgG antibody concentration in blood (Kirkegaard *et al.* 2010, Sonne *et al.* 2010, 2017). These effects correlated with contaminant concentrations.

In addition to the dog, the exposure of pregnant ewes to mixtures of chemicals contained within a sewage sludge derived fertiliser (bio-solids) is widely considered as a real-life model of human exposure to chemical mixtures. A wide range of reproductive and other biological effects have been reported in developing foetuses, in addition to the offspring of mothers exposed during pregnancy. These include effects on the foetal testis, ovary, hypothalamus, and pituitary (Paul *et al.* 2005, Bellingham *et al.* 2009, Fowler *et al.* 2009, Lea *et al.* 2016*a*).

Timing of exposure

Exposure to chemicals is likely to occur throughout an individual's life, beginning *in utero*, continuing postpartum, throughout adolescence, adulthood, and gametogenesis (Fig. 3). The intrauterine environment is where the foetus is most susceptible to the exposure of endocrine disruptive ECs, due to an endocrine mediated period of growth and development (Kot *et al.* 2019).

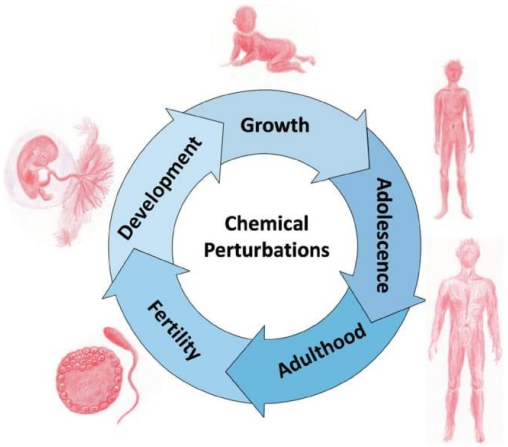

Figure 3 Exposure to chemicals can occur throughout all points in life. Each point of exposure can have a significant impact on developmental processes further in life.

The placenta is an interface at which substrate transport between mother and foetus is mediated, although some ECs have the ability to cross the placental barrier through active transplacental transfer or passive diffusion, depending on their chemical composition (Bommarito *et al.* 2017, Cabrera-Rodríguez *et al.* 2019). A recent report measuring the concentrations of trace elements and heavy metal ions found higher concentrations of lead (Pb) in the foetal membrane and umbilical cord than in the placenta (Kot *et al.* 2019). This is suggestive of an accumulative nature of Pb and demonstrates the ability of Pb, and other chemicals, to cross the placental barrier. Mechanisms underlying this passage, however, remain unidentified (Bommarito *et al.* 2017). In mice, the exposure to particulate air pollution from industrialised areas during pregnancy has been shown to induce morphological changes to the placenta (Veras *et al.* 2008). Changes to the integrity of the placenta could lead to a higher rate of chemical diffusion from mother to foetus, although this remains speculative at this time.

Although susceptibility to the adverse effects of ECs remains high *in utero,* new-borns are still at risk of endocrinological alterations through EC exposure (Wineland *et al.* 2019). In humans, mini-puberty occurs 1 to 12 weeks postpartum and incorporates a hormonal surge, inducing gonocyte development (Hadziselimovic *et al.* 2005). Exposure to ECs with endocrine disrupting properties during this time, such as via fatty breast milk, could have additional adverse effects on sexual development and maturation (Pajewska-Szmyt *et al.* 2019).

Breast milk is suggested as a main EC exposure route to new-borns (reviewed in Pajewska-Szmyt *et al.* 2019). Due to the lipophilic properties of some ECs including PCBs, Polychlorinated dibenzo-dioxins (PCDDs), and polychlorinated dibenzo-furans (PCDFs), primary deposition occurs in lipid droplets in adipose tissue, including in mammary tissue (Lee *et al.* 2017). During milk production, free fatty acids and lipoprotein stores are utilised, along with the release of ECs that have accumulated there (Pratt *et al.* 2012). Despite the lower affinity of chemicals such as BPA and other bisphenol analogues to adipose tissue, concentrations are detected in breast milk samples (Deceuninck *et al.* 2015). Detectable concentrations in breast milk have decreased following more stringent regulations of chemical usage (Pratt *et al.* 2012, Fång *et al.* 2013). Geographical variation is also present within breast milk samples where significant differences in POP congener type and concentration have been reported evident between countries (Antignac *et al.* 2016). Ingestion of chemicals by breastfed infants during mini-puberty could induce adverse effects on endocrinology and genitourinary development, having both immediate and chronic effects.

ECs are reported to adversely affect reproduction through endocrinological interactions, compromising gametogenesis (Daoud *et al.* 2017, Ianos *et al.* 2018). It has also been suggested that EC exposure may, in some cases, manifest long after initial exposure due to epigenetic modifications originating from exposure at the critical window of genitourinary development in the foetus and new-born (Thankamony *et al.* 2016, Bommarito *et al.* 2017). By having the potential to interact with DNA transcription, and more specifically the molecular intricacies involved in gametogenesis, adverse effects will be present throughout an individual's life, with potential multi- and even transgenerational effects. Consequently, EC-induced adverse trends in semen quality and male fertility, as highlighted previously, could result from a combination of epigenetic modifications originating from early life exposure, cumulative concentrations of high lipophilic substances, and acute exposure to chemicals at the point of spermatogenesis, although this is only speculative.

A clear difference between the dog and human is the period of exposure due to differences in longevity. Although this may contribute to the noise observed in the human data, chemical effects are also influenced by diet, adiposity, the physico-chemical properties of the contaminants, mixture effects, metabolism, and possibly other factors that are less understood. It is therefore noteworthy that despite these many complex influences, the parallel between-species trends in reproductive function remain.

Female reproductive development and function

Historically, concerns over declining trends in male reproductive health have preceded those in the female

and this purely reflects the relative ease of empirical study in the male. Since there are similarities in the early ontogeny of the developing male and female reproductive tracts, it has been suggested that early developmental perturbations in the female may manifest as impaired fecundity and/or altered reproductive function in later life (Buck-Louis *et al.* 2011). A key reproductive process that has particular sensitivity to environmental change is folliculogenesis. In the human, this highly regulated process that follows meiotic arrest of the oocyte occurs *in utero*, and the number of oocytes that determine female reproductive lifespan is set prior to birth. In contrast, a majority of follicle development in the dog and mouse occurs after birth, thus providing experimental access to the very earliest stages of development that are difficult to access in the human (Reynaud *et al.* 2012, Sarraj & Drummond 2012).

In the human, the dysregulation of foetal ovarian development due to maternal smoking has been linked with early menopause (Tawfik *et al.* 2015) and there is increasing evidence of a link between premature ovarian insufficiency and exposure to environmental toxicants (reviewed in Vabre *et al.* 2017). In addition, widespread environmental pollutants, such as Bisphenol A, have been reported as potential contributors to the pathogenesis of polycystic ovarian syndrome (PCOS) (Palioura & Diamanti-Kandarakis 2015, Hu *et al.* 2018, Soave *et al.* 2020). These observations provide support for the concept of 'ovarian dysgenesis syndrome (ODS)' with additional linkages to fertility, ovarian cancer, and PCOS.

In the dog, although there is little evidence of an environmental impact on female fertility, easy access to ovaries removed at spaying does provide a means of assessing chemical contaminants directly in the gonad. In this regard, our own studies have shown that known environmental toxicants are present in the dog ovary, in some cases at higher concentrations than detected in the testis. Similar contaminants have also been detected in milk from lactating bitches (Fig. 4). Furthermore, contaminants comprising PCB congeners, PBDE congeners, and plastic-derived phthalates have been detected in commercial dog food (Lea *et al.* 2016*b*).

This raises the possibility that the perturbation of female reproductive development may occur through exposure to contaminants in the diet delivered in milk to the pups and/or via food to the mature bitch. As alluded to earlier, the partitioning of maternal lipophilic chemicals into her own high fat content milk provides a natural mechanism by which her chemicals in adipose tissue are off-loaded (Lehmann *et al.* 2015). The problem is that the primary source of nutrition for the neonate also becomes a source of exposure to the 'maternal legacy' of lipophilic chemical contaminants. For example, in the polar bear, PCB concentrations in the suckling cubs are higher than in the adults and the concentration found in the milk exceeds maternal dietary intake (Polischuk

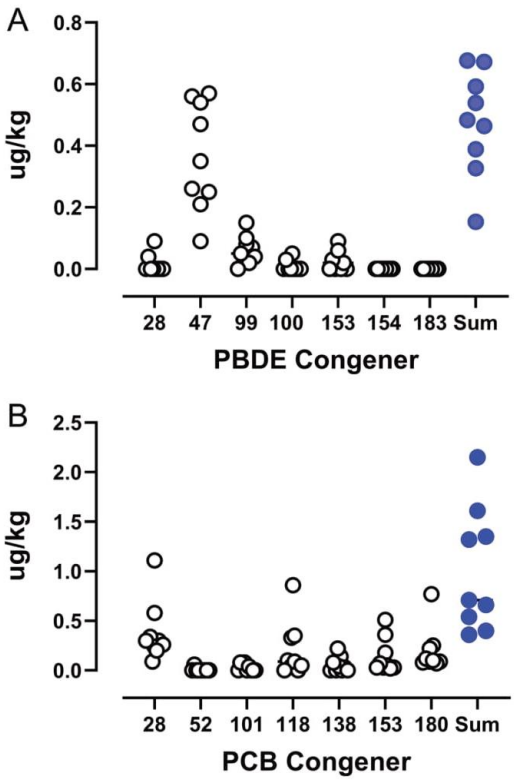

Figure 4 Contaminants detected in the milk from lactating bitches. While all chemicals present are of concern, PBDE 47 (A) is the predominant flame retardent congener. Each value represents a different analysed sample.

et al. 2002, Bytingsvik *et al.* 2012, Sonne *et al.* 2020). In the killer whale, greater concentrations of ECs have been found within the calves of lactating females, indicative of transfer from mother to offspring (Haraguchi *et al.* 2009). Our data showing that both PCB and PBDE congeners are detectable in the milk of whelping bitches supports this concept and these same chemical types have been detected in human milk (Needham *et al.* 2011, Garí *et al.* 2019, Pajewska-Szmyt *et al.* 2019).

With regard to maternal environment, it has been reported that litters of Arctic maternal sled dogs fed seal blubber as a source of environmental toxicants exhibit a skewed sex ratio in favour of females (Sonne *et al.* 2010). In a separate study, we have reported that pups generated in a breeding programme of assistance dogs also exhibit a temporal trend towards an altered sex ratio in favour of females. Since stud dogs from the same programme exhibit a temporal decline in semen quality that is thought to reflect exposure to chemical

contaminants, it is possible that the feminisation of the litters may be similarly mediated (Lea *et al.* 2016*b*). Although direct chemical effects on the dog ovary have not been determined, chemicals detected in the ovary do appear to perturb early follicle development in a post-natal mouse ovary explant culture model *in vitro* (M.E. Van der Mescht, unpublished observation). Furthermore, in the sheep bio-solids model described previously, late gestation female lambs exposed to chemical mixtures via the mother exhibit a dysregulation in early stage follicle (primordial/transitional) development (Lea *et al.* 2016*a*).

Conclusion and future remarks

Environmental pollution remains a critical issue of global concern. There is now an overwhelming body of evidence to suggest that both male and female reproductive health is being adversely affected through exposure to mixtures of environmental contaminants. Understanding the mechanisms that underlie these effects is fraught with complexity, particularly in terms of selecting the appropriate tools to investigate environmentally relevant 'chemical cocktail' effects on the human. An invaluable experimental strategy is to identify a species in which environmental chemical exposures induce biological effects which approximate those reported in the human. In this regard, the domestic dog that shares our everyday household environment constitutes a valuable species for evaluating such effects on human reproductive health. We postulate therefore that, in combination with *in vivo* models, such as bio-solid exposed sheep, *in vitro* rodent studies, and human biomonitoring, the sentinel household dog provides an invaluable contribution to our understanding of toxicant effects on human male and female reproductive well-being.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

R G L conceived and designed the review article. R N S, I T H, and R G L contributed equally to the writing of this manuscript. R N S and R G L created the figures for the manuscript. M V D M, A B, and G C W E provided information, data, and in-depth comments that developed this review. R N S, I T H, and R G L edited the manuscript for grammar and content.

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Appendix E: Environmental chemicals: A concern for stallion fertility?

ENVIRONMENTAL CHEMICALS - A CONCERN FOR STALLION FERTILITY?

With Imogen Harris, PhD researcher at Hartpury University in collaboration with Stallion AI Services Ltd

From plasticisers to pesticides, there is a growing list of products containing toxic environmental chemicals. The question is, how might they affect our breeding stallion's reproductive health and performance?

The environmental impact that human processes are having on species across the globe is a growing area of concern. A reduction in reproductive health and efficiency forms part of this narrative.

In a time where fertility across species is impaired, it is essential to draw our attention to the impact of human led industries on the reproductive health and performance of our breeding stallions.

Exposure to environmental chemicals (ECs) from industrial processes are associated with declining reproductive trends. However, our understanding of the toxicity of ECs on testicular health and function in breeding stallions remains limited.

ECs are produced in extensive manufacturing, agricultural and pharmaceutical processes, leaching into the environment and impacting the surrounding ecosystem. EC uses include flame-retardants, solvents, pesticides, insecticides and plasticisers for foodstuff packaging.

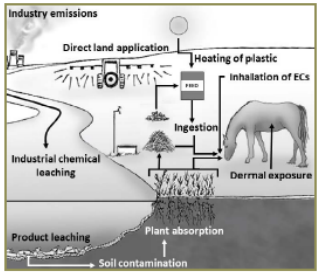
EC exposure occurs through ingestion of contaminated feeds, inhalation of EC particles and absorption through the skin. ECs can disrupt the endocrine system and mimic and block hormonal responses, including that of the reproductive system. Currently there are 800-1,000 chemicals produced with known or suspected endocrine disrupting properties [1].

ECs have been detected in soils and grasses across livestock agricultural land, raising concern over stallion exposure (figure 1). Alfalfa, a common horse feed, is efficient at removing ECs from contaminated soils. However, a link between feeding alfalfa to horses and EC exposure is unknown. Considering foodstuff packaging materials, attention could be turned to reducing the use of plastic wrappings for feeds, supplements and haylages, to minimise stallion EC exposure.

Current research suggests horses are expo sed to a range of ECs. We now need to determine if and how exposure might compromise the reproductive performance and success of breeding stallions.

Exposure is likely to occur throughout life, beginning in the womb, continuing postpartum, adolescence and adulthood, affecting the development and function of the testis [2].

Figure 1: Potential routes of EC exposure (authors own)



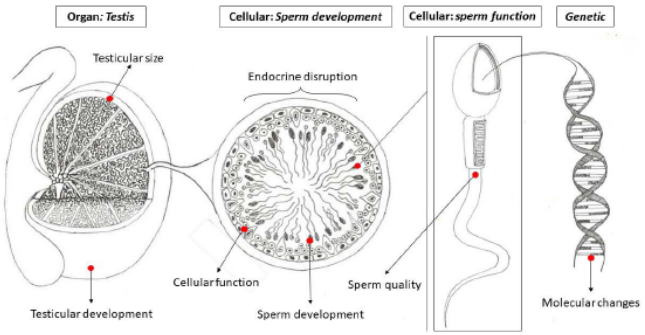


Figure 2: The differing levels at which ECs may impair male reproductive health and function (authors own)

Consequences for stallion fertility

The effects of ECs on testicular health have been heavily reported in other species. EC exposure is associated with declining semen quality in dogs and humans, testicular malformations in sheep, decreased testicular weight in porpoises, and even population declines in killer whales. The effects of ECs on testicular health and function can manifest at multiple levels; as an organ, on tissue structures, and on a cellular and a genetic level (figure 2).

Current research in rodents and sheep suggest that ECs have the ability to affect the way genes function, impacting their offspring. This raises concern over the current reproductive status of these breeding animals and the reproductive performance of their progeny. Given the extensive range of ECs, their worldwide presence and reproductive toxicity, furthering this area of research within the equine industry is essential for the sustainability of the breeding stallion.ting the development and function of the testis [2].

References : 1. Godfray HCJ, et al. A restatement of the natural science evidence base on the effects of endocrine disrupting chemicals on wildlife. Proc R Soc B. 2019; 286(1897).
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What can we do?

#1 Use further research to help inform equine management practices and product development, to minimise exposure to ECs that could impact stallion fertility.

#2 Work towards making the equine industry more 'environmentally friendly' for the good of the planet and our breeding stallions.

Research at Stallion AI Services

Current research carried out with Stallion AI Services aims to assess trends in semen quality and determine the effects of ECs on stallion reproduction. Advancing this area of research is vital for the sustainability of the breeding stallion in a changing environment.

HARTPURY UNIVERSITY

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Appendix F: Reprotoxic environmental chemicals (ECs) in the herbivorous equine model: testicular contamination and routes of exposure.

Reprotoxic environmental chemicals (ECs) in the herbivorous equine model: testicular contamination and routes of exposure

Imogen T Harris*, Alison Z Pyatt, Kathryn Nankervis, Zulin Zhang, and Rebecca N Sumner

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1. Introduction

ECs are associated with reproductive perturbations in multiple species (1). Carnivorous diets are associated with EC biomagnification, with lower levels detected in herbivorous species. To our knowledge, stallion specific contamination levels remain unknown.

2. Research aims

- To analyse stallion testis for ECs; Polychlorinated biphenyls (PCBs), Polybrominated diphenyl ethers (PBDEs) and Polycyclic aromatic hydrocarbons (PAHs).
- To analyse equine food-stuff materials as potential routes of EC exposure.

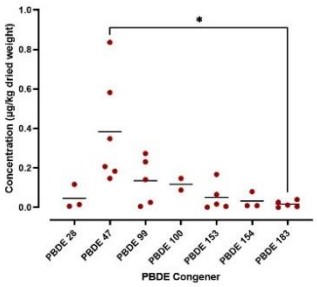
3. Methods

- Thoroughbred stallion testis (n=6), soil (n=2), grass (n=2) and horse feeds (n=6) were analysed via gas chromatography and Mass Spectrometry for PAHs, PCBs and PBDEs.
- Data were analysed (Kruskal-Wallis and Dunn's: testis and feed; two way-ANOVA: soil and grass) to determine differences between chemical congener concentrations (GraphPad Prism 9).

4. Results

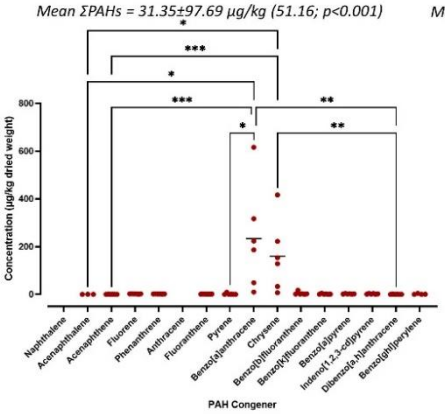
4.1 Testicular PBDE concentrations (µg/kg DW)

Mean ΣPBDEs = 0.11±0.089 µg/kg (15.45; p<0.05)



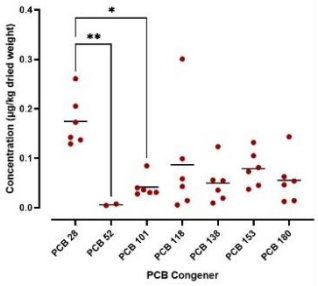
4.2 Testicular PAH concentrations (µg/kg DW)

Mean ΣPAHs = 31.35±97.69 µg/kg (51.16; p<0.001)



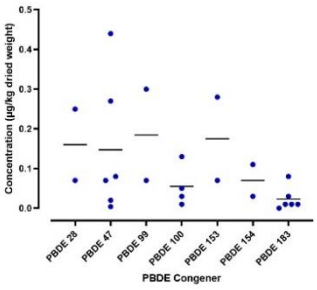
4.3 Testicular PCB concentrations (*µg/kg DW*)

Mean ΣPCBs = 0.07±0.044 µg/kg (18.01; p<0.05)



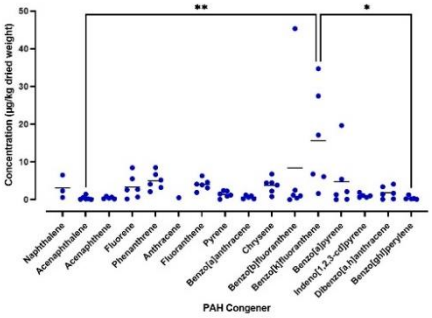
4.4 PBDE concentrations in pooled feed (µg/kg DW)

Mean ΣPBDEs = 0.1±0.11 µg/kg (7.84; p>0.05)



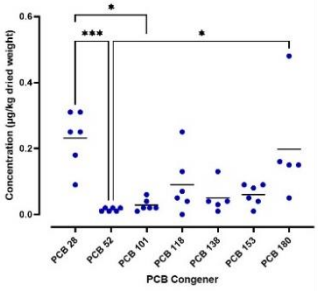
4.5 PAH concentrations in pooled feed (µg/kg DW)

Mean ΣPAHs = 3.73±7.17 µg/kg (41.96; p<0.001)



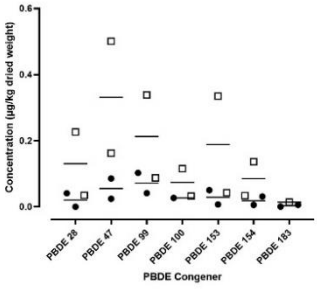
4.6 PCB concentrations in pooled feed (µg/kg DW)

Mean ΣPCBs = 0.1± 0.1 µg/kg (24.59; p<0.001)



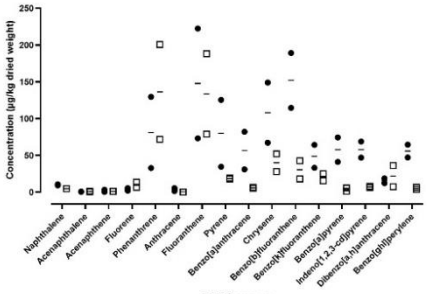
4.7 PBDEs in soil and grass (µg/kg DW)

Mean ΣPBDEs in soil (●) and grass (□)= 0.03±0.03 and 0.16±0.15 µg/kg (F(6,12)=0.4958; p>0.05)



4.7 PAHs in soil and grass (µg/kg DW)

Mean ΣPAHs in soil (●) and grass (□)= 55.33±54.48 and 28.51±48.8 µg/kg (F(15,31)=1.199; p>0.05)



4.7 PCBs in soil and grass (µg/kg DW)

Mean ΣPCBs in soil (●) and grass (□)= 0.08±0.07 and 0.41±0.37 µg/kg (F(6,14)=2.107; p>0.05)

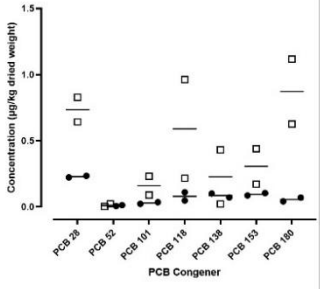


Figure legend. Each point is the individual value, and a single line represents the mean (±S.D.). (*) represent significant (p<0.05) pairwise comparisons. Concentrations are presented in dry weights (DW).

5. Conclusions

- **Testis were contaminated with ECs at concentrations reported to perturb reproductive health (2).**
- Ingestion of contaminated feeds is a probable exposure route of ECs in stallions.

6. References

1. Sumner RN, Harris IT, Van Der Mescht M, Byers A, England GCW, Lea RG. The dog as a sentinel species for environmental effects on human fertility. Reproduction. 2020;159(6):265–276.
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Appendix G: Temporal trends in stallion sperm quality: furthering the debate on declining male fertility through the equine model.

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Temporal trends in stallion sperm quality: furthering the debate on declining male fertility through the equine model

Imogen T Harris*, Alison Z Pyatt and Rebecca N Sumner.

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1. Introduction

The global equine industry promotes stallions to be selected as sires based on performance, with little regard to sperm quality. In humans, research is indicative of a decline in sperm quality over the last 70 years, with trends paralleled in the dog[1,2]. Developing an equine model could advance knowledge in the equine sector, whilst contributing to human fertility debates. Here we present updates on a novel inquiry to evaluate temporal trends within a population of breeding stallions.

2. Research aims

- To determine temporal trends in stallion sperm quality in a population of breeding stallions.
- To determine suitability of the equid as a comparative model for trends in human semen quality.

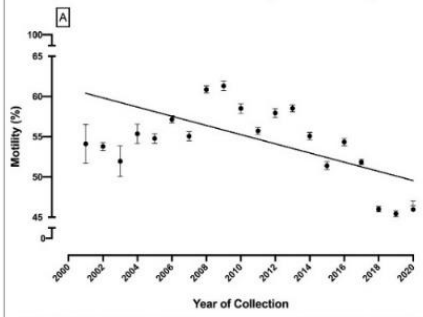
3. Methods

- Data on 11,387 ejaculates; from 1,036 stallions were obtained from a U.K. breeding facility (2001-2020).
- Semen analysis methods included microscopy (X100 lens).
- Temporal trends in raw motility, density and volume were obtained using linear regression (IBM SPSS, 26).

4. Results

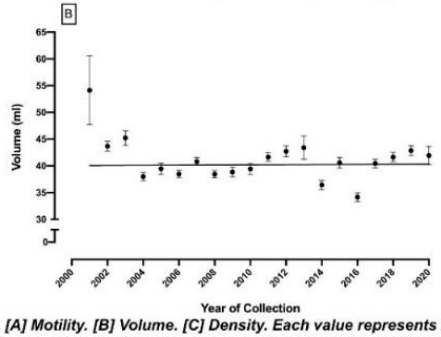
4.1 Raw Sperm Motility

A significant decline of ≈10% (*B*= -0.663 (*p*≤0.001))



4.2 Raw Semen Volume

Remained consistent (*B*= -0.003 (*p*≤0.001))



4.3 Raw Sperm Density

A slight overall increase (*B*= -0.003 (*p*≤0.05))

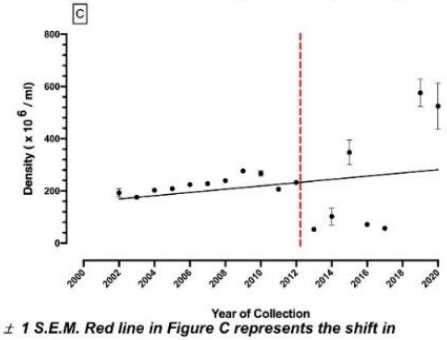


Figure legend Temporal trends in motility, volume and density. [A] Motility. [B] Volume. [C] Density. Each value represents mean ± 1 S.E.M. Red line in Figure C represents the shift in analysis techniques from a SpermaCue to a Nucleocounter. Black line denotes linear regression. B = regression coefficient

5. Conclusions

- **Adverse trends in stallion sperm motility supports research in other species indicative of declining sperm quality[1,2,3,4].**
- Limited inclusion of effect modifiers including inbreeding coefficients, makes it hard to isolate a single causative factor influencing adverse trends, although a common aetiology in other species is suggested to be exposure to reprotoxic environmental chemicals.

6. Further work

- Further analysis of effect modifiers using a multiple linear regression model.
- Elucidate the potential environmental aetiologies underlying the adverse trends in stallion sperm quality.
- Further develop the equine model for use within the reproductive sciences sector.

7. References

[1] Levine H, Jorgensen N, Martino-Andrade A, Mendiola J, Weksler-Derri D, Mindlis I, *et al.* Temporal trends in sperm count: a systematic review and meta-regression analysis. Human Reproduction Update. 2017 May; 23(6): 646-659. Available from: https://pubmed.ncbi.nlm.nih.gov/28981654/ doi: 10.1093/humupd/dmx022

[2] Lea RG, Byers AS, Sumner RN, Rhind SM, Zhang Z, Freeman SL, *et al.* Environmental chemicals impact dog semen quality in vitro and may be associated with a temporal decline in sperm motility and increased cryptorchidism. Sci Rep. 2016 Aug; 6(31281). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4977511/ doi: 10.1038/srep31281

[3] Wahl RL and Reif, JS. Temporal trends in bull semen quality: A comparative model for human health? Environmental Research. 2009 January; 109: 273-280. Available from: https://www.sciencedirect.com/science/article/abs/pii/S0013935108002429 doi:10.1016/j.envres.2008.10.012

[4] Chang S, Nazem T, Douniko D, Lee J, Bar-Chama N, Shamonki JM, *et al.* Eleven year longitudinal study of US sperm donors demonstrates declining sperm count and motility. Fertility and Sterility. 2018 September; 110(4): E54-E55. Available from: https://www.fertstert.org/article/S0015-0282(18)30762-3/fulltext doi: https://doi.org/10.1016/j.fertnstert.2018.07.170

Appendix H: Standard stallion semen quality assessment methods and parameters in the equine breeding sector.

Parameter	Method of analysis
Sperm motility	10µl of semen on a pre-warmed slide (37°C), with a 22x22mm cover slip is analysed by phase contrast microscopy by eye. Objective analysis methods for parameters including total and progressive motility include Computer Assisted Sperm Analysis (CASA) systems, which analyse kinematic values to define motile sperm cells. 60-70% progressive motility is considered sufficient to define a fertile stallion (Hernández-Avilés and Love, 2021).
Semen volume	The sperm-rich fraction of semen is weighed using the standard conversion of 1g to 1ml (Whigham *et al.,* 2014). Typical stallion ejaculate volume is 60ml to 120ml (Multigner *et al.,* 1999).
Sperm concentration	20µl of diluted ejaculate added to a device such as a SpermaCue or NucleoCounter, where cells are counted and concentration is determined with the following equation: *Sperm concentration = 5(sperm count in 5 squares) x dilution factor x 10^4* Automated technologies including Computer Assisted Sperm Analysis (CASA) systems can also be used to determine sperm concentration. Stallions with concentration values of 182 ± 111 x10^6/ml, and 165 ± 126 x10^6/ml have been defined as having high and low fertility, respectively (Jasko *et al*., 1992).
Morphology	Includes the morphological analysis of the head, mid-piece, and tail of the sperm. This is undertaken using nigrosine and eosin stain whereby a 10µl of semen would be pipetted into a 1.5ml eppendorph containing an equal ratio of Nigrosine to Eosin. Phase contrast microscopy is then used for the visual assessment of morphology and sperm membrane integrity. CASA systems also have functions to analyse morphology. Spermatozoon consist of a midpiece, principal piece, and end piece, being approximately 60um in length (Brito, 2007). The head of stallion spermatozoon are oval, with the anterior third being the widest part of the head piece (Brito, 2007). Deviations around this appearance would define a spermatozoon as morphologically abnormal although there is variation for this parameter ranging from 5% to 8% across stallions (Dott, 1975). To define a stallion as fertile, the individual should present a minimum of 1 x 10^9 morphologically normal, progressively motile sperm (Hernández-Avilés and Love, 2021).

Appendix I: Articles utilised within the evidence synthesis test-list (Chapter 3).

Appendix I1; List of articles included within the evidence synthesis test-list.

Appendix I2; Reference list of articles included within the evidence synthesis.

Appendix I1; List of articles included within the evidence synthesis test-list.

Systematic review protocol test-list bibliography (citations)
Aurich *et al*., 2020; Consuegra *et al*., 2020; Hernández-Avilés *et al*., 2020; de Lima, Freitag and Kozicki, 2020; Loureiro *et al*., 2020; Mislei *et al*., 2020; Novello *et al*., 2020; Ruiz *et al*., 2020; Burger, Dolivo and Wedekind, 2015; Gibb *et al*., 2015; Heutelbeck *et al*., 2015; Lemasson *et al.*, 2015; Love *et al*., 2015; Mráčková, Zavadilová and Sedlinská, 2015; Muñoz *et al*., 2015; Rezagholizadeh *et al*., 2015; Albrizio *et al*., 2010; Alghamdi *et al.*, 2010; Hoogewijs *et al*., 2010; Len *et al*., 2010; Morrell *et al*., 2010; Nuñez, Adelman and Rubenstein, 2010; Ponthier *et al*., 2010; Wrench *et al*., 2010; Kuisma *et al*., 2006; Pesch, Bergmann and Bostedt, 2006; Brinsko *et al*., 2005; Gamboa and Ramalho-Santos, 2005; Kirk, Squires and Graham, 2005; Turner, Casas-dolz and Schlingmann, 2005; Quintero-Moreno *et al*., 2003; Sutovsky *et al.,* 2003; Labbe *et al*., 2001; Li, 2000; Parker *et al*., 2000; Stradaioli *et al*., 2000; Oristaglio Turner, McDonnell and Hawkins, 1995.

Appendix I2; Reference list of articles included within the evidence synthesis.

Systematic review protocol test-list bibliography (references)

Albrizio, M., Lacalandra, G. M., Micera, E., Guaricci, A. C., Nicassio, M. and Zarrilli, A. (2010) Delta opioid receptor on equine sperm cells: Subcellular localization and involvement in sperm motility analyzed by computer assisted sperm analyzer (CASA), *Reproductive Biology and Endocrinology*, **8**, pp. 1–11.

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Appendix J: Definitions used for the categorisation of breeds within the evidence synthesis.

Breed category	Original label
Warmblood	Warmblood, German Warmblood, Swedish Warmblood, Dutch Warmblood, PRE Andalusian, Andalusian, Spanish purebred, Lusitano, Hanoverian, Holstein, Oldenburg, Aztec, Quarter horse, Mangalarga Marchador, Brazilian jumping horse, Criollo breed, Saddlebred, Marwari, Silesian, Trotter, Old Kladruber, Franches-Montagnes, Lipizzaner, Standardbred, East Bulgarian Warmblood
Hotblood	Arabian, Thoroughbred
Coldblood	Polish Coldblood, Icelandic
Miniature and Pony type	Shetlands, Miniature Horse, Miniature and Shetland Cross, Miniature Brazilian Pony, Brazilian Pony, Palouse Pony, Caspian, American Miniature, Pony Type, Welsh Pony
Mixed breeds	Studies that included breeds which fell in-between categories were defined as 'mixed-breeds'. For example, including a mixture of hotblood and warmblood breeds.

Appendix K: References utilised within the final meta-regression analysis within the evidence synthesis (Chapter 3).

K1 Citation list of studies (n=230) included in the final regression analysis

K2 Reference list of studies (n=230) included in the final regression analysis

Appendix K1; Citation list of studies (n=230) included in the final regression analysis.

Publication year	Citation
2021	Al-Kass *et al*., 2021; Halo *et al*., 2021; Ivanova *et al*., 2021; Papin *et al*., 2021; Tongu *et al.*, 2021
2020	Albrizio *et al*., 2020; Catalán, *et al*., 2020; Catalán *et al*., 2020; Consuegra *et al*., 2020; Crespo, Gosálvez *et al*., 2020; Crespo, Quinones-Perez *et al*., 2020; Griffin *et al*., 2020; Guasti *et al*., 2020; Hernández-Avilés *et al.*, 2020a; Hernández-Avilés *et al.*, 2020b; Jeannerat *et al*., 2020; Kohne *et al*., 2020; Loureiro *et al*., 2020; Nery *et al*., 2020; Novello *et al*., 2020; Papa *et al*., 2020; Prell *et al*., 2020; Whitesell *et al*., 2020
2019	Atroshchenko *et al*., 2019; Brasileiro *et al*., 2019; Buss, Aurich and Aurich, 2019; Cavalero *et al*., 2019; Consuegra, Crespo, Bottrel *et al*., 2019; Consuegra, Crespo, Dorado *et al*., 2019; Delgado-Bermúdez *et al.*, 2019; Gonzalez-Castro *et al*., 2019; Hernandez-Aviles *et al*., 2019; Len *et al*., 2019; Muller, 2019; Ortega-Ferrusola *et al*., 2019; Ortiz-Rodriguez *et al.*, 2019; Papas *et al*., 2019; Serafini *et al*., 2019; Shore, 2019; Treulen *et al*., 2019; van Dorland *et al*., 2019
2018	Aurich *et al*., 2018; Cabrera *et al*., 2018; Consuegra *et al*., 2018; Ellerbrock *et al*., 2018; Ferrer and Miller, 2018; Gautier *et al*., 2018; Halo *et al*., 2018; Johannisson *et al*., 2018; Kass *et al*., 2018; Larentis *et al*., 2018; Nowak *et al*., 2018; Pérez-Marín *et al*., 2018; Perez-Marin *et al*., 2018; Varela *et al*., 2018; Wu *et al*., 2018
2017	Berlinguer *et al*., 2017; Bucci, Giaretta, *et al*., 2017; Bucci, Spinaci, *et al.*, 2017; Darr *et al*., 2017; de la Torre *et al*., 2017; de Oliveira *et al*., 2017; Giaretta *et al*., 2017; Hidalgo *et al*., 2017; Jung and Yoon, 2017; Leisinger *et al.*, 2017; London *et al*., 2017; Morrell *et al*., 2017; Neuhauser, Säcker and Handler, 2017; Ortiz-Rodriguez *et al*., 2017; Schmidt *et al*., 2017; Šichtař *et al*., 2017; Soni *et al*., 2017; Tejpal *et al*., 2017; Wach- Gygax *et al*., 2017
2016	Aurich *et al*., 2016; Balao da Silva *et al.*, 2016; Barrier Battut *et al.*, 2016; Bucci *et al*., 2016; Darr *et al*., 2016; Ellerbrock *et al*., 2016; Figueiredo, 2016; Freitas *et al*., 2016; Gibb *et al*., 2016; Goedde, 2016; Martins *et al*., 2016; Merkl *et al*., 2016; Oldenhof *et al*., 2016; Orsztynowicz *et al*., 2016; Puglisi *et al*., 2016; Restrepo-Betancur, Cantero-Nanclares and Montoya-Paez, 2016; Riccio *et al*., 2016; Roach *et al*., 2016; Rossi, Falomo and Mantovani, 2016; Schrammel *et al*., 2016; Swegen *et al*., 2016; Voge *et al*., 2016
2015	Burger *et al*., 2015; Crespo *et al*., 2015; Davolli, 2015; Hayden *et al*., 2015; Heutelbeck *et al*., 2015; Miller *et al*., 2015; Miro *et al*., 2015; Muñoz *et al*., 2015; Neuhauser, Dörfel and Handler, 2015; Ramires Neto *et al*., 2015; Rezagholizadeh *et al*., 2015; Waheed, Ghoneim and Abdou, 2015; Yeste *et al*., 2015
2014	Balao da Silva *et al*., 2014; Burns and Herickhoff, 2014; Campos *et al*., 2014; Florez-Rodriguez *et al.*, 2014; Gallardo Bolaños *et al*., 2014; Giaretta *et al*., 2014; Gracia-Calvo *et al*., 2014; Hartwig *et al*., 2014; Ing *et al*., 2014; Kiser *et al*., 2014; Krakowski *et al*., 2014; Morrell *et al.*, 2014; Morrell and Johannisson, 2014; Pozor *et al*., 2014; Whitesell *et al*., 2014
2013	Balao da Silva *et al*., 2013; Krakowski *et al.*, 2013; Len *et al*., 2013; Monteiro *et al*., 2013; Morrell *et al.*, 2013; Neto *et al.*, 2013; Pasing *et al*., 2013; Ponthier *et al*., 2013; Ramires-Neto *et al*., 2013a; Ramires-Neto *et al.*, 2013b; Rosenberg *et al*., 2013; Waheed, El-Bahr and Al-haider, 2013
2012	Alvarenga *et al*., 2012; Balao da Silva *et al*., 2012; Bolaños *et al.*, 2012; Bliss *et al*., 2012; Campos, 2012; Daigneault *et al*., 2012; Dean *et al*., 2012; Edmond *et al*., 2012; Ferrer *et al*., 2012; Fioratti *et al.*, 2012; Janett *et al.*, 2012; Love *et al.*, 2012; Mawyer *et al*., 2012; Morrell *et al*., 2012; Oldenhof *et al*., 2012; Ortgies *et al*., 2012; Stuhtmann *et al.*, 2012
2011	Balao Da Silva *et al*., 2011; Contri *et al.*, 2011; Gibb *et al*., 2011; Gutiérrez-Cepeda *et al*., 2011; Love, 2011; Monteiro *et al*., 2011; Morrell *et al.*, 2011; Morrell, Wienen and Wallgren, 2011; Parlevliet, Lynn and Paccamonti, 2011; Pozor *et al*., 2011; Stuhtmann, 2011
2010	Albrizio *et al*., 2010; Cavinder *et al*., 2010; Giannoccaro *et al*., 2010; Hoogewijs *et al.*, 2010; LeFrapper, Walston and Whisnant, 2010; Len *et al.*, 2010; Minervini *et al*., 2010; Morrell *et al*., 2010; Morrell, Rodriguez-Martinez and Johannisson, 2010; Naughton, 2010; Veronesi *et al*., 2010
2009	Edmond, 2009; Foster, 2009; Mansour, 2009; Ortega-Ferrusola *et al*., 2009; Peters *et al*., 2009; Seale, 2009; Webb and Dean, 2009

2008	Brum, Sabeur and Ball, 2008; Deichsel *et al*., 2008; Grady, 2008; Hidalgo *et al.*, 2008; Len, 2008; Waite *et al*., 2008
2007	Dietz *et al.*, 2007
2006	Janett *et al*., 2006; Pagl *et al*., 2006; Ricker *et al*., 2006; Sieme, Knop and Rath, 2006
2005	Albrizio *et al*., 2005; Almeida and Ball, 2005; Brinsko *et al*., 2005; Janett *et al*., 2005
2004	Brinsko *et al*., 2004
2003	Baumber *et al*., 2003; Brinsko *et al*., 2003; Nie, Johnson and Wenzel, 2003
2002	Ball and Vo, 2002; Bruemmert *et al.*, 2002; Carver and Ball, 2002; Devireddy *et al*., 2002; Hinrichs *et al.*, 2002; Limone *et al*., 2002; Macpherson *et al*., 2002; Nie, Wenzel and Johnson, 2002; Sieme, Echte and Klug, 2002
2001	Ball and Vo, 2001; Bennett-Wimbush *et al*., 2001; Rigby *et al.*, 2001
2000	Brinsko *et al*., 2000
1998	Navarro *et al*., 1998
1997	Reilas *et al*., 1997
1996	Heitland *et al*., 1996; Immegart, 1996
1995	Bedford *et al*., 1995; Heitland *et al.*, 1995
1994	Malmgren *et al*., 1994; Metcalf *et al*., 1994
1993	Jasko *et al.*, 1993; Webb, Ams and Pool, 1993
1992	Jasko *et al.*, 1992; Kayser *et al*., 1992; Parlevliet *et al.*, 1992
1991	Jasko, Lein and Foote, 1991; Varner, Vaughan and Johnson, 1991

Appendix K2; Reference list of studies (n=230) included in the final regression analysis

References (n=230) utilised within the final meta-analysis for chapter 1

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Appendix L: Statistical code used for the meta-regression analysis (Genstat)

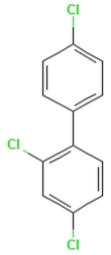
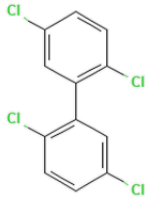
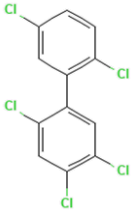
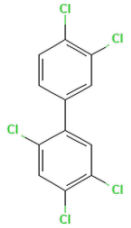
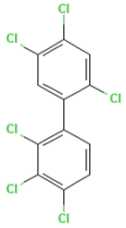
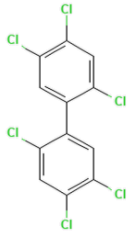
```
CALC Wt = (1/(BOUND(Final_SEM;1)**2))/MEAN(1/(BOUND(Final_SEM;1)**2))
MODEL [WEIGHTS=Wt] motility_mean
FIT   [FPROB=yes; TPROB=yes] Final_year
RKEEP FITTED = Fit

PREDICT [COMBINATIONS=full; ADJUST=equal; ALIAS=ignore] Final_year;
LEVELS=!(1985,1986,1987,1988,1989,1990,1991,1992,1993,1994,1995,1996,1997
,1998,1999,2000,2001,2002,2003,2004,2005,2006,2007,2008,2009,2010,2011,20
12,2013,2014,2015,2016,2017,2018,2019)
PREDICT [PRINT=*;COMBINATIONS=full; ADJUST=equal; ALIAS=ignore; SE=TSE] \
        Final_year; LEVELS=Final_year

CALC Size = SQRT(Wt)
PEN 1; METHOD=point; SYMBOL='circle'; COLOUR='grey'; CFILL='match';
SIZE=Size
PEN 2; METHOD=line; SYMBOL='none'; COLOUR='red'; THICK=2
YAXIS 3; TITLE='Progressive motility (%)'
XAXIS 3; TITLE='Year of collection'
FRAME 2; YUPPER=0.93; YLOWER=0.8; XLOWER=0.11
DGRAPH [W=3; TITLE='Weighted regression of PMOT against year of
collection'] \
   Y=motility_mean,Fit; X=Final_year; PEN=1,2; DESC='Observed (size ~
weight)','Linear fit'

CALC casa_STR_RMV = MVREPLACE(casa_VAP; MEAN(casa_VAP))
CALC casa_VAP_RMV = MVREPLACE(casa_STR; MEAN(casa_STR))
MODEL [WEIGHTS=Wt] motility_mean
FIT  [FPROB=yes] Final_year
STEP [INRATIO=4; OUTRATIO=4; MAXCYCLE=8; FPROB=yes; NOMESSAGE=alias] \

Data_type,Breed,Fertility_group,Location,Season_of_collection,Hemisphere,
\
     Extender,Centrifugation,CASA_model,casa_STR_RMV,casa_VAP_RMV
RDISPLAY [PRINT=accumulated; FPROB=yes]

DROP  [PRINT=accumulated; FPROB=yes] Final_year

PREDICT [COMBINATIONS=full; ADJUST=equal; ALIAS=ignore] CASA_model
PREDICT [COMBINATIONS=full; ADJUST=equal; ALIAS=ignore] Hemisphere
PREDICT [COMBINATIONS=full; ADJUST=equal; ALIAS=ignore] Location
PREDICT [COMBINATIONS=full; ADJUST=equal; ALIAS=ignore] Breed
PREDICT [COMBINATIONS=full; ADJUST=equal; ALIAS=ignore] Final_year;
LEVELS=!(2000,2001,2002,2003,2004,2005,2006,2007,2008,2009,2010,2011,2012
,2013,2014,2015,2016,2017,2018,2019)

RCHECK [RMETHOD=deviance] residual; composite

CALC Year = Final_year - MIN(Final_year)
CALC YearQuadratic,YearCubic = Year**(2,3)
MODEL [WEIGHTS=Wt] motility_mean
FITINDIVIDUALLY [PRINT=#,accumulated; FPROB=yes]
Year,YearQuadratic,YearCubic

FITINDIVIDUALLY [PRINT=#,accumulated; FPROB=yes] POL(Year;3)
RGRAPH

RKEEP FITTED = Fit
PEN 1; METHOD=point; SYMBOL='circle'; COLOUR='grey'; CFILL='match';
SIZE=Size
PEN 2; METHOD=line; SYMBOL='none'; COLOUR='red'; CFILL='match'; THICK=2
YAXIS 3; TITLE='Progressive motility'
XAXIS 3; TITLE='Year of collection'
```

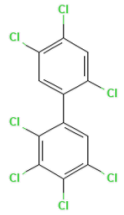
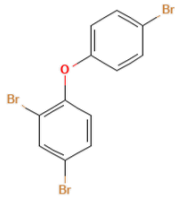
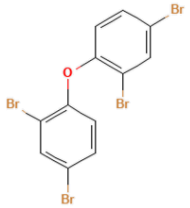
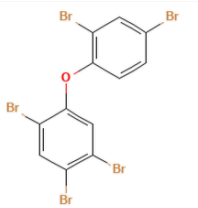
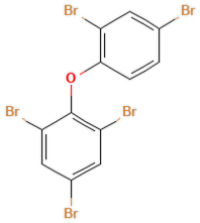
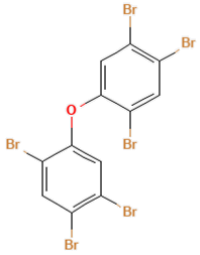
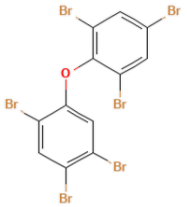
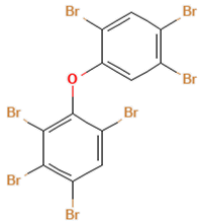
```
DGRAPH [W=3; TITLE='Weighted multiple regression of PMOT against year of
collection'] \
   Y=motility_mean,Fit; X=Final_year; PEN=1,2; DESC='Observed (size ~
weight)','Model fit'
```

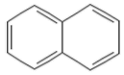
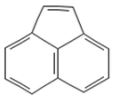
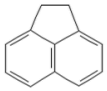
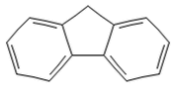
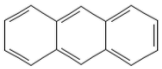
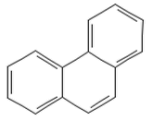
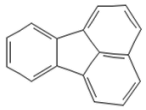
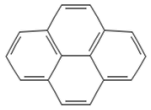
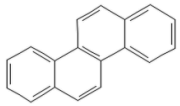
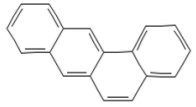
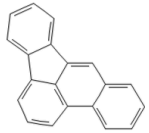
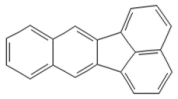
Appendix M: Definitions for categorisation of groups used in objective B (Chapter 4).

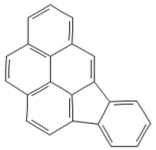
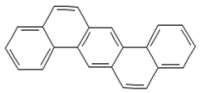
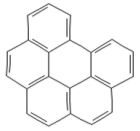
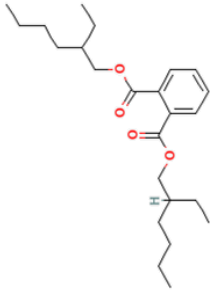
Variable	Category	Original label
Breed	Warmblood	American Curly, American Paint, American Quarter, Andalusian, AES, Appaloosa, Belgian Warmblood, British Sport Horse, British Warmblood, Cleveland Bay, Danish Warmblood, Dutch Warmblood, German Warmblood, Hackney, Hanoverian, Holstein, Hungarian Warmblood, Irish Draught, Irish Sport Horse, Kinsky, Knabstrupper, KWPN, Lusitano, Morgan, Oldenburg, Paint Horse, Pintabian, Quarter Horse, Scottish Warmblood, Selle Francais, Spanish Mustang, Sport Horse, Standardbred, Swedish Warmblood, Trakehner, Warmblood, Westphalian
	Hotblood	Arabian, Anglo Arab, Thoroughbred, Akhal Teke
	Coldblood	Clydesdale, Drum horse, Friesian, Gypsy Cob, Icelandic, Percheron, Shire, Suffolk Punch, Traditional Cob, Traditional Coloured
	Cross breed	Cross bred, part bred
	Pony type	British riding pony, British sports pony, Connemara, Dales pony, Dutch Welsh, Eriskay, Fell pony, German Rein Pony, German riding pony, Highland pony, Native, New Forest Pony, Polo Pony, Riding Pony, Scottish Sports Pony, Welsh Mountain Pony, Wester EMS
Discipline	American	Western, Reining
	Breeding	Breeding
	Endurance	Endurance, Eventing, Racing, Polo, Multidisciplinary
	Flatwork	Dressage, Showing
	Harness	Harness racing, Trotting, Driving
	Jumping	Show Jumping, Working hunter

Appendix N: Classification of Σ7PCBs, Σ7PBDEs, Σ16PAHs, and DEHP, including molecular structures, formulas and weights and CAS registration codes.

Congener	Structure	MF*	MW (g/mol) **	CAS***	Congener	Structure	MF*	MW (g/mol) **	CAS***
PCB 28		$C_{12}H_7Cl_3$	257.5	7012-37-5	**PCB 52**		$C_{12}H_6Cl_4$	292.0	35693-99-3
PCB 101		$C_{12}H_5Cl_5$	326.4	37680-73-2	**PCB 118**		$C_{12}H_5Cl_5$	326.4	31508-00-6
PCB 138		$C_{12}H_4Cl_6$	360.9	35065-28-2	**PCB 153**		$C_{12}H_4Cl_6$	360.9	35065-27-1

PCB 180		$C_{12}H_3Cl_7$	395.3	35065-29-3	**PBDE 28**		$C_{12}H_7Br_3O$	406.89	41318-75-6
PBDE 47		$C_{12}H_6Br_4O$	485.8	5436-43-1	**PBDE 99**		$C_{12}H_4Br_6O$	564.7	60348-60-9
PBDE 100		$C_{12}H_4Br_6O$	564.7	189084-64-8	**PBDE 153**		$C_{12}H_4Br_6O$	643.6	68631-49-2
PBDE 154		$C_{12}H_4Br_6O$	643.6	207122-15-4	**PBDE 183**		$C_{12}H_3Br_7O$	722.5	207122-16-5

Naphthalene		$C_{10}H_8$	128.2	91-20-3	**Acenaphthalene**		$C_{12}H_8$	152.2	208-96-8
Acenaphthene		$C_{12}H_{10}$	154.2	83-32-9	**Fluorene**		$C_{13}H_{10}$	166.2	86-73-7
Anthracene		$C_{14}H_{10}$	178.2	120-12-7	**Phenanthrene**		$C_{14}H_{10}$	178.2	85-01-8
Fluoranthene		$C_{16}H_{10}$	202.3	206-44-0	**Pyrene**		$C_{16}H_{10}$	202.3	129-00-0
Chrysene		$C_{18}H_{12}$	228.3	218-01-9	**Benzo[a]anthracene**		$C_{18}H_{12}$	228.3	56-55-3
Benzo[b] fluoranthene		$C_{20}H_{12}$	252.3	205-99-2	**Benzo[k]fluoranthene**		$C_{20}H_{12}$	252.3	207-08-9

Benzo[a]pyrene		$C_{20}H_{12}$	252.3	50-32-8	**Indeno[1,2,3-cd]pyrene**		$C_{22}H_{12}$	276.3	193-39-5
Dibenzo[a,h] anthracene		$C_{22}H_{14}$	278.3	53-70-3	**Benzo[ghl]perylene**		$C_{22}H_{12}$	276.3	191-24-2
DEHP		$C_{24}H_{38}O_4$	390.6	117-81-7					

**MF; Molecular formula*
***MW; Molecular weight (g/mol)*
****CAS; Chemical Abstracts Service registry numbers for specific chemical identification*
Structural images have been extracted from the PubChem chemical database (PubChem, 2022).

Appendix O: Number of stallions and number of samples contributing to each year of collection.

Total Motility (%)					
Year of collection	**2001**	**2002**	**2003**	**2004**	**2005**
Stallion /sample number	4 / 17	31 / 375	4 / 23	16 / 69	52 / 409
Year of collection	**2006**	**2007**	**2008**	**2009**	**2010**
Stallion / sample number	82 / 660	88 / 660	94 / 789	75 / 559	54 / 390
Year of collection	**2011**	**2012**	**2013**	**2014**	**2015**
Stallion / sample number	82 / 850	71 / 581	60 / 591	89 / 684	81 / 762
Year of collection	**2016**	**2017**	**2018**	**2019**	**2020**
Stallion / sample number	74 / 696	93 / 783	94 / 788	100 / 823	30 / 177
Concentration ($x10^6$/ml)					
Year of collection	**2001**	**2002**	**2003**	**2004**	**2005**
Stallion / sample number	*	2 / 14	27 / 189	42 / 403	56 / 403
Year of collection	**2006**	**2007**	**2008**	**2009**	**2010**
Stallion / sample number	82 / 665	89 / 662	93 / 755	74 / 527	54 / 381
Year of collection	**2011**	**2012**	**2013**	**2014**	**2015**
Stallion / sample number	81 / 827	35 / 296	1 / 4	3 / 8	5 / 24
Year of collection	**2016**	**2017**	**2018**	**2019**	**2020**
Stallion / sample number	2 / 5	1 / 11	*	2 / 7	2 / 4
Volume (ml)					
Year of collection	**2001**	**2002**	**2003**	**2004**	**2005**
Stallion / sample number	4 / 17	33 / 388	30 / 206	54 / 464	56 / 433
Year of collection	**2006**	**2007**	**2008**	**2009**	**2010**
Stallion / sample number	82 / 664	89 / 657	93 / 760	73 / 548	54 / 394
Year of collection	**2011**	**2012**	**2013**	**2014**	**2015**
Stallion / sample number	81 / 832	70 / 559	58 / 571	90 / 674	81 / 734
Year of collection	**2016**	**2017**	**2018**	**2019**	**2020**
Stallion / sample number	73 / 685	92 / 757	95 / 785	100 / 815	30 / 179
Total Sperm Output ($x10^6$)					
Year of collection	**2001**	**2002**	**2003**	**2004**	**2005**
Stallion / sample number	*	2 / 14	27 / 188	41 / 390	56 / 400
Year of collection	**2006**	**2007**	**2008**	**2009**	**2010**
Stallion / sample number	82 / 665	89 / 654	93 / 752	74 / 536	54 / 385
Year of collection	**2011**	**2012**	**2013**	**2014**	**2015**
Stallion / sample number	80 / 829	35 / 297	1 / 4	4 / 8	4 / 15
Year of collection	**2016**	**2017**	**2018**	**2019**	**2020**
Stallion / sample number	2 / 5	*	*	2 / 6	2 / 6

Appendix P: Feed types and respective ingredients of feedstuffs analysed in objective C (chapter 5).

Feed type	Feed ingredients	Oil content	Packaging
Alfalfa, high oil	Alfalfa, rape seed oil	12%	Plastic
Stud cubes	Wheatfeed, Extracted sunflower, Barley, Oats, Unmolassed sugar beet, Oatfeed, Grass, Cane molasses, Wheat, Calcium carbonate, Rapeseed oil, Dehulled soya bean meal (genetically modified), Rice bran, Sodium chloride, Fructose oligosaccharides, Magnesium oxide, Mannan oligosaccharides	4.5%	Paper
Foal cubes	Micronised Wheat, Micronised Soya Beans, Whey, Distillers' Grains, Soya Bean Meal, Molasses, Micronised Linseed, Calcium Carbonate, Dicalcium Phosphate, Vitamins and Minerals	7%	Paper
Racehorse cubes	Micronised Wheat, Nutritionally Improved Straw, Wheatfeed, Distillers' Grains, Micronised Soya Beans, Molasses, Soya Oil, Calcium Carbonate, Vitamins and Minerals, Calcined Magnesite, Sodium Chloride	6%	Plastic

Appendix Q: Limit of detections for the chemical analysis (GC-MS) of testicular, soil, grass and feed samples

Sample type	PCBs	PBDEs	PAHs	DEHP
Testicular	ΣPCBs: 0.50 µg/kg	PBDE 28, PBDE 47, PBDE 99, PBDE 100: 0.02 µg/kg; PBDE 153, PBDE 154, PBDE 183: 0.50 µg/kg	Naphthalene, Acenaphthalene, Acenaphthene, Fluorene, Anthracene, Benzo[a]anthracene, Chrysene, Benzo[k]fluoranthene, Benzo[a]pyrene, Benzo[ghl]perylene: 1.00 µg/kg; Phenanthrene, Fluoranthene, Benzo[b]fluoranthene, Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene: 5.00 µg/kg; Pyrene: 15.00 µg/kg	DEHP: 0.05 µg/kg
Soil	ΣPCBs: 0.01 µg/kg	PBDE 28, PBDE 47: 0.01 µg/kg; PBDE 99: 0.04 µg/kg; PBDE 100, PBDE 154: 0.02 µg/kg; PBDE 153: 0.03 µg/kg; PBDE 183: 0.05 µg/kg	Naphthalene, Fluorene, Fluoranthene, Pyrene: 3.00 µg/kg; Acenaphthalene, Phenanthrene, Benzo[a]anthracene, Chrysene: 2.00 µg/kg; Acenaphthene: 4.00 µg/kg; Anthracene: 5.00 µg/kg; Benzo[b]fluoranthene, Benzo[k]fluoranthene, Benzo[a]pyrene, Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene, Benzo[ghl]perylene: 1.00 µg/kg	DEHP: 0.05 µg/kg
Grass	ΣPCBs: 0.02 µg/kg	PBDE 28, PBDE 47, PBDE 99, PBDE 100: 0.02 µg/kg; PBDE 153, PBDE 154, PBDE 183: 0.50 µg/kg	Naphthalene, Acenaphthalene, Acenaphthene, Fluorene, Anthracene, Benzo[a]anthracene, Chrysene, Benzo[k]fluoranthene, Benzo[a]pyrene, Benzo[ghl]perylene: 1.00 µg/kg; Phenanthrene, Fluoranthene, Benzo[b]fluoranthene, Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene: 5.00 µg/kg; Pyrene: 15.00 µg/kg	DEHP: 0.05 µg/kg
Feedstuff	ΣPCBs: 0.02 µg/kg	PBDE 28, PBDE 47, PBDE 99, PBDE 100: 0.02 µg/kg; PBDE 153, PBDE 154, PBDE 183: 0.50 µg/kg	Naphthalene, Acenaphthalene, Acenaphthene, Fluorene, Anthracene, Benzo[a]anthracene, Chrysene, Benzo[k]fluoranthene, Benzo[a]pyrene, Benzo[ghl]perylene: 1.00 µg/kg; Phenanthrene, Fluoranthene, Benzo[b]fluoranthene, Indeno[1,2,3-cd]pyrene, Dibenzo[a,h]anthracene: 5.00 µg/kg; Pyrene: 15.00 µg/kg	DEHP: 0.05 µg/kg.