Received: 13 March 2023 | Accepted: 29 December 2023

DOI: 10.1111/evj.14057

ORIGINAL ARTICLE

Equine Veterinary Journal WILEY

Does inbreeding contribute to pregnancy loss in Thoroughbred horses?

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Funding information
Horserace Betting Levy Board; Thoroughbred Breeders Association; Royal Veterinary College; The Alborada Trust

Abstract

Background: Excessive inbreeding increases the probability of uncovering homozygous recessive genotypes and has been associated with an increased risk of retained placenta and lower semen quality. No genomic analysis has investigated the association between inbreeding levels and pregnancy loss.

Objectives: To compare genetic inbreeding coefficients (*F*) of naturally occurring Thoroughbred Early Pregnancy Loss (EPLs), Mid and Late term Pregnancy Loss (MLPL) and Controls. The *F* value was hypothesised to be higher in cases of pregnancy loss (EPLs and MLPLs) than Controls.

Study design: Observational case–control study.

Methods: Allantochorion and fetal DNA from EPL ($n = 37$, gestation age 14–65 days), MLPL ($n = 94$, gestational age 70 days–24 h post parturition) and Controls ($n = 58$) were genotyped on the Axiom Equine 670K SNP Genotyping Array. Inbreeding coefficients using Runs of Homozygosity (FROH) were calculated using PLINK software. ROHs were split into size categories to investigate the recency of inbreeding.

Results: MLPLs had significantly higher median number of ROH (188 interquartile range [IQR], 180.8–197.3), length of ROH (3.10, IQR 2.93–3.33), and total number of ROH (590.8, IQR 537.3–632.3), and F_{ROH} (0.26, IQR 0.24–0.28) when compared with the Controls and the EPLs ($p < 0.05$). There was no significant difference in any of the inbreeding indices between the EPLs and Controls. The MLPLs had a significantly higher proportion of long (>10 Mb) ROH (2.5%, IQR 1.6–3.6) than the Controls (1.7%, IQR 0.6–2.5), $p = 0.001$. No unique ROHs were found in the EPL or MLPL populations.

Main limitations: SNP-array data does not allow analysis of every base in the sequence.

Conclusions: This first study of the effect of genomic inbreeding levels on pregnancy loss showed that inbreeding is a contributor to MLPL, but not EPL in the UK

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Thoroughbred population. Mating choices remain critical, because inbreeding may predispose to MLPL by increasing the risk of homozygosity for specific lethal allele(s).

KEYWORDS
abortion, fetus, homozygosity, horse, mare, miscarriage

1 | INTRODUCTION

Inbreeding (the mating of related individuals) is a common practice in the livestock industry because individuals with desirable traits are highly prized as breeding stock. The descendants of these individuals therefore make up a greater proportion of the population. In the Thoroughbred breeding industry, with a focus on racing potential, 97% of 10 118 individuals studied could be traced to a single stallion, Norther Dancer.[1] The inbreeding coefficient (*F*) is the probability that a pair of alleles at a specific locus will be identical-by-descent[2]; thus, increasing the risk of uncovering undesirable recessive phenotypes. Historically, pedigree data have been used to estimate inbreeding levels, this however is limited particularly by missing or incorrect data.[3–5] Relevant genomic estimations such as Runs of Homozygosity (ROH) are considered preferable, and have the additional benefit of indicating inbreeding trends.[5] Over time, mutations break up longer ROH into shorter ROH, and thus short ROHs can estimate inbreeding that happened in the distant past, whilst longer ROH indicate a more recent occurrence of inbreeding.

In horses, inbreeding has been associated with an increased risk of retained placenta[6] and lower semen quality.[7–10] Fertility scores and foaling rates have been shown to have either no association,[11,12] a weak association,[13] or a significant association[14] with inbreeding levels. Gestation length is not associated with inbreeding in horses.[15–21] Only a single study using pedigree data to calculate the inbreeding coefficient (FPED) has investigated any link between inbreeding and pregnancy loss, finding both increased FPED and mare age to be significant contributors to increased risk of early abortion at <5 months gestation in Norwegian Trotters.[22] To date no genomic analysis has been completed to determine any association between inbreeding levels and pregnancy loss in horses. Around 5%–10% of equine pregnancies end in early pregnancy loss (EPL; up to 65 days gestation),[23] and a further 7.3% of equine pregnancies are lost between Day 70 of gestation and 24 h post parturition (mid and late term pregnancy loss [MLPL]).[24] The underlying causes of pregnancy loss differ between early and mid to late gestation.[24–27] A biobank of naturally occurring EPLs, created using recent advances in methodologies to collect tissue samples,[28] and MLPLs, have allowed investigation of the FROH for the pregnancy itself rather than that of the parents. It was hypothesised that the inbreeding coefficient would be higher in cases of pregnancy loss (both EPLs and MLPL) than Controls. This project specifically aimed to compare the estimated genetic inbreeding coefficient using ROH between cases (naturally occurring EPLs and MLPL) and Controls.

2 | MATERIALS AND METHODS

2.1 | Anonymity

Anonymity was maintained by coding the names of veterinarians, stud farms, mares and stallions, with the codes maintained in a password protected Microsoft Excel database.

2.2 | Sample collection

Sample collection and processing of the EPLs has been reported previously.[28,29] In brief, following confirmation of pregnancy failure before 65 days post ovulation (no heartbeat/collapsed vesicle), conceptuses were recovered by uterine lavage by the attending veterinarian during the 2013–2021 breeding seasons.[28] Successfully flushed conceptuses were then placed in sterile transport media and stored at 4°C until being transported on ice to the laboratory for assessment and dissection within an hour of arrival. Placentae from cases of abortion, stillbirth, or perinatal death within 24 h of parturition were obtained following submission for diagnostic investigation at a Newmarket based diagnostic laboratory during the 2017–2020 breeding seasons. Approximately 5 × 5 mm sections of allantochorionic tissue were taken and stored in 1.5 mL of DNAgard (Biometrica) and stored at room temperature for up to 6 months. When allantochorion was not available, sections of fetal gluteal muscle measuring 5 × 5 mm were dissected and stored following the same protocol.

The control group were adult UK Thoroughbreds ($n = 58$ mares, all over 3 years old). Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood following collection from the jugular vein of Thoroughbred mares ($n = 5$) from the institutional research herd as previously described.[30] PBMC pellets were then snap frozen in liquid nitrogen and transferred to −80°C. Hair samples from 53 Thoroughbreds, across eight UK stud farms, were submitted anonymously by the attending veterinarians between 2017 and 2021. The eight stud farms represented a sub population of the stud farms which had submitted EPLs and MLPLs. Aside from the name of the stud farm that the sample came from, no

additional clinical data was collected beyond the individual fitting the criteria of being a registered Thoroughbred, over 3 years of age.

2.3 | DNA extraction

DNA from frozen tissues, tissue stored in DNAgard and PBMCs were extracted using QIAGEN DNeasy Blood and Tissue kit (Qiagen Sciences), following manufacturer's guidelines. Briefly, tissue or cells were incubated at 56°C overnight in 180 µL buffer ATL and 20 µL proteinase K (600 mAU/mL). Tissues were then incubated at room temperature for 2 min with 28 U RNase A as recommended by the manufacturer then passed through a spin column, before elution with 100 µL Buffer AE provided in the kit.

Intact roots from 15 hairs were lysed in a mix of 300 µL cell lysis solution and 5 µL proteinase K at 37°C overnight. To the supernatant, 100 µL protein precipitation solution (PPS) was added then vortexed and incubated on ice for 10 min. Following centrifugation for 3.5 min at 16 000*g*, the supernatant was added to 300 µL isopropanol and mixed by inverting 40 times, then centrifuged again for 3.5 min at 16 000*g*. The supernatant was discarded and 300 µL 70% ethanol added, vortexed for 45 s, then centrifuged for 3.5 min at 16 000*g*. The supernatant was discarded, and the pellet dried overnight at room temperature. Once dried, the pellet was resuspended in 20 µL of hydration solution.

All DNA was quantified using a DeNovix Spectrophotometer (DeNovix), measuring quantity (ng/µL) and quality (A260/A230 and A260/A280). DNA quality was confirmed to have no effect on the inbreeding values calculated.

2.4 | Genotype preparation and SNP pruning

The resulting .CEL files generated from all samples (Cases and Controls) hybridised to the Axiom™ Equine 670K SNP Genotyping Array were imported into Axiom Analysis Suite (AxAS, v5.0.1.38), with SNP probe locations based on EquCab3.0 reference genome. Following the 'Genotyping' workflow, genotype data were exported as a .vcf file. SNP quality control (QC) settings were kept as default as recommended by the manufacturer. Only SNP probes that met AxAS 'Best and Recommended' (i.e., passed all internal programme QC metrics) were included in the exported .vcf file.

As there appears to be little consensus on the filtering steps required for ROH analyses, SNPs were not filtered based on Hardy–Weinberg Equilibrium (HWE), Minor Allele Frequency (MAF), or Linkage Disequilibrium (LD), the latter two being in accordance with recently published guidelines.[31] The removal of rare variants may artificially inflate or deflate calls, potentially missing critical ROHs. To reduce the calling of ROHs that were in LD, the minimum length of ROH was set to 1 Mb for analysis of groups within this study. Only diploid samples were tested, with aneuploid and polyploid individuals removed prior to analysis.

2.5 | ROH detection in PLINK

The .vcf files generated above were then used to identify ROHs in PLINK v1.90[32] using the options as previously described.[33,34] The options used were as follows: minimum SNP density = one SNP per 50 kb, maximum gap length = 100 kb, minimum length per ROH = 1 Mb, minimum number of homozygous SNPs = 80, maximum number of heterozygous SNPs per ROH = 1, maximum number of missing SNPs per ROH = 2. Only autosomes were included in this analysis.

2.6 | ROH analysis

The total number of ROHs per individual (N_{ROH}), the average length ROH an individual possessed (L_{ROH}), and the total length of all ROHs (S_{ROH}) were next calculated for each sample using the outputs generated in PLINK. The genomic inbreeding coefficient (F_{ROH}) was calculated by dividing the S_{ROH} by the total autosomal genome length (L_{AUTO}[35]). The autosomal length for EquCab3.0 was 2 281 300 kb (2280.9 Mb) as calculated from values on ENSEMBL (http://www.ensembl.org/Equus_caballus/Location/Chromosome?r=25%3A1-1000).

$$F_{ROH} = \frac{S_{ROH}}{L_{AUTO}}.$$

The number of short-ROHs (1–2 Mb) and long-ROHs (>10 Mb) (in similarity with Grilz-Seger et al.[33]) for each category were calculated per individual and the percentage of ROHs in each group per individual were then compared between groups.

2.7 | Unique ROH

To identify any candidate ROHs associated with pregnancy loss, ROHs detected in EPLs, and separately MLPLs, were combined into .csv files and compared to all ROHs detected in Controls using *bedtools intersect* pipeline.

2.8 | Data analysis

Normality of the data was assessed in GraphPad Prism (v9.1.2, https://www.graphpad.com/) using the Shapiro–Wilk normality test. In all cases, the normality tests failed, and therefore Kruskal–Wallis with post hoc Dunn's test were used to identify statistical differences between groups, with significance set at $p < 0.05$. The median and interquartile ranges are presented throughout.

3 | RESULTS

3.1 | ROH differ between mid and late pregnancy loss cases and Controls

The EPLs ($n = 37$) were obtained from 22 stud farms and the observed gestation ages ranged from 14 to 68 days. The MLPLs ($n = 94$) came from 42 stud farms, for 9/94 cases the stud farm was unavailable. The observed gestational age in the MLPL group ranged from 86 days gestation to 24 h post parturition. The ROHs of EPLs and MLPLs were compared to each other and with Controls ($n = 58$). The EPLs and Controls did not significantly differ in median values of $N_{ROH}/L_{ROH}/S_{ROH}/F_{ROH}$ (Figure 1A–D).

The MLPLs had significantly higher values for all four metrics ($N_{ROH} = 188$ [IQR 180.8–197.3], $L_{ROH} = 3.10$ [IQR 2.93–3.33], $S_{ROH} = 590.8$ [IQR 537.3–632.3] and $F_{ROH} = 0.26$ [IQR = 0.24–0.28]) when compared with both the EPLs and the Controls, $p < 0.05$ (Figure 1A–D). MLPLs were further explored as Abortions (70–300 days of gestation, $n = 74$) and Stillbirths (301 days of gestation to 24 h post parturition, $n = 16$) and no significant difference found between the groups in any of the inbreeding indices, $p > 0.05$. Four MLPLs were excluded from this additional analysis as, although they could be categorised as a MLPL based on the crown rump length of the fetus, only estimated gestational ages were available.

3.2 | MLPLs show a higher degree of recent inbreeding

Previous work has shown that shorter ROHs (smaller than 0.5 Mb) are indicative of historical inbreeding from 50 to 100 generations ago, that is, before the establishment of the Thoroughbred breed.[36] The EPLs had a significantly lower median percentage of short ROH (1–2 Mb; 47.3%, IQR 42.1–50.2) than the Controls (48.1%, IQR 46.5–54.3, $p = 0.02$; Figure 2A). The MLPL had significantly higher percentages of long ROH (>10 Mb; 2.5%, IQR 1.6–3.6) compared to the Controls (1.7%, IQR 0.6–2.5), $p = 0.001$ (Figure 2B), but were not significantly different from the EPLs (1.8%, IQR 1.2–3.1), $p = 0.3$. There was no significant difference in the percentages of short length ROH between MLPLs (48.8, IQR 44.8–51.8) and either EPLs (47.3%, IQR 42.1–50.2) or Controls (48.1, IQR 46.5–54.3), $p = 0.2$ and 0.8 respectively (Figure 2A).

3.3 | No ROHs were found to be specific to pregnancy loss

In total, 9682 ROHs were found across 58 Controls, 6460 ROHs were found across 37 EPLs and 16 395 ROHs were found across 94 MLPL. To investigate whether specific ROHs may be lethal, the ROH call lists from EPLs, and separately the MLPLs, were compared with the ROH call list from the Controls. No ROH calls came up as unique between

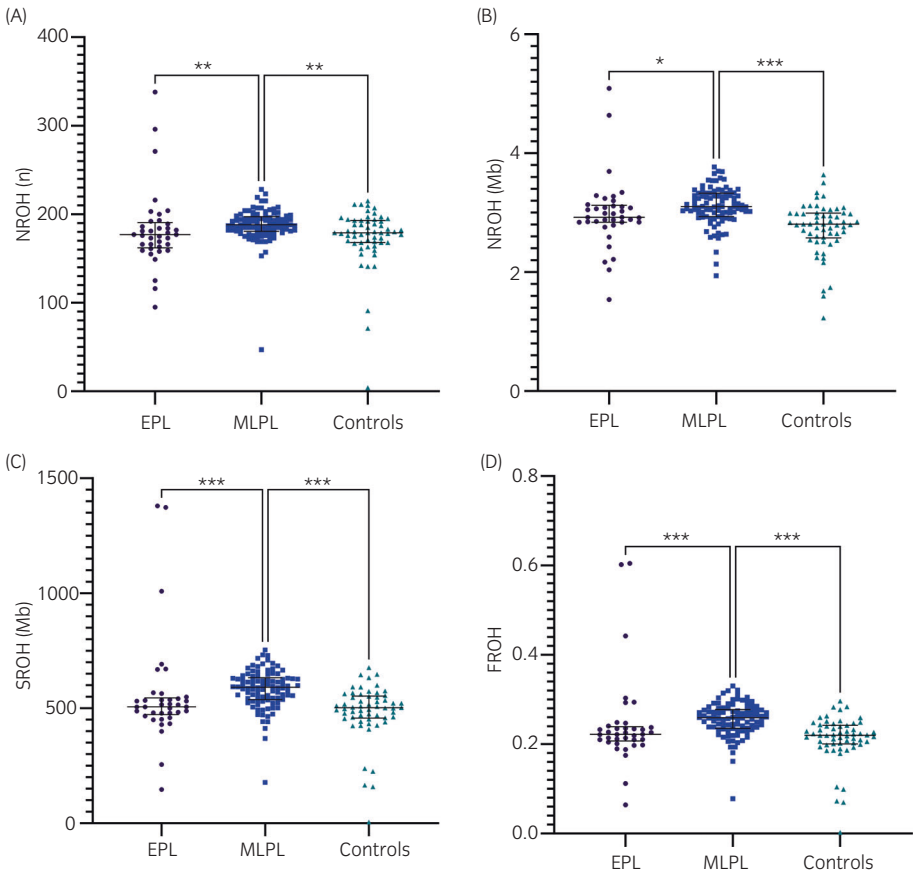

FIGURE 1 Runs of homozygosity (ROH) analysis of Thoroughbred EPLs and MLPLs compared with breed matched Controls. Thoroughbred Early Pregnancy Losses (EPL, $n = 37$) were compared with Mid and Late Term Pregnancy Losses (MLPL, $n = 94$) and breed matched Controls ($n = 58$). The MLPLs had significantly increased median (A) NROH = number of ROH ($p < 0.001$), (B) SROH = sum of ROH ($p < 0.001$), (C) LROH = average length of ROH ($p < 0.001$) and (D) FROH = inbreeding coefficient ($p < 0.001$). Black line = median and interquartile range. Kruskal–Wallis with Dunn's multiple comparisons test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

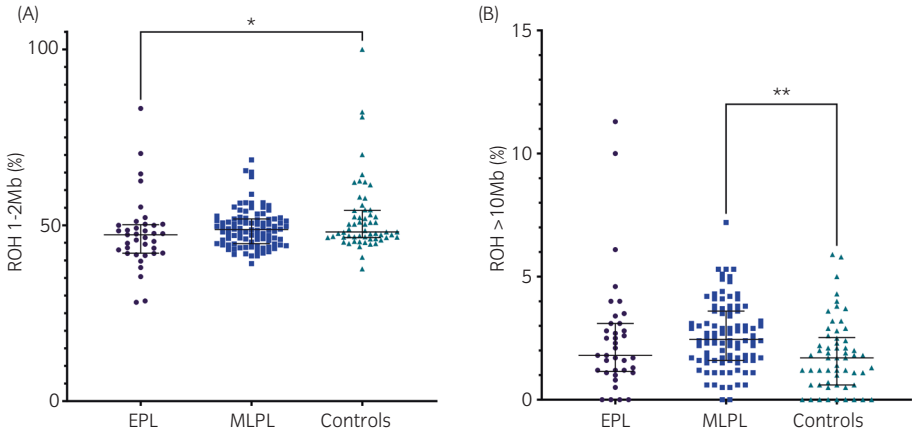

FIGURE 2 Runs of homozygosity (ROH) size analysis of Thoroughbred EPLs, MLPLs and breed matched Controls. Early Pregnancy Loss (EPL; $n = 37$), Mid and Late Term Pregnancy Loss (MLPL, $n = 94$) and breed matched Controls ($n = 58$) and the proportion of (A) short-ROHs (1–2 Mb), and (B) long-ROHs (>10 Mb). Black line = median and interquartile range. Kruskal–Wallis with Dunn's multiple comparisons test (*$p = 0.02$; **$p = 0.001$).

the EPL and Controls, the MLPL and Controls, or the EPLs and MLPLs.

4 | DISCUSSION

Approximately 5%–10% of confirmed equine pregnancies fail before 65 days of gestation,[23] with a further 7.3% failing before the end of the first day of life.[24] To date, no study has specifically investigated any link between genetic inbreeding metrics and pregnancy loss in the mare. This study found that pregnancies lost in mid and late gestation (MLPLs), from Thoroughbred mares in the UK, had significantly higher inbreeding metrics than UK adult Thoroughbred horses, with the proportion of long ROH (an indicator of recent inbreeding) also increased in these lost pregnancies. Contrary to the initial hypothesis, pregnancies lost early in gestation (EPLs) were found to show no significant difference in inbreeding metrics compared to UK adult Thoroughbred horses. No ROHs were found to be unique to the EPL or MLPL cohort.

Higher inbreeding metrics will be associated with an increased risk of the individual inheriting a deleterious homozygous mutation. Examples of homozygous single point mutations that are known to result in pregnancy loss and other congenital abnormalities include congenital hepatic fibrosis,[37] congenital hydrocephalus,[38] and warmblood fragile foal syndrome,[39] the latter recently described as a cause of pregnancy loss for the first time in a Thoroughbred.[40] The findings of our study further underpin the importance of continued research into identifying and characterising fatal mutations, and with new mutations arising all the time, continued surveillance is important. SNP mutations have been associated with abortion and stillbirth,[38–40] but to date none have been identified as causes of lethality in EPLs. Whilst the presence of defective recessive alleles in homozygous status could still contribute to EPL as a less common or rare phenomena, our data support the hypothesis that SNP mutations are more likely to cause lethality in mid to late gestation. It should also be noted that as we only explored diploid cases, we cannot understand the effects of inbreeding on aneuploidy and other chromosomal abnormalities from this data.

Whilst inbreeding theoretically increases the risk of the offspring inheriting the same deleterious mutation from both parents, practically the link may not be as linear as expected. Thoroughbreds were ranked 3rd amongst 37 horse breeds for inbreeding coefficient but 9th for genomic mutational load (genetic burden due to accumulation of deleterious mutations).[41] The protein-coding mutational load is even more nuanced, with almost all the 37 breed groups studied overlapping, regardless of their inbreeding levels. The relatively lower mutational load of Thoroughbreds may in part be due to the breeding practice of selecting for racing potential. Individuals born with a poor phenotype would either not enter racing or have a poor performance on the track so would be unlikely to enter the breeding stock. Likewise, MLPL may act as a successful natural purging step, preventing the individual from entering the national herd in the first place and reproducing.

The MLPLs were found to have a significantly higher proportion of long (>10 Mb) ROH than the Controls. Longer ROHs are indicative of more recent inbreeding as consanguineous matings are more likely to share a greater number of alleles. Over time, heterozygosity can be reintroduced to the population through mutations which break up ROHs into smaller runs. The presence of the higher percentage of long ROH in the MLPL group follows the same trend as the regression analysis of FROH over five decades by McGivney et al.[1] In Great Britain, the number of stallions registered for covering has almost halved in the last 10 years, from 285 stallions in 2011 to 147 stallions in 2021,[42,43] restricting the choice for breeders. Whilst Thoroughbred breeders make careful selection of their matings and breeding choices, the effects of this decline should be under continued scrutiny by the industry to prevent the Thoroughbred populations from suffering an inbreeding depression.

There is limited comparative data available. Todd et al.[20] explored inbreeding levels using pedigree data of Australian Thoroughbreds and found no significant association of the mare, stallion or conceptus' inbreeding coefficients with the foaling rate. Klemetsdal and Johnson[22] also used pedigree data, this time in Norwegian Trotters, and observed that the inbreeding coefficient of the potential offspring (i.e., the pregnancy) was not a significant contributor to foaling rate (proportion of covers resulting in a live foal) in their modelling.

Klemetsdal and Johnson[22] also explored predictors of early abortion (pregnancy loss prior to Month 5 of gestation). Whilst they reported that a 1% increase in a mare's inbreeding coefficient was associated with a 1.27% increase in early abortion frequency, they found that the inbreeding coefficient of the pregnancy itself was not significantly associated with early abortion.[22] This study period only partially overlaps the phenotypes we explored and uses pedigree derived inbreeding coefficients rather than genomic data. Our data suggests that inbreeding exerts an effect on pregnancy loss from Day 70 of gestation all the way through to 24 h post parturition.

There are limitations to this study as the sample sizes are relatively small, it is restricted to one breed and we used a mixture of DNA sources, from the placenta and fetus in the pregnancy losses, and hair and PBMC in the Controls. The samples were submitted from numerous stud farms across the UK; however, it is acknowledged that self-selection bias in the farms and veterinarians who chose to submit material may affect the results. Further, the cause of the loss may in some cases, reduce or preclude the availability of tissue, for example sampling of EPLs is reliant on products of conception being available for collection via uterine lavage and submitted for analysis. Similarly, some causes of MLPL may not be submitted to a diagnostic laboratory for post mortem examination if investigation is not perceived to be required, for example an intrapartum stillbirth from distal limb contractions. This opens up the potential for bias in the phenotypes assessed in this study. Further, non-diploid EPL and MLPL samples were excluded from the analysis due to the possibility of inflated or reduced F_{ROH} coefficients related to the ploidy status that could have impacted the results. Given the high proportion of chromosome wide copy number variants in EPLs,[29] this would have disproportionately affected this phenotype and be a source of bias. It would be of interest to repeat this analysis with different breeds and with larger sample sizes. Whilst other factors such as year of sampling and DNA quality could plausibly impact the results, the inclusion of multiple breeding seasons, the exclusion of failed probes and only individuals that had a SNP call rate of >98% will have minimised their influence.

In conclusion, we observed higher inbreeding metrics in UK Thoroughbred pregnancies lost in mid and late gestation compared to the adult population, evidencing that lack of heterogeneity is a contributor to pregnancy failure after the early pregnancy period. We hypothesise that this is due to an increase in the occurrence of homozygous recessive alleles, highlighting that studies into the role of specific gene mutations are both required and warranted. Although no significant differences were observed in the inbreeding metrics between the EPL and the UK Thoroughbred adults, we recognise a bias in the phenotypes of the losses in this group. Our data highlights the importance of cognisance in mating decisions in the Thoroughbred industry, and continued work in the laboratory to identify possible deleterious mutations.

AUTHOR CONTRIBUTIONS

Jessica M. Lawson: Data curation; formal analysis; investigation; methodology; resources; software; visualization; writing – original draft. **Charlotte A. Shilton:** Data curation; formal analysis; investigation; methodology; resources; software; visualization; writing – original draft. **Victoria Lindsay-McGee:** Investigation; writing – review and editing. **Androniki Psifidi:** Investigation; writing – review and editing. **D. Claire Wathes:** Conceptualization; funding acquisition; writing – review and editing. **Terje Raudsepp:** Conceptualization; funding acquisition; methodology; supervision; writing – review and editing. **Amanda M. de Mestre:** Conceptualization; funding acquisition; supervision; writing – original draft; writing – review and editing.

ACKNOWLEDGEMENTS

Anne Kahler, Belinda Rose and Daniel Hampshire for collecting and processing the EPLs.

FUNDING INFORMATION

Project grant awarded by the Thoroughbred Breeders Association, Horserace Betting Levy Board, the Alborada Trust, and partial PhD studentship funding from the Royal Veterinary College's Paul Mellon Trust for Equine Research.

CONFLICT OF INTEREST STATEMENT

No competing interests have been declared.

DATA INTEGRITY STATEMENT

Amanda M. de Mestre, Charlotte A. Shilton and Jessica M. Lawson take responsibility for the integrity of the data and the accuracy of the data analysis.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/evj.14057.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request and are not publicly available due to privacy and ethical consent restrictions.

ETHICAL ANIMAL RESEARCH

All conceptus recoveries from clinical cases of pregnancy loss under ethics approval from the Clinical Research and Ethical Review Board at the Royal Veterinary College (URN:2012-1169 and URN:2017-1660-3). Sampling of research herd approved by Ethics Committee of the Royal Veterinary College, London (HO licence PPL 70/8577).

INFORMED CONSENT

Informed consent was obtained from all participating stud farms.

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How to cite this article: Lawson JM, Shilton CA, Lindsay-McGee V, Psifidi A, Wathes DC, Raudsepp T, et al. Does inbreeding contribute to pregnancy loss in Thoroughbred horses? Equine Vet J. 2024. https://doi.org/10.1111/evj.14057