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ORIGINAL ARTICLE

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Insulin, but not adiponectin, is detectable in equine saliva using an automated, commercial assay

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Abstract

Background: The hormones insulin and adiponectin are commonly measured in equids because of their involvement in endocrinopathic laminitis. These are currently measured in serum/plasma, although jugular venipuncture can cause stress in some animals and may be impossible in needle-shy individuals. However, both hormones can be measured in saliva in other species.

Objectives: To determine whether [insulin] and [total adiponectin] are detectable in equine saliva using automated assays and whether saliva collection is associated with changes in stress indicators. Additionally, the correlation between serum and salivary [insulin] was investigated.

Study design: In vivo experiment.

Methods: Paired blood and saliva samples were collected from eight adult ponies at multiple time-points ($n = 45$ paired samples). [Insulin] and [total adiponectin] were measured using automated assays validated for equine serum/plasma. Blink rates and heart rates were determined, using video recordings and a wearable heart rate monitor respectively, to assess the effects of sample collection on stress indicators compared with a control situation without a stressful stimulus.

Results: [Total adiponectin] was undetectable in saliva. However, salivary [insulin] was measurable with acceptable inter-assay (1.3 ± 0.9%) and intra-assay (1.1 ± 0.6%) variability. Blink and heart rates during saliva collection did not differ significantly from those in the control setting. Serum and salivary [insulin] were not significantly correlated.

Main limitations: Small sample size comprising native UK ponies; potential sampling bias as animals were recruited to the study partly based on their behaviour during blood sampling; saliva collected from unfasted animals.

Conclusions: Insulin is measurable in equine saliva using an automated assay currently available in the UK, but further validation and the determination of specific diagnostic thresholds are required. Saliva collection was not associated with changes in stress indicators. Further research is therefore needed to determine the potential of equine saliva as a non-invasive alternative to blood for insulin determination.

KEYWORDS
adiponectin, horse, insulin, laminitis, refinement, saliva

1 | INTRODUCTION

Equine endocrinopathic laminitis is a common, painful and potentially devastating condition that can affect horses, ponies and donkeys. Although multiple risk factors are recognised, insulin and adiponectin concentrations are currently the most important predictors of endocrinopathic laminitis risk.[1,2] These two hormones are commonly measured in equids, using either serum or plasma obtained via jugular venipuncture. However, blood sampling requires veterinary intervention that is invasive, may cause stress to some animals, and may even be impossible to perform in needle-shy individuals. In contrast, saliva is an alternative biological sample that can be obtained easily without the need for specialist training or equipment. Salivary insulin can be measured in humans,[3] dogs[4] and pigs,[5] and is reported to be significantly correlated with serum concentrations in humans. Adiponectin is detectable in human[6] and dog[7] saliva, and salivary and serum adiponectin concentrations are significantly correlated in both species. Equine saliva is already used to measure cortisol[8] and antibodies indicating tapeworm burden,[9] however the measurement of insulin and adiponectin in equine saliva has not yet been investigated.

The primary aims of this study, therefore, were: (i) to determine whether equine salivary total adiponectin and insulin concentrations could be detected using automated assays currently available to measure these hormones in blood in the UK, and (ii) to compare indicators of stress during saliva collection and jugular venipuncture in ponies using non-invasive measures previously described for use in equids including spontaneous blink rate,[10,11] heart rate and heart rate variability.[12,13] Additionally, the correlation between serum and salivary insulin concentrations was investigated.

2 | MATERIALS AND METHODS

2.1 | Animals

This study was performed using eight healthy UK native-breed ponies (four mares and four geldings, 5–18 years, 210–450 kg) undergoing another study. Blood samples were collected as part of this other study and no additional samples were collected specifically for the current study.

2.2 | Sample collection and analysis

Ponies remained at pasture throughout the study and during sample collection. Paired blood and saliva samples were collected on the same day and during the same sampling session, at several time-points (no more than once every 2 weeks) over a 14-week period resulting in 45 paired samples. For each pony, the saliva sample was collected first, followed by the blood sample a few minutes later. Blood samples were collected via jugular venipuncture into both plain tubes and EDTA-coated tubes. Samples in plain tubes were left to clot at room temperature for at least 30 min before centrifuging at 3000 × *g* for 5 min to obtain serum. Blood samples collected into EDTA-coated tubes were inverted several times and placed on ice before centrifuging at 500 × *g* for 5 min to obtain plasma.

Saliva was collected using EquiSal® saliva swabs (Austin Davis Biologics). The swab was inserted into the interdental space and moved back and forth over the tongue until a sufficient volume (500 μL) was collected as indicated by the swab colour indicator changing from white to pink. Swabs containing saliva were placed into the tubes provided containing 2 mL EquiSal® preservation buffer. Samples were taken to the lab within 2 h and centrifuged at 3000 × *g* for 2 min at room temperature to extract all saliva before storing at −80°C until further analysis.

All samples were then submitted to a commercial laboratory (Liphook Equine Hospital Laboratory) for measurement of serum insulin (Immulite 2000 xpi; Siemens)[14] and plasma total adiponectin concentrations (via immunoturbidimetric assay; ImmunoDiagnostics),[15] and to Bell Equine Veterinary Clinic for measurement of serum insulin concentration (Tosoh AIA-360; Tosoh Bioscience).[1] Saliva samples were analysed using the same assays used for analyte measurement in serum and plasma. In addition, three saliva samples were selected to determine the intra-assay ($n = 3$) and inter-assay repeatability

TABLE 1 Description of the four situations in which blink and heart rates were recorded.

Situation	Description
Control	The pony is at rest in his/her normal environment at pasture with usual paddock mates and is loosely restrained by a handler using a headcollar.
Jugular venipuncture	The pony is loosely restrained by a handler using a headcollar while blood is collected from the jugular vein by the usual investigator.
Sham jugular venipuncture	The pony is loosely restrained by a handler using a headcollar while the usual investigator pretends to collect a blood sample. The vein is raised and a syringe is used to mimic blood sampling but no needle is inserted.
Saliva collection	The pony is loosely restrained using a headcollar and a swab is inserted in the mouth to collect saliva.

($n = 2$) of insulin measurements (% coefficient of variation ± standard deviation).

2.3 | Assessment of stress indicators during saliva collection and jugular venipuncture

Two indicators of stress (spontaneous blink rate[11] and heart rate[12,16]) were recorded in seven of the ponies when exposed to four situations (Table 1). These assessments were performed on the last sample collection day. One of the ponies was removed from the larger study at an early stage and therefore was not included in the assessment of stress indicators. To measure blink rate, a 1-min video of the left eye of each pony was recorded during each treatment. The video was then played back at 0.20× speed and the number of full blinks, half blinks and eyelid twitches were recorded.[11] To measure heart rate, each pony was fitted with a heart rate monitor (Polar H10, v3.1.1, Polar Electro Oy).[16,17] Ponies were previously habituated to wearing the monitor. Habituation involved fitting the monitor on each animal in the field and leaving it on for a few minutes to ensure all ponies became used to wearing the monitor, were comfortable to move naturally while wearing it, and did not show any fear or aversion. This was done three to five times with each pony over a period of 2 weeks prior to data collection until the animal was able to remain undisturbed during placement of the monitor and continued grazing with it fitted.

Once habituated, animals were fitted with the monitor 5 min before starting data collection. The heart rate (bpm) and RR interval (ms) were recorded over 3 min during each situation using the Polar Equine app (v.1.2.1, Polar). The root mean square of successive differences between beats (RMSSD) was then calculated using the following equation[12,13]:

$$\mathrm{RMSSD} = \sqrt{\frac{\sum_{i=1}^{n-1} (RR_{i+1} - RR_i)^2}{N-1}},$$

where RMSSD is the root mean square of successive differences between beats and RR is the interval between successive heart beats.

2.4 | Statistical analysis

Salivary insulin concentrations were adjusted to account for dilution with the preservation buffer during sample collection. Data normality was assessed using visual inspection of histograms and Shapiro–Wilk test. Correlations between serum and saliva insulin concentrations were determined using Spearman's correlation coefficient. Salivary [insulin] was also calculated as a percentage of serum [insulin]. The number of blinks, heart rate, RR interval and RMSSD were compared between the control and the other three situations using a mixed-effects model followed by Dunnett's *post-hoc* test. All analyses were performed using Prism v.9.1.2 (GraphPad) and $p \leq 0.05$ was considered to indicate statistical significance.

3 | RESULTS

3.1 | Measurement of salivary insulin and total adiponectin

The range of [total adiponectin] was 2.4–20.3 μg/mL in plasma (data not shown). However, total adiponectin was undetectable in all saliva samples.

Salivary insulin was undetectable using the Immulite 2000 xpi analyser (limit of detection: 2 μIU/mL). However, salivary insulin was measurable in all samples using the Tosoh AIA-360 analyser and the inter- and intra-assay variability was acceptable (Table 2). The range of serum and salivary [insulin] measured using this analyser was 10.4–133.1 and 6.5–135.5 μIU/mL, respectively.

3.2 | Comparison of stress associated with blood and saliva sample collection

There were no significant changes in any stress indicator during saliva collection when compared with the control situation (Figure 1). There was a significant decrease in the numbers of half-blinks during sham (21.8 ± 5.9 blinks/min, $p < 0.05$) and real venipuncture (17.9 ± 3.6 blinks/min, $p < 0.01$) compared with the control (28.1 ± 5.2 blinks/min), but no change in other stress indicators (Figure 1).

3.3 | Correlation between serum and salivary insulin concentrations

Serum and salivary [insulin] were not significantly correlated (Spearman's $\rho = 0.27$, $p = 0.08$). As a percentage of serum [insulin], the median salivary [insulin] was 66.2% (interquartile range: 41.9%–98.2%; Figure 2).

4 | DISCUSSION

The aim of this study was to determine whether [insulin] and [total adiponectin] could be detected in equine saliva using commercial assays currently available in the UK. Saliva collection presents several potential advantages with regard to welfare, especially in animals that

TABLE 2 Inter- and intra-assay variability of salivary [insulin] measurement using the Tosoh A360 analyser.

Sample	Mean sample concentration (μIU/mL)	% coefficient of variation ± SD	
		Inter-assay ($n = 2$)	Intra-assay ($n = 3$)
1	121.5	0.2	0.4
2	56.5	2.5	1.9
3	22.5	1.1	1.1
Mean ± SD		1.3 ± 0.9	1.1 ± 0.6

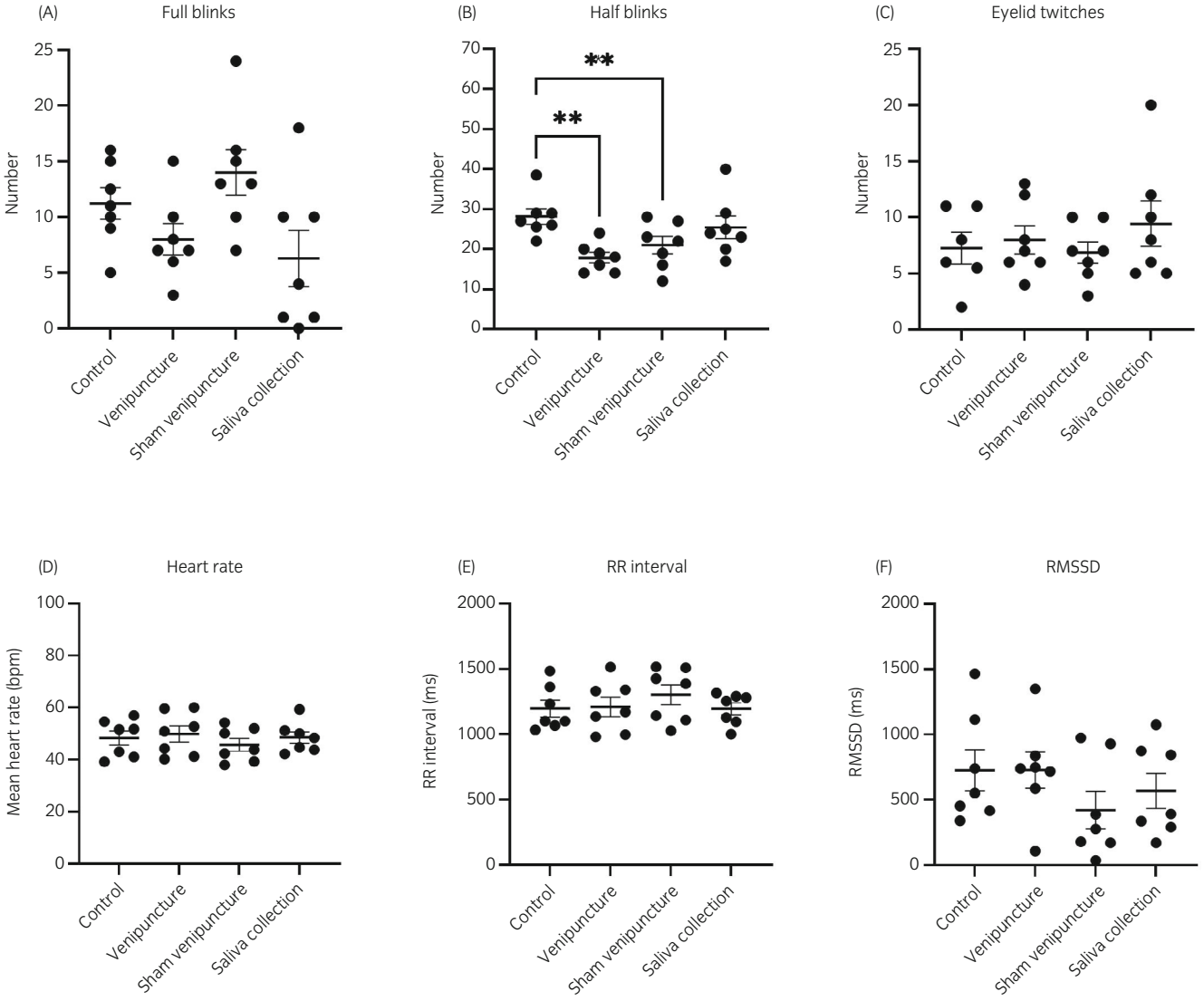

FIGURE 1 Indicators of stress recorded during control, venipuncture, sham venipuncture and saliva collection. (A–C) Number of blinks, (D) heart rate and (E, F) heart rate variability indicators. Lines and bars represent means ± SEM ($n = 7$ ponies). RMSSD, root mean square of successive differences between beats. $^{**}p < 0.01$.

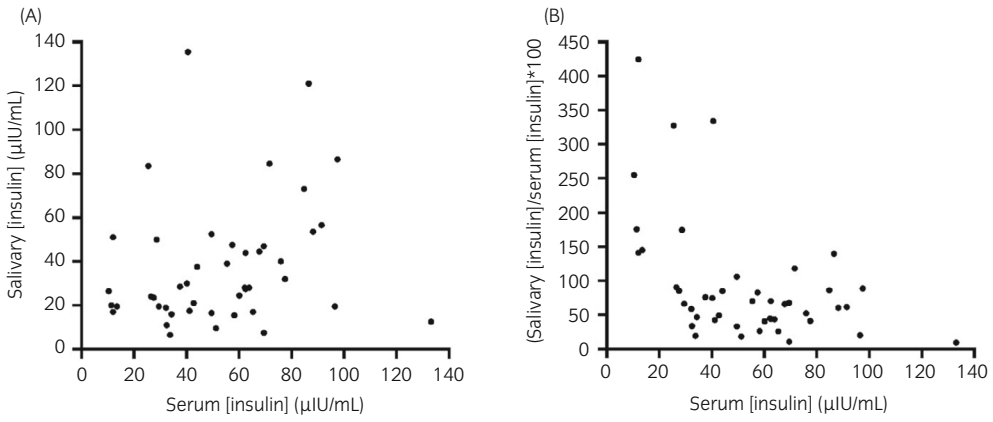

FIGURE 2 (A) Salivary versus serum insulin concentrations measured in matched samples and (B) salivary [insulin] as a percentage of serum [insulin] versus serum [insulin]. Data presented are from $n = 8$ ponies and $n = 7$ time-points ($n = 45$ paired blood and saliva samples in total).

are refractory to blood sampling and in cases requiring frequent sampling or long-term monitoring. This would be considered an example of Refinement in line with the principles of the 3Rs applied to animal research.[18] The use of saliva to measure insulin and total adiponectin would allow horse-owners to collect and send samples to their veterinary practitioner, after an initial consultation, without requiring the

veterinarian to physically visit the animal multiple times simply to collect a blood sample. This would be cost-effective and particularly advantageous in areas without convenient access to a veterinarian (so-called 'veterinary deserts') and in cases of animals requiring repeated monitoring and sampling.

[Insulin] was detectable in all saliva samples using the Tosoh AIA-360 analyser, but not the Immulite 2000 xpi. The Tosoh A360 is thought to offer greater sensitivity than the Immulite 2000 xpi analyser at low concentrations (limits of detection: 0 and 2 μIU/mL, respectively),[19] which may have contributed to its ability to detect salivary insulin. Alternatively, it is possible that the epitope used for detection by the Immulite 2000 xpi was masked or altered in saliva, thus rendering its measurement impossible with this analyser. Furthermore, it is also possible that the Tosoh A360 analyser detected other closely related molecules in addition to or instead of insulin, such as insulin-like growth factors, which are measurable in saliva in rats[20] and humans.[21,22] There are no data available on possible cross-reactivity with molecules other than equine insulin using either analyser with equine serum. The use of an orthogonal analysis method (such as liquid chromatography–tandem mass spectrometry) would therefore be of value to confirm the identity of the molecule measured by this assay in equine saliva. The first aim—to determine whether insulin is detectable in saliva—was therefore achieved, although it should be acknowledged that this study did not include a full validation of the Tosoh A360 assay for saliva as this was beyond the scope of this work. However, future studies may proceed with full assay validation, including dilutional parallelism, spiked recovery and sample stability assessment.

The second aim was to assess and compare indicators of stress recorded during saliva and blood collection. Previous research has shown that in stressful situations, horses show a decrease in spontaneous blink rates and a concomitant increase in eyelid twitches.[11] Saliva collection was not associated with any changes in blink rates in the present study compared with a control situation, in agreement with a previous report.[10] Although there were no increases in eyelid twitches recorded during venipuncture, the significant decrease in half-blinks during sham and real venipuncture suggests that saliva collection may be a less stressful alternative to blood sampling with a lesser negative impact on welfare. In addition to heart rate as an indicator of stress, RR interval and RMSSD are two measures of heart rate variability that provide insight into sympathetic and vagal activity.[12,13] There was also no significant change in any of these indicators during saliva collection, further indicating that this was not associated with increased stress in ponies. It must be noted that venepuncture was also not associated with any changes in heart rate or heart rate variability in these ponies. This is likely because the ponies in this study had undergone regular blood sampling as part of the other larger study, were well habituated to this procedure, and were in fact selected for inclusion in the other study in part because of their behaviour during blood sampling.

Finally, the concentrations of insulin determined in saliva and in serum were compared. Preliminary results from this study indicate that although insulin is detectable in equine saliva, salivary [insulin] is not significantly correlated with serum [insulin]. Salivary and blood insulin concentrations have been reported to correlate in some (human[3,6]) but not all species (dogs[4] and pigs[5]), although the reasons for these differences remain unclear. The proportion of salivary [insulin] calculated as a percentage of serum [insulin] was variable, suggesting that the composition of the saliva may vary, perhaps according to time of day,[23,24] feeding status[25] and hydration,[26,27] as suggested by research in other species, which in turn may influence the percentage recovery of insulin from saliva. Further work is therefore required to investigate factors affecting the variability in salivary [insulin] and as a consequence, the conditions for the collection of saliva samples may require standardisation to ensure insulin measurements are reliable and reproducible. The influence of feeding status on salivary insulin concentrations should be investigated in particular to determine whether equids should be fasted before saliva collection. This may include fasting animals for a short period (e.g., 30 min[9]) or rinsing out the animal's mouth with water to remove food remnants[28] prior to sample collection. In the present study, samples were collected from unfasted ponies at pasture to make the collection process as simple as possible for the target user (horse-owner) and as minimally stressful as possible for the animals involved.

Diagnostic thresholds would need to be determined for salivary insulin specifically and these may be different to serum insulin threshold values. The potentially greater variation in salivary insulin measurements compared with serum measurements may mean that salivary [insulin] should be assessed as ranges or risk bands. Indeed, the variability of salivary insulin measurements could be linked to the ability of saliva to respond quickly to changing physiological states, which may be considered an advantage provided it can be elucidated and used judiciously in assessing the metabolic state of an animal. The need to determine new reference ranges for insulin in a biological sample other than blood therefore should not deter future studies into the use of this sample, which presents several benefits in terms of animal and owner experience.

Salivary total adiponectin was undetectable using the only commercial assay validated for use with equine plasma samples currently available in the UK. Previous studies have successfully measured salivary adiponectin in humans[6] and dogs,[7] although there are no data available for other species. It is likely that the inability to detect salivary adiponectin in the present study is associated with the assay used. For example, the epitope necessary for detection may be masked in saliva. Adiponectin may also be secreted into the saliva with post-translational modifications that are not recognised by the antibody used, as research in humans has indicated differences in the adiponectin isoforms present in saliva and serum.[29] It is also possible that salivary adiponectin concentrations are too low to be detected with the current assay, as salivary [adiponectin] is reported to be approximately 1000 times lower than in serum in both humans[30,31] and dogs.[7] Finally, the saliva collection method may also play a role in downstream detection of adiponectin, as differences have been reported between samples collected into a tube or using the Salivette® system in humans.[30] Therefore, it may be possible to measure equine salivary adiponectin using other sample collection

systems or assays, such as an ELISA previously validated for measurement of high-molecular weight adiponectin in equids[32] that is currently unavailable in the UK.

Limitations of this study include the small sample size (eight animals), all of which were native UK pony breeds. In addition, this study only included animals that were co-operative during blood and saliva sampling, which may have introduced sampling bias. The sampling methodology involved collecting multiple samples from a small number of ponies, rather than collecting single samples from a large population of animals. This is because the present study was designed to fit alongside a larger study to maximise the use of blood samples that were already being collected and analysed. The overarching aim of the present study was to investigate the possible refinement of methods by using a non-invasive biological sample and the study was therefore designed to avoid collecting additional blood samples.

In conclusion, insulin, but not adiponectin, was measurable in equine saliva with low inter- and intra-assay variability using one of the automated assays currently available in the UK. Saliva collection was not associated with changes in indicators of stress in ponies. Salivary and serum insulin concentrations were not significantly correlated and saliva [insulin] was a variable percentage of serum [insulin]. This suggests that various factors may influence the measurement of insulin in this biological fluid. Standardised collection conditions and specific diagnostic threshold values for salivary insulin therefore remain to be determined and the measurement of equine salivary insulin should be investigated in a larger cohort to validate the use of this biological sample, which may hold potential as a non-invasive alternative to blood.

AUTHOR CONTRIBUTIONS

Marine Barnabé and Nicola Menzies-Gow contributed to study design, study execution, data analysis and interpretation, preparation of the manuscript and final approval of the manuscript. Jonathan Elliott and Patricia Harris contributed to study design, data interpretation, preparation of the manuscript and final approval of the manuscript. Marine Barnabé had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis.

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CONFLICT OF INTEREST STATEMENT

Patricia Harris is an employee of Waltham Petcare Science Institute.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/evj.14019.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Zenodo at https://zenodo.org/record/8370563, reference number 8370563.

ETHICAL ANIMAL RESEARCH

This study was approved by the Royal Veterinary College Animal Welfare and Ethical Review Board (2020-135N) and the Clinical Research Ethical Review Board (URN 2022-2113-2) and was conducted partly under a UK Home Office licence (PP5634400).

INFORMED CONSENT

Not applicable.

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