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Original Article

Three ways to find a fluke: Evaluating diagnostic methods for *Fasciola hepatica* surveillance in Swedish sheep

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ABSTRACT

Infections with *Fasciola hepatica* are most prevalent in south-western Sweden, but climate change may facilitate further spread, as both the parasite and its snail host thrive in humid, mild conditions. Sheep are particularly susceptible, resulting in decreased productivity and economic losses as well as clinical disease and death. Routine field diagnosis in Sweden currently relies on sedimentation of faecal samples, although semi-quantitative PCR and coproantigen ELISA (cELISA) are available alternatives. This study presents the first Swedish comparison of diagnostic performance using composite faecal samples – each representing three ewes – collected from 73 flocks in spring ($N = 47$) and autumn ($N = 58$). Sedimentation, quantitative PCR (qPCR), and cELISA (evaluated at 2% and 8% optical density thresholds) were compared for their ability to detect *F. hepatica*. Farmers' parasite control practices were also surveyed. Concordance among methods was assessed using Cohen's kappa (κ). Sedimentation and qPCR showed substantial agreement (κ = 0.72), qPCR and cELISA 8% cut-off showed considerable agreement (κ = 0.61), sedimentation and cELISA showed moderate agreement (κ = 0.56), while cELISA 2% cut-off naturally identified more positive samples but did not correlate with the other methods. These findings suggest that cELISA has potential as an adjunct diagnostic tool, particularly for detecting early infections, but false-negative results in pooled samples limit its suitability as a replacement for sedimentation or qPCR in routine testing. Survey responses indicated that most farmers were aware of parasite risks and followed general sampling and deworming guidelines, though specific attention to fluke control was limited.

1. Introduction

The common liver fluke *Fasciola hepatica* is a digenean trematode that primarily infects ruminants but also a range of other mammals, including humans (Beesley et al., 2018). Transmission requires lymnaeid snails as intermediate hosts and is favoured by mild temperatures, high rainfall, and swampy pastures – conditions that promote the development of both the parasite and snail populations (Relf et al., 2011). In Sweden the main intermediate host is the mud snail *Galba truncatula* (Novobilsky et al., 2013). Seasonal fluctuations in incidence are well understood in northern Europe (Bloemhoff et al., 2015), and climate change is expected to increase prevalence mainly by lengthening grazing seasons and thus the transmission window (Fox et al., 2011). Several European countries have documented increasing numbers of fasciolosis outbreaks in recent years (Bosco et al., 2015; Fairweather, 2011; Scott et al., 2005; Skuce and Zadoks, 2014).

Detection of *F. hepatica* or parasite-related lesions at slaughter or necropsy provides information at the animal or herd level (Alvarez Rojas et al., 2014), but these are *post-mortem* procedures. Alternatives are needed that can be used in live animals to monitor infection levels but also to evaluate the efficacy of flukicides. Faecal sedimentation, the most widely used diagnostic method in Sweden, allows the detection of parasite eggs but has significant limitations (Arifin et al., 2016). The eggs are only detectable 10–12 weeks after infection, which often delays diagnosis until winter in animals in the first season (Alvarez Rojas et al., 2014). Secondly, shedding is intermittent, leading to false negative results, while the persistence of eggs after treatment can lead to false positive results (Gordon et al., 2012). Finally, egg counts are generally

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low and do not correlate with parasite load or disease severity (Kahl et al., 2023).

Although sedimentation remains a practical tool, it is also labour intensive, requires time-consuming procedures and trained personnel, and carries the risk of misidentification due to morphological similarity to rumen flukes (Wenzel et al., 2019). Modified techniques such as the Flukefinder® method (https://flukefinder.com/) have shown improved egg recovery (Kahl et al., 2023), but more efficient approaches are still required for large-scale monitoring and testing the efficacy of flukicides (Flanagan et al., 2011; Gordon et al., 2012).

Molecular and serological methods such as quantitative (q)PCR and coproantigen (c)ELISA may offer improved sensitivity, specificity and suitability for herd-level surveillance. For example, qPCR is a highly sensitive and specific method for detecting *Fasciola* DNA. Most assays target multicopy ribosomal or mitochondrial genes using TaqMan probe chemistry, achieving detection limits below one egg equivalent per sample (Alasaad et al., 2011). Unlike sedimentation, qPCR enables quantification through cycle threshold (Ct) values. Although qPCR requires specialised equipment and standardisation, it is the most reliable molecular tool for laboratory-based diagnosis and monitoring of *F. hepatica* infection., while its routine use for large-scale screening may be limited by practical constraints. Additionally, the coproantigen enzyme-linked immunosorbent assay (ELISA) is a sensitive and specific technique for detecting *Fasciola* infection by identifying parasite-derived antigens in faeces. Unlike antibody-based assays, it confirms active infection (Martínez-Sernández et al., 2016; Mezo et al., 2004). The test uses specific monoclonal antibodies against *Fasciola* excretory-secretory antigens to capture antigens present in faeces, with enzyme-linked detection and colorimetric measurement (Valero et al., 2009). It is non-invasive and can also detect early infection during the prepatent period. Despite some dependence on antibody specificity and potential cross-reactivity (Gordon et al., 2012), cELISA remains a reliable diagnostic and epidemiological tool for monitoring fasciolosis in livestock. To our knowledge, this method has only been validated for use in individual faecal samples, somehow a disadvantage in livestock applications where pool/composite samples are used for economic reasons.

The aim of this study was to compare sedimentation and qPCR for the detection of *F. hepatica* eggs, as well as cELISA with two different cut-offs, for diagnosing *F. hepatica* infection in composite samples from Swedish sheep flocks. The study also investigated, farmers' routines regarding faecal sampling and deworming and gathered information on previous known exposure to fluke infection.

2. Materials and methods

2.1. Flock selection

In February 2024, a call for voluntary participation in this study was distributed to sheep farmers with flocks affiliated with Farm & Animal Health in south-west Sweden. Farmers were asked to submit faecal samples from their flocks, which were tested with faecal sedimentation for *F. hepatica* free of charge. A total of 42 flocks in the regions of Skåne, Halland and Västra Götaland participated, each submitting individual faecal samples from three randomly selected ewes. Sampling took place between mid-February and mid-March 2024 and all analyses were performed at Vidilab (Enköping, Sweden).

In September 2024, the same farmers were asked to submit new faecal samples, preferably from the same ewes or from animals within the same grazing group. Twenty-six flocks complied with this request. To maximise the number of samples available for the coproantigen ELISA, an additional 31 faecal samples from southern and central Sweden were requested from Vidilab in the autumn. These additional samples were submitted between mid-September and mid-October 2024.

2.2. Questionnaire survey

In September 2024, a questionnaire was sent to the 42 flocks that had participated in the spring sampling. The questionnaire collected information on the farmer's routine faecal sampling and anthelmintic treatment, as well as the flock's history of *F. hepatica* (Appendix 1).

2.3. Sedimentation technique

At least one pooled faecal sample from each flock, consisting of the faeces of three ewes, was analysed according to Vidilab's established routine (Vidilab AB, 2021). Two grams of faeces per animal (a total of 6 g per pooled sample) were homogenised in 84 ml of water using a hand blender. The suspension was filtered through double gauze to remove coarse debris and transferred to a sedimentation tube. Excess suspension was retained for subsequent qPCR analysis. The tube was left to stand for 20 min. The supernatant was then aspirated until 2–3 cm of liquid remained. The tube was refilled with water up to the rim. This washing step was repeated about four times at 20 min intervals until the sample was sufficiently clear for microscopic examination.

The final suspension of approximately 10 ml was poured into a petri dish and a drop of detergent was added to reduce the surface tension. The samples were examined under a standard light microscope (Leica) at 40× magnification. The results were recorded as positive or negative for *F. hepatica* eggs.

2.4. Coproantigen ELISA

Coproantigen detection was carried out using a commercial ELISA kit (BIO K 201 – Monoscreen AgELISA *Fasciola hepatica*, Bio-X Diagnostics S. A., Rochefort, Belgium) according to the manufacturer's instructions with minor modifications, i.e. testing pooled samples and overnight incubation (see following lines).

On the first day, faecal suspensions were prepared by mixing approximately 0.7 g faeces with 2 ml of buffer, centrifuged at 1000*g* for 10 min, and the supernatants were stored at 4 °C overnight. In the spring, each sample was prepared by pooling equal amount of faeces from three sheep from the same flock. In the autumn, the same procedure was repeated but samples from 28 pools were analysed also individually.

On the second day, supernatants were incubated on antibody-coated plates (each plate having 47 samples and a control). Reagents used for the further steps were wash buffer, biotin-conjugated anti-*F. hepatica* antibody, avidin peroxidase, tetramethylbenzidine (TMB) substrate, and stop solution. Optical density (OD) was measured at 450 nm. Relative OD values were calculated in Excel and classified as positive or negative using the recommended 8% and 2% thresholds.

2.5. Polymerase chain reaction

The excess suspension from the sedimentation step (described in Section 2.3) was prepared for DNA extraction. The samples were centrifuged at 706*g* for 5 min and the supernatant was discarded. To further clean up from small particles, saturated saline was added, mixed and centrifuged again at 706*g* for 5 min. After discarding supernatant, phosphate-buffered saline (PBS) was added, and the procedure was repeated three times. After the final wash, the samples were allowed to sediment for 20 min and the supernatant was removed. One millilitre of the sediment was transferred to a bead beating tube containing a tungsten bead (3 mm, Qiagen). Samples were then disrupted with a TissueLyser II (Qiagen) at 30 Hz for 2 × 1 min, followed by proteinase K digestion at 55 °C for 10 min. Finally, DNA extraction was performed according to the standard Vidilab protocol. DNA was then subsequently extracted using magnetic beads on the TANbead® 4800 robotic platform (Taiwan Advanced Nanotech Inc.).

For qPCR, a master mix was prepared with PerfeCTa ToughMix

(Quantabio), ultrapure water and primer-probe mix (FhF 12,5 μM: 5′-TTG GTA CTC AGT TGT CAG TGT G-3′, FhR 12,5 μM: 5'-AGC ATC AGA CAC ATG ACC AAG-3′, Probe:FhP 5 μM: 5′-[FAM] ACC AGG CAC GTT CCG TCA CTG TCA CTT T [BHQ1]-3′) as described by Alasaad et al. (2011). Twenty-two microlitres of the master mix was pipetted into each well and 3 μl of DNA sample was included. Positive and negative controls were added. The plates were sealed and run in a qPCR instrument (BioRad Opus 96). Finally, samples were categorised as positive for *F. hepatica* DNA (positive cut-off = Ct < 35).

2.6. Statistical analysis

Diagnostic results and questionnaire responses were compiled in Microsoft Excel. Agreement between diagnostic methods was assessed using Cohen's kappa coefficient, which was calculated and interpreted according to DATAtab (https://datatab.net/tutorial/cohens-kappa).

3. Results

3.1. Sedimentation

A total of 105 pooled faecal samples (from three ewes per pool) originating from 65 flocks were analysed during spring and autumn. *Fasciola hepatica* eggs were detected in 12 samples from 11 flocks (Table 1).

3.2. Coproantigen ELISA

All samples analysed by sedimentation were also tested with cELISA. The results were interpreted using two optical density (OD) thresholds: 2% and 8%. At the 8% threshold, 8 samples were positive, while more samples ($N = 24$) tested positive at the 2% threshold (Table 1).

Of the 58 pooled samples collected in autumn, 28 were additionally analysed as 83 individual faecal samples. Using the 8% threshold, four individual samples tested positive; two of these were from pools that had also tested positive, while the other two were from pools that had tested negative. At a threshold of 2%, 26 individual samples were positive, all but three of which were also represented in positive pooled samples.

On one ELISA plate (plate 3), the OD of the positive control was slightly below the minimum value of 0.800 recommended by the manufacturer, raising the possibility that some samples on this plate were incorrectly categorised. The results from this plate was therefore interpreted with caution.

3.3. Polymerase chain reaction

A total of 78 pooled faecal samples were analysed by qPCR in spring and autumn. Of these, 9 samples (11.5%) tested positive for *F. hepatica* DNA (Table 1).

3.4. Comparison of diagnostic methods

Agreement between diagnostic methods was assessed using Cohen's kappa coefficient. Agreement between sedimentation and cELISA (8% cut-off) was moderate (κ = 0.56). In contrast, agreement between sedimentation and cELISA at the 2% cut-off was poor (κ = −0.02). qPCR showed substantial agreement with cELISA at the 8% cut-off (κ = 0.61), but only fair agreement with cELISA at the 2% cut-off (κ = 0.34). The strongest agreement was observed between qPCR and sedimentation (κ = 0.72) (Table 2).

Table 1
The number of positive and negative samples collected in spring, autumn and overall for sedimentation, the cELISA with two different threshold values, and qPCR.

Diagnostic method	Spring		Autumn		Total	
	Pos	Neg	Pos	Neg	Pos	Neg
Sedimentation	10	37	2	56	12	93
cELISA 8%	5	42	3	55	8	97
cELISA 2%	9	38	15	43	24	81
qPCR	7	24	2	45	9	69

3.5. Geographical distribution

The flocks that tested positive for *F. hepatica* were mainly located in south-west of Sweden (Skåne, Halland and Västra Götaland), with additional isolated cases found in central Sweden (Fig. 1A). A similar geographical distribution was observed in the herds that reported *F. hepatica* infection in the questionnaire survey (Fig. 1B).

3.6. Questionnaire survey

A total of 33 sheep farmers answered the questionnaire. Flock sizes ranged from 4 to 500 ewes, with an average of 57. Small flocks (<40 ewes) were the most common. Overall, 84.8% of respondents reported taking faecal samples for routine parasitological analysis (coproscopic examination excluding *F. hepatica*). Of these, 54.5% sampled both ewes and lambs annually, 30.3% sampled only ewes annually, while 6.1% sampled irregularly, usually when disease was suspected. When asked about the results of recent faecal examinations, 36.4% reported trichostrongylid eggs with *Haemonchus contortus*, 24.2% reported trichostrongylid eggs without *H. contortus* and 12.1% reported negative results.

Sixteen flocks reported previous cases of *F. hepatica*. Of these, 37.5% stated that the diagnosis was made solely at slaughter, 12.5% by faecal examination only and 43.8% by a combination of faecal samples and slaughterhouse findings. Several flocks reported previous slaughter findings labelled with at least one of the codes for parasitic liver lesions: large liver fluke (code 79/80), small liver fluke (code 81/82), parasitic liver damage (code 83/84) and other liver damage (code 87/88), with varying frequencies. Five farmers stated that their current slaughterhouse does not provide them with slaughter findings.

In 2024, 81.8% of flocks reported administering anthelmintics. Most treatments targeted only gastrointestinal nematodes. Only five flocks reported treatment specifically against *F. hepatica*, and one of these flocks remained positive for the parasite in autumn. The most reported anthelmintic was ivermectin (10 flocks), followed by albendazole (7), triclabendazole (2), fenbendazole (1) and levamisole (1). After treatment, faecal examinations were carried out in 21.2% of the flocks, but only to monitor the efficacy of nematode control.

4. Discussion

This study assessed the agreement between sedimentation, semi-quantitative PCR (qPCR), and coproantigen ELISA (cELISA) with two different cut-offs for detecting *Fasciola hepatica* in composite faecal samples from each of three ewes in the same grazing group. Overall, the results showed variable agreement among methods, reflecting their differing sensitivities, diagnostic principles, and susceptibility to pre-patent infections and sample variability. Taken together the

Table 2
Combined seasons (Spring + Autumn). Cohen's kappa and 95% confidence intervals for diagnostic method comparisons and different thresholds for the coproantigen enzyme linked immunosorbent assay (cELISA).

	κ -value	±95% CI	Agreement
Sedimentation vs. cELISA 8%	0.56	(0.47–0.65)	Moderate
Sedimentation vs. cELISA 2%	−0.02	(−0.11–0.07)	No
cELISA 8% vs. qPCR	0.61	(0.52–0.70)	Substantial
cELISA 2% vs. qPCR	0.34	(0.25–0.43]	Fair
Sedimentation vs. qPCR	0.72	(0.63–0.81)	Substantial

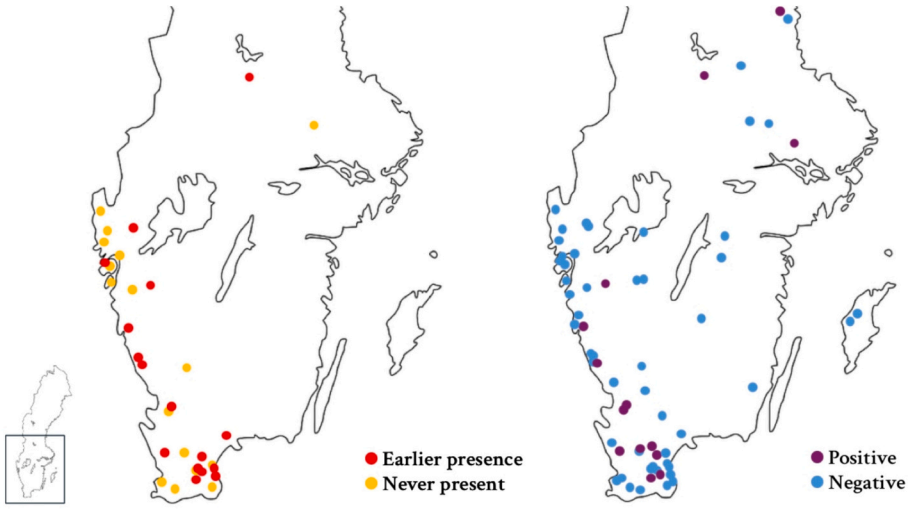

Fig. 1. The geographical distribution of, A) positive and negative flocks based on the results of sedimentation, PCR and/or cELISA 8%, and B) flocks that reported having or not having *F. hepatica* in the flock in the survey study.

discrepancies observed here likely reflect biological and methodological factors, including infection timing, and intermittent egg shedding.

Agreement between sedimentation and cELISA cut-off 8% was moderate ($\kappa = 0.56$). Six samples positive by sedimentation were negative by cELISA, while two samples positive by cELISA were negative by sedimentation. Similar findings were reported by Arifin et al. (2016), who observed a positive correlation between faecal egg counts and cELISA results. A key explanation for cELISA-positive but sedimentation-negative samples is the infection stage. Eggs of *F. hepatica* are not shed until 10–12 weeks post-infection (Mezo et al., 2004; Munita et al., 2019). As ruminants are primarily exposed in late summer in Sweden (Novobilský et al., 2014), autumn samples may have represented pre-patent infections detectable only by antigen-based assays. This pattern was evident in our study, where cELISA-positive (cut-off 2%) but sedimentation-negative samples were mainly collected in autumn. Another explanation for sedimentation-negative results is the intermittent shedding of eggs by *F. hepatica*, which can lead to false negatives in single sampling events (Alvarez Rojas et al., 2014; Fairweather, 2011; Gordon et al., 2012; Kahl et al., 2023). Consistent with this, FarmStat data from 2021 showed clear seasonal variation, with the highest prevalence (11%) in the second quarter and none detected in the third (Reneby and Jonasson, 2023). This pattern aligns with the present study, where most positives occurred in spring due to infection previous pasture season and few in autumn possibly during the parasite prepatent period.

Unexpectedly, some samples were positive by sedimentation but negative by cELISA 8% cut-off. As cELISA is generally considered more sensitive and capable of detecting low antigen concentrations (Mezo et al., 2004), this discrepancy was surprising. A likely explanation is the pooling of samples prior to cELISA analysis, a procedure that is not described in the kit instructions but that it was adopted in the present study to conform the assay to routine practice and allow comparison with pooled sedimentation and PCR results. In parallel testing, weakly positive individual samples often became negative when pooled, indicating a dilution effect that likely reduced antigen detectability. Since the amount of faecal material analysed with the cELISA is smaller compared to the sedimentation, this dilution effect has a greater impact on this method. A potential correction factor that might lead to obtain similar results with the cELISA and with the sedimentation might be using the 2% cut-off (see Table 1).

Using a lower optical density (OD) threshold of 2% in cELISA markedly increased the number of positives, particularly in autumn. However, the agreement with sedimentation and qPCR was poor. Palmer et al. (2014) reported high diagnostic performance with a sheep-adapted cut-off (23% of the manufacturer's recommended value), highlighting the importance of species-specific cut-offs. The inconsistent use of cut-off values across studies complicates direct comparisons and underscores the need for further validation (Kelley et al., 2021).

In our study qPCR detected slightly fewer positives than sedimentation. This is consistent with Arifin et al. (2016) who reported greater sensitivity for sedimentation than qPCR, emphasising that sample volume, consistency, and as DNA extraction procedures can affect results. However, this contrasts with previous findings of somewhat higher sensitivity for qPCR (Robles-Pérez et al., 2013; Shahzad et al., 2012). Nevertheless, agreement between qPCR and sedimentation was substantial ($\kappa = 0.72$). Agreement between qPCR and cELISA (8% cut-off) was also substantial ($\kappa = 0.61$). At this threshold three samples were qPCR-positive but cELISA-negative, while two were cELISA-positive but qPCR-negative. As cELISA can detect antigens from immature flukes, prepatent infections may explain qPCR-negative but cELISA-positive outcomes (Martínez-Sernández et al., 2016). Both cELISA-positive/qPCR-negative samples in this study were collected in autumn, supporting this interpretation. Additionally, differences in faecal consistency between cattle and sheep and heterogeneity of faecal composition could contribute to inconsistent results across diagnostic methods (Arifin et al., 2016). Misidentification of *F. hepatica* eggs and those of rumen flukes (*Calicophoron/Paramphistomum* spp.) may also explain some sedimentation positives not confirmed by other methods (Wenzel et al., 2019). While rumen flukes are common in Western Europe (Huson et al., 2017), their prevalence in Sweden remains uncertain.

Geographically, *F. hepatica* foci were concentrated in south-western Sweden, consistent with earlier reports. The detection of positive flocks in central Sweden suggests a gradual northward spread (Reneby and Jonasson, 2023). Given the parasite's adaptability and the influence of climate change, further expansion is likely (Fairweather, 2011; Fox et al., 2011). The farmer questionnaire revealed that participating flocks were larger than the national average. Although Swedish sheep farms remain small by international standards, smaller flock sizes facilitate closer animal management, potentially enabling earlier recognition of clinical signs (Stafford and Gregory, 2008). Most farmers reported good sampling routines for nematodes, in line with recommendations to test ewes pre-pasture and lambs regularly during pasture season. Furtherer, routine testing for *F. hepatica* was rare and typically initiated only after abattoir findings or clinical suspicion. This reliance on *post-mortem* detection likely explains why most historical diagnoses were made at slaughter. Introducing routine testing for *F. hepatica* in high-risk areas

could improve early detection and allow more targeted anthelmintic use.

Regarding parasite control, ivermectin was the most frequently used anthelmintic, consistent with its efficacy against trichostrongylids including *Haemonchus contortus* (González Canga et al., 2009). Albendazole use was also widespread, even among herds without known *F. hepatica* infections. Although albendazole has broad-spectrum activity and is effective only against mature stages of *F. hepatica* at higher doses, resistance has been reported (Novobilsky et al., 2012), warranting cautious use. Among five flocks treated for *F. hepatica*, one remained positive after triclabendazole treatment in autumn, possibly reflecting emerging resistance, which is a growing concern in Europe (Howell and Williams, 2020). No farmer reported using closantel, which is effective against both adult and some immature flukes; however in Sweden, this substance is only available for cattle. Only 21.2% of farmers conducted post-treatment faecal testing, although follow-up monitoring every two years is recommended, especially in herds with recurrent high *H. contortus* egg counts (Höglund et al., 2019). Post-treatment testing is essential to detect emerging resistance and guide management strategies.

5. Conclusions

Our findings highlight the need to strengthen *F. hepatica* surveillance in Swedish sheep by improving diagnostics as a complement to the recommended follow-up of data from slaughter. The three diagnostic methods – sedimentation, cELISA, and qPCR – demonstrated varying detection capacities across infection stages and sample types. With adjusted sensitivity, cELISA could be adapted for pooled faecal samples, making it suitable for routine and cost-effective use. Equally important is improving farmer engagement and integrating *F. hepatica* testing into existing parasite monitoring programmes. These measures would promote earlier detection, improve treatment efficacy, and support more sustainable parasite control.

CRediT authorship contribution statement

Matilda Felländer: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Giulio Grandi:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Mikael Juremalm:** Writing – review & editing, Supervision, Investigation. **Bitte Ljungström:** Writing – review & editing, Supervision, Resources. **Katarina Gustafsson:** Writing – review & editing, Conceptualization. **Johan Höglund:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Ethics approval

All animal-related activities were approved by the Uppsala Animal Ethics Committee (permit Dnr 5.8.18–12,184/2023) and conducted in accordance with Swedish animal welfare legislation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vprsr.2026.101469.

Data availability

The data from this study are protected by intellectual property rights and include information subject to the General Data Protection Regulation (GDPR). Access to the data may be granted upon reasonable request to the corresponding author, subject to applicable legal, ethical, and contractual restrictions, and the completion of appropriate agreements.

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