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Gastrointestinal parasite community structure in horses after the introduction of selective anthelmintic treatment strategies

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ABSTRACT

A relatively new method to study the species richness and diversity of nematode parasites in grazing animals is to perform deep sequencing on composite samples containing a mixture of parasites. In this work, we compared species composition of strongyles in two groups of horses as a function of egg count and age, based on a DNA barcoding approach. Faecal egg counts and larval cultures were obtained from nearly 300 horses, i.e., domestic horses (n = 167) and trotters (n = 130) sampled nationwide. The second internal transcribed spacer region (ITS2) of strongyle nematodes in the larval cultures was first amplified using barcoded universal primers and then sequenced on the PacBio platform. Subsequently, bioinformatic sequence analysis was performed using SCATA to assign operational taxonomic units (OTU). Finally, species occurrence and composition were assessed using R. ITS2 sequences were found in the majority (89%) of larval samples. Sequencing yielded an average of 140 (26 to 503) reads per sample. The OTUs were assigned to 28 different taxa, of which all but three could be identified as species. The average relative abundance of the seven most abundant species (all Cyathostominae) accounted for 87% of the combined data set. The three species with the highest prevalence in both horse groups were *Cyathostomum catinatum, Cylicocyclus nassatus* and *Cylicostephanus calicatus*, and they were frequently found in different combinations with other species regardless of horse group. Interestingly, this result is largely consistent with a previous Swedish study based on morphological analysis of adult worms. In addition, two migratory strongylids (*Strongylus vulgaris* and *S. edentatus*) occurred in few domestic horses and trotters. Except for *C. minutus* and *C. nassatus*, which decreased with age, and *C. catinatum* and *S. vulgaris*, which increased, no specific trends were observed with respect to horse age. Taken together, these results are broadly consistent with data obtained before the introduction of selective targeted treatment in Sweden in 2007. All in all, our results suggest that this treatment strategy has not led to a significant change in strongyle nematode community structure in Swedish horses. The study also confirms that nemabiome analysis in combination with diversity index analysis is an objective method to study strongyle communities in horses.

1. Introduction

Nematodes of the family Strongylidae are commonly found as adult worms in the large intestine of grazing horses and are known to cause significant welfare, health and management problems in horses worldwide (Nielsen et al., 2007). They are all plug-feeders that attach to the intestinal mucosa and have a direct life cycle whereby horses become infected on pasture by ingesting infective third-stage larvae (L3), which develop from eggs that are excreted in the faeces (Matthews, 2011). However, it is a very diverse group with dozens of species broadly classified into two main subfamilies: Strongylinae (strongylins) and Cyathostominae (cyathostomins), based on the shape of the buccal capsule and on their body size (Lichtenfels et al., 2008).

Because the clinical implications and treatment strategies for members of subfamily Strongylinae vary widely, it is important to understand their specific characteristics. Of the strongylins, *Strongylus vulgaris* is the best known and has attracted attention primarily because it can cause life-threatening colic and other serious health problems (Reinemeyer and Nielsen, 2009). This is due to its complex life cycle, as it migrates through the arteries, causing damage and thrombosis that can lead to necrotic lesions in the intestine (Duncan and Pirie, 1975). In contrast, cyathostomins are non-migratory. This is the predominant group which

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consist of numerous species that vary in their clinical impact. These include weight loss and severe, persistent enteritis primarily due to mass reactivation of inhibited larvae in the intestinal mucosa (Corning, 2009; Love et al., 1999). Their ability to lie dormant more or less deeply at certain sites in the intestinal mucosa varies from species to species (Lyons et al., 2000). This could mean that some species are more pathogenic and more difficult to control than others, but this certainly needs to be investigated further.

Since the eggs of all strongylids are similar in shape and size, and only some L3 can be identified to the genus until recently, species composition could only be determined from morphological criteria of adult worms found either in the digesta at necroscopy of horses (Drudge and Lyons, 1977), or after their expulsion in the faeces after anthelmintic treatment (e.g. Osterman Lind et al., 2003). While there are several species of both migratory (genus *Strongylus*) and non-migratory strongylins (in four genera), there are also at least 40 valid species of cyathostomins belonging to more than ten genera in equids (Lichtenfels et al., 2008). Usually, only a few species make up the majority of those found (Ogbourne, 1978; Reinemeyer et al., 1984). However, less is known about how the different species are influenced by horse- and farm-specific covariates and whether they prefer certain climatic conditions. In addition, there is little information on whether larval cyathostominosis is predominantly caused by particular species and how they respond to anthelmintic treatments. This highlights the need to improve the diagnostic tools used in equine parasitology.

Previous studies on nematodes in horses in Sweden have mainly focused on the patterns of excretion of strongyle egg in faeces in relation to strategic or selective treatment strategies with anthelmintics (Alm et al., 2023; Nilsson et al., 1989; Osterman Lind et al., 2023; Osterman Lind et al., 2007; Osterman Lind et al., 1999; Tydén et al., 2019), while data on species composition and abundance have so far only been collected at necropsy (Höglund et al., 1997) or by using the worm expulsion method (Osterman Lind et al., 2003). Since the introduction of molecular methods focusing on the Internal Transcribed Spacer-2 region (ITS2) in the rDNA gene region, the possibilities for studying the composition of nematode communities have expanded as they allow non-invasive sampling (Hung et al., 1999). In this way, for example, information on the occurrence of *S. vulgaris* could be obtained ten years after the use of prescription anthelmintics in Swedish horses (Tydén et al., 2019).

Although the reverse line blot method was an initial attempt to identify immature stages of cyathostomins using molecular techniques (Cwiklinski et al., 2012; Traversa et al., 2007), it has not been used in large-scale studies because it is considered too labour-intensive. One of the most recent advances in nematode biodiversity research is the introduction of deep amplicon sequencing of so-called nemabiomes, a powerful, DNA barcoding method that we have already used in other hosts of veterinary interest (Halvarsson et al., 2022; Halvarsson and Höglund, 2021). As next-generation sequencing technologies enable synchronous sequencing of a large number of species in a large number of samples, and the approach has already shown promise for identifying strongylids from collected samples in equine faecal cultures (e.g. Poissant et al., 2021), we decided to apply the same approach to samples from Swedish horses.

Knowledge of species composition in nematode communities improves the ability to develop more specific and effective control measures (Nielsen et al., 2014). This is particularly important given the widespread resistance to anthelmintics in equine strongylid populations, especially among cyathostomins (Kaplan, 2002). In this study, we compare, for the first time, the species composition and abundance of strongyles in Swedish domestic horses and trotters, partly in relation to their age, using the nemabiome barcoding approach.

2. Material and methods

2.1. Samples

As part of the routine veterinary examination, faecal samples were collected from domestic horses. The samples were sent to Vidilab AB, a private diagnostic laboratory, where faecal egg counts (FEC) were performed and L3 larvae were obtained by coproculture for identification of *S. vulgaris*. From trotters, faecal samples were collected upon inspection at slaughter in abattoir in central Sweden. A total of 297 samples were analysed (n = 167 domestic horses and n = 130 trotters), of which 263 provided sequences (n = 136 domestic horses and n = 127 trotters). For the domestic horses, we also determined the year of birth. By combining this with the sample year, we were able to calculate their age. Domestic horses were sampled in 2017, while trotters were sampled at slaughter between 2015 and 2017, with the majority in 2016. Total DNA from the larval cultures was extracted using the Nucleospin DNA tissue kit (Macherey-Nagel) according to the manufacturer's protocol.

2.2. Molecular methods

The samples were amplified with the universal primer pair NC1/NC2, which targets the ITS2 region (Gasser et al., 1993). These primers were combined with unique 8 bp barcodes. This allowed for sample demultiplexing in the bioinformatics pipeline, after sequencing the pooled PCR products. In brief, 50 μl PCR reactions were performed in duplicates. PCR products were purified using Agencourt AMPure XL magnetic beads (Beckman Coulter, IN, USA). The purified PCR products were then pooled in equal amounts and then sequenced using SMRT cell V3 RSII technology on the Pacific Biosciences platform at SciLifeLab, Uppsala, Sweden. For full details, please refer to Halvarsson and Höglund (2021).

2.3. Bioinformatics

The sequencing reads were processed in the SCATA bioinformatics pipeline (http://scata.mykopat.slu.se). In the pipeline, sequences with a quality score of less than 20 or a score of less than 10 at a given position are removed. The remaining sequences were then demultiplexed based on the tag sequences, followed by the removal of tags and primer sequences. To account for systematic sequencing errors in homopolymer regions (Laehnemann et al., 2016), these were collapsed to 3 bp if they could not be assigned to an operational taxonomic unit (OTU) based on differences in these regions (Lindahl et al., 2013). Sequences were compared for similarity using USEARCH (Edgar, 2010) with a minimum length similarity of 85%. A sequence was assigned to an OTU if the sequence deviated less than 0.25% from other sequences in the OTU cluster (Kõljalg et al., 2013). The clustering threshold was optimised by including the taxonomic nematode ITS2 database (v1.2.0) (Avramenko et al., 2015; Workentine et al., 2020) as reference during test runs of the pipeline.

Pairwise alignment was performed with a mismatch penalty of 1, an opening gap penalty of 0 and a gap extension penalty of 1. Read counts were converted to a relative proportion of the total read counts for each sample. Post-SCATA analysis removed samples with fewer than 25 reads, OTUs with one or two reads and less than 0.5% of the total number of reads. OTUs not representing parasitic nematodes were omitted after a manual NCBI BLAST search for each OTU. Species assignment to an OTU was considered if the identity percentage in the BLAST search was $\geq$ 98.5% of the reference sequence in the NCBI database. Based on the species assignment, OTUs with the same species assignment were grouped to represent the respective species in the final dataset for the statistical analyses.

2.4. Statistics

Statistical analyses were performed in R v4.3.1 (R Core Team, 2020). Species richness was calculated by summing all parasite species per individual. To investigate whether a few parasite species dominated the community, Simpsońs diversity index and Shannon-Wiener index were calculated using the [vegan] v. 2.6.4 package (Oksanen et al., 2019), based on relative abundances after standardising the number of reads. In its normal form, Simpson diversity ranges from 0 to 1, where 0 represents infinite diversity and 1 represents no diversity. Therefore, this index is presented in its inverse to enhance readability. An UpSet diagram, created with the package [ComplexUpset] v1.3.3 (Krassowski, 2020; Lex et al., 2014), visualises the most frequent community compositions with a cut-off of n = 3 hosts. To determine the correlation in detection rate between the specific *S. vulgaris* analysis and detection with PacBio sequencing, Cohenś kappa was calculated using the package [psych] v2.3.6 (William Revelle, 2023). Plots were visualised using [ggplot2] v3.4.2 (Wickham, 2016).

3. Results

3.1. Faecal egg counts

On average, 39% of horses were below the egg detection level for the used method (FEC < 50 eggs per gramme of faeces, EPG), with arithmetic strongyle egg counts ranging from 500 ± 613 to 324 ± 464 EPG in domestic horses and trotters, respectively. The maximum number of eggs in the domestic horses was 2500 and 4800. Strongyle egg shedding (FEC) was similar in the two categories of horses (Fig. 1A), but decreased with age in the domestic horses (Fig. 2A). Unfortunately, the age data for the trotters were missing.

3.2. Species diversity and richness

After quality filtering and OTU clustering, 36926 reads were selected from 263 samples among which 136 were from domestic horses and 127 were from trotters. The reads yielded 254 OTUs of nematode parasites, and all but three were identified to species level using NCBI BLAST (S1). On average, 140 reads were obtained per sample (ranging from 26 to 503). After performing sequence clustering, OTUs were grouped into species based on NCBI BLAST similarity, and 28 unique taxa were found (Table 1 and Tab. S1). Species richness and two measures of alpha diversity were similar between the two horse categories (Fig. 1C-E). Young domestic horses as well as individuals older than 20 years had a greater species richness (Fig. 2B), while using the same measures of alpha diversity no differences were observed in comparison to age (Fig. 2C-D).

3.3. Relative abundance

The number of horses infected with the different taxa identified is shown in Table 1. There were no differences between the two groups of horses, except for *Cyathostomum pateratum*, which was slightly more abundant in trotters (i.e. non-overlapping 95% confidence intervals). The average abundance of the seven most common species accounted for 87% in the combined data set (*Coronocyclus coronatus, Cyathostomum catinatum, Cylicocyclus nassatus, Cylicostephanus calicatus, C. goldi, C. longibursatus, C. minutus*). Together with *Strongylus edentatus* and *S. vulgaris*, the relative abundances of these species are shown in

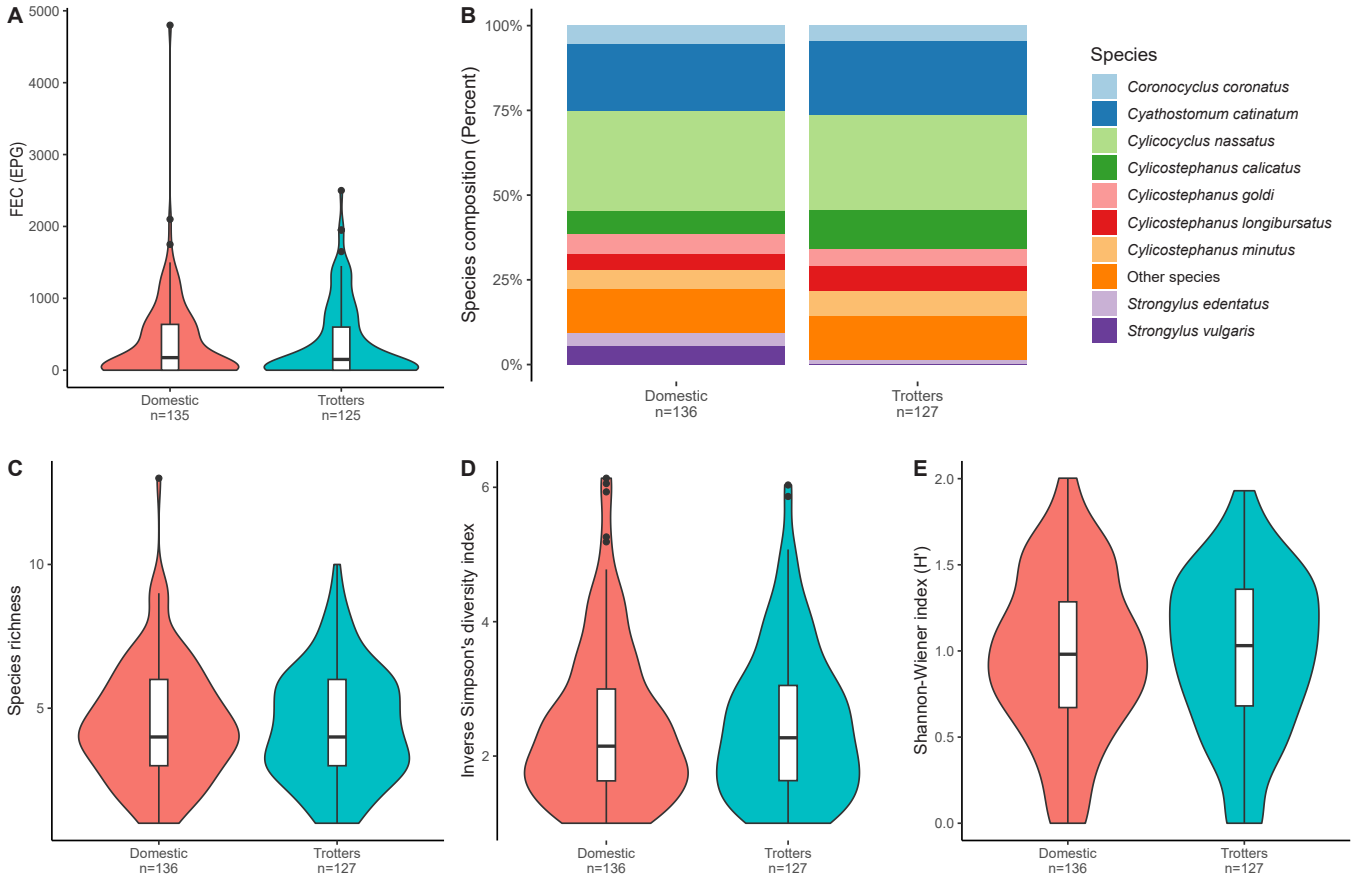

Fig. 1. (A) Violin plot depicting FEC (EPG) for domestic horses and totters. (B) Bar plot summarizing species composition. (C) Species richness for the two horse categories. (D) Inverse Simpson's diversity index and (E) and Shannon-Wiener index H'. (D) and (E) are two different measurements of alpha diversity for the host species. Boxplots within the violin plots display median values.

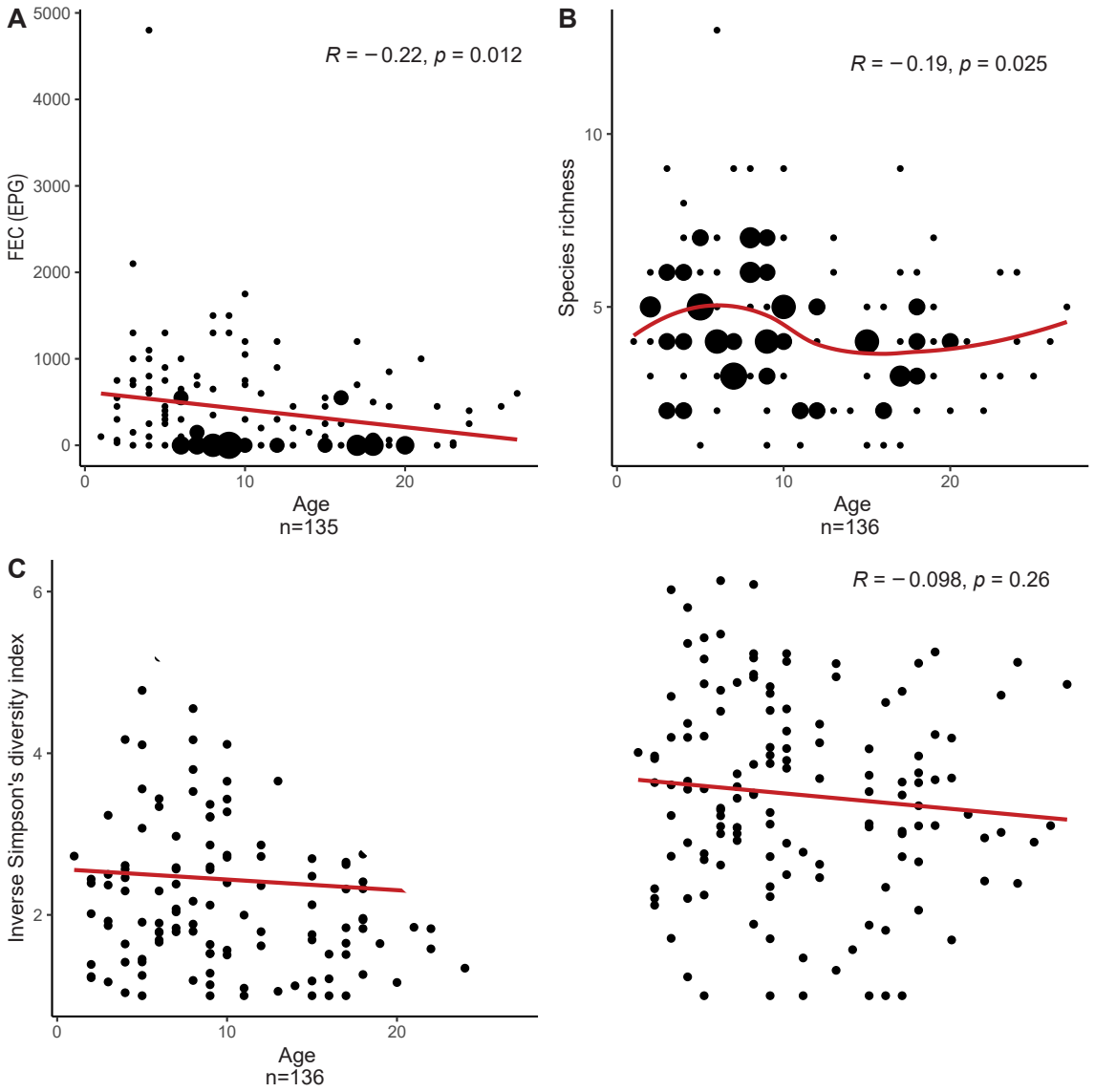

Fig. 2. Correlation between the age of the domestic horses and (A) FEC (EPG), (B) species richness, (C) alpha diversity using inverse Simpson's diversity index, and (D) alpha diversity using Shannon-Wiener index. EPG decreased with age, and species richness indicates higher richness in young and old horses. No difference was found in alpha diversity measures.

(Fig. 1B) and a correlation between them and host age is shown in (Fig. 3). The main trends are that the relative abundance of *C. catinatum* ($R_{Pearson} = 0.22$, $p = 0.0088$) and *S. vulgaris* ($R_{Pearson} = 0.34$, $p < 0.001$) increases with age (Figs. 3B and 3I), while *C. nassatus* ($R_{Pearson} = -0.29$, $p < 0.001$) and *C. minutus* ($R_{Pearson} = -0.25$, $p = 0.003$) decrease (Figs. 3C and 3G).

The three species with the highest prevalence were *C. catinatum, C. nassatus* and *C. calicatus*, and they were found either alone or in combination with other species [Fig. 4]. Comparison of the test used to analyse the presence of *S. vulgaris* with the ability of the SCATA pipeline to detect *S. vulgaris* using the PacBio data showed moderate agreement (McHugh, 2012) with a Cohen's Kappa test (K= 0.56, CI: 0.41 lower, 0.71 upper, n = 260). In addition to the increase in fractional abundance of *S. vulgaris* with age (Fig. 3I), the proportion of positive domestic horses also increased with age (GLM, z = 2.69, df = 134, P = 0.007), using pipeline prevalence data.

4. Discussion

This study presents new and important data, revealing both significant differences and similarities in species composition of strongylids between domestic horses and trotters in Sweden. Interestingly, we found that the highly pathogenic species *S. vulgaris* is about ten times more common in domestic horses than in trotters. Overall, however it is only present in small quantities. This is noteworthy, especially considering that selective anthelmintic treatment has been adopted in Sweden since 2007. Otherwise, the relative abundance of species composition was quite similar between the two horse groups. Eight species in four genera (*Coronocyclus, Cyathostomum, Cylicocyclus and Cylicostephanus*) were the most abundant. Among these *Cyathostomum catinatum* dominated, followed by *Cylicocyclus nassatus* and *Cylicostephanus calicatus*, which accounted for about half of the total species abundance in both horse categories. Apart from a slightly higher species richness in younger and older horses, no clear correlations were found. For instance, some species increased (*C. catinatum* and *S. vulgaris*) or decreased (*Cylicostephanus minutus* and *C. nassatus*) with horse age, total species diversity remained

Table 1
The number of horses infected with the different parasites in the two groups of horses.

	Domestic (N=136)			Trotters (N=127)				
	Infected	Prevalence	±95% CI	Infected	Prevalence	±95% CI	Total	Refs
Cyathostominae								
Coronocyclus coronatus	49	36%	8%	46	36%	8%	36%	1,2,3,4,5,6
Coronocyclus labiatus	19	14%	6%	7	6%	4%	10%	1,2,3,4,5,6
Cyathostomum catinatum	97	71%	8%	88	69%	8%	70%	1,2,3,4,5,6
Cyathostomum labratum	3	2%	2%	1	1%	2%	2%	
Cyathostomum pateratum	22	16%	6%	39	31%	8%	23%	1,2,3,4,5,6
Cyathostomum sp.	17	13%	6%	4	3%	3%	8%	6
Cylicocyclus ashworthi	16	12%	5%	21	17%	6%	14%	1,2,3,4,5,6
Cylicocyclus insigne	8	6%	4%	7	6%	4%	6%	1,2,3,4,5,6
Cylicocyclus leptostomum	13	10%	5%	21	17%	6%	13%	1,2,3,4,6
Cylicocyclus nassatus	91	67%	8%	83	65%	8%	66%	1,2,3,4,5,6
Cylicostephanus bidentatus	6	4%	3%	2	2%	2%	3%	2,4
Cylicostephanus calicatus	47	35%	8%	60	47%	9%	41%	1,2,3,4,5,6
Cylicostephanus goldi	53	39%	8%	35	28%	8%	33%	1,2,3,4,5,6
Cylicostephanus longibursatus	42	31%	8%	53	42%	9%	36%	1,2,3,4,5,6
Cylicostephanus minutus	34	25%	7%	52	41%	9%	33%	1,2,3,4,5,6
Cylicostephanus sp1	18	13%	6%	14	11%	5%	12%	5
Cylicostephanus sp2	3	2%	2%	2	2%	2%	2%	6
Gyalocephalus capitatus	3	2%	2%	1	1%	2%	2%	1,2
Parapoteriostomum euproctus	2	1%	2%	0	0%	0%	1%	2
Parapoteriostomum mettami	1	1%	1%	0	0%	0%	0%	1,2
Petrovinema poculatum	1	1%	1%	2	2%	2%	1%	1,2
Poteriostomum imparidentatum	4	3%	3%	2	2%	2%	2%	2,5
Strongylinae								
Strongylus edentatus	19	14%	6%	5	4%	3%	9%	2,5
Strongylus vulgaris	18	13%	6%	2	2%	2%	8%	2,5
Craterostomum acuticaudatum	5	4%	3%	2	2%	2%	3%	1,2,5,6
Triodontophorus brevicauda	7	5%	4%	2	2%	2%	3%	2
Triodontophorus serratus	3	2%	2%	2	2%	2%	2%	1,2,5
Trichostrongylinae								
Trichostrongylus axei	0	0%	0%	3	2%	3%	1%	2

1 = Osterman et al.2003, 2 = Poissant et al. 2021, 3 = Nielsen et al. 2022, 4 = Malsa et al. 2023, 5 = Abbas et al. 2023, 6 = Boisseau et al. 2023

independent of FEC.

The findings have important implications for the design of sustainable and effective methods of parasite control on horse farms worldwide. In a previous study, a 2.9-fold increased risk of *S. vulgaris* infection was observed on Swedish farms that relied solely on strongyle FEC for treatment compared to farms that also used larval cultures or conducted regular deworming without prior diagnosis (Tydén et al., 2019). Surprisingly, *S. vulgaris* was detected by PCR in 28% of horses and 61% of farms in the same study. This is in stark contrast to studies conducted before the introduction of the selective treatment strategy in Sweden. For example, in a 1986 field study, based on morphological identification of third-stage larvae, only low numbers were found in 11% of herds (Nilsson et al., 1989). Furthermore, adults and larvae of *S. vulgaris* were found in only 4% of horses slaughtered in central Sweden between 1992 and 1993 (Höglund et al., 1997). In another nationwide study conducted a few years later, *S. vulgaris* was found in 14% of the farms (Osterman Lind et al., 1999).

Due to the different inclusion criteria and diagnostic methods, it is admittedly problematic to compare the results of the different studies. However, the combination of results suggests that infection rates of *S. vulgaris* were much lower in the past when horses were dewormed regularly and not tested before treatment. It is therefore of great interest that the results of the present study, in which only 8% of all horses were infected with *S. vulgaris*, are not only in better agreement with the historical situation than the results of Tydén et al. (2019), but also show that only a few (2%) of the trotting horses were infected. Interestingly, the same trend was also observed for *S. edentatus*, while *S. equinus* was not present at all. The prevalence of *S. vulgaris* in the domestic horses increased significantly with age in the present study. In contrast, samples from trotters, whose age is unfortunately not known, were taken at slaughter. Since trotters are often culled at a young age when they are no longer able to perform, it can be assumed that this discrepancy was at least partly age-related, although this explanation remains speculative. However, as the horses studied here overall reflect the role of these parasites under current husbandry conditions, the selective deworming strategy seems to work satisfactorily for strongylins. This is also in line with the results of a recent study on horses participating in a nationwide parasite surveillance programme of the National Veterinary Institute in Sweden (Osterman Lind et al., 2023).

In addition to strongylins, we have identified 26 other unique taxa, three of which have just been assigned to a genus. Most, 85%, belong to the subfamily Cyathostominae, in which seven species account for 87% of the total records. In addition, three species of non-migratory strongylins (*Triodontophorus brevicauda*, *T. serratus* and *Craterostomum acuticaudatum*), whose classification is still uncertain (Lichtenfels et al., 2008) as well as the generalist *Trichostrongylus axei* from the family Trichostrongylidae were recorded in small numbers. Although the occurrence of one species (*C. pateratum*) differed slightly between domestic horses and trotters, the overall species structure was similar, as no significant difference in species richness and diversity was found between the two groups of horses. This confirms firstly that cyathostomins were the predominant group, which is consistent with common knowledge. Secondly, it shows that almost all species identified are specific to horses, and that the potential risk of cross-transmission from other hosts (wildlife and grazing livestock) is insignificant. This contrasts with a recent Scottish study in which seven trichostrongylids were found, albeit in relatively small numbers (Sargison et al., 2022).

When we compare our ITS2 nemabiome dataset for the subfamily Cyathostominae with the results of similar studies, we find mostly similarities, but also some differences. For example, in our study, *C. catinatum* and *C. nassatus* were the two most abundant species, occurring in about 70% of horses, followed in descending order by *C. calicatus, C. longibursatus, C. minutus* and *C. coronatus*. For obvious reasons, these species occurred in different combinations in the same hosts (Fig. 4). The dominance of *C. catinatum* and *C. nassatus* in our study is consistent with the records of a group of horses in the USA tested

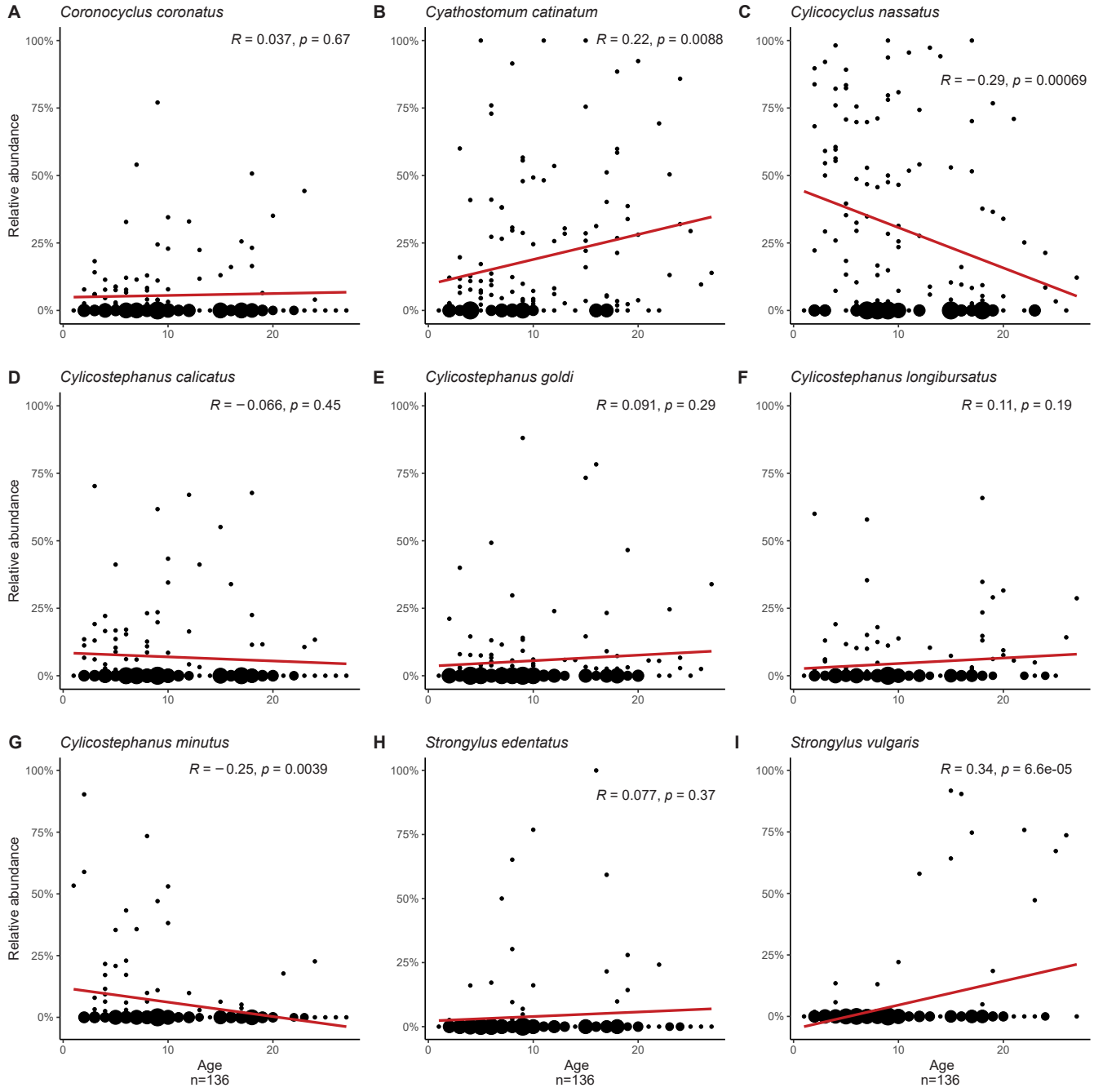

Fig. 3. Correlation plots between the age of domestic horses and relative abundance of the seven most common cyathostomins (A-G) and strongylins (H-I). Significant decreases in relative abundance with age were found for *C. nassatus* (C) and *C. minutus* (G), while significant increases were found for *C. catinatum* (B) and *S. vulgaris* (I).

before treatment (Nielsen et al., 2022). Although we identified more Cyathostominae (15 versus 22 taxa), two species (*C. brevicapsulatus* and *C. radiatus*) detected at low levels in the above study were not present in our study. Additionally, *C. insigne*, which was thought to be less sensitive to ivermectin (Nielsen et al., 2022), was only detected in 6% of horses by us. This was the case even though this drug has been used relatively intensively for several decades, suggesting that ivermectin is still effective against this species in Sweden. Furthermore, our results are in agreement with those of two wild horse populations from Canada, where *C. catinatum* and *C. nassatus* were among the most common taxa (Poissant et al., 2021). In contrast, *C. longibursatus*, followed by *C. nassatus*, was predominant in a longitudinal study of six Scottish ponies, which as in the previous study, had fewer cyathostomins than our study. However, as in our study, it also contained some taxa that could not be classified to species level (Sargison et al., 2022). This is consistent with a recent Australian cross-sectional study that found 17 taxa of Cyathostominae (including one unidentified taxa), and the three most abundant species were *C. longibursatus*, *C. nassatus* and *C. coronatus* (Abbas et al., 2023). Finally, in a French study investigating the anthelmintic effect of a sainfoin (*Onobrychis viciifolia*) feed as a possible alternative method to control cyathostomin populations in horses and its interaction with ivermectin, 13 species were detected before treatment (Malsa et al., 2022). Although *C. nassatus* but also *C. catinatum* were detected in large numbers, *C. minutus*, followed by *C. ashworthi*, was the most abundant

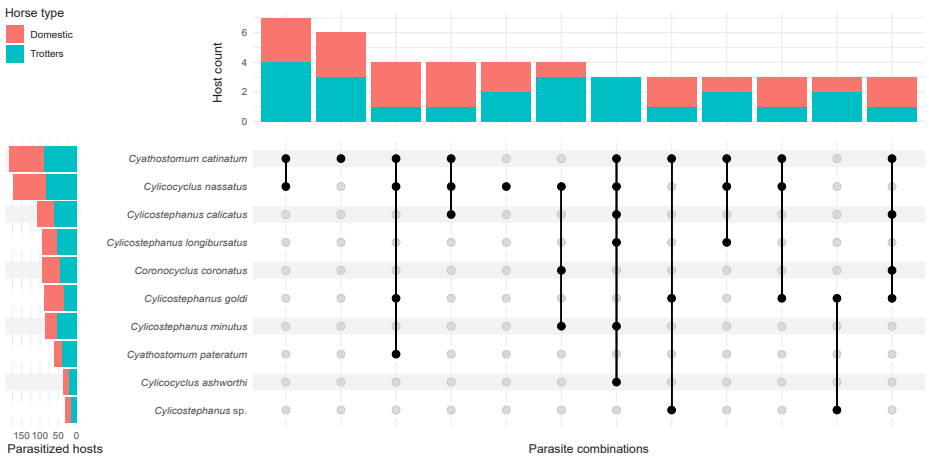

Fig. 4. Upset diagram displaying parasite combinations found in at least n = 3 horses. The most common combination (*Cyathostomum catinatum* and *Cylicocyclus nassatus*) was found in seven horses. The most frequently identified parasite, *C. catinatum*, was found in 185 horses.

species. This is in contrast to another French study in which 14 species (including three unidentified taxa) were found and in which *C. nassutus*, followed by *C. minutus*, were the predominant species (Boisseau et al., 2023). All in all most taxa in all these studies could be assigned to species but, as several authors point out, it will be necessary in the future to sequence morphologically identified specimens to further improve species delineation using nemabiome analysis in horses. It may also be useful to use genetic markers other than the ITS-2 locus and proteomic tools to distinguish between certain cryptic cyathostomins (e.g. Bredtmann et al., 2017).

In summary, the above nemabiome studies have identified a total of 11 unique species within Cyathostominae found in all studies (i: ***Coronocyclus coronatus****, C. labiatus,* ii: ***Cyathostomum catinatum****, C. pateratum,* iii: *Cylicocyclus ashworthi, C. insigne,* ***C. nassatus,*** and iv: ***Cylicostephanus calicatus, C. goldi, C. longibursatus*** and ***C. minutus***). Eight of these species (highlighted in **bold**) were classified as medium to high abundance species in a recent global meta-analysis where identification was based on worm morphology, while the other three species were classified as medium to low abundance species (Bellaw and Nielsen, 2020). This is largely consistent with our current results, with one exception (i.e. *C. longibursatus* which was moderately abundant). Nevertheless, the combined equine nemabiomes suggest that these eight species can survive in a wide range of climatic and management conditions. On the other hand, many studies have reported substatial intraindividual differences in species diversity and abundance between different horses. In addition to the records in the present study, see e.g. (Boisseau et al., 2023; Malsa et al., 2022). The most likely reason why more species were documented overall in our study is that we included hundreds of horses from across the country, as opposed to fewer horses in most other studies. This probably explains the discovery of rare species such as *C. bidentatus, C. labratum* and *G. captitatus* in our study, This view is also supported by the results of the cross-sectional study by Abbas et al. (2023). Otherwise, no specific age trends were observed in our study, except for *C. minutus* and *C. nassatus*, whose relative abundance in domestic horses increased with age, while *C. catinatum* decreased.

The sequencing technology and pipelines used to generate the final datasets (nemabiomes) also differed in some aspects from study to study. However, in all studies, the majority of amplicon sequences could be assigned to a species or genus, and generally with a high degree of confidence, suggesting that the species in the reference sequence library were sufficiently genetically distinct. As Sargison et al. (2022) noted, the final taxonomy files have remarkable similarities, although their study and that of Poissant et al. (2021) used different methods and bioinformatics software to assign horse nemabiomes. This observation aligns with a previous study on the composition of nemabiomes from sheep samples analysed using three different bioinformatics pipelines. While minor differences were found between the respective pipelines, the overall picture remained very consistent regardless of the pipeline used (Baltrušis et al., 2022).

Comparison of our current nemabiome data set with a data set on the species composition of 12 untreated horses aged 1 to 5 years at a stud farm where adult worms were collected and morphologically identified a few days after deworming with various anthelmintics in November 1997 (Osterman Lind et al., 2003), revealed several interesting observations. Notably, the 18 species identified by Osterman Lind et al. (2003) were non-migratory strongylids, mainly from the subfamily Cyathostominae. All but one (*Coronocyclus labratus*) were also detected in the current nemabiome study. Firstly, this was the case although some species (*C. acuticaudatum, T. serratus, P. poculatum* and *P. mettami*) were represented in low ($\leq$ 3%) abundance in both studies. Secondly, the two most abundant species in both studies were *C. catinatum* and *C. nassatus*. Third, *C. longibursatus, C. leptostomum, C. minutus* and *C. calicatus* were relatively abundant in both studies. This confirms the accuracy of our metabarcoding approach. All in all, this suggests that the species composition of the strongylid communities in Swedish horses has remained relatively stable over several decades, despite the fact that anthelmintics based on the principle of selective treatment have been used for many years.

5. Conclusions

In this study, we compared nemabiomes in domestic horses and trotters. We found that *C. catinatum* and *C. nassatus*, followed by five other taxa of Cyathostominae, were the most frequently identified species in both groups of horses. This is consistent with an earlier study from the late 1990 s based on morphological identification of adult worms. We also detected two stongylins (including the highly pathogenic *S. vulgaris* and the less pathogenic *S. edentatus*), with the former parasite occurring in similarly low numbers as before the introduction of selective treatment. However, in contrast to the non-migratory strongylids, significant differences were found with a lower incidence in trotters. The combination of these results suggests that current parasite control is effective and that species diversity and abundance have remained relatively stable over several decades. In summary, we can conclude that nemabiome analysis is a powerful tool, as we were able to identify 28 unique taxa, most of which could be assigned to species level. Accordingly, this tool can be used in future applications to reveal

differences in species-specific relative abundance and overall species richness, and it should ideally also be included in future routine parasite diagnostics.

CRediT authorship contribution statement

Hägglund Sara: Project administration, Investigation. **Peter Halvarsson:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. **Grandi Giulio:** Writing – review & editing, Methodology, Conceptualization. **Höglund Johan:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

We hereby declare that the information disclosed is accurate and that I am not aware of any other situation of actual, potential or apparent conflict of interest. I undertake to inform you of any change in these circumstances, including if a problem arises in the course of the meeting or the work itself.

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Animal welfare statement

No ethical permissions were necessary for this study as the samples were collected from killed animals or sent in for routine veterinary diagnostics.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.110111.

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