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## Generalist nematodes dominate the nemabiome of roe deer in sympatry with sheep at a regional level



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### ABSTRACT

The growth of livestock farming and the recent expansion of wild ungulate populations in Europe favor opportunities for direct and/or indirect cross-transmission of pathogens. Comparatively few studies have investigated the epidemiology of gastro-intestinal nematode parasites, an ubiquitous and important community of parasites of ungulates, at the wildlife/livestock interface. In this study, we aimed to assess the influence of livestock proximity on the gastrointestinal nematode community of roe deer in a rural landscape located in southern France. Using ITS-2 rDNA nemabiome metabarcoding on fecal larvae, we analysed the gastrointestinal nematode communities of roe deer and sheep. In addition, we investigated *Haemonchus contortus* nad4 mtDNA diversity to specifically test parasite circulation among domestic and wild host populations. The dominant gastrointestinal nematode species found in both the roe deer and sheep were generalist species commonly found in small ruminant livestock (e.g. *H. contortus*), whereas the more specialised wild cervid nematode species (e.g. *Ostertagia leptospicularis*) were only present at low frequencies. This is in marked contrast with previous studies that found the nemabiomes of wild cervid populations to be dominated by cervid specialist nematode species. In addition, the lack of genetic structure of the nad4 mtDNA of *H. contortus* populations between host species suggests circulation of gastro-intestinal nematodes between roe deer and sheep. The risk of contact with livestock only has a small influence on the nemabiome of roe deer, suggesting the parasite population of roe deer has been displaced by generalist livestock parasites due to many decades of sheep farming, not only for deer grazing close to pastures, but also at a larger regional scale. We also observed some seasonal variation in the nemabiome composition of roe deer. Overall, our results demonstrate significant exchange of gastrointestinal nematodes between domestic and wild ungulates, with generalist species spilling over from domestic ungulates dominating wild cervid parasite communities.

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## 1. Introduction

Pathogens impact the health and production of livestock (Gunn and Irvine, 2003). The associated costs of treatments and of production loss can have far-reaching economic and social consequences for the animal industry (Martin et al., 2011; Charlier et al., 2020). Since the early 20th Century until now, populations of wild ungulates have grown and expanded across the entire European continent (Linnell and Zachos, 2010). For example, the roe deer population has faced a massive decline during the late

19th–early 20th Centuries and was very fragmented, whereas now roe deer occupy approximately 74% of the European continent (Linnell and Zachos, 2010). It was observed that approximately 70% of the land mass of Europe is home to at least two wild native ungulate species (Linnell et al., 2020), whilst the number of domestic livestock has remained constant (Eurostat, 2020. Livestock population in numbers. <https://ec.europa.eu/eurostat/web/products-eurostat-news/-/ddn-20200923-1> (accessed 2 April 2022)). Consequently, the likelihood of co-existence of wild and domestic animals is, on the whole, increasing across Europe but varies considerably at the regional scale. The actual extent of habitat sharing, both spatial and/or temporal, will depend on many different aspects including the species involved, home range size and the accessibility of pastures of domestic ungulates to wild ungulates. Or conversely, domestic species can exploit the habitats of wild species, as seen with alpine pastures (Berentsen et al., 2014; Carrasco-Garcia et al. 2016). In such situations, direct and/or indirect transmission of mutual pathogens can occur among populations (Dobson et al., 2008). Mutual pathogens are of major concern and can have drastic consequences for livestock and wildlife populations, and for humans when it implies zoonoses (e.g. avian influenza, tuberculosis, brucellosis; Rahman et al., 2020). Thus, understanding the epidemiology of pathogens at the wildlife-livestock-human interface is of prime importance for pathogen control strategies (Gortazar et al., 2015).

Worldwide, gastrointestinal nematodes are responsible for major health impacts on livestock with important economic outcomes (Hoberg et al., 2001; Roeber et al., 2013). Gastrointestinal nematodes are capable of infecting several host species when grazing on pastures contaminated with infective larval stages (Hoberg et al., 2001; Walker and Morgan, 2014). The same species of gastrointestinal nematodes are able to infect common ungulate species such as roe deer and cattle (e.g. *Trichostrongylus axei*) or sheep (e.g. *Haemonchus contortus*) (Winter et al., 2018). Such parasites are referred to as generalist parasites because they are able to survive and reproduce in more than one host species (Walker and Morgan, 2014) and their geographical distribution and presence in wildlife is often attributed to livestock grazing activities (Hoberg et al., 2004). Specialist parasites can develop in a limited number of closely related hosts (e.g. *Cooperia curticei*, a specialist of sheep). Characterisation of the gastrointestinal communities of six ungulate species (*Capreolus capreolus*, *Cervus elaphus*, *Ovis aries*, *Ovis musimon*, *Rupicapra rupicapra*, *Capra ibex*) living in the Italian alps (Zaffaroni et al., 2000), revealed the dominance of specialist over generalist nematodes in the communities of nematodes from the different hosts. However, our knowledge of the capabilities of each parasite species to infect a host species is currently outdated (Wyrobisz-Papiewska et al., 2018). Conceivably, a parasite could have been mislabeled as a specialist due to a lack of investigation in a variety of host species, or because it has not yet encountered another compatible host, e.g., due to geographical or behavioral barriers.

Because domestic and wild ungulates are phylogenetically related, interspecies transmission of helminths among species is possible (Walker and Morgan, 2014). Therefore, the sympatry of ungulate species increases the risk of cross transmission between wild and domestic hosts. In such a situation, transmission of gastrointestinal nematodes has been proven in several countries, using different methodologies such as in the Italian alps with the genetic survey of *H. contortus* using nad4 mtDNA, internal transcribed spacer 1 (ITS1) and ITS2 sequencing and microsatellites (Cerutti et al., 2010), in the Iberian Peninsula with necropsy and visual identification of nematode species (Pato et al., 2013), and in the United States with ITS2 metabarcoding (Barone et al., 2020). ITS2 sequencing has been shown to be accurate in detecting and identifying gastrointestinal nematode species present in fecal

samples from sheep (Redman et al., 2019) and roe deer (Beaumelle et al., 2021). However, the extent of cross-host transmission of gastrointestinal nematodes remains unclear (Walker and Morgan, 2014; Morgan et al., 2019). In a context where roe deer are well adapted to peripheral habitats around agricultural lands (Morellet et al., 2011), a better understanding of nematode epidemiology at the domestic and wildlife interface is needed to determine the relative role of domestic and wild populations in the maintenance and transmission of gastrointestinal nematode species, and the best strategies for long-term management of these populations.

The aim of this study was to investigate the gastrointestinal nematode parasite communities from roe deer and domestic sheep inhabiting the same rural area. Roe deer, *Capreolus capreolus*, is a common and widely distributed ungulate species in various landscapes of Europe from open agricultural areas to forests, from plains to mountains (Linnell and Zachos, 2010). Roe deer share several gastrointestinal nematode species with sheep (at least 21 species according to Winter et al. (2018)), making these two host species very appropriate for study of the community of nematodes at the interface of wild and domestic ungulates. In order to determine the gastrointestinal nematode diversity, we used a next-generation sequencing metabarcoding approach with the sequencing of ITS2 rDNA to identify gastrointestinal nematode species (Avramenko et al., 2015) and nad4 mtDNA to study the genetic diversity of *H. contortus*, a highly pathogenic gastrointestinal nematode species of sheep (Roeber et al., 2013). Here, we investigated the relative abundance of gastrointestinal nematode species and the genetic structure of a *H. contortus* population in sheep and in roe deer. Then, we investigated variations in the nemabiomes of roe deer with respect to their spatial proximity to livestock pastures, considering the seasonal life-cycles of parasites.

## 2. Materials and methods

### 2.1. Study areas and sampling

The study area is part of the long-term socio-ecological research platform ZA PYGAR, designed to study landscape dynamics and multiple ecosystem services (Ouin et al., 2021). In an ~120 km<sup>2</sup> area located in south-western France (Aurignac, 43°13'N, 0°52'E), roe deer behavior (e.g. movement of GPS collared individuals) and landscape heterogeneity have been monitored for 25 years. The landscape is composed of a mosaic of crops (including mostly wheat, maize, sorghum, rye grass and lucerne), forest, woodlots, hedgerows and fenced livestock pasture (cattle, horses and sheep). The most abundant wild ruminant is the roe deer with an estimated population density of 4–8 deer/100 ha in most parts of the landscape and up to 34 deer/100 ha in the forest sectors (Hewison et al., 2007). Sheep farms include herds of 18–300 sheep, covering a total of 3.5 km<sup>2</sup> of sheep pasture. Each sheep farm controls gastrointestinal parasites with different direct and indirect strategies including treatments with anthelmintics such as macrocyclic lactones and (pro-)benzimidazoles, and rotational grazing. Roe deer have been observed to enter fenced pastures when domestic ungulates are grazing (unpublished results). However, they often go along the pastures or spend time in pastures when the sheep or cattle are not there. Sheep pastures are large and pastures of the same farm are adjacent to each other and connected to the sheep pen.

Between 17 October 2018 and 19 February 2019, roe deer fecal samples were either individually collected rectally from immobilised individuals captured in winter (see Malagnino et al. (2021) for details of the procedure) or directly harvested from the ground by prospecting in both forest or open sectors of the area

(Fig. 1). Fresh sheep fecal samples were collected from the ground in the fenced pasture of each sheep farm. Only fresh feces that were >2 m distant from each other were collected for the environmental samples from roe deer to limit the probability that feces belonged to the same individual. Feces from roe deer were never collected from the ground of sheep pastures but in their vicinity. Samples were collected in plastic bags, sealed after air removal, and analysed within 48 h in the parasitology laboratory of the National Veterinary School of Toulouse (ENVT, France). Samples were not refrigerated during transport as storage at a cold temperature can affect the development of some parasite species (O'Connor et al., 2006). We sampled 201 fecal samples from roe deer and 211 fecal samples from eight sheep farms (we collected 18–34 sheep fecal samples per farm).

## 2.2. Parasitological analyses

The number of eggs per gram of feces (epg) was counted following a modified McMaster protocol (Raynaud et al., 1970) with a salt solution (NaCl;  $d = 1.2$ ). Fecal egg counts were performed on individual fecal samples for roe deer, while we pooled sheep feces into 2–4 mixed feces per farm (total of 25 pools; Supplementary Fig. S1) and performed composite fecal egg counts for sheep. We did not count the epg for 15 roe deer fecal samples as the material amount was limited. Coprocultures were set up with ~100 g of sheep feces and ~10 g of roe deer feces. Individual larval cultures were incubated for 12 days at  $24 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$  and humidified every 2 days with tap water to ensure the feces retained sufficient moisture. L3s were recovered by filling the beaker with tap water at room temperature ( $\pm 25 \text{ }^\circ\text{C}$ ) and inverting it on a Petri dish (MAFF, 1986). In a first step, L3s were collected in a volume of 40–45 mL of tap water, then centrifuged (10 min at 2,264g) to obtain a final suspension of 5 mL. These suspensions were stored at  $4 \text{ }^\circ\text{C}$  until counting and molecular processing. Genomic DNA was extracted from samples with greater than 20 L3s and performed using the Qiagen DNeasy® powerSoil kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with an elution volume of 50–150  $\mu\text{L}$  (50  $\mu\text{L}$  for <500 L3s, 100  $\mu\text{L}$  for 500–1000 L3s and 150  $\mu\text{L}$  for >1000 L3s).

## 2.3. High throughput sequencing analyses

To determine the gastrointestinal nematode species composition, we amplified ITS2 rDNA (NC1: 5'-ACGCTGGTT CAGGGTTGTT-3' and NC2: 5'-TTAGTTCTTTCTCCGCT-3') with 1:10 diluted DNA and each sample was tagged with a unique combination of barcode identifiers to allow pooling into a single 100 ng amplicon library, following the protocol of Avramenko et al. (2015). The library was run on an Illumina MiSeq Desktop Sequencer. We processed the raw sequence reads through a DADA2-based pipeline (Callahan et al., 2016; see <https://www.nemabiome.ca> for specific details). Following Beaumelle et al. (2021), the gastrointestinal nematodes were identified with three different methods of assignment: BLASTn (Altschul et al., 1990) against the NCBI database (13 April 2021), AssignTaxonomy (Wang et al., 2007; Callahan et al., 2016) and IDTaxa (Murali et al., 2018) against the nematode ITS2 rDNA database 1.1.0 (Workentine et al., 2020). We chose to attribute a confidence level to taxonomic identification at species level: high or moderate confidence if the three or two methods, respectively, were congruent (Supplementary Table S1). The resulting classification of amplicon sequence variants (ASVs) was processed further to remove singletons, potential contaminants and samples with less than 1000 reads, following with the procedure set out in Taberlet et al. (2018). For more details, see the procedure of Beaumelle et al. (2021). Only gastrointestinal nematode ASVs were included in the analyses (e.g. ASVs classified as *Vare-*

*strongylus capreoli*, *Cystocaulus ocreatus* and *Neostrongylus linearis* were removed from the dataset before analysis).

For the *H. contortus* population genetic study, a partial nad4 fragment (~307 bp) was amplified using the primers HD4\_F (5'-GTTATTGAGAATTTTATCATA-3') and ND4\_R (5'-TGAAAGTCA GAAAATATTATAC-3'). PCR set-up and cycling parameters were the same as those used for the ITS2 amplicon except an annealing temperature ( $T_a$ ) of  $54 \text{ }^\circ\text{C}$  was used. Nad4 amplicons were sequenced on an Illumina MiSeq Desktop Sequencer and were generated with the DADA2-based pipeline (Callahan et al., 2016) following the same process as the ITS2. We only kept amplicons identified as *H. contortus* based on a similarity of >97% and a query coverage of 100% against NCBI database sequences.

## 2.4. Roe deer spatial data

To investigate how the proximity of domestic animals influences the nemabiome of roe deer, the proportion of home range that overlaps with sheep or cattle pastures for each roe deer was estimated. To do that, we first calculated the area of home ranges of roe deer that have been monitored in our study area with GPS collars (one location every 6 h) between 2005 and 2017, as presented by Malagnino et al. (2021). We used the GPS locations recorded from November to December to index the spatial behavior of adult roe deer in autumn and winter, a period corresponding to non-territorial and non-reproductive stages for this species. We estimated the home range using the fixed kernel method (Worton, 1989) with the Least Squares Cross Validation (LSCV) method for the smoothing factor  $h$  (Seaman et al., 1998) implemented in the R package adehabitatHR (Calenge, 2006), and considering the 90% kernel density contour (see Malagnino et al. (2021) for details on home range calculation). The study area is characterised by a gradient of opening of the landscape, from open landscape with few wooded habitats to closed forest. Because the sectors where we collected the feces are distributed according to this gradient (Fig. 1), we calculated the averaged home range size of roe deer by sector. Then, for each fecal sample collected from the ground, we estimated a circular virtual home range, considering: (i) the feces location as the centroid of the home range and (ii) its size as the average home range size calculated from GPS-tracked roe deer in the same sector. The radius around each fecal sample was then estimated at 458–553 m, and the mean home range size varied between 66 and 96 ha depending on the sector (Fig. 1). For feces directly collected during the capture of roe deer from 11 January 2019 to 15 February 2019 ( $n = 52$  out of 201 fecal samples), we used the locations recorded by their GPS collar during a period from 10 days after the capture (to avoid a capture effect on spatial behavior, Morellet et al., (2009) ) to the end of March (before a potential dispersion event) to calculate their median centroid and then placed a circular virtual home range as we had done for fecal pellets collected from the ground. Finally, we assessed whether the virtual home range of each roe deer overlapped sheep or cattle pastures. We qualified roe deer as being either at high risk of contact with pasture or low risk of contact with pasture when their potential home range overlapped pasture or not, respectively, irrespective of the percentage of overlap.

## 2.5. Statistical analyses on measures of parasitic community

All analyses were carried out using R 3.6 (R Core team, 2020. <https://www.R-project.org/>). To compare the nemabiomes of roe deer and sheep, we only considered the presence or absence of gastrointestinal nematode (GIN) species in analyses. We compared the mean richness of nemabiomes (number of ASVs in nemabiomes) in roe deer and sheep using a Wilcoxon rank-sum test, and we tested the significance of the association between the host (roe deer or

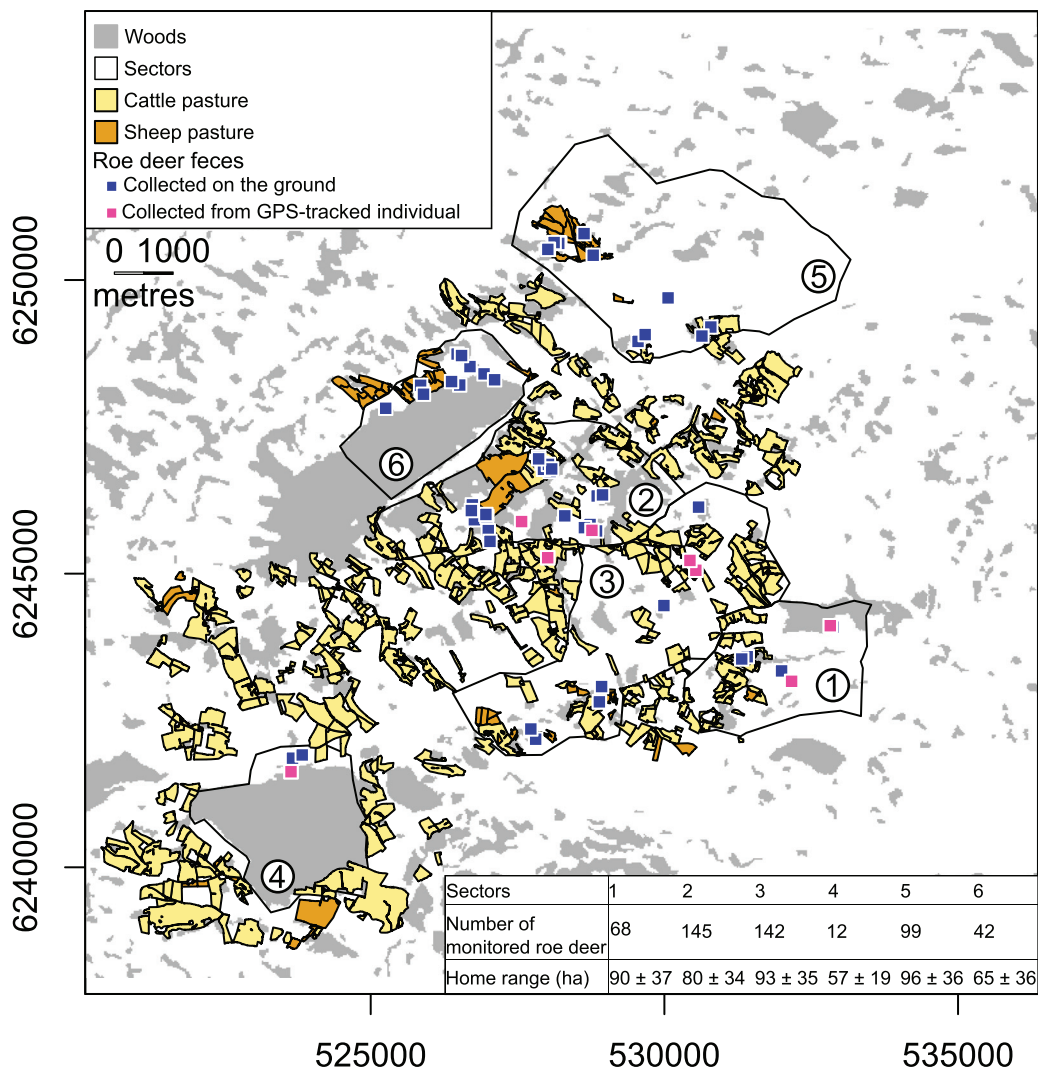


Fig. 1. Localization of sectors, roe deer samples, sheep and cattle pastures in the study area of Aurignac, France. Each sector has a number (1–6). Mean ± S.D. home range size of roe deer in November–December in each sector is provided in the table.

sheep) and the occurrence of gastrointestinal nematode species with the function *multipatt* (association function: “r.g”) in the package *indicspecies* (C  ceres and Legendre, 2009).

To depict the differences in GIN communities between roe deer and sheep, and among farms, a phylogenetic analysis of ASVs was conducted using a maximum likelihood tree with the HKY+G (Hasegawa et al., 1985) model according to *ModelTest* function (Posada and Crandall, 1998; Schliep, 2011). We visualised the ASV phylogeny with the *ggtree* package (Yu et al., 2017), with *Bunostomum* sp. as the root.

We tested the variation in roe deer nemabiomes according to the presence of livestock pasture in their virtual home range using linear models with alpha (Shannon index: consider richness and evenness) and beta (weighted UniFrac index in *phyloseq* package, (McMurdie and Holmes, 2013)) diversity as dependent variables. The UniFrac index incorporates information on the relative relatedness of community members by incorporating phylogenetic distances between observed organisms in the computation. For both alpha and beta diversity, we considered a set of independent variables that we considered would influence GIN diversity, the date of sampling, and the overlap of roe deer home range with sheep and cattle pastures, i.e., high versus low risk. For alpha diversity, we used a linear model to test which variables influence the diversity

in roe deer nemabiomes. As beta diversity is a distance value between pairs of samples, we adapted the variables for comparison of each pair. We counted the number of days between pairs of roe deer samples because the nemabiome varies temporally. Pairs of roe deer samples were categorised as low risk of contact if the two samples did not overlap sheep pasture, as high risk of contact if the two samples overlapped sheep pasture, and as low and high risk of contact if one sample had sheep pasture in its home range but not the other. The geographic distances between pairs of samples were added to the model to control for potential spatial autocorrelation. We used a linear mixed model (R package *lme4*, (Bates et al., 2015)) with samples as random effect to control multiple comparisons among samples (Clarke et al., 2002; Row et al., 2017).

We used a model selection approach to select the independent variables that significantly influence the alpha and beta diversity. It resulted in 13 and 26 competitive models for alpha and beta diversity, respectively. We then ranked the models for each set of candidate models using the Akaike information criterion with a correction for small sample sizes (AICc) and calculated  $\Delta$ AICc and AICc weights. We selected the model with the lowest AICc value. Models with  $\Delta$ AICc < 2 were considered equivalent, and in this case, we considered the most parsimonious one, i.e. the model with the lowest degree of freedom.

## 2.6. Statistical analyses on measures of genetic populations of *H. contortus*

All analyses were carried out using R 3.6 (R Core team, 2020, <https://www.R-project.org/>) unless otherwise specified. The nad4 amplicon was used to study the population genetic structure of *H. contortus* among sheep farms and roe deer with high/low risk of contact with sheep pasture. The sequences were aligned with the function *AlignSeqs* of the package DECIPHER (Wright, 2016). We assessed the diversity of nad4 haplotypes per sample as the proportion of nad4 haplotypes in the samples weighted by the number of *H. contortus* L3s. We estimated this number as the proportion of *H. contortus* obtained with the ITS2 multiplied by the number of L3s used for DNA extraction. We set up the data by grouping the samples together into populations belonging to each sheep farm or roe deer category. All genetic indices were computed with Arlequin 3.5 (Excoffier and Lischer, 2010) and a Kimura 2p model (Kimura, 1980). We computed the haplotype diversity, the mean number of pairwise differences and the nucleotide diversity (average over loci) for each population. We measured the genetic differentiation among populations with the pairwise  $\Phi_{ST}$ , the  $F_{ST}$  analog for DNA sequence data, based on 1,000 permutations for statistical significance. In addition, an analysis of molecular variance (AMOVA) was carried out to characterise population differentiation of *H. contortus* between groups of host species (sheep and roe deer) and among populations.

## 2.7. Data accessibility

The DADA2 pipeline and the ASVs analysed during the current study are available in MendeleyData (DOI: [10.17632/mj36n-hxm2n.1](https://doi.org/10.17632/mj36n-hxm2n.1)). The roe deer sample information and the sequence data generated with the high throughput sequencing are available in the NCBI Short Read Archive under project accession number PRJNA857182 (SUB11761160).

## 3. Results

### 3.1. Parasite material

The median epg for roe deer (50; 95% interquartile range (IQR): 0,2787.5;  $n = 186$ ) and sheep (75; 95% IQR: 0,820;  $n = 25$ ) feces were similar (Mann–Whitney  $U$  test;  $W = 2696.5$ ,  $P = 0.18$ ). The epg for roe deer significantly decreased between 17 October 2018 and 19 February 2019 (Pearson's  $r$  test;  $r_{184} = -0.36$ ,  $P < 0.001$ ).

Because we extracted DNA from samples estimated to have at least 20 L3s and thereafter, samples with at least 1000 reads, we analysed the ITS2 rDNA nemabiome of 77 roe deer fecal samples and 21 pools of sheep feces (total of 166 fecal samples) (Figs. 2, 3). We obtained 11/77 samples of roe deer with less than 100 L3s (Fig. 2). The median number of L3 used for DNA metabarcoding for roe deer (360; 95% IQR: 29,1080;  $n = 77$ ) was lower than for sheep (960; 95% IQR: 540,1632;  $n = 21$ ) (Mann–Whitney  $U$  test;  $W = 1377$ ,  $P < 0.001$ ). Among roe deer, there was no significant correlation between the Shannon index and the number of L3s used for DNA extraction (Pearson's  $r$  correlation;  $r_{75} = -0.14$ ,  $P = 0.23$ ), suggesting no influence of the number of L3s on our results. We identified 91 ASVs assigned to 16 different taxa either at genus (25%) or species (75%) levels. We could not determine the species level for some ASVs in the genera *Bunostomum*, *Cooperia*, *Ostertagia* and *Trichostrongylus* (Supplementary Table S1). We managed to sample most of the taxonomic diversity present in the study area as shown by the taxa accumulation curves rapidly reaching a plateau (Supplementary Fig. S2). There were 35 ASVs only found in

one sample and one fecal sample of roe deer included seven unique ASVs (this sample had no ASVs in common with the other samples).

Within the 98 samples of sheep and roe deer, 68 samples were positive for *H. contortus* based on the ITS2 data. From those samples, we obtained the nad4 data for 18 sheep samples and 41 roe deer samples. We identified 513 different nad4 ASVs with a minimum read relative frequency of 0.1%.

### 3.2. Diversity of gastrointestinal nematodes in sheep and roe deer

There was a higher richness in the roe deer population (17 taxa and 83 ASVs) than in the sheep population (12 taxa and 45 ASVs) (Fig. 4), but we observed a higher mean richness in sheep samples ( $16.7 \pm 4.5$  ASVs (min: 8, max: 26)) than in roe deer samples ( $7.1 \pm 3.6$  ASVs (min: 1, max: 18)) (Mann–Whitney  $U$  test;  $W = 1539$ ,  $P < 0.001$ ). In this study area, the most abundant nematodes are generalist species (i.e., *H. contortus* and *T. axei*), that were detected in almost all sheep feces (i.e., in 90.5% and 85.7% of the samples, respectively;  $n = 21$ ). *Haemonchus contortus* and *T. axei* were detected in 63.6% and 68.8% of the samples of roe deer ( $n = 77$ ) with a mean read relative frequency of 47% and 29%, respectively. *Chabertia ovina*, *Trichostrongylus colubriformis*, *Cooperia fuelleborni*, *Teladorsagia circumcincta*, *Cooperia curticei* and *Cooperia* spp. were predominantly observed in sheep (IndicSpecies test;  $P < 0.001$ ) while *Spiculopteria spiculoptera* and *Ostertagia leptospicularis* were specific to roe deer in this study (IndicSpecies test; all  $P$ -values  $< 0.05$ ) and were never found in sheep (Fig. 3). Generalist nematode species have higher occurrence and proportion than specialist species in roe deer. In addition, we noted the presence of *Ostertagia ostertagi*, a strongyle from cattle, in four roe deer fecal samples (Fig. 3).

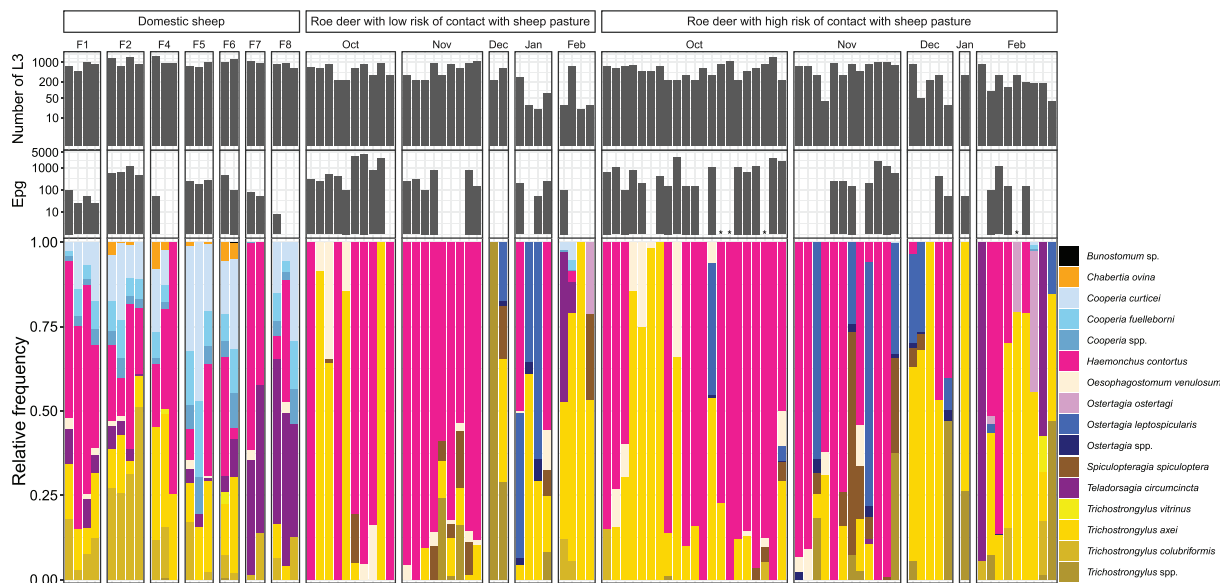
Considering the ITS2 rDNA, roe deer were infected by the same ASVs as domestic sheep. In addition, the most common ASVs in sheep were also the ASVs with the highest prevalence in roe deer feces (Fig. 4).

Considering the nad4 mtDNA of *H. contortus*, roe deer and sheep were infected by populations with similar haplotype diversity (from 0.934–0.972), mean number of pairwise differences (4.615–6.593) and nucleotide diversity (0.015–0.021) except for the sheep from farm 8 which had lower haplotype diversity (0.882) (Table 1). All  $\Phi_{ST}$  values were low ( $< 0.10$ ) and the  $\Phi_{ST}$  between roe deer and sheep were all  $< 0.047$  (Table 2). The majority of molecular variance was explained by the variation within *H. contortus* populations (98.20%) (Table 3).

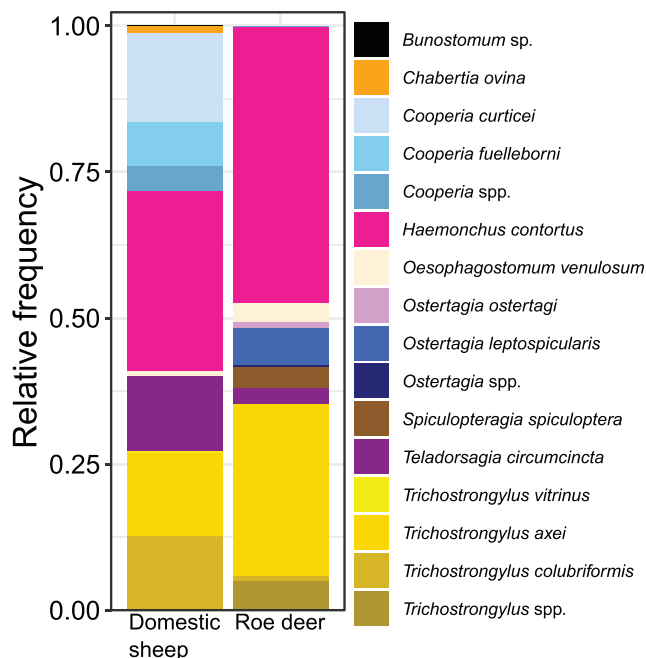
### 3.3. Differences among the roe deer nemabiomes according to their proximity to pastures

The selected linear model for alpha diversity only relies on the day of sampling (Supplementary Table S2), with an increase in diversity over the sampling period ( $\beta = 0.003 \pm 0.001$ ,  $P = 0.003$ , adjust  $R^2$  of the model = 0.10).

The selected linear mixed model for beta diversity included the interaction between the sampling period and the risk of contact with sheep pasture (Supplementary Table S3, Table 4, Fig. 5) and had a conditional  $R^2$  of 0.20. The risk of contact with cattle was not retained in the selected model, neither for explaining alpha diversity nor for beta diversity (Table 4). The nemabiome changed significantly over the sampling period as a higher number of days between sampling dates resulted in higher dissimilarity between nemabiomes, even more so when roe deer at low risk of contact with sheep pastures were compared with roe deer with high risk of contact (Fig. 5). On the other hand, there was no significant difference between roe deer nemabiomes according to their proximity to pasture without the interaction with time (e.g. the most



**Fig. 2.** Fecal egg counts, numbers of L3s used for DNA extraction and relative frequencies of gastrointestinal nematode amplicon sequence variants at taxa levels in samples of roe deer and sheep faeces collected in 2018 and 2019. Each taxon is defined by one color (pattern). The data are split based on host species and month for roe deer and farm (F1–F8) for sheep. There were four roe deer fecal samples for which fecal egg counts were not available (\*).



**Fig. 3.** Mean relative frequencies of gastrointestinal nematode amplicon sequence variants at taxa levels in roe deer and sheep. Each taxon is defined by one color (pattern). The mean nemabiome of roe deer is based on 77 samples and the sheep nemabiome is based on 21 pools of sheep feces (total of 166 fecal samples).

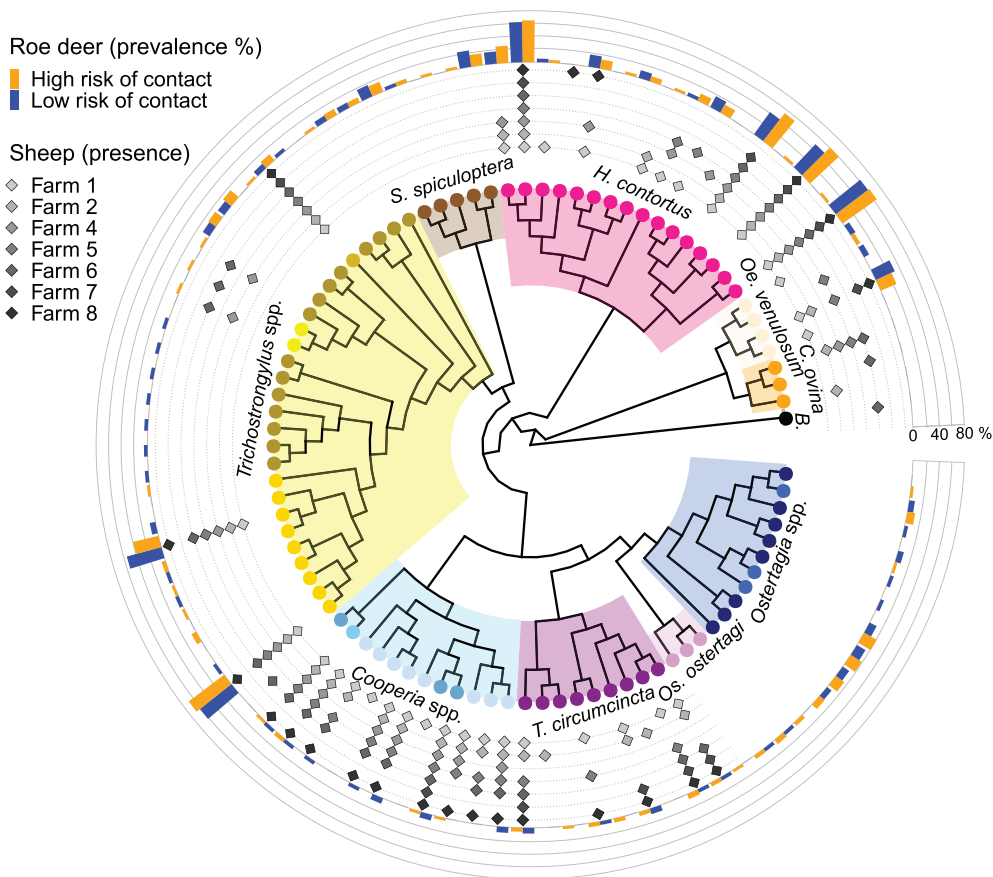
abundant ASV assigned to *H. contortus* has a prevalence of 62% and 65% and the most abundant ASV assigned to *S. spiculoptera* has a prevalence of 24% and 17% in roe deer with low and high risk of contact with sheep pasture, respectively; Table 4, Fig. 4).

We noted that sheep-specific parasites *T. colubriformis*, *T. vitrinus*, *C. curticae* and *Teladorsagia circumcincta* infected seven, one, four and five roe deer, respectively. There were too few samples to test the occurrence of these sheep-specific parasites in roe deer. Nevertheless, eight roe deer infected by those parasites had sheep pasture in their potential home range.

#### 4. Discussion

Using ITS2 rDNA nemabiome metabarcoding, we aimed to precisely assess the gastrointestinal nematode communities infecting both sheep and roe deer living in sympatry in southern France and estimated the influence of living in sympatry on their parasite communities. In both species, we identified 16 taxa corresponding to 10 nematode genera. Two species in particular, i.e., *H. contortus* and *T. axei*, prevailed in the study area; each of them was detected in more than 63% of roe deer and 85% of sheep. Overall, we found a high level of similarity between the nemabiomes of sheep and roe deer living in this agriculture area, regardless of the levels of contact between roe deer and sheep pastures. In a second step, we analysed the *H. contortus* nad4 mtDNA gene to assess the genetic relatedness of *H. contortus* populations within sheep farms and roe deer. The low nad4 mtDNA genetic differentiation among *H. contortus* from sheep and roe deer suggested long-term and regular cross-transmission of strongyles among sheep and roe deer.

Domestic sheep from Aurignac are infected by gastrointestinal nematode species commonly found in other sheep herds in Europe, identified using either morphological or genetic criteria (Zaffaroni et al., 2000; Taylor et al., 2015; Redman et al., 2019; Halvarsson and H  glund, 2021). We also observed a high similarity in the sheep nemabiomes among farms, which was not surprising given their close geographical proximity. The highly pathogenic parasite *H. contortus* (Roeber et al., 2013) was detected in all sheep farms, with a low to medium relative abundance (from 14% to 52%). It is also worth noting that the period of sampling of sheep farms (end of October to early November) corresponds to a great climatic suitability for *H. contortus* development in south-western France (Rose et al., 2016). This level of infection (up to 52% in our study) and the high level in Halvarsson and H  glund (2021) (up to 60%) raise concerns about the control of *H. contortus* in sheep farms in Europe, and it confirms the high adaptation of *H. contortus* to different climatic conditions (Queiroz et al., 2020). Furthermore, climatic models predict changes in the seasonal dynamic and spatio-temporal distribution of *H. contortus* within the next century (Rose et al., 2016). The less pathogenic, but still of concern, *T. colubriformis*, *Cooperia* spp., *T. axei* and *T. circumcincta* (Roeber et al., 2013) were also found with significant mean relative abun-



**Fig. 4.** Maximum likelihood tree of ITS2 rDNA amplicon sequence variants in samples of sheep and roe deer. Amplicon sequence variants present in sheep farms are specified by diamonds. Prevalences (%) of amplicon sequence variants in roe deer samples are given for roe deer with and without risk of contact with sheep pasture. Each taxon is defined by one color. Taxa of the same genus/species are grouped within the same highlight color. B., *Bunostomum* sp.; C., *Chabertia*; Oe., *Oesophagostomum*; H., *Haemonchus*; S., *Spiculoptera*; T., *Teladorsagia*; Os., *Ostertagia*.

**Table 1**  
Genetic population diversity index of the partial nad4 gene for all sheep farms and roe deer with low/high risk of contact.

Host population	n	H	Hd	k	π
Sheep	<b>6,042</b>	<b>241</b>	<b>0.979</b>	<b>5.865 ± 2.803</b>	<b>0.019 ± 0.010</b>
Farm 1	1,531	42	0.937	5.572 ± 2.678	0.018 ± 0.010
Farm 2	1,253	90	0.969	5.748 ± 2.754	0.019 ± 0.010
Farm 4	1,008	22	0.934	4.615 ± 2.267	0.015 ± 0.008
Farm 5	396	32	0.943	5.696 ± 2.735	0.019 ± 0.010
Farm 6	391	46	0.966	6.593 ± 3.121	0.021 ± 0.011
Farm 7	1,053	69	0.945	6.125 ± 2.916	0.020 ± 0.011
Farm 8	410	17	0.882	5.918 ± 2.831	0.019 ± 0.010
Roe deer	<b>17,755</b>	<b>367</b>	<b>0.968</b>	<b>6.423 ± 3.042</b>	<b>0.021 ± 0.011</b>
High risk of contact	11,405	275	0.972	6.419 ± 3.040	0.021 ± 0.011
Low risk of contact	6,350	159	0.956	6.366 ± 3.018	0.021 ± 0.011

n, estimated number of L3s; H, number of haplotypes; Hd, haplotype diversity; k, mean number of pairwise differences; π, nucleotide diversity (average over loci).

dances (11%, 27%, 14%, 11%, respectively, over the whole study area) and were present in almost all sheep farms (six out of seven farms). In comparison with other sheep farms in Europe (Burgess et al., 2012; Evans et al., 2021; Halvarsson and H oglund, 2021), the prevalence and relative abundance of *Cooperia* spp., *T. axei* and *T. colubriformis* were high, while *T. circumcincta* and *T. vitrinus* were found in lower abundance. This observation may result from the climatic conditions which can be particularly warm and dry in south-western France and hence, less favourable to *T. circumcincta* and *T. vitrinus* compared with *T. axei*, *T. colubriformis* and *H. contortus* (O’Connor et al., 2006). It might also partially result from the strategies used by breeders to control helminth infection, e.g., peri-

ods of treatment, family, and duration of anthelmintic effectiveness.

In roe deer we detected specialist parasites of deer, *Ostertagia leptospicularis* and *Spiculoptera spiculoptera* but in much lower relative abundance and prevalence than the generalist parasites *H. contortus* and *T. axei*, also detected in domestic sheep. Our results contrast with the results obtained for other populations of roe deer (Zaffaroni et al., 2000; Pato et al., 2013; Beaumelle et al., 2021), where specialist parasites of deer outnumbered the generalist parasites. In particular, the study of two isolated populations of roe deer in France (Beaumelle et al., 2021), following the same method to analyse samples, showed very different nemabiomes. In fact, *H. contortus* was not detected in one of the populations

**Table 2**

Pairwise  $\Phi_{ST}$  (based on Kimura 2-Parameters distance) of the partial nad4 gene between *Haemonchus contortus* of sheep farms and roe deer with high/low risk of contact. All pairwise values are significantly different from 0 ( $P < 0.05$ ).

Sheep farm		Sheep farm							Roe deer
		F1	F2	F4	F5	F6	F7	F8	HR
Sheep farm	F1								
	F2	0.025							
	F4	0.050	0.039						
	F5	0.047	0.041	0.091					
	F6	0.035	0.024	0.046	0.054				
	F7	0.048	0.040	0.044	0.079	0.053			
	F8	0.057	0.054	0.089	0.095	0.070	0.029		
	Roe deer	HR	0.014	0.014	0.031	0.046	0.028	0.016	0.030
	LR	0.016	0.023	0.040	0.047	0.031	0.023	0.036	0.008

F1–F8, Sheep farms 1–8; HR, roe deer with a high risk of contact with sheep pasture; LR, roe deer with a low risk of contact with sheep pasture.

**Table 3**

Analysis of molecular variance results of percentage of variation and fixation indices. Groups are sheep and roe deer. Populations are the seven sheep farms and roe deer with low/high risk of contact. Analyses were carried out using conventional  $F$ -statistics (estimating  $F_{ST}$ ,  $F_{SC}$  and  $F_{CT}$ ). Subscripts S, C and T stand for samples, groups and total, respectively.

Source of variation	Percentage of variation	Fixation indices
Among groups	–0.30	$F_{CT} = -0.003$
Among population within groups	2.09	$F_{SC} = 0.021$
Within population	98.20	$F_{ST} = 0.018$

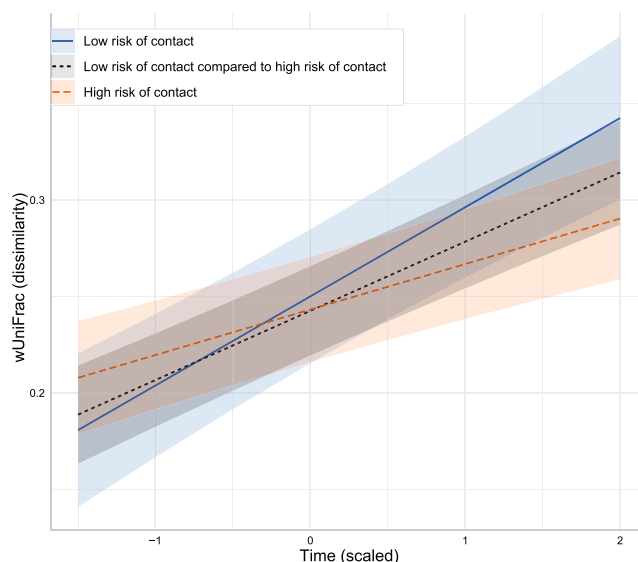
**Table 4**

Coefficient of the best generalised mixed model selected for weighted UniFrac distance for all roe deer samples pairs. For the variable relative to the contact of roe deer with sheep pasture, the level of reference is roe deer with low risk of contact with sheep pasture.  $P$ -values are based on conditional  $F$ -tests with Kenward-Roger approximation for the degrees of freedom.

Predictors	Estimates	95% CI	$P$
Intercept	0.25	0.21/0.29	<0.001
Roe deer with high and low risk of contact	–0.01	–0.03/0.02	0.533
Roe deer with high risk of contact	–0.01	–0.05/0.04	0.749
Time	0.05	0.03/0.06	<0.001
Roe deer with high and low risk of contact $\times$ Time	–0.01	–0.02/0.00	0.136
Roe deer with high risk of contact $\times$ Time	–0.02	–0.04/–0.01	<0.001

CI, confidence interval.

and at low abundance and prevalence in the other population; and the prevalence of *T. axei* was <13% in both populations. In the roe deer population we studied here, the level of *H. contortus* infection appeared to be excessively high but similar to a previous study on roe deer living in sympatry with domestic sheep in Hungary (Csivincsik et al., 2017). To our knowledge, there is no study about the pathogenicity of *H. contortus* in roe deer, but it was observed to provoke pathological response i.e., depression of feed intake and elevation of pepsinogen concentration, a marker of abomasum wound in red deer *Cervus elaphus* (Johnston et al., 1984), and to be responsible of extensive mortality in white-tailed deer *Odocoileus virginianus* in Florida (USA) (Eve and Kellogg 1977). Regarding the pathogenesis of *H. contortus* in livestock, the high abundance values of *H. contortus* observed may raise concerns for roe deer, and further investigation should be performed to properly evaluate its effect on the health of roe deer. In addition to the nematodes usually found in roe deer, we found specialist nematodes of domestic sheep in roe deer (*T. circumcincta*, *T. colubriformis* and *C. curticei*). In a few samples, we also observed cattle specialist nematodes in roe deer, e.g. *Ostertagia ostertagi*, that suggest possi-



**Fig. 5.** Dissimilarity (weighted UniFrac distance) between roe deer nemabiomes depending on time and proximity to sheep pasture. Confidence intervals (95%) are displayed around each regression curve.

ble transmission of a cattle parasite to roe deer in the study area (Verheyden et al., 2020). More surprisingly, we noticed the presence of *C. fuelleborni* in both sheep and roe deer. This observation is intriguing because this parasite has a distribution limited to Africa, e.g., in African buffalo *Syncerus caffer* and in Topi *Damaliscus korrigum* (Albrechtova et al., 2020 and references therein). We cannot, however, exclude an error of assignment during the bioinformatic treatment of data, due to the low level of genetic differentiation between *C. curticei* and *C. fuelleborni* at the ITS2 gene. A visual investigation of adult nematodes collected from slaughtered sheep in farms where this parasite has been identified could conclusively determine the presence of *C. fuelleborni* or related species among sheep and roe deer in south-western France.

At the population level, we found roe deer nemabiomes to be more diverse than sheep nemabiomes, i.e., there were twice as many ASVs in the roe deer population than in sheep. In contrast, individual samples of roe deer exhibited a lower individual richness than pooled samples of sheep, i.e., there were twice as many ASVs in individual sheep than in individual roe deer. The transmission of gastrointestinal nematodes from sheep to roe deer may have contributed to this higher diversity in roe deer populations, while anthelmintic treatments might have reduced the species diversity in domestic sheep (Halvarsson and Hoglund, 2021). Roe deer populations are not constrained by fences in contrast to

sheep, resulting in parasite flow among overlapping and neighboring roe deer and during natal dispersion (Debeffe et al., 2012). In particular, in this study area, there are one-third of juvenile roe deer which undertake natal dispersion with a mean dispersal distance of 4.7 km (Debeffe et al., 2012). In addition, because nematodes have a free-living phase and can live in the environment for several weeks before being ingested, direct host contact is not necessarily required for pathogen transmission among hosts. Thus, although roe deer may be territorial (Maublanc et al., 2012), transmission of gastrointestinal nematodes among roe deer, even with a low level of home range overlap, certainly occurs. Large scale movements can explain the higher gastrointestinal nematode diversity at the population level (Teitelbaum et al., 2018). The lower individual richness in roe deer samples could be explained by the difference in sampling methods (individual versus pool) between roe deer and sheep. In addition, we observed a high variability among roe deer nemabiomes that can be explained by individual factors such as age, sex, and sociality of individuals from which samples were collected (Ezenwa and Worsley-Tonks, 2018; Beaumelle et al., 2021). Such information was, however, not available for most individuals and has not been evaluated here. The lack of spatial structure for parasites of roe deer (either with nad4 gene of *H. contortus* populations (see below) or with the nemabiome of roe deer according to their distance to sheep pastures) confirmed the high circulation of gastrointestinal nematodes among roe deer, and between roe deer and sheep. It is noteworthy that the most common ASVs of generalist nematodes were shared between sheep and roe deer. We hypothesise that, over many decades of sheep farming in the region, the typical roe deer parasite population has been slowly displaced by generalist livestock parasites, not only for deer leaving close to pastures, but at a much larger scale. Our study detected a slight spatial structure of nemabiomes among the roe deer populations, and this spatial structure was influenced by the proximity to sheep pasture, in interaction with the season. We did not find more similarity between nemabiomes relative to the proximity of roe deer to sheep pastures. It seems that the nemabiomes of roe deer living in the proximity of sheep pasture are more stable over time compared with the nemabiomes of roe deer living in remote areas. This result suggests a bottleneck effect near sheep pastures, probably due to the strong selection pressure associated with long-term repeated use of anthelmintics in livestock, and to the transmission of sheep parasites to roe deer.

There was a low level of genetic differentiation among the nad4 gene of *H. contortus* populations from different farms but no difference between host species. Low genetic structure among nematodes populations of different host species living in sympatry were also observed, e.g. in co-grazing system in the Alps (Cerutti et al., 2010) and in the Palouse prairie in Montana, USA (Archie and Ezenwa, 2011). However, this is the first time, to our knowledge, that such high gene flow between wild and domestic ungulates in an area where domestic ungulates are in fenced enclosures has been reported. As we observed a population structure of *H. contortus* among sheep farms, it indicates that the avoidance of contact between hosts can lead to a genetic divergence between *H. contortus* populations. However, we observed a lower population structure of *H. contortus* among sheep farms than what was observed between *H. contortus* populations in goat farms in south-western France (Silvestre et al., 2009). The latter results were, however, observed in goat farms where the very low movement of individuals among populations could explain the higher genetic differentiation among parasite populations. To our knowledge, sheep from farms in the present study never grazed the same pasture, we can therefore hypothesise that either wildlife participates in the flow of *H. contortus* among sheep herds or that farmers may exchange sheep among herds.

With this study, we demonstrated the influence of livestock agriculture on the nemabiome communities of roe deer. We hypothesised that livestock farming has an impact on the surrounding ecosystem by modifying parasitic communities of wildlife. In this agricultural area, the nemabiome more typical of roe deer appears to have been largely replaced by generalist parasites found in livestock. In addition, the lack of spatial structure for the nemabiome and nad4 gene also suggest the strong influence of livestock farming on the ecosystem, not only in the vicinity of pastures, but also at a wider scale. Following Csivincsik et al. (2017), our results suggest that livestock activities have more effect on roe deer nemabiomes than the opposite (V  zquez et al., 2009; Verheyden et al., 2020). Gastrointestinal nematode resistance to anthelmintics is a major threat for livestock, and it can reach high levels of resistance, especially in sheep farms (Papadopoulos et al., 2012; Ram  nke et al., 2016; Cazajous et al., 2018; Charlier et al., 2022). In our study area, long-term use of anthelmintics may have selected lineages of gastrointestinal nematode species resistant to anthelmintics. Notably, the most abundant parasite species of sheep (*H. contortus*, *T. axei*, *T. colubriformis*, *T. circumcincta* and *C. curticei*) are all known to develop resistance to anthelmintic treatment (Avramenko et al., 2019). These species were highly abundant in our roe deer and anthelmintic-resistant nematodes have been observed in domestic sheep with high prevalence (Papadopoulos et al., 2012) and in wildlife, including roe deer (in United Kingdom; Chintoan-Uta et al., 2014; in Hungary; Csivincsik et al., 2017). It remains to be determined if wild ungulates can be an additional factor in the maintenance of anthelmintic resistance or if they participate in the dilution of resistant nematode species for livestock (Martin et al., 2011; Brito et al., 2013; Forteau et al., 2020; Brown et al., 2022).

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpara.2022.07.005>.

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