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# Impact of supplemental winter feeding on ruminal microbiota of roe deer *Capreolus capreolus*

Sara Ricci, Robin Sandfort, Beate Pinior, Evelyne Mann, Stefanie U. Wetzels and Gabrielle Stalder

S. Ricci and G. Stalder ✉ ([gabrielle.stalder@vetmeduni.ac.at](mailto:gabrielle.stalder@vetmeduni.ac.at)), Res. Inst. of Wildlife Ecology, Dept of Interdisciplinary Life Sciences, Univ. of Veterinary Medicine, Austria, Savoyenstraße 1, AU-1160 Vienna, Austria. SR also at: Univ. of Camerino, Camerino (MC), Italy – R. Sandfort, Inst. of Wildlife Biology and Game Management, Univ. of Natural Resources and Life Sciences Vienna, Vienna, Austria. – B. Pinior, Inst. for Veterinary Public Health, Dept for Farm Animals and Veterinary Public Health, Univ. of Veterinary Medicine, Vienna, Austria. – E. Mann and S. U. Wetzels, Inst. of Milk Hygiene, Milk Technology and Food Science, Dept for Farm Animal and Public Health in Veterinary Medicine, Univ. of Veterinary Medicine, Vienna, Austria.

European ungulates such as the roe deer face seasonally varying climatic conditions as well as food availability and quality. In some European countries, including Austria, it is common practice to provide game animals with supplemental feeding in winter. In this study we investigated if supplemental feeding significantly affects the composition of the bacterial rumen community. The rumen microbial composition of eight adult female roe deer was analysed by Illumina MiSeq 16S rRNA gene amplicon sequencing. Animals from a study area with supplemental feeding sites were compared to individuals relying on natural feed. Furthermore, the microbial community composition of different ruminal compartments (liquid phase, solid phase and wall) was compared. Our results revealed a significant qualitative difference between the microbiota composition of the two populations studied. Easily fermentable supplemental feeding promoted the proliferation of phylotypes correlated with conditions of acidosis in domestic ruminants, suggesting a possible similar adaptation and a hypothetical negative effect on health status also in roe deer. The results furthermore confirmed that in roe deer, like in other ruminant species, the most represented phyla are *Firmicutes* (63.2%) and *Bacteroidetes* (23.5%), and that the ruminal microenvironments influence the microbial community composition, with the lowest species richness and variation in the epimural microbiota.

Keywords: 16S rRNA gene sequencing, *Capreolus capreolus*, roe deer, rumen bacterial microbiota, supplemental feeding

Roe deer *Capreolus capreolus* populate wide areas of Europe, across a wide range of environmental conditions and habitat types. In contrast to other wild herbivores such as the common red deer *Cervus elaphus* they are considered concentrate selectors, equipped with a digestive tract, that is adapted to process highly digestible forages (Hofmann 1989). Their gastrointestinal tract allows for fast ruminal passage of the ingesta, rapid rate of fermentation and therefore less rumination as well as further fermentation in the hindgut (Drożdż 1979, Hofmann 1989). As highly selective feeders, they are especially sensitive to dietary changes (Toigo et al. 2006, Parker et al. 2009). Nevertheless, during the winter months roe deer, like other wild ruminants, have to adapt their nutritional and physiological strategies to the seasonal variation of lower food quality and availability (Gębczyńska 1980, Tixier et al. 1997, Arnold et al. 2015, Krasnov et al. 2015).

The practice of winter feeding is widely used in many parts of Europe and North America, in order to prevent population decline during cold winters, usually for hunting reasons (Milner et al. 2014). Furthermore, feeding sites are used to help increase reproductive rates, to prevent damages to forests and plantations, to move animals from an area to another, and to help the animals maintain a high body mass (Peek et al. 2002, Gundersen et al. 2004, Toigo et al. 2006, Parker et al. 2009). The easily accessible and high energy feed influences spatial distribution and behaviour of wild ungulates (Ossi et al. 2017) and also poses a number of potentially negative effects, such as higher risk of disease transmission, higher browsing pressure around feeding sites, health effects such as increased disease transmission and manipulation of natural selection (Mysterud 2010, Mathisen et al. 2014, Sorensen et al. 2014).

However, knowledge of the impact of supplemental winter feeding on the physiology, respectively gastrointestinal microorganisms of their wild ruminant host, is currently lacking. Host diet is known to be an important factor shaping the microbial ruminal population and consequently impact

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their physiology and health (Fernando et al. 2010, Taschuk and Griebel 2012, Henderson et al. 2015, Delgado et al. 2017, Petri et al. 2018). The study of the microbiota of the gastrointestinal tract, and especially of the ruminal microbial composition, is therefore essential in order to understand the physiological requirements of wild ruminants and the potential health impact of their diet (Hobson and Stewart 1997, Mackie et al. 2000, Kim et al. 2011, Li et al. 2014, Henderson et al. 2015, Hird 2017).

The aim of this study was to describe the ruminal bacterial composition of free-ranging roe deer and to investigate if supplemental winter feeding affects their rumen microbiota composition. Therefore, we investigated the ruminal microbiota composition of two different roe deer populations in winter, of which one had access to feeding sites and one relied on natural feed.

## Material and methods

### Study area and sample collection

The study was approved and conducted in accordance with current laws of Austria, the regulations of the institutional Ethics Committee and the institutional Good Scientific Practice Guidelines of the University of Veterinary Medicine, Vienna.

The study area (2600 ha) is located in the mountains of Styria, Austria, between 800 and 1300 m a.s.l. The area is mostly covered by managed forest with only 2% open areas. As part of a larger research project, winter feeding was stopped in 2012/2013 in the study area and ad libitum supplementary feeding was provided from mid-September until April in the control sites. Home range and feeding site use of roe deer was monitored via GPS-collars applied on the animals.

Feed contained pellets consisting of non-fibrous carbohydrates, proteins and a smaller amount of less digestible fibers such as cellulose and lignin (Table 1), and hay.

Table 1. Composition of the feed provided to the animals, analysed by Futtermittellabor der Landwirtschaftskammer Niederösterreich (Wieselburg). Ingredients quantity is expressed in grams per kilo and energy supply is expressed in megajoules.

| Nutrients                 | Ingredients per kg of fresh feed (g kg <sup>-1</sup> ) | Ingredients per kg of dry matter (g kg <sup>-1</sup> ) |
|---------------------------|--|--|
| Dry matter                | 905  | 1000   |
| Total proteins            | 130  | 144  |
| Total fat                 | 39   | 43   |
| Total fiber               | 81   | 90   |
| Undigested fiber          | 185  | 204  |
| Cellulose and lignin      | 86   | 95   |
| Lignin                    | 18   | 20   |
| Non fibrous carbohydrates | 502  | 555  |
| N-free extracts           | 606  | 670  |
| Starch                    | 429  | 474  |
| Ashes                     | 49   | 54   |
| <b>Energy supply</b>      | <b>MJ</b>  | <b>MJ</b>  |
| Convertible energy        | 11.66  | 12.89  |
| Net energy                | 7.33   | 8.10   |

Eight healthy (n = 4 in study area and n = 4 in control site) free-ranging adult females were shot during the hunting season according to the respective hunting law, in early winter (between 25 October and 15 November 2017). The whole gastrointestinal tract was immediately removed after death. Therefore, the rumen was ligated between the distal part of the oesophagus and the cardia region. The distal part of the rectum was severed and the whole gastrointestinal tract was carefully placed into a clean plastic bag. Each sample was labelled with the date, the location and the age of the animal and was subsequently frozen at -20°C and transported to the laboratory.

### Sample preparation and DNA extraction

To collect the aliquots for DNA extraction, two samples were taken from rumen wall, rumen fluid and solid digesta of each animal through a 1) incision 6 cm caudal from the transverse groove on the ventral sac of the parietal surface and 2) incision 3 cm cranial to the transverse groove, on the ventral sac in order to provide adequate distance to the rumen orifice and connecting GI-tract. Liquid and solid phase were collected in sterile tubes through the incisions; two 1 × 1 cm pieces of rumen wall were cut and put in a sterile tube. The tube was filled with 1× phosphate-buffered saline (PBS) and inverted 10 times, to rinse the tissue. Each section was then placed in a 2 ml Eppendorf tube with a clean pair of tweezers. A new scalpel and clean tweezers were used for each animal. Rumen wall samples were prepared for extraction replicating the procedure described by Wetzels et al. (2016). Clean and disinfected instruments were used for each sample.

Sample preparation for DNA extraction from the solid phase of the rumen content was modified from the protocol described by Kong et al. (2010). A total of 250 mg of solid phase was put in Lysing Matrix A tubes (<www.mpbio.com/product.php?pid=116910050&country=223>) and 750 µl of 0.4 M potassium phosphate buffer were added. The tubes were then put in the bead beater for 30 s and the bead beating was repeated three times, to completely homogenize the material. The samples were kept on ice and were then centrifuged for 10 min at 10 000 rpm. The supernatant was discarded and 500 µl of Pre-lysis buffer (20 mM Tris/Cl, 2 mM EDTA, 1% Triton-X 100) were added. The samples were then stirred using a pipette and vortexed until the pellet was resuspended before heating it at 95°C for 5 min on thermoblock (Eppendorf Thermomixer F 1.5, Vienna, Austria), slightly shaking (900 min<sup>-1</sup>). The tubes were centrifuged again at 14 000 rpm for 5 min. The supernatant (circa 600 µl) was separated and stored on ice in a 2 ml tube for later use. A total of 1200 µl of 0.4 M potassium phosphate was added to the tube to resuspend the pellet and then two enzymes were added: 100 µl of 100 mg ml<sup>-1</sup> lysozyme and 10 µl of 2.5 U µl<sup>-1</sup> mutanolysin (M9901-5KU Mutanolysin from Streptomyces). The samples were vortexed until the pellet was resuspended completely and incubated at 37°C for 30 min, shaking at 300 min<sup>-1</sup>. After the incubation, 20 µl of 20 mg ml<sup>-1</sup> Proteinase K (Proteinase K recombinant PCR grade) were added and the samples were incubated again for 1 h at 56°C, shaking at 300 min<sup>-1</sup>.

Finally, the samples were placed in the bead beater again for three cycles of 45 s, and then centrifuged at 14 000 rpm

for 3 min. The obtained supernatant (circa 1300 µl) was pooled with the other supernatant kept on ice following the steps described above. A total of 250 µl of the supernatant collected was used in the extraction kit.

Liquid phase of the rumen content samples was not pre-processed before extraction and 250 µl of liquid were used in the kit after vortexing briefly.

PowerSoil DNA Isolation Kit was used for DNA extraction for all samples, following the manufacturer's instructions with some modifications: horizontal vortexing was prolonged to 20 min for rumen papillae, to increase mechanical lysis, and shortened to 1 min for the products of the solid phase pre-processing; pre-heated (70°C) diethylpyrocarbonate-treated water (50 µl) was used for elution of DNA, instead of solution C6, for all samples. The samples were finally centrifuged at 10 000 rpm for 1 min and the DNA extracted was stored at -20°C until further processing. Each sample was extracted in duplicates (A and B), pooled after DNA extraction and a negative extraction control was included for each sample type and processed in the same way as the rumen samples. Qubit 2.0 Fluorimeter and Real-Time PCR software ver. 4.10 were used to determine the quantity of DNA obtained from the extractions. The Real-Time PCR was performed using EvaGreen dye, following the protocol developed by Müller et al. (2013).

### DNA sequencing and sequence analysis

DNA extracted from each sample (A and B) was pooled in a single tube. A total of 24 samples (eight rumen wall samples, eight liquid phase samples, eight solid phase samples) and three negative controls were sent to Microsynth NGS laboratories (Microsynth AG, Balgach Switzerland) to be sequenced using Illumina MiSeq sequencing platform. V3-V4 hypervariable regions of bacterial 16S rRNA genes were amplified (primers 341F\_ill: CCTACGGGNGGCWGCAG and 802R\_ill: GACTACHVGGGTATCTAATCC, Klindworth et al. 2012) to generate a product of 460 bp. Library was prepared adding barcodes and Illumina adaptors to the PCR products and the Nextera XT Sample Preparation Kit (Illumina) was used according to the manufacturer's recommendations. Equimolar pools of samples were sequenced using a 300 bp paired-end reads protocol for Illumina MiSeq sequencing platform. PCR, library preparation, sequencing and sequence trimming and stitching of corresponding overlapping paired-end were performed by Microsynth.

The sequences obtained were processed and analysed using the software package QIIME ver. 1.9.1 (Caporaso et al. 2010). A total of 2 733 545 reads were checked for chimeric sequences by comparing against the reference Gold database (<<http://drive5.com/uchime/gold.fa>>), resulting in a total of 2 711 116 reads passing the quality control. Microbial taxonomy was assigned by clustering reads with UCLUST ver. 1.2.22q using Greengenes ver. 13.8 as reference database (based on a 97% similarity threshold), for a total of 4546 OTUs detected (OTUs with less than 10 sequences for OTU were excluded). Sequences were furthermore checked for the presence of contaminants using 'Decontam' package in R (Davis et al. 2018; <<https://github.com/benjjneb/decontam>>) and filtered afterwards, using a threshold of  $p \leq 0.1$ . Eventually, chloroplasts and mitochondria OTUs

were excluded from the database, resulting in 4520 remaining OTUs.

The results discussed in this paper are presented according to GreenGenes ver. 13.8 database taxonomy. The most representative sequences for the 50 most abundant OTUs for each feeding group were additionally compared for microbial taxonomy assignment manually, using NCBI BLASTn ver. 2.8.0 against 16S ribosomal RNA sequences (Bacteria and Archaea) and RDP 11.5 classifier databases. The best hit was selected excluding the uncultured strains and on the basis of the highest identity percentage for BLAST, of the highest S\_ab scores (percentage of shared seven-mers) for RDP and, in case of equality between more strains, on the basis of the habitat information, if available.

### Statistical analysis

The hypothesis tested were if feeding groups (fed, unfed) and sample type (rumen wall, liquid phase, solid phase) have an effect on rumen microbiota.

Alpha diversity indices (Shannon, Simpson, Chao1, observed species, abundance-based coverage, Simpson's evenness) were calculated with the function `alpha_diversity` of QIIME after samples were normalized to the lowest read count per sample (48 659). The Venn diagram showing the shared OTUs between the three rumen compartments was created using BioVenn web application (Hulsen et al. 2008). Through the function `beta_diversity_through_plots` within QIIME, weighted and unweighted UniFrac analyses were performed. Results obtained were then used to perform principal coordinates analysis and shown in PCoA plots. Beta diversity indices were compared between groups (fed, unfed) and among sample types (rumen wall, liquid phase, solid phase) using ANOSIM method (r test, 999 permutations).

The effects of three independent variables (feeding group (i.e. fed versus unfed) and sample type (i.e. rumen wall, liquid phase, solid phase)) on the abundance of diversity indices (n = 6), phyla (n = 20), families (n = 96), genera (n = 119) and OTUs (n = 4520) was analysed as follows. The normality distributions of the microbiome data and the corresponding residuals were analysed with 1) the tests for multivariate data with the function MANOVA, 2) corresponding histograms, 3) residual plots and 4) the Shapiro-Wilk tests as well as the Anderson-Darling test for multivariate normality.

We used a one way (univariate) ANOVA with a Tukey post hoc test to investigate the effect of the independent variables on the alpha diversity. Depending on the distribution of the residuals different transformations were used (i.e. log transformation, square root transformation). To analyze the hypothesis of the effect of the feeding groups and of sample type on rumen microbiota regarding alpha diversity the individuals were additionally considered as random factors in the linear-mixed model. In nine cases, a non-normal distribution was identified and thus, we applied a Kruskal-Wallis  $\chi^2$  test with a multiple pairwise comparison based on Dunn's test and for two sample groups, we have used the non-parametric Wilcox test.

The beta diversity of phyla, families, genera and OTUs were not normal distributed and thus we used a permutational multivariate analysis of variance (PERMANOVA,

formerly ‘nonparametric MANOVA’) with the ADONIS function and permutations from 999 to 5000. To analyse whether independent variables had a significant effect on the composition of phyla, families, genera and OTUs, we calculated the Bray–Curtis dissimilarity matrix with the R function `vegdist` of the `vegan` package. The distance matrix was further applied as response variable. We applied a pairwise ADONIS function to identify pairwise differences between the independent variables. The multivariate homogeneity of group dispersions was performed with the `betadisper` function, followed by a permutation-based test of multivariate homogeneity of group dispersions with a pairwise comparison of group mean dispersions. Subsequently, the pairwise comparison with the ADONIS function, the Kruskal–Wallis test and pairwise test for multiple comparison of mean rank sum (Dunn’s test), adjusted with Benjamini–Hochberg was used to identify statistically significant differences between (each individual) phylum, family, genus and OTU abundance and independent variables regarding fed versus unfed and sample type (rumen wall, liquid phase, solid phase). Data are considered significant at  $p \leq 0.05$ . The statistical analysis was implemented using the R statistical computing environment ([www.r-project.org](http://www.r-project.org)) using the package `lme4`, `dplyr` (Wickham et al. 2019), `vegan` and `pairwiseAdonis` (Oksanen et al. 2018, Martinez Arbizu 2019).

### Accession numbers

Sequencing data are available in BioProject SRA database under the accession number PRJEB29211.

## Results

### Sequence analysis of the microbiota of the rumen in roe deer

The results presented in this section include the analysis of the samples of both feeding groups and all the three rumen compartments.

In total 4520 OTUs were detected, 11 of which classified as Archaea, belonging to phylum *Euryarchaeota*, and representing 0.2% of the dataset. A high percentage of amplicons could not be assigned to any taxonomic classification, representing 5% of the whole dataset (233 OTUs in total). The rest of the OTUs were classified as Bacteria.

Bacterial and Archaeal reads were processed together and assigned to 20 different phyla, of which the most abundant were respectively *Firmicutes* (relative abundance: 63.2%), *Bacteroidetes* (relative abundance: 23.5%), *TM7* (relative abundance: 4.8%) and *Actinobacteria* (relative abundance: 4.0%) (Fig. 1A). A total of 96 families and 119 genera were identified. The four most abundant families were *Lachnospiraceae*, *Prevotellaceae*, *Ruminococcaceae* and *Mogibacteriaceae* and the most abundant genera detected were *Prevotella*, *Butyrivibrio*, *Anaerostipes*, *Coprococcus*, *Ruminococcus* and *Blautia* (Fig. 1B). The overall most abundant OTU (OTU1) was classified as *Prevotella* sp., followed by OTU2 unclassified at the genus level, assigned to family *Mogibacteriaceae*. OTU3 was classified as *Anaerostipes* sp., and was followed by OTU4 and OTU5, both not assigned

at genus level and belonging to family *Ruminococcaceae*. The 50 most abundant OTUs are shown in the Supplementary material Appendix 1 Table A1. The 50 most abundant OTUs for each feeding group were furthermore compared for taxonomy assignment with two other databases (NCBI and RDP), and the findings are shown in Supplementary material Appendix 1 Table A2, A3.

### Differences in microbial community composition between the two feeding groups

The two different feeding groups (called fed and unfed from now on) had different most abundant OTUs (Fig. 1). The most represented OTUs for the fed group were OTU3 (*Anaerostipes* sp.), OTU16 (*Prevotella* sp.), OTU8 (family *Ruminococcaceae*) and OTU21 (family *Coriobacteriaceae*).

For the unfed group, the most abundant OTUs were OTU1 (*Prevotella* sp.), OTU2 (family *Mogibacteriaceae*), OTU6 (family *Mogibacteriaceae*) and OTU4 (family *Ruminococcaceae*).

Analysis of similarities found significant differences between the fed and unfed group only in unweighted analysis ( $p = 0.01$ ), while in the weighted analysis the two feeding groups were not significantly different ( $p = 0.10$ ). The results of the analysis of similarities are shown in PCoA representing UniFrac analysis output (Fig. 2). In PCoA, showing weighted UniFrac analysis for the fed and unfed group, the samples are not clustered by diet, while in the graph showing unweighted UniFrac the samples of the unfed population appear less disperse than the fed samples (Fig. 2A–B). ANOSIM and ADONIS for the two populations was also calculated within the single sample type, but no significant differences emerged.

Nevertheless, regardless of the sample type, three phyla (*Firmicutes*, *Planctomycetes* and *SRI*) and the group of unassigned OTUs were found to be significantly different between the two populations. In addition, there was a trend for phylum *Bacteroidetes* ( $p = 0.09$ ), but not a significant difference.

In particular, phyla *Firmicutes*, *Planctomycetes* and *SRI* were significantly enriched in the unfed group, while unassigned OTUs and *Bacteroidetes* were enhanced in the fed group (fold changes respectively 0.3, 8.1, 2.4,  $-0.6$ ,  $-0.4$ ).

Families *Campylobacteraceae* and *Oxalobacteraceae* were significantly different between the two groups: the first was enriched in fed group (fold change =  $-0.5$ ), and the second in the unfed group (fold change = 1.1).

In total, eight genera were different between the two groups: *Veillonella*, *Anaerovibrio*, *Moryella* and *CF231* were enriched in the fed population (fold changes between  $-1.0$  and  $-0.9$ ), *Acinetobacter*, *PSB-M-3*, *Roseburia* and *Coprococcus* were more abundant in the unfed group (fold changes between 1.8 and 4.8).

A total of 348 OTUs showed a significant difference between the two populations. In particular, 18 of the 100 most abundant OTUs showed significant differences between the feeding groups. Of these, eight were enriched in the fed group and showed a fold change above 2.0: OTU31 (order *Clostridiales*, fold change = 13.5), OTU32 (genus *Coprococcus*, fold change = 8.3), OTU53 (family *Lachno-*

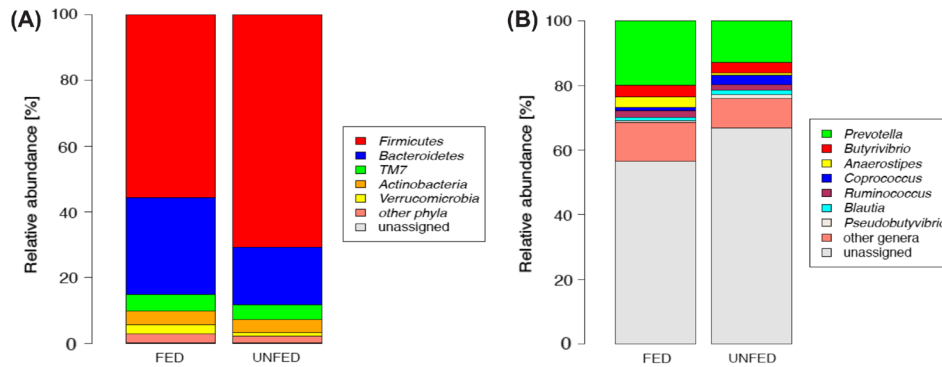


Figure 1. Taxonomic classification of 16S rRNA gene sequence reads parted by fed versus unfed population. Data represent average of OTUs relative abundances from replicate libraries for each category. (A) bacterial phyla. (B) bacterial genera in the rumen of roe deer. Phyla and genera with less than 1% relative abundance were grouped together (depicted as 'other'). Sequences that could not be assigned are depicted as 'unassigned'.

*spiraceae*, fold change=7.3), OTU57 (genus *Coprococcus*, fold change=43.5), OTU59 (family *Mogibacteriaceae*, fold change=12.6), OTU72 (family *Lachnospiraceae*, fold change=3.0), OTU78 (family *Lachnospiraceae*, fold change=3.18) and OTU91 (genus *Pseudobutyrvibrio*, fold change=61.3). Although showing lower fold changes values (between -0.95 and -1.00), OTU21 (genus *Prevotella*), OTU25 (Unassigned), OTU70 (genus *Prevotella*) and

OTU100 (family *Ruminococcaceae*) were enriched in the fed population.

### Differences in microbial community composition among sample types

In the UniFrac analysis performed on the three different rumen compartments, the unweighted UniFrac PCoA

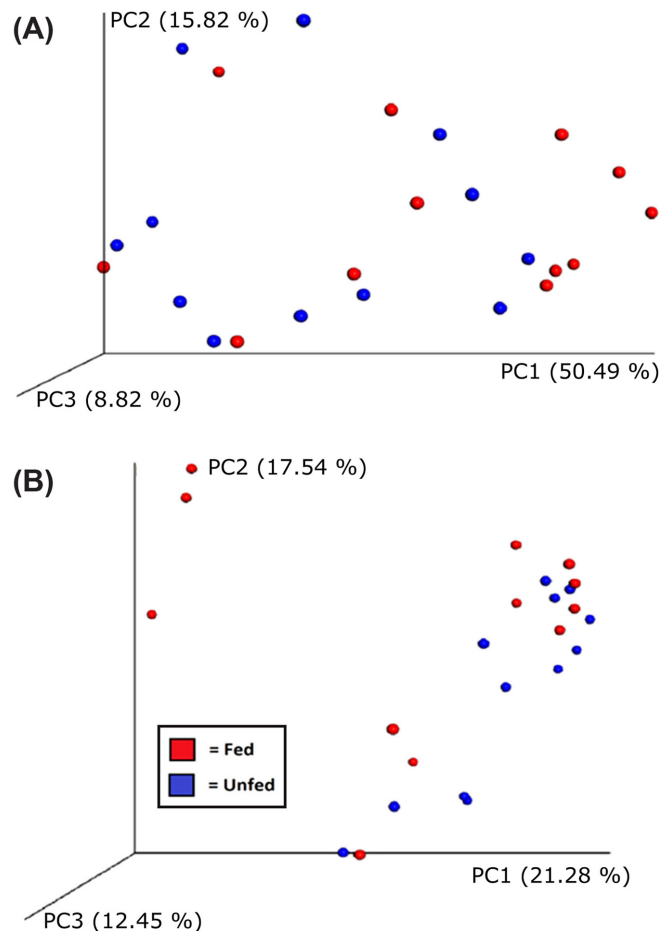


Figure 2. PCoA plot based on (A) weighted UniFrac distance matrices and (B) unweighted UniFrac distance matrices for the two feeding groups. UniFrac distances were calculated for all OTUs. Each point represents values from one sample with colors expressing population affiliation.

shows very visible clusters of the sample types, while the weighted UniFrac has more evenly distributed patterns, even though the three different rumen compartments emerge to be still clearly separated, especially the solid phase (Fig. 3). In fact, as confirmed by ANOSIM and ADONIS analysis, the three different rumen compartments showed a significant difference both in weighted and unweighted analysis ( $p=0.001$ ).

Sample type had a significant impact on phyla, genera and OTU relative abundances, but not on families, as shown in Table 2.

Among the three sample types, 12 phyla showed a significant shift. In particular, in rumen wall samples, phyla *Synergistetes* and *Verrucomicrobia* and unassigned OTUs were significantly enriched, while phylum *Cyanobacteria* was significantly lower.

Phyla *Bacteroidetes* and *Chloroflexi* were significantly enriched in the liquid and solid phase; phylum *Actinobacteria* was significantly less represented in the liquid phase and phyla *Spirochaetes* and *Fibrobacteres* were significantly reduced in the solid phase.

While only four families showed a significant difference between the rumen compartments, 48 genera and 40 of the 100 most abundant OTUs, showed a significant shift.

### Analysis of diversity and evenness (alpha diversity) for the two feeding groups

Species richness estimators (Chao1, observed species and abundance-based coverage (ACE)) mean values were higher in unfed group than in fed group, but these differences, although showing a trend, were not statistically significant.

Shannon, Simpson and Simpson index-based measure of evenness show very similar mean values between the two groups. The three indices were not significantly different between groups, neither when compared among the single individuals.

On the contrary, species richness estimators (Chao1, observed species and ACE) showed significant differences between some individuals when compared amongst each other, in particular 1–4, 1–5 and 1–6 (1,4 = fed 5,6 = unfed), with unfed individuals having the highest values, thus a higher species richness (Table 3).

### Analysis of diversity and evenness (alpha diversity) for the rumen compartments

When analysing the diversity indices associated with sample type, rumen wall showed significant differences both with

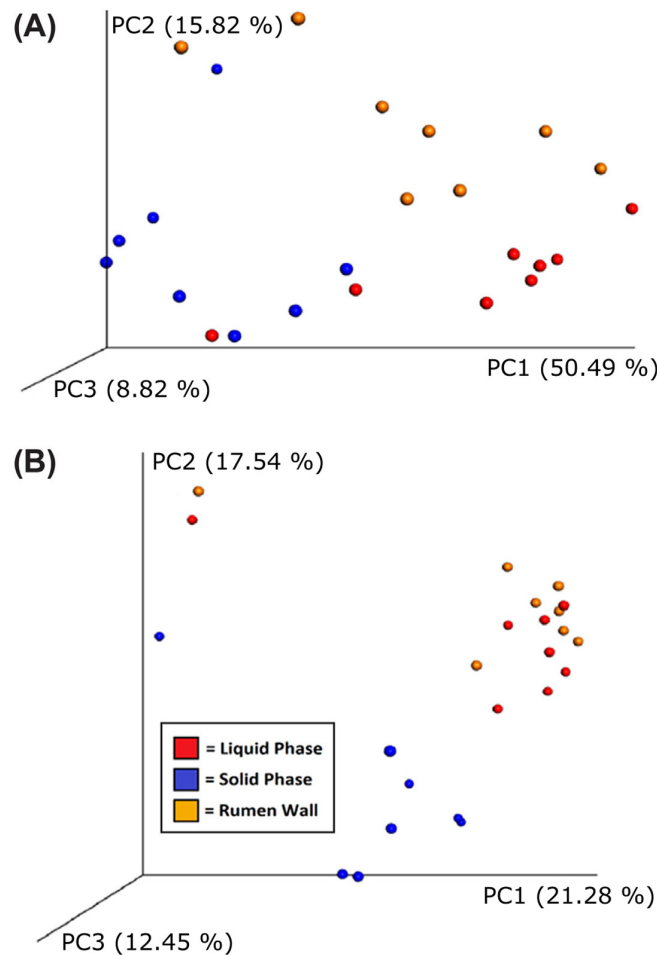


Figure 3. PCoA plot based on (A) weighted UniFrac distance matrices and (B) unweighted UniFrac distance matrices for the three sample types. UniFrac distances were calculated for all OTUs. Each point represents values from one sample with colors expressing rumen compartment.

Table 2. p-values for the pairwise PERMANOVA test for phyla, families, genera and OTUs relative abundances on the three rumen compartments (Wall=rumen wall, Liquid=liquid phase and Solid=solid phase). Significant values are marked with \*.

| Category            | p-value |
|---------------------|---------|
| Phyla               |         |
| Wall versus Liquid  | 0.047*  |
| Wall versus Solid   | 0.003*  |
| Liquid versus Solid | 0.004*  |
| Families            |         |
| Wall versus Liquid  | 0.591   |
| Wall versus Solid   | 0.391   |
| Liquid versus Solid | 0.897   |
| Genera              |         |
| Wall versus Liquid  | 0.003*  |
| Wall versus Solid   | 0.003*  |
| Liquid versus Solid | 0.003*  |
| OTUs                |         |
| Wall versus Liquid  | 0.003*  |
| Wall versus Solid   | 0.003*  |
| Liquid versus Solid | 0.003*  |

liquid phase and solid phase. In particular, rumen wall has been associated with lower species richness indices (Fig. 4).

Considering the diversity indices calculated for the three rumen compartments within the single feeding groups, no significant differences were found for the fed group. On the contrary, in the unfed group, the sample types demonstrated significant differences, as shown in Table 4.

In particular, Chao1 and ACE indices showed significant differences between rumen wall and the other two compartments, while Shannon and observed species indices differed only between liquid phase and rumen wall. Simpson and Simpson's evenness indices did not show significant differences in the unfed group (Table 5).

## Discussion

To the authors' best knowledge, this is the first study that investigates the ruminal microbiota composition of two different roe deer populations fed on different diets during winter. Although there is a lack of studies investigating European roe deer *Capreolus capreolus* ruminal microbiota, the results of this work are in accordance with the findings

of similar studies, investigating the microbiota of Siberian roe deer *Capreolus pygargus* (Li et al. 2014), other wild cervids (elk *Cervus canadensis*, white tailed deer *Odocoileus virginianus* and reindeer *Rangifer tarandus tarandus*) (Gruninger et al. 2014, Salgado-Flores et al. 2016) as well as other wild and domestic ruminant species (Kim et al. 2011, Henderson et al. 2015). The two most abundant phyla, for both the fed and unfed populations, were *Firmicutes* and *Bacteroidetes*, which were also found in all the previous mentioned studies. Also, at the genus level the core microbiota present in roe deer were comparable to other ruminant species (Henderson et al. 2015). The most abundant genera found in this study were *Prevotella*, *Butyrivibrio*, *Anaerostipes*, *Coprococcus* and *Ruminococcus*, which are, in different proportions, also the most common genera found in 32 different species of ruminants (Henderson et al. 2015). In cattle rumen microbiota adapt to diet changes of the host and especially high grain diet can determine a shift in microbial composition of ruminal environment (Belanche et al. 2012, Wetzels et al. 2017). Albeit differences in the GI-tract anatomy, metabolism and feeding habits, might suggest a different microbiota composition in domestic livestock and wild ruminants, it has been shown that ruminants belonging to the family *Cervidae* and *Bovidae* fed concentrate diets had very similar microbiological community structure in the rumen (Henderson et al. 2015). In a recent work by Menke et al. (2019), it was demonstrated that supplemental feeding in winter shapes gut microbiota in red deer *Cervus elaphus*. Hence, it seems plausible that highly fermentable supplemental feeding in winter may affect roe deer ruminal microbiota, as confirmed by the current study. A remarkable finding was the significant reduction of phylum *Firmicutes* relative abundance as well as a slightly higher abundance of *Bacteroidetes* in the fed group. Similarly, other studies found a decrease in *Firmicutes* together with an increase of *Bacteroidetes* in cattle fed a carbohydrate-rich diet (Fernando et al. 2010, Wetzels et al. 2017).

The phylum *Planctomycetes* (which relative abundance was only 0.02% in this work) also differed between the two populations, being enriched in the unfed group. This phylum has been described in previous studies, but even though it is known to be involved in nitrogen-fixing processes, its role in the ruminal environment still remains unclear (Kim et al. 2011, Delmont et al. 2018). Another overall low abundant

Table 3. Mean values ( $\pm$ SD) of diversity indices and species richness indicators per individual of the microbiota of all the three rumen compartments. The group receiving supplemental feeding (Fed) was composed by individuals 1, 4, 7 and 8, while the group without supplemental feeding (Unfed) was composed by individuals 2, 3, 5 and 6.

|              | Shannon         | Simpson         | Chao1          | Observed_species | ACE            | Simpson_e       |
|--------------|-----------------|-----------------|----------------|------------------|----------------|-----------------|
| Fed          |                 |                 |                |                  |                |                 |
| Individual 1 | 7.03 $\pm$ 0.80 | 0.97 $\pm$ 0.02 | 1284 $\pm$ 276 | 1023 $\pm$ 270   | 1236 $\pm$ 272 | 0.05 $\pm$ 0.03 |
| Individual 4 | 8.04 $\pm$ 0.35 | 0.99 $\pm$ 0.01 | 2159 $\pm$ 373 | 1748 $\pm$ 311   | 2143 $\pm$ 380 | 0.05 $\pm$ 0.02 |
| Individual 7 | 7.59 $\pm$ 0.30 | 0.98 $\pm$ 0.00 | 1525 $\pm$ 256 | 1268 $\pm$ 186   | 1517 $\pm$ 228 | 0.05 $\pm$ 0.02 |
| Individual 8 | 7.38 $\pm$ 0.58 | 0.97 $\pm$ 0.02 | 1869 $\pm$ 152 | 1452 $\pm$ 184   | 1840 $\pm$ 182 | 0.03 $\pm$ 0.01 |
| Mean Fed     | 7.51 $\pm$ 0.60 | 0.98 $\pm$ 0.01 | 1709 $\pm$ 420 | 1373 $\pm$ 346   | 1684 $\pm$ 426 | 0.05 $\pm$ 0.02 |
| Unfed        |                 |                 |                |                  |                |                 |
| Individual 2 | 7.50 $\pm$ 0.53 | 0.98 $\pm$ 0.01 | 1851 $\pm$ 373 | 1395 $\pm$ 298   | 1833 $\pm$ 366 | 0.05 $\pm$ 0.01 |
| Individual 3 | 7.84 $\pm$ 0.61 | 0.99 $\pm$ 0.00 | 1935 $\pm$ 280 | 1460 $\pm$ 215   | 1908 $\pm$ 224 | 0.06 $\pm$ 0.02 |
| Individual 5 | 7.77 $\pm$ 1.29 | 0.97 $\pm$ 0.03 | 2253 $\pm$ 495 | 1817 $\pm$ 422   | 2232 $\pm$ 491 | 0.04 $\pm$ 0.03 |
| Individual 6 | 7.90 $\pm$ 0.16 | 0.97 $\pm$ 0.00 | 2371 $\pm$ 251 | 1852 $\pm$ 221   | 2316 $\pm$ 297 | 0.02 $\pm$ 0.01 |
| Mean Unfed   | 7.75 $\pm$ 0.67 | 0.98 $\pm$ 0.02 | 2103 $\pm$ 383 | 1631 $\pm$ 334   | 2072 $\pm$ 373 | 0.04 $\pm$ 0.02 |



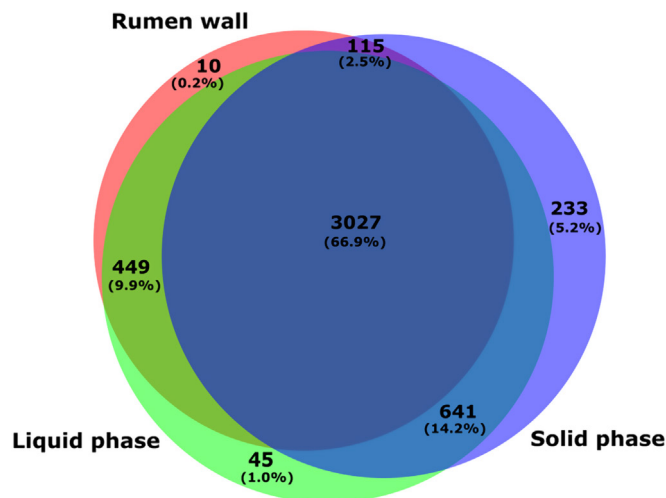


Figure 4. Venn diagram of total numbers and percentages of shared OTUs between the three ruminal compartments (rumen wall = red, solid phase = blue, liquid phase = green).

phylum *SRI* (relative abundance 0.13%) showed significant differences between the two feeding groups. On the contrary, phylum *TM7* showed a relative high abundance in roe deer ruminal microbiota (4.75%), but it was not found to be significantly different between the two studied groups. The result is unusual as the relative abundance of this phylum is lower in similar studies (Kim et al. 2011, Li et al. 2014). Both phyla *SRI* and *TM7* belong to the so-called 'candidate phyla': these are phyla for which cultivated representatives are not available or extremely difficult to obtain. Since for most of these phylotypes only the 16s rRNA gene information is available, their precise role in the ruminal environment is still unknown (Ley et al. 2008, Davis et al. 2009, Campbell et al. 2013, Hanada et al. 2014, He et al. 2015, Youssef et al. 2015). Therefore, an explanation for the significant shift or the high abundance of this phyla in roe deer rumen microbiota cannot be hypothesized. Nevertheless, their occurrence is interesting as it confirms that roe deer rumen microbiota have not been fully characterized and understood yet. This finding is furthermore strengthened by the fact that many amplicons in this work could not be assigned with a precise taxonomy, and were therefore classified as 'unassigned', especially in the fed group. In addition, in the GreenGenes taxonomy comparison with the NCBI and RDP databases, many OTUs couldn't be assigned to taxa isolated from ruminal environment or revealed very low percentage values of identity, probably because the greatest part of rumen microbiota is still represented by uncultured

microorganisms (Edwards et al. 2004, Creevey et al. 2014, Seshadri et al. 2018, Zehavi et al. 2018). At the family level, the families *Campylobacteraceae* and *Oxalobacteraceae* were significantly different in the two groups. In other studies, the presence of the family *Campylobacteraceae* was lower in animals fed with high concentrate grain diets: on the contrary, in our study this family increased in the fed population. It is suggested that family *Campylobacteraceae* is not susceptible to acid environments and that its function is protein degradation (Wetzels et al. 2017, Petri et al. 2018). On the other hand, bacteria belonging to the family *Oxalobacteraceae* contain a sequence to code for urease enzyme in their genome, which is necessary to convert non-proteinic nitrogen (Jin et al. 2017). The higher abundance of this family in the unfed group may suggest that the unfed population had probably a lesser intake of proteins, and especially nitrogen, due to their cellulose rich diet, while the higher abundance of family *Campylobacteraceae* in the fed group is probably due to the protein supplement in their diet (Orpin et al. 1985). Representatives of the Genus *Coprococcus*, including OTU32 and OTU57, were more abundant in the unfed group. This is probably because bacteria belonging to this genus are likely to facilitate fermentation on specific secondary plant compounds (such as polyphenols), and this is relatable to the fibrous diet of the unfed population (Patel et al. 1981). Both fed and unfed population had significantly enriched representatives of the family *Ruminococcaceae* (OTU4 and OTU100). An even distribution of this family, as well as

Table 4. Mean values ( $\pm$ SD) of diversity indices and species richness indicators per rumen compartment in the two feeding groups. Rw = rumen wall, Lp = liquid phase of the rumen content, Sp = solid phase of the rumen content, Fed = group receiving supplemental feeding, Unfed = group without supplemental feeding.

|         | Shannon         | Simpson         | Chao1          | Observed_species | ACE            | Simpson_e       |
|---------|-----------------|-----------------|----------------|------------------|----------------|-----------------|
| Fed     |                 |                 |                |                  |                |                 |
| MEAN Rw | 7.36 $\pm$ 0.84 | 0.98 $\pm$ 0.02 | 1531 $\pm$ 389 | 1229 $\pm$ 340   | 1500 $\pm$ 391 | 0.05 $\pm$ 0.02 |
| MEAN Lp | 7.85 $\pm$ 0.41 | 0.98 $\pm$ 0.01 | 1856 $\pm$ 452 | 1492 $\pm$ 344   | 1832 $\pm$ 459 | 0.05 $\pm$ 0.02 |
| MEAN Sp | 7.31 $\pm$ 0.46 | 0.98 $\pm$ 0.02 | 1741 $\pm$ 467 | 1398 $\pm$ 298   | 1720 $\pm$ 475 | 0.04 $\pm$ 0.02 |
| Unfed   |                 |                 |                |                  |                |                 |
| MEAN Rw | 7.25 $\pm$ 0.80 | 0.97 $\pm$ 0.02 | 1760 $\pm$ 272 | 1365 $\pm$ 219   | 1733 $\pm$ 225 | 0.03 $\pm$ 0.02 |
| MEAN Lp | 8.25 $\pm$ 0.31 | 0.99 $\pm$ 0.01 | 2350 $\pm$ 159 | 1820 $\pm$ 162   | 2308 $\pm$ 159 | 0.06 $\pm$ 0.03 |
| MEAN Sp | 7.76 $\pm$ 0.51 | 0.98 $\pm$ 0.01 | 2197 $\pm$ 433 | 1708 $\pm$ 431   | 2175 $\pm$ 439 | 0.04 $\pm$ 0.02 |

Table 5. p-values of diversity indices and species richness indicators compared among rumen compartments (Wall=rumen wall, Liquid=liquid phase and Solid=solid phase). Significant values are marked with \*.

| p-values            | Shannon | Simpson | Chao1   | Observed_species | ACE     | Simpson_e |
|---------------------|---------|---------|---------|------------------|---------|-----------|
| Solid versus Liquid | 0.451   | 0.634   | 0.598   | 0.648            | 0.676   | 0.334     |
| Wall versus Liquid  | 0.038*  | 0.801   | <0.001* | 0.006*           | <0.001* | 0.086     |
| Wall versus Solid   | 0.425   | 0.789   | 0.016*  | 0.073            | 0.014*  | 0.759     |

of the *Lachnospiraceae* family, was found in other studies (Kim et al. 2011, Henderson et al. 2015). In the present study, four OTUs (OTU53, OTU74, OTU78 and OTU72) classified as *Lachnospiraceae* were significantly increased in the unfed population. Members of this family are suggested to interact with archaeal populations in the methanol metabolism and are involved both in starch and fiber degradation (Kim et al. 2011, Biddle et al. 2013, Henderson et al. 2015, Deusch et al. 2017). Furthermore, three of the 100 most abundant OTUs classified as belonging to order *Clostridiales* (OTU14, OTU31 and OTU63) were significantly higher in the unfed population, confirming the findings of Henderson et al. (2015), who showed that unclassified *Clostridiales* were more abundant in bovines fed forage. However, this was less significant in other ruminant species fed the same diet. OTU67, classified as genus *Butyrivibrio*, was also enriched in the unfed group. Similar findings were detected in other studies, suggesting that bacteria belonging to this genus might be susceptible to pH changes or substrate availability (Fernando et al. 2010, Petri et al. 2013). An analogous assumption can be made regarding OTU91 (genus *Pseudobutyrvibrio*), since genera *Butyrivibrio* and *Pseudobutyrvibrio* are closely related (Kim et al. 2011). In the fed group, two OTUs classified as belonging to genus *Prevotella* (OTU21 and OTU70), showed significant enrichment. This is in accordance to previous studies, in which *Prevotella* was associated with diets containing high concentrations of easily fermentable substrates (Petri et al. 2013, Henderson et al. 2015, Schären et al. 2017). Members of this genus are known to produce propionate and to particularly digest sugars, starch and pectin (Hobson and Stewart 1997, Russell and Rychlik 2001, Krause et al. 2003). The findings discussed up to this point agree with the results of the analysis of similarities: the unweighted UniFrac analysis showed a significant difference between the two different roe deer populations, while the weighted analysis was not significant. This means that the differences between the two groups were mainly qualitative and not quantitative, with a composition differing especially at the OTU level: the lower abundant OTUs differed greatly between the two groups but the highly abundant OTUs were mainly similarly present (Lozupone et al. 2007). According to the diversity indices, there is no statistical difference between the two roe deer populations. Although in the fed group the species richness indices are slightly reduced compared to the unfed population, and some individuals of the two groups show significant differences, it cannot be stated that supplemental feeding reduces species richness, as it is known from cattle (Plaizier et al. 2017).

The significant differences of the microbiota composition of the three different rumen compartments especially in the unfed group in this study, have also been found in previous studies in cattle (Kong et al. 2010, Liu et al. 2016,

Schären et al. 2017) and wild ruminants such as elk (Gruninger et al. 2014). It seems that rumen wall microbiota are associated with the lowest species richness and that a time from several days to weeks is required for a significant change in the microbial community associated with rumen papillae (Wetzels et al. 2016, Schären et al. 2017, Mann et al. 2018). The sampling time in the present study was from one to two months after the provision of supplemental feeding. Therefore, the animals probably had enough time to adapt to the diet shift, and the results are representative of the microbiota adaptation to high grain diet. The lower species richness and the significant differences in the microbial composition of the rumen content found in this work indicate that the microbial populations of the solid and liquid phase of the rumen digesta might be more susceptible to diet-induced changes (Schären et al. 2017). Easily fermentable feed contributed to shift the microbial community towards a composition similar to recognized pathological conditions in cattle, such as subacute ruminal acidosis. This might be supported by the work of Ritz et al. (2013) who compared ruminal pH of roe deer with and without supplemental feeding, and found a pH drop in the ruminal environment in fed animals. Also, studies performed post-mortem on several wild ruminants fed high grain diets in zoos revealed the presence of disease characteristics compatible with subacute ruminal acidosis (Ritz et al. 2013, Schilcher et al. 2013, Gattiker et al. 2014).

In conclusion the results of this study suggest that providing roe deer with easily fermentable supplemental feeding in winter significantly affects the microbial population and induces changes similarly to domestic ruminant species. A larger sample size in future studies would be desirable and would allow for more robust conclusions on negative effect on animals' health status. The high abundance of unclassified bacterial strains at any taxonomic level found in this study furthermore shows the need to generate more knowledge of the ruminal microbiota in wild ruminants. This study generates fundamental baseline data on the diversity and composition of gut microbiota in the European roe deer that will complement previous research and create a starting point for future research to understand the potential causes of alterations to the ruminal microbial communities and their physiological and pathophysiological dynamics in this species.

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Supplementary material (available online as Appendix wlb-00572 at <[www.wildlifebiology.org/appendix/wlb-00572](http://www.wildlifebiology.org/appendix/wlb-00572)>). Appendix 1.