



Original Research Paper

## Molecular study of dietary diversity of the Exmoor-ponies (*Equus ferus caballus*)

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### Abstract

Much is still to learn about the reintroducing of horses in rewilding in regards to their forage on woody species as horses are often categorized as true grazers and dwellers of the open habitat. Relative amount of woody vegetation and different woody species ingested by Exmoor-ponies (*Equus ferus caballus*) in the Rewilding Mols project area in the Mols Bjerge National Park, Denmark, during winter were determined using macroscopic analysis of faeces and DNA metabarcoding. This showed an increase in the relative amount of woody vegetation ingested during winter (December – February) peaking at 35.2% in January. The three most dominating woody species were common heather (*Calluna vulgaris*), common oak (*Quercus robur*), and scots pine (*Pinus Sylvestris*) with a total relative abundance in the diet throughout the entire study period of '10%', '10%' and '6%', respectively. No direct debarking by the Exmoor-ponies was observed nor evidence of debarking on trees in the area (data not shown). The current forage on woody vegetation during winter was concluded not to be significant in order to trigger canopy dieback and eventually cause retrogressive succession. If the goal for a specific rewilding project includes creation of clearings in forest stands one should reconsider the choice of Exmoor-ponies.

**Keywords:** Exmoor-ponies, Rewilding, Retrogressive succession, Macroscopic analysis, DNA metabarcoding.

### Introduction

Rewilding, here defined as the “passive management of ecological succession with the goal of restoring natural ecosystem processes and reducing the human control of landscapes,” is increasingly practiced as a tool in nature conservation and management with potential for enhancing biodiversity (Navarro & Pereira, 2015; Carey, 2016; Svenning et al., 2016). Trophic rewilding including the reintroduction of robust horse breeds, such as the Exmoor-pony (*Equus ferus caballus*), as ecological proxies for the extant tarpan is increasingly practised throughout Europe (Linnartz & Meissner, 2014; Carey, 2016; Allen et al., 2017). Multiple studies (Gill, 2006; Hester et al., 2006; Vera, 2009; Laskurain et al., 2013; Sandom et al., 2013; Vermeulen, 2015; Cornelissen, 2017; Fløjgaard et al., 2017) have already indisputably shown that large grazers, such as different species of deer, cattle and horses, can have considerable effects on forest development by influencing different

stages of ecological succession. By grazing, trampling and wallowing horses are capable of creating disturbances in their habitat to prevent overgrowth of shrubs and trees and hinder forest succession (Sandom et al., 2013; Vermeulen, 2015; Fløjgaard et al., 2017). As so many plant and animal species are dependent on the forest clearings, letting horses roam in the forests could potentially create these clearings and increase landscape heterogeneity and thereby biodiversity (Laskurain et al., 2013; Vermeulen, 2015; Fløjgaard et al., 2017). However, there is currently a lack of knowledge in regards to whether the disturbances the horses make are sufficient to open already established forest and reconvert them to open biotopes – the process of retrogressive succession (Kuiters et al., 2006; Vera, 2009; Vermeulen, 2015; Cornelissen, 2017). Horses are generally thought of as true grazers and dwellers of the open habitats and knowledge about their forage on woody species is limited (Cornelissen & Vulink, 2001; Kuiters et al., 2006; Vera et al., 2006; Klich, 2017).

Debarking and uprooting of trees by large herbivores can result in forest clearings but this behaviour is dependent on the specific species and the physical conditions the animal is exposed to (Vermeulen, 2015; Cornelissen, 2017). As a true grazer, horses prefer to forage on grasses and herbaceous vegetation (Buttenschön, 2007). During winter, however, horses are forced to search for alternative food sources such as twigs, branches and bark depending on the varying hardness of the winter (Moen et al., 2006; Vermeulen, 2015; Köhler et al., 2016). It is therefore during this period that the effect of horse grazing on the landscape can be expected to be greatest and thus capable of creating a mosaic pattern of open grassland, bushes and forests (Vermeulen, 2015; Fløjgaard et al., 2017). When winter is harsh, arrives early, and lasts long the probability of the herbivores browsing on woody species increases (Gudmundsson & Dyrmondsson, 1994; Vermeulen, 2015; Fløjgaard et al., 2017). However, with climate change and annual rises in global temperature the likelihood of these harsh winters decreases (IPCC, 2014; Navarro & Pereira, 2015).

In order for rewilding to be successfully implemented as a nature management practise, much is still to be learned about what specific species are ideal to reintroduce in various projects with different goals and how they respond to different environmental factors (Kuiters et al., 2006; Corlett, 2016; Nogués-Bravo et al., 2016; Svenning et al., 2016)

## **Materials and Methods**

### ***Study site***

The study was carried out in Mols Bjerger National Park in the Southern part of Djursland, Denmark (56°17'N; 10°29'E). Mean annual precipitation is approximately 600 mm and the mean annual temperature is 8.4 °C (DMI, 2018). The national park was established in 2009 and constitutes 180 km<sup>2</sup> including the city of Ebeltoft as well as various villages and holiday cottage areas. The protected nature area is located centrally in the national park and make up 2500 ha (Miljø- og Fødevareministeriet, 2018; Nationalpark Mols Bjerger, 2018b). Within this area, The Natural History Museum Aarhus owns a highly biodiverse natural hotspot where an area of 120 ha is fenced off from the rest of the national park, named Rewilding Mols at The Mols Laboratory (Naturhistorisk Museum Aarhus, 2018). Within this area, Exmoor-ponies roam freely together with Galloway-cattle (*Bos taurus galloway*) as part of a rewilding project launched in November 2016 with increasing winter populations of the two large herbivore species counting 13 and 13 in 2016, 18 and 22 in 2017, and 27 and 32 in 2018, respectively.

The national park has a high variety of habitat types many of which are included in EU's Habitats Directive. Within the Rewilding Mols project area, three relatively dry and open landscapes dominate; heath with approximately 55 ha, dry grassland with 23 ha, and meadow with 6 ha. In addition to these open habitat types, approximately 36 ha of deciduous and coniferous forest with patches of alder (*Alnus* spp.), ash (*Fraxinus* spp.) and pine (*Pinus* spp.) and dominated by oak (*Quercus* spp.) and beech (*Fagus* spp.) resides within the fenced-off area. The entire nature area of the Mols Laboratory is a Natura 2000 area.

## **Field work**

The study period ranged from December 11th 2017 to February 9th 2018 with faeces samples collected on seven days in December (12th-15th and 20th-22nd) denoted “December 1” and “December 2” respectively, five days in January (15th-19th) denoted “January” and five days in February (5th-9th) denoted “February” making a total of 17 days. The dominant mare of the Exmoor-herd was equipped with a GPS-collar (Vectronic Aerospace Wildlife) in December 2017, which was used to locate the herd. Two faeces samples were collected each day, one in the morning and one in the afternoon except for two days where only one sample was collected in total. This made a total of 32 samples. Samples were collected from the first horse to defecate after the herd had been localized. Samples were kept in plastic bags, freezed immediately after and kept frozen until preparation for analysis. Parts from plant species that were observed eaten by the horses were also collected, identified in the field if possible, frozen immediately after collection and kept frozen until analysis in order to create a reference library to assist the macroscopic analysis.

Temperature and precipitation were not monitored in the field. In order to investigate whether temperature and precipitation affected the dietary choice, this data was later drawn from the weather archives of The Danish Meteorological Institute from the specific area and study period (DMI, 2018).

## **Laboratory work**

### ***Reference plants***

Plant parts collected in the field were cut into smaller pieces and crushed lightly in a mortar with a few drops of water. The crushed plant parts were fixed on a microscopic slide with glycerine, and analysed in the microscope (Axioskop, Zeiss, Denmark) ranging from 10x to 40x magnifications for characteristics in shape and size of tracheid, parenchyma and stomata in order to distinguish between woody plant material and non-woody plant material. Photos were taken through the microscope to compare with photos of semi- or undigested plant parts from the faeces samples (Appendix 1).

### ***Macroscopic analysis***

In the laboratory, 2 grams from each faeces sample were transferred to Eppendorf tubes and kept frozen until DNA analysis. Samples collected from the same day were pooled in the same tube. Faeces samples for macroscopic analysis were then thawed overnight in the refrigerator (5°C), transferred to large petri dishes (145x20 mm) (Greiner bio-one, Hungary) with water and a few drops of dishwashing soap and stored in the refrigerator. After seven days each sample was rinsed with water through a sieve with 1 mm mesh (Endecotts LTP, England) and transferred to new petri dishes with added soap. During the period of the macroscopic analysis, samples yet to be analysed were rinsed weekly and transferred to new petri dishes with added soap to prevent the growth of mould.

A subsample of approximately 1 mL were extracted at random from each sample with a disposable pipette with the tip cut off and transferred to an Eppendorf tube. The Eppendorf tube was shaken gently for five seconds in order to resuspend the plant material before six drops were transferred with a disposable pipette with the tip cut off from the Eppendorf tube to a microscopic slide, which had been divided into six 2x1.3 cm squares. For each square, the amount of woody plant parts and non-woody plant parts was estimated on the basis of different morphological characteristics in tracheid, parenchyma and stomata (Appendix 1). Further subsamples were analysed until the difference between the accumulated mean percentages of woody plant material in the subsamples was less than 5 % in three subsamples in a row. Thus, five to ten subsamples were extracted and analysed from each sample.

To assess the change in the amount of woody plant material in faeces samples throughout the study period, the average proportion of woody species in the periods (December 1, December 2, January and February) were analysed using a one-way ANOVA and Tukey's t-test performed using the software PAST

(<https://folk.uio.no/ohammer/past/>). To test for differences in the amount, relative amount, abundance and relative abundance of woody vs. non-woody plants species present in the faeces samples throughout the study period, pairwise X2 tests were performed.

### ***DNA-extraction***

PCR and Subsamples for metabarcoding analyses were preserved and stored at -20°C until DNA extraction performed using the DNeasy Blood & Tissue kit (QIAGEN GmbH) following manufacturer instructions (QIAGEN GmbH). Approximately 1-2g from each of the faecal subsample was transferred to a 1.5ml Eppendorf tube, already containing 180 µl Buffer ATL. 20µl proteinase K was added and then vortexed. The tubes were incubated at 56°C for 1 hour and occasionally vortexed until the faecal material was completely lysed. 200 µl Buffer AL was then added and the tubes were subsequently incubated at 56°C for 10 minutes, after which 200 µl ethanol (96-100 %) was added, and the tubes were subsequently vortexed again. The mixture was transferred into a DNeasy spin column placed in a 2 mL collection tube and centrifuged at 8000 rpm for 1 minute. The flow-through and the collection tube were discarded and the spin column was placed in a new 2 mL collection tube. The same identical process was repeated after the addition of 500 µl Buffer AW1 and then again after addition 500 µl Buffer AW2. The only exception being the last centrifuge, which was performed once at 14.000 rpm for 3 minutes. The spin column was transferred to a new collection tube and ultimately the DNA was eluted by adding 100 µl Buffer AE, incubated for 1 minute at room temperature (15-25°C) and centrifuged at 8000 rpm for 1 minute. The spin column was discarded and the extracted DNA was stored at -20°C until quantification and amplification could proceed.

Quantification was performed using tapestation D1000 to check the quality of the DNA and DNA concentration was measured with qubit HS.

### ***PCR***

IlluminaMiSeq platform was prepared using the fusion PCR method. Fusion primers were prepared based on the target sequence of primers c and d to enable unidirectional sequencing of the trnL (UAA) intron located in the chloroplast DNA (Taberlet et al., 2007). The length of the fragment amplified was 750 bs. Both primers were fitted with an adaptor sequence at the 5'-end and the g primers with a sample specific barcode sequence, in accordance with the manufacturer's instructions for Ion Amplicon Preparation. DNA amplifications were carried out in a final volume of 50 µL, including 45µL Platinum1 PCR SuperMix High Fidelity (Invitrogen), 1 µL template DNA and 200 nM of each fusion primer. The PCR reaction involved 2 min incubation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 68°C, and ended with 10 min incubation at 68°C. All PCR runs included a negative control, without sample material. All PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Inc.) in accordance with the manufacturer's instructions.

### ***Sequence analysis***

Sequencing reads were quality checked and trimmed using NGS QC Toolkit (Patel & Jain, 2012), merged using FLASH (v1.2.7) (Magoč & Salzberg, 2011) and formatted for use with the UPARSE workflow, which was used to screen for chimeric sequences (Edgar, 2013). Reads were then de-replicated and clustered into Operational Taxonomic Units (OTUs) at 98% sequence similarity using Usearch 7 (<https://www.drive5.com/usearch/manual7/>). Taxonomy was assigned by blasting the sequences with the Genbank database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

## **Results**

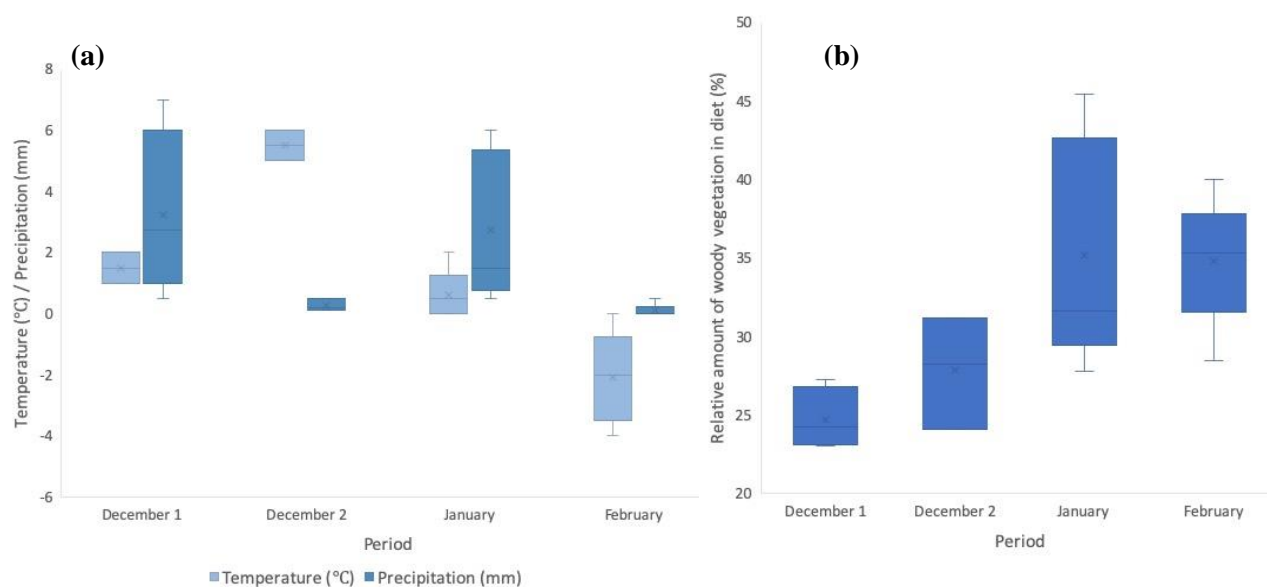
### ***Weather***

December 2 was significantly the warmest period with an average air temperature of 5.5 °C whilst February significantly was the coldest period with average air temperature of -2.1 °C ( $p < 0.05$ ) (fig. 1(a)). Furthermore,

these were the periods with the least amount of precipitation, with 0.27 mm in December 2 and 0.1 mm in February (fig. 1(a)). The difference in temperature between December 1 and January was not significant ( $p > 0.05$ ). In December 1 precipitation fell as rain (3.25 mm) while in January (2.74 mm) it fell as snow.

### Macroscopic analysis

The average relative amount of woody vegetation in faeces increased significantly between December 1 and January and December 1 and February ( $p < 0.05$ ) with the highest average relative amount of woody vegetation in the diet occurring in January ( $35.2 \pm 3.3\%$ ) (fig. 1(b)). This corresponds with January being the period receiving the most snowfall (fig. 1(a)). There is considerable variation in January, which causes relatively large differences in the values for the mean and median. This corresponds to the high variation in snowfall also occurring in January (fig. 1(a)). The average relative amount of woody vegetation in the diet was  $34.8 \pm 1.9\%$  in February, which is slightly less than the relative amount for January. However, the median relative amount is highest in February (35.3%) where the temperature is also the lowest. The lowest relative amount of woody vegetation in the diet occurred in December 1 with  $24.7 \pm 1\%$ .



**Figure 1.** (a) Mean temperature (°C) and precipitation (mm) in the four periods. The y-axis shows both the temperature (°C) and precipitation (mm). (b) Relative amount of woody vegetation in the diet of Exmoor ponies during the winter of 2017-2018. The y-axis shows the relative amount of woody vegetation in the diet in %. The x-axis shows the four periods; December 1 (12.12.17-15.12.17), December 2 (20.12.17-22.12.17), January (15.01.18-19.01.18), and February (05.02.18-09.02.18).

## DNA

### Sequence filtering

Sequencing yielded a total of 3027 reads, while the number of reads per sample varied from 31 to 315 (Mean =  $161.647 \pm 69.123$  S.D). Reads with a value less than 10 were excluded leaving 6 to 87 (Mean =  $43.941 \pm 18.936$  S.D) reads per sample. 782 sequences were unidentified and 1604 sequences were identified as bacteria using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), leaving 641. The BLAST output for these 641 sequences was crosschecked with a reference plant list from the Rewilding Mols area. This list, however, is not complete and species identified in the samples but not occurring on the list were crosschecked using

“Dansk flora” by Frederiksen & Rasmussen (2006) for likelihood of occurring in the known nature types in the area. The BLAST output for 27 sequences was non-native species and therefore excluded. Due to several sequences belonging to the same species, a total of 36 different sequences were ultimately identified at the species (22) or genus (14) level in the samples, and separated between woody (14) and non-woody (22) taxa (table 1).

Common apple (*Malus pumila*) is only present in December 1 while silver birch (*Betula pendula*), common oak (*Quercus robur*), common heather (*Calluna vulgaris*), scots pine (*Pinus sylvestris*) and beech (*Fagus spp.*) are present throughout the study period (except for beech, which was absent in December 2). *Vaccinium* (*Vaccinium spp.*) is present in December 2 and January while raspberry (*Rubusidaeus*) and juniper (*Juniperus spp.*) are present from December 2 to February. Common ash (*Fraxinus excelsior*) is only present in December 2, common alder (*Alnusglutinosa*) and black crowberry (*Empetrum nigrum*) are only present in January, and alder buckthorn (*Frangula alnus*) and willow (*Salix spp.*) are only present in February.

**Table 1.** Woody and non-woody species/genera in the faeces samples identified using metabarcoding

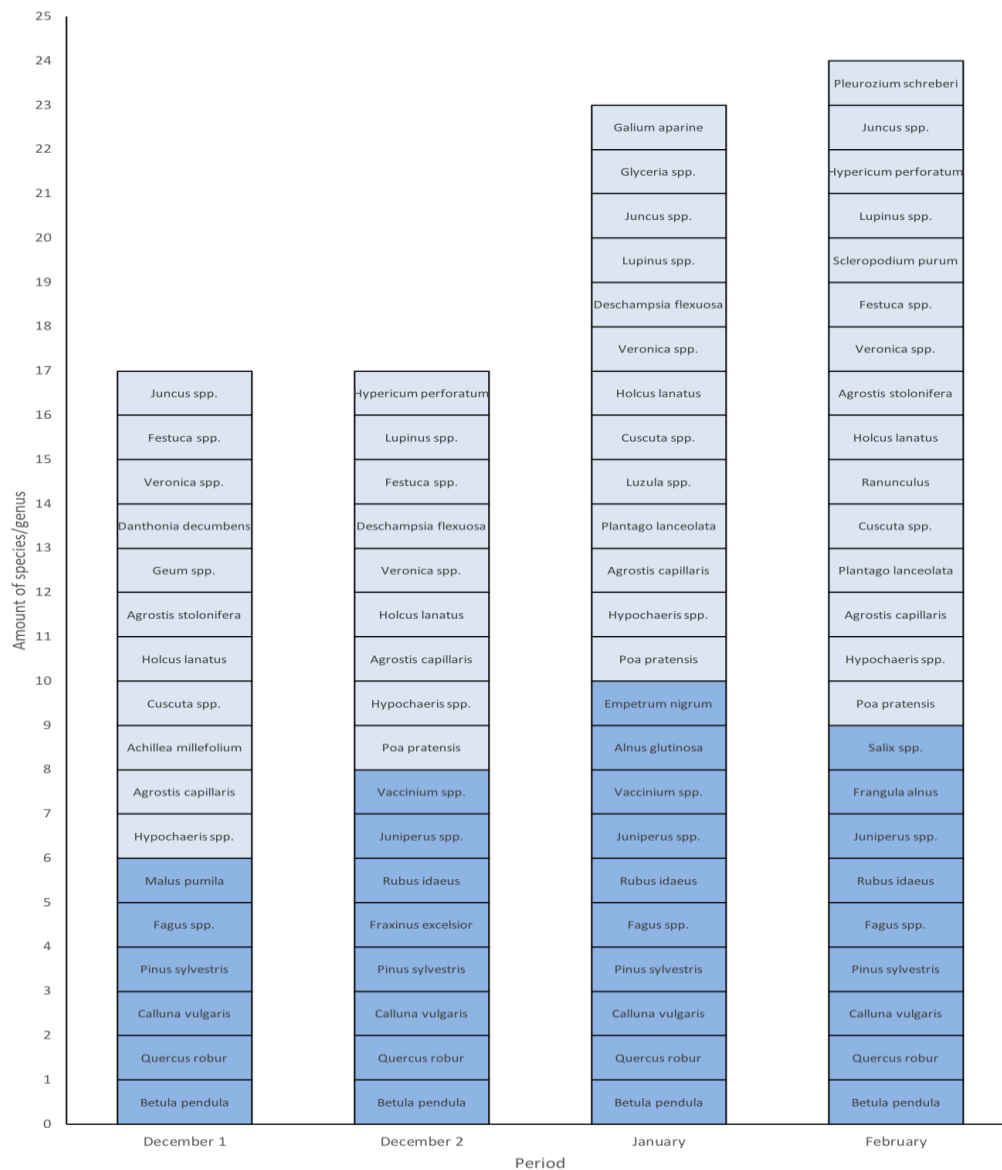
Woody species	Non-woody species
Alder buckthorn ( <i>Frangula alnus</i> )	Bluegrass ( <i>Poa pratensis</i> )
Silver birch ( <i>Betula pendula</i> )	Cat's ear ( <i>Hypochaeris spp.</i> )
Common oak ( <i>Quercus robur</i> )	Common bent ( <i>Agrostiscapillaris</i> )
Heather ( <i>Calluna vulgaris</i> )	Ribwort plantain ( <i>Plantagolanceolata</i> )
<i>Vaccinium</i> ( <i>Vaccinium spp.</i> )	Common yarrow ( <i>Achilleamillefolium</i> )
Scots pine ( <i>Pinus sylvestris</i> )	Wood rush ( <i>Luzulaspp.</i> )
Beech ( <i>Fagus spp.</i> )	Dodder ( <i>Cuscutaspp.</i> )
Common apple ( <i>Malus pumila</i> )	Buttercup ( <i>Ranunculus</i> )
Raspberry ( <i>Rubusidaeus</i> )	Tufted grass ( <i>Holcuslanatus</i> )
Common ash ( <i>Fraxinus excelsior</i> )	Creeping bent ( <i>Agrostis stolonifera</i> )
Juniper ( <i>Juniperus spp.</i> )	Avens ( <i>Geum spp.</i> )
Common alder ( <i>Alnusglutinosa</i> )	Heath grass ( <i>Danthoniadecumbens</i> )
Crowberry ( <i>Empetrum nigrum</i> )	Bird's eyes ( <i>Veronica spp.</i> )
Willow ( <i>Salix spp.</i> )	Wavy hair-grass ( <i>Deschampsiaflexuosa</i> )
	Fescue grass ( <i>Festuca spp.</i> )
	Neat feather-moss ( <i>Scleropodiumpurum</i> )
	Lupin ( <i>Lupinus spp.</i> )
	St. John's wort ( <i>Hypericum perforatum</i> )
	Rushes ( <i>Juncus spp.</i> )
	Mannagrass ( <i>Glyceria spp.</i> )
	Red-stemmed feathermoss ( <i>Pleuroziumschreberi</i> )
	Goosegrass ( <i>Galiumaparine</i> )

### Amount and relative amount of woody species

The  $X^2$  tests showed no statistical significance in any of the comparisons of the amount of species/genera, relative amount of species/genera, abundance of species/genera or relative abundance of species/genera between woody and non-woody species throughout the study period. For this reason, the review of the results from the DNA-analysis will be purely descriptive.

Both the amount and relative amount of different woody species/genera were lowest in December 1 with six different species/genera constituting ‘35’ % of total plant species found in the faeces (Figure 2 and 3). This corresponds with December 1 being the period in which the relative amount of woody vegetation in the diet was the lowest ( $24.7 \pm 1\%$ ) (Figure 1(b)). January was the period with the highest amount of woody

species/genera (10), which correlates with January having the highest relative amount of woody vegetation in the diet and being the period receiving the most snow. However, January was not the period where the relative amount of woody species/genera was highest, which was December 2 with ‘47’ % compared to ‘46’ % in January (Figure 3). Even though December 2 had the highest relative amount of woody species/genera, it had the second lowest amount of different woody species/genera (8) (Figure 2). February was the period with the second highest amount of woody species/genera (9) but second lowest relative amount of woody species/genera (‘37’ %) (Figure 2 and 3).

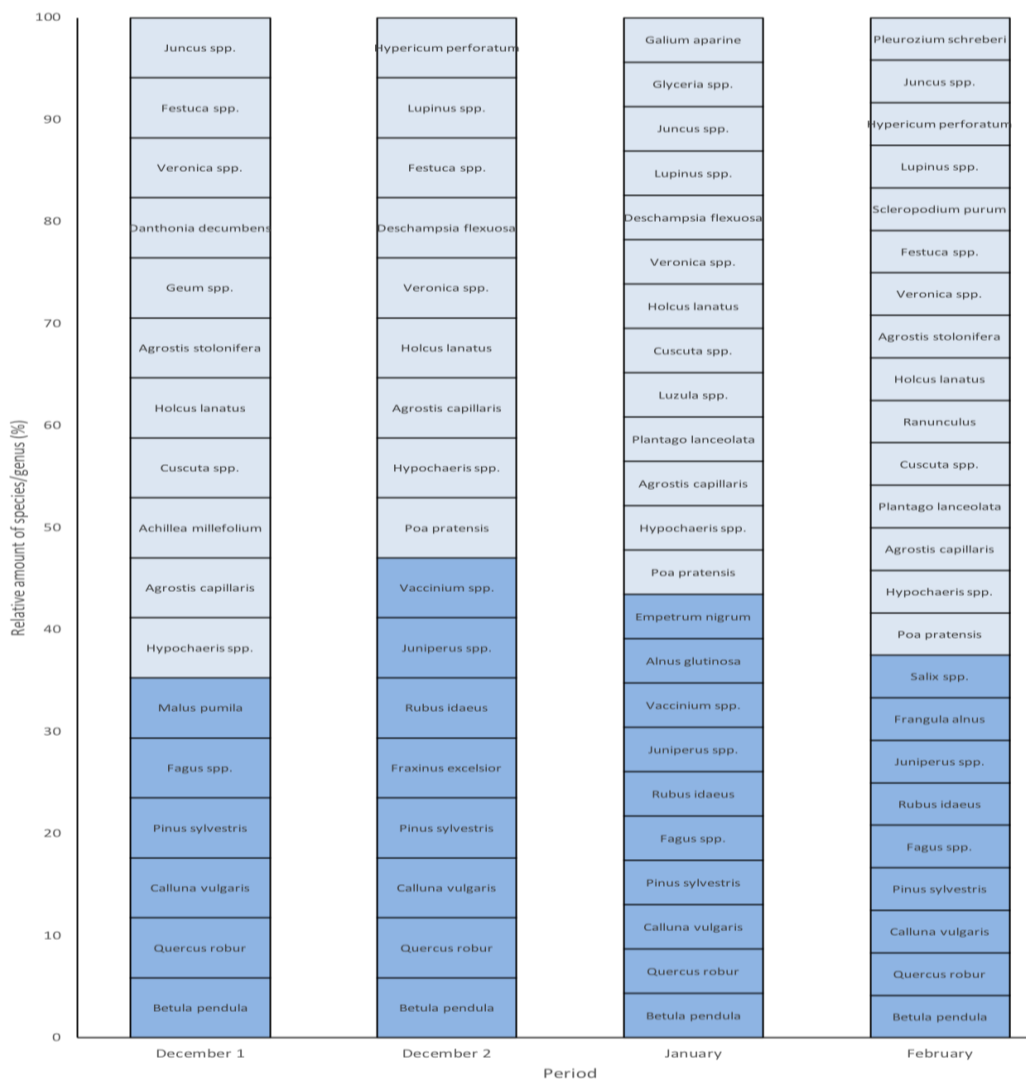


**Figure 2.** Development in amount of species/genera ingested throughout the study period. The y-axis shows the amount of species/genera and the x-axis shows the four periods: December 1 (11.12.17-15.12.17), December 2 (20.12.17-22.12.17), January (15.01.18-19.01.18), and February (05.02.18-09.02.18). Woody species are coloured dark blue and non-woody species are coloured light blue.

### Relative abundance of woody species/genera

The abundance of woody species/genera increased throughout the study period reaching its highest level in February (Appendix 2). The three most dominating woody species were common heather (*C. vulgaris*), common oak (*Q. robur*) and scots pine (*P. sylvestris*) with a total relative abundance throughout the entire study period of ‘10’%, ‘10’% and ‘6’%, respectively. Of the non-woody species, only common bent (*Agrostis capillaris*) had an equally high relative abundance of ‘10’%.

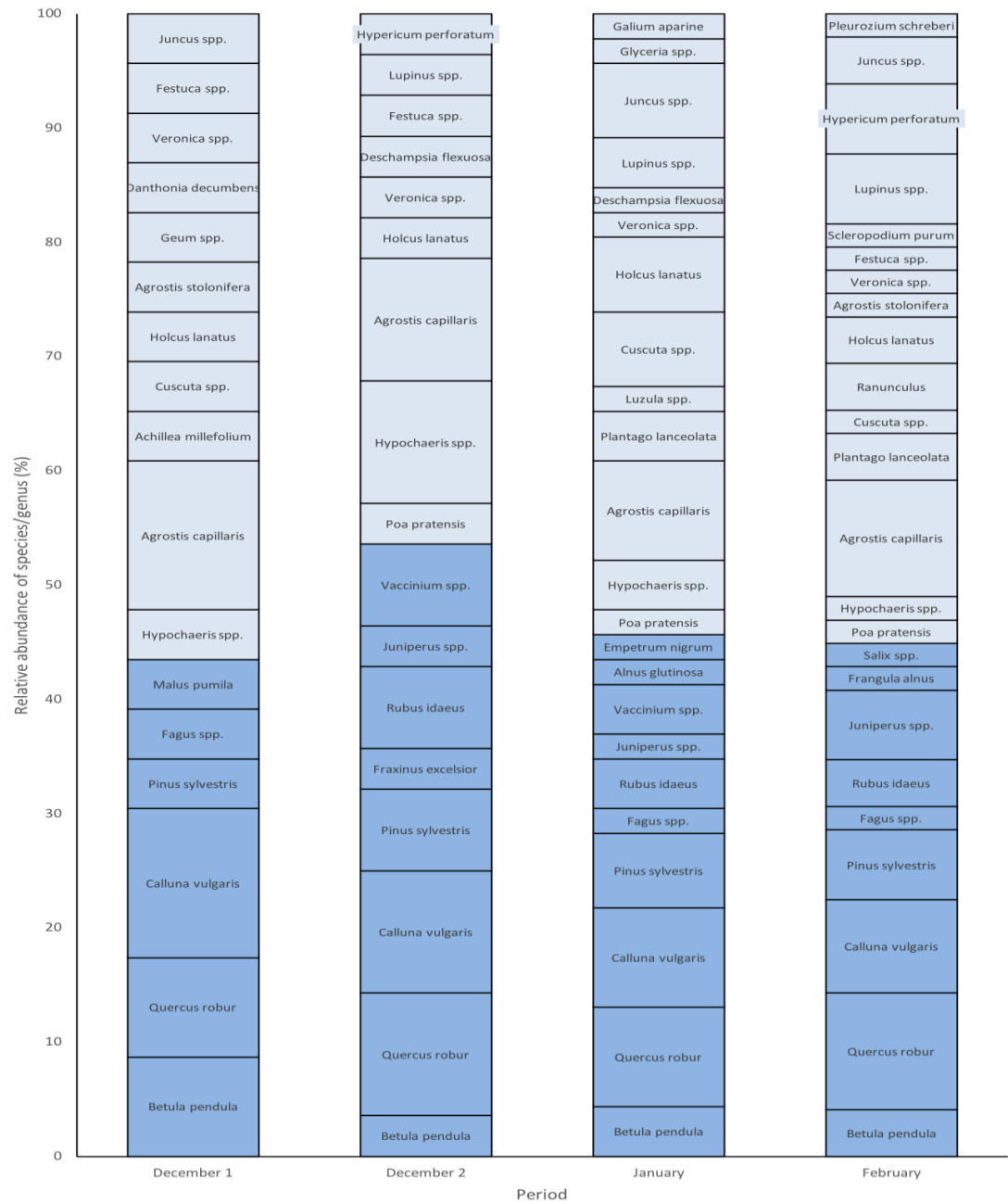
The relative abundance of woody species/genera was lowest in December 1 with ‘43’ % (Figure 4), correlating with this being both the period in which the ponies ingested the least amount of woody vegetation and the period with the least amount of different woody species/genera. The highest relative abundance of species occurred in December 2 with ‘52’ % (Figure 4) with the relative abundance of common oak (‘10’ %), scots pine (‘7’ %), raspberry (‘7’ %) and vaccinium (‘7’%) being the highest compared to the rest of the periods.



**Figure 3.** Development in relative amount of species/genera ingested throughout the study period. The y-axis shows the relative amount of species/genera in % and the x-axis shows the four periods: December 1 (11.12.17-



15.12.17), December 2 (20.12.17-22.12.17), January (15.01.18-19.01.18), and February (05.02.18-09.02.18). Woody species are coloured dark blue and non-woody species are coloured light blue.



**Figure 4.** Development in relative abundance of species/genera ingested throughout the study period. The y-axis shows the relative abundance of species/genera in % and the x-axis shows the four periods: December 1 (11.12.17-15.12.17), December 2 (20.12.17-22.12.17), January (15.01.18-19.01.18), and February (05.02.18-09.02.18). Woody species are coloured dark blue and non-woody species are coloured light blue.

## Discussion

### *Relative amount of woody vegetation*

The proportion of woody vegetation in the Exmoor-ponies' diet significantly increased from the beginning of the study period until it peaked in January with 35.2% of woody vegetation in the diet (Figure 1(b)). Studies by Putman et al. (1987) and Buttenschøn (2007) showed that easily digested grasses comprise 80-90% of the summer diet but it decreases to approximately 50% in winter. When preferred vegetation become limited or inaccessible during winter, for example due to snow cover, a compensatory increase in browsed woody plants make up a larger proportion of the diet (Linnartz & Meissner, 2014; Vermeulen, 2015). Even though the intake of woody vegetation was highest in January, the proportion of woody plants in the diet did not reach the aforementioned 50% found in other studies. Putman et al. (1987) did not report about the weather conditions in their study period but the difference in diet proportion between studies could be attributed to the very mild winter in this study with average temperature of 2.2 °C. From December 2 to February, the temperature significantly decreased with December 2 being the warmest period and February being the coldest period (Figure 1(a)). Despite February being the coldest period, the proportion of woody vegetation in the diet was highest in January. This could be due to the fact that close to no precipitation fell in February compared to 2.74 mm in January (Figure 1(a)). December 1 had the highest amount of precipitation but because of average temperature of 1.5 °C this did not fall as snow compared to the precipitation falling in January, where the temperature was 0.6 °C. This demonstrates how snow cover could affect diet selection of the Exmoor-ponies in winter.

### *Species ingested and significance in effect of Exmoor-ponies on vegetation development*

The three most dominating woody species throughout the entire study period were common heather (*C. vulgaris*), common oak (*Q. robur*) and scots pine (*P. sylvestris*) (Figure . 4). The presence of scots pine in the diet throughout the study period is cause for wonder. Scots pine has a relatively high amount of tannins, which decreases digestibility and is unpalatable to some herbivores. This often causes horses to avoid this species (Kuiters & Slim, 2003; Kuiters et al., 2006; Buttenschøn, 2007; Barbehenn & Constabel, 2011). Kuiters et al. (2006) investigated selective bark-stripping by free-ranging horses and even though *P. sylvestris* covered 28% of the woodland area investigated in Veluwezoom National Park, Netherlands, scots pine showed no signs of damage from bark-stripping by horses. In contrast to horses, other herbivores such as deer (*Cervus spp.*) and moose (*Alces alces*) often prefer bark of coniferous species, especially scots pine (Kullberg & Bergström, 2001; Kuiters et al., 2006). However, as deer and moose are both ruminants, similarities between these herbivores and the hindgut-fermenting horse are hard to draw (Kullberg & Bergström, 2001).

If pine was the only food resource available in January, where snowfall was highest, or February, where temperature was lowest, one could argue that the cold, extreme weather was forcing the ponies to look for alternative food sources and compromising on the quality of these. That was however, not the case. Because environmental and abiotic factors such as water availability and soil nutrients are of great significance on the nutritional value and production of chemicals in plants (Palo, 1984; Giertych et al., 2006; Buttenschøn, 2007), further studies on scots pine specifically in the Rewilding Mols area could have been beneficial to help explain this trend. However, this was not the scope for this study.

The dwarf shrub common heather (*C. vulgaris*) dominates heathlands and attracts many species of butterflies and insects and is thus important for biodiversity (Kuiters et al., 2006; Buttenschøn, 2007; Critchley et al., 2013). However, it is important for heather to constantly rejuvenate, which grazing can contribute to (Buttenschøn, 2007). Critchley et al. (2013) showed that cover of heather continuously increased and developed best throughout an eight year study period when grazed by cattle compared to grazing by sheep. Sheep generally prefer and select for heather possibly causing an intensive grazing pressure on heather, which can be detrimental to its development. In contrast, cattle only consume *C. vulgaris* if other more palatable

species are unavailable (Critchley et al., 2013). As heather is also defended from herbivory by chemical substances (Vera et al., 2006), horses could exercise the same behaviour as cattle in regards to only consuming heather when more preferred species become unavailable for example during winter. This prevents the horses from performing an intensive, and thus harmful grazing pressure on heather. Several studies report that intensive grazing is detrimental to the distribution of *C. vulgaris* (Mitchell et al., 2008; Critchley et al., 2013). However, the grazing pressure of the Exmoor-ponies and Galloway-cattle roaming the Rewilding Mols area is within the recommended grazing pressure of 0.3-0.5 large livestock per. hectares for heathlands (Buttenschøn, 2014). The effect of the ponies grazing of heather is therefore arguably predominantly positive and could contribute to increasing biodiversity of this specific biotope.

Together with beech (*Fagus spp.*) and common alder (*A. glutinosa*), common oak (*Q. robur*) makes up the climax vegetation of late successional stages in Denmark (Buttenschøn, 2007; Bobiec et al., 2011; Staun & Friis Møller, 2015). Common oak is generally a preferred, palatable species to ingest by several herbivores and for this reason, seed and sapling commonly need to grow in cover of thorny species from families such as *Rosaceae* and *Juniperus* in order to be protected from herbivory – the mechanism of associational resistance (Kuiters & Slim, 2003; Vera et al., 2006; Buttenschøn, 2007; Bobiec et al., 2011). From December 2 to February, raspberry (*R. idaeus*) and juniper (*Juniperus spp.*) constituted '4%' and '3%', respectively, of the relative abundance of woody species. The ingestion of raspberry and juniper could be associated with the intake of oak due to the fact that oak saplings generally grow in the cover of unpalatable and thorny shrubs such as raspberry and juniper (Olf et al., 1999). The intake of these chemically and physically armed species could be unintentional with the real goal being foraging on the oak residing within them (Kuiters & Slim, 2003; Vera et al., 2006; Buttenschøn, 2007).

The Exmoor-ponies browsing on oak (and other early pioneer species such as silver birch (*B. pendula*) and willow (*Salix spp.*) also included in the diet) can have a largely positive effect on biodiversity and contribute in keeping the open grassland open, as shown by many previous studies (Gill, 2006; Hester et al., 2006; Sandom et al., 2013; Vermeulen, 2015; Cornelissen, 2017; Fløjgaard et al., 2017). Grazing by livestock, such as horses, are believed to create and sustain favourable conditions for spontaneous regeneration of oak (Bobiec et al., 2011).

The question of whether the Exmoor-ponies reintroduced to the Rewilding Mols area over time will provide enough disturbance in the established forest stands to cause retrogressive succession is largely dependent on their forage on late successional species, such as beech, common alder, and common ash (Buttenschøn, 2007). All mentioned species are capable of seedling growth in light-limited environments and they can outcompete early, more light-demanding species such as silver birch and willow (Bakker et al., 2004). Beech was the late successional species with the highest total relative abundance in the Exmoor's diet of '2%' and presence in all period except December 2 (Figure . 3). Ash and alder were only present in the periods December 2 and January, respectively, with a total relative abundance of '1%' for ash and '0.5%' for alder for the entire study period. For the Exmoor-ponies to have a significant effect in regards to causing crown dieback of these late-successional species, debarking is the most effective behaviour to achieve this (Cornelissen & Vulink, 2001; Kuiters et al., 2006; Vermeulen, 2015). Debarking may potentially impair growth, and could result in partial or total crown dieback eventually changing forest structure (Kuiters et al., 2006; Vermeulen, 2015). As this study was based upon macroscopic analysis of faeces and DNA metabarcoding, it is not possible to distinguish between whether the Exmoor-ponies foraged on the tree leaves, branches or debarked. For this to have been possible, visible observations in the field could have been included throughout the period. For one week prior to the study period (in November) and in December 1 and 2, visual observations were performed in different time intervals in the period from 8 a.m. to 16 p.m. During these observations, no debarking was observed and browsing on twigs or leaves were only observed a total of 14 times during the entire observation period (data not shown). Furthermore, trees with signs of debarking were not observed in the entire nature area. For this reason, it is reasonable to argue that the results of the DNA metabarcoding identifying different woody

species/genera in the diet of the Exmoor-ponies can be a false positive of the assumption that the ponies are foraging directly on the tree leaves, twigs, branches or bark. During the study period, it was noted that plenty of leaf litter were lying on the ground and there is thus a potential risk that the intake of woody species originates from the horses ingesting leaf litter when grazing and not because they are directly browsing on the trees. If this is the case more than direct foraging on trees, the potential effect that the ponies can have on the forest succession is considerably limited.

Linnartz & Meissner (2014) stated that horses love to debark species such as poplars (*Populus* spp.), willows and beech. However, studies by Kuiters et al., (2006) and Klich (2017) accentuated that not much is known about horses foraging on woody vegetation. Klich (2017) showed that only nine out of 20 possible tree species were debarked by horses in Głogów Forest District, Poland, with willow being the most debarked species. Kuiters et al. (2006) studied debarking of beech by free-ranging horses and found that beech was much more susceptible to bark-stripping than any other tree species occurring in the area of the Veluwezoom National Park, Netherlands. Other common trees in the research site, such as common oak, silver birch, and scots pine showed no signs of debarking. Both studies showed a correlation between trees debarked and diameter at breast high (D.B.H), concluding that horses have a tendency to select for younger individuals. Due to the former management practice of the Rewilding Mols area and the general prioritization on the open grassland biotopes, not much disturbance has happened previously in the beech dominated forest stands of the nature area. It is therefore reasonable to assume that the age of the beech trees and possibly other late successional species are predominately of an older age and that the chances of debarking are thus limited. Kuiters et al. (2006) suggest that without debarking horses, it would be expected that beech as a shade-tolerant and long-lived species would increase in dominance. However, as the Exmoor-ponies browse on young stands of the shade-tolerant and long-lived species they may reduce their regeneration and prevent any increase in their overall distributional range.

Rowan (*Sorbus aucuparia*) and fir (*Abies* spp.) were also debarked to some extent by the horses in the studies by Kuiters et al. (2006) and Klich (2017), respectively. Both these species/genera are present in the rewilding Mols area but was not foraged by the Exmoor-ponies in this study. Studies show that horses have the capacity to adapt to debarking of trees (Buttenschön, 2007). For this reason, studying the development in debarking of the Exmoor-ponies in the Rewilding Mols area over the next decade could show an increase in debarking and thus potential for retrogressive succession.

## Conclusion

This study showed an increase in the relative amount of woody vegetation in the diet of Exmoor-ponies reintroduced to the nature area of Rewilding Mols at The Mols Laboratory as a rewilding management tool peaking in January with 35.2%. This increase occurred even though very little snow cover limited the ponies' preferred diet of grasses. However, other studies have shown that woody vegetation constitute up to 50% of the diet of horses during winter time causing the relative amount of woody vegetation foraged in this study to seem limited. This study suggests that the Exmoor-ponies are able to keep the open biotopes open. This is based on their forage of common heather (*C. vulgaris*) and early successional species such as silver birch (*B. pendula*), willow (*Salix* spp.), and especially common oak (*Q. robur*). The limited foraging on late successional species such as beech (*Fagus* spp.), common alder (*A. glutinosa*) and common ash (*F. excelsior*) and the lack of evidence of debarking suggest that Exmoor-ponies are not capable of causing crown dieback in the established forest stands of the Rewilding Mols area. Together with the limited intake of woody vegetation compared to reports from other studies and limited forage on late successional species, this study concludes that the Exmoor-ponies cannot cause retrogressive succession under current winter weather scenarios and general availability of food resources. However, due to evidence of adaptation to debarking and as population size in the area increase, it is not possible to conclude if the Exmoor-ponies will continue to have limited effect on the forest dynamics. Further studies should therefore be carried out on the Exmoor-ponies in

regards to this question and many others revolving around the new science of rewilding as a management practice.

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## Author Contributions

Field work, laboratory analysis and script were performed by Marie Hagstrup. Macroscopic analysis, DNA-extraction, PCR and sequence analysis were performed by Mie BechLukassen. Dan Bruhn, Kent Olsen and Marie Hagstrup, CinoPetoldi elaborated and contributed to writing the manuscript

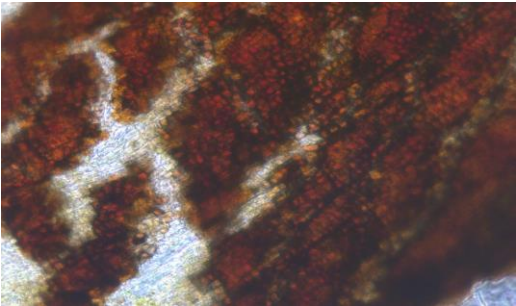
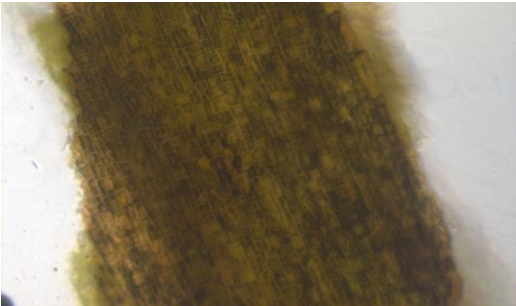
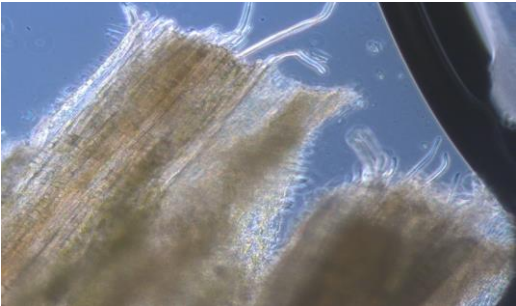
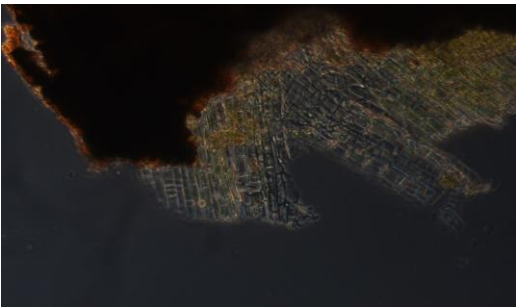
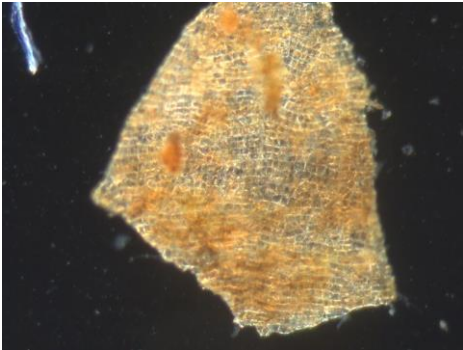
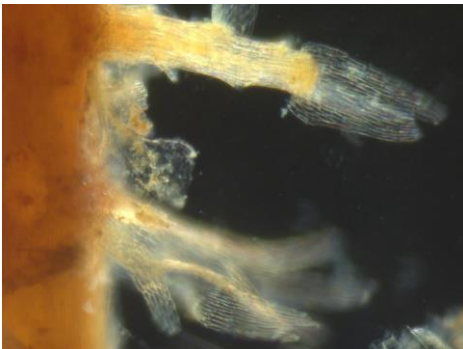
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Appendix 1: Reference plants

Woody species	
Reference plant	Faeces
   	 



Non-woody species	
Reference plant	Faeces
   	  

Appendix 2: Abundance of species/genera



**Figure 5.**Development in abundance of species/genera ingested throughout the study period. The y-axis shows the abundance of species/genera and the x-axis shows the four periods: December 1 (11.12.17-15.12.17), December 2 (20.12.17-22.12.17), January (15.01.18-19.01.18), and February (05.02.18-09.02.18). Woody species are coloured dark blue and non-woody species are coloured light blue.