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Meta-barcoding reveals high contribution of shrubs and trees in the diet of the European Bison (*Bison bonasus*) on Bornholm, Denmark.

- Local adaptation or global misconception?

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Meta-barcoding reveals high contribution of trees and shrubs in the diet of the European bison (*Bison bonasus*) on Bornholm, Denmark – Local adaptation or global misconception?

Titel / undertitel:
Meta-barcoding afslører en høj andel af træer og buske i den Europæiske bisons (*Bison bonasus*) føde på Bornholm, Danmark – Lokal tilpasning eller global misforståelse?

Subject description:
This study investigates the food preferences of the European bison (*Bison bonasus*) under semi-natural conditions in Almindingen, Bornholm. Through meta-barcoding of dung samples the items consumed are mapped and analysed in order to assess the impact of the bisons on the nature they inhabit.

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ABSTRACT

Food preferences of the European bison (*Bison bonasus*) have only been studied inside the old habitats of the Białowieza forest and the Caucasian Mountains. A successful restoring of the endangered species and the increased interest in the bison in a management perspective has lead to their introduction in several new habitats across Europe.
In 2012, seven European bisons were introduced to an enclosure in Almindingen on Bornholm, Denmark, as a contribution to the restoration of the natural dynamics of the woodland. The intention was to establish favourable environments for increased biodiversity in which the bison would assist by creating a more open forest.
Dietary preference and niche partitioning play important roles in terms of how grazers will contribute to the shaping of their habitat. However, the diet preferences of the European bison outside the old habitats are yet to be explored.
This study was set to investigate the food preferences of the European bison in Danish nature. Through the novel approach of meta-barcoding, I examined the plant content in 39 dung samples collected from the bisons on Bornholm from June to August 2015. I identified 6 new species and 25 new genera that have not been identified in the bison diet before. Incorporating vegetation data from the enclosure exposed a positive selection for several graminoids that are not frequently selected by the existing wild and domesticated herbivores in Danish nature.
Unexpectedly, the results revealed a high proportion of trees and shrubs (48.6-51.9 %) in their summer diet. This is inconsistent with previous studies stating that the European bison predominantly relies on herbaceous vegetation in the vegetative season. These results could therefore be a sign of diet plasticity, reflecting their ability to adapt to new environments and may indicate that the bison's impact on forest openness could be more substantial than previously assumed. As the open forest is identified as a valuable habitat for many endangered species, the European bison may thus prove a valuable instrument in conservation ecology.

Keywords

Food preferences • Diet plasticity • European bison • *Bison bonasus* • Ecosystem engineer • Meta-barcoding • trnL

RESUMÉ

Hidtidige studier af den europæiske bisons (*Bison bonasus*) fødepræferencer er udelukkende blevet foretaget i Białowieza-skoven og i Kaukasus-bjergene.
En succesfuld reetablering af den truede art samt en øget interesse for bisonen som naturplejer har medført, at de introduceres til adskillige nye habitater rundt om i Europa.
I 2012 blev syv europæiske bisoner udsat i en hegning i Almindingen på Bornholm, Danmark, i et forsøg på at reetablere skovens naturlige dynamikker. Hensigten var at skabe en mere lysåben skov, hvorved bisonen ville bidrage til at skabe mere gunstige forhold for en øget biodiversitet.
Fødepræferencer og udnyttelse af forskellige nicher spiller en vigtig rolle for hvorledes en græsser påvirker vegetationen i sit habitat. Til trods for dette er bisonens fødevalg endnu ikke blevet udforsket udenfor de gamle habitater.
Dette studie undersøger den europæiske bisons fødevalg i den danske natur. Ved anvendelse af meta-barcodning, en ny genbaseret teknologi, undersøger jeg planteindholdet i 39 fækalieprøver, der er indsamlet fra bisonerne på Bornholm fra juni til august i 2015.
Prøverne indeholdt 6 nye plantearter og 25 nye planteslægter, der ikke før er blevet identificeret som bisonføde. Vegetationsdata fra indhegningen afslørede i sammenhold med fødeanalysen en positiv selektering for græsser, der ellers sjældent indtages af de øvrige ungulater i Danmark.
Mod forventning viste resultaterne, at bisonens sommerføde indeholdte en høj andel af træer og buske (48,6-51,9 %). Dette er i modstrid med tidligere studier, der angiver at bisonens føde i den vegetative sæson primært består af græsser og urter. Resultaterne fra dette forsøg kunne være et tegn på bisonens evne til at tilpasse sig til nye fødeemner, og kunne foruden indikere at bisonen bidrager til at holde skoven åben i større grad end før antaget.
Adskillige truede arter er knyttet til de åbne skovområder, og bisonen kunne derved vise sig at være et værdifuldt værktøj i naturbevarelse.

Nøgleord

Fødepræferencer • Fødeplasticitet • Europæisk bison • *Bison bonasus* • Ecosysem engineer • Meta-barcoding • trnL

CONTENTS

PREFACE

This 45 ECTS-point MSc project investigates the food preferences of the European bison (*Bison bonasus*), a novel contribution to Danish nature management practices.
Based on meta-barcoding technology, the food preferences of the bison population on Bornholm, Denmark are examined. The results are discussed in relation to previous findings concerning use of habitat, diet preferences and feeding plasticity. Finally, the impact of the European bison on forest openness is evaluated.

The thesis is written as an article and therefore this part does not provide details of methodology nor the initial experimental work, in order to present a successful laboratory protocol.
The reader is referred to the section Supplementary methods*,* to gain a better understanding of the underlying experimental work and methods applied in the study. The section includes both the experimental background, the experimental results and the subsequent considerations that determined the methodology applied. Furthermore, details of the sampling regime, success in amplification, taxonomic assignation and calculation of Electivity index (D) can be found in the Supporting material section.

There are many people without whom this thesis would not be presented in the way it is today.
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INTRODUCTION

The European Union declared an intention to actively halt the decline of biodiversity before 2020 (European Commission 2011). Biodiversity (or biological diversity), defined as "*the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part: this includes diversity within species, between species and of ecosystems"* (UN Rio Convention, 1992), is experiencing unprecedented losses due to a variety of causes including shift in land use and rapid degradation of natural areas. In a Danish report assessing conservation strategies, the forest was identified as a key target for biodiversity conservation. To ensure habitats for endangered species, an approach to restore or resemble the natural dynamics of the forest prevails (Petersen et al. 2012; Natur- og Landbrugskommissionen 2013).

Large herbivores are suggested to contribute to a balanced and more diverse ecosystem, assisting in the creation of a more open forest (Svenning 2002; Vera 2000). In May 2012, seven European bisons (subspecies *Bison bonasus bonasus*) were introduced to an enclosure in Almindingen on Bornholm. The purpose was to create a more dynamic and open forest (Brandtberg & Dabelsteen 2013), and thus increase the potential for increased biodiversity.
The reintroduction of the European bison is one of the biggest rewilding projects in Denmark (2016). Critics question the benefits from such projects and raise concern of the possible consequences of introducing new species (Nogués-Bravo et al. 2016). However, in order to meet the EU's 2020 goal, questions on whether the current composition of grazers can deliver the variation to the extent desired have been raised (Buttenschøn 2007). The conflict outlines the importance of gaining more knowledge about the effect and possibilities of the European bison in a nature management perspective.

The European bison, also known as the wisent, is Europe's largest terrestrial mammal. 11,500 years ago, it populated most parts of Denmark and Northern Europe (Aaris-Sørensen 1998). The population suffered a drastic decline as a consequence of hunting activities and fragmentation of natural habitats. The final extinction of the last wild-living populations occurred in the Caucasian mountains in 1927 (spp. *caucasicus*), and in 1919 in Poland for the lowland bison (spp. *bonasus*). The existing population of European bisons of today was restored from 54 animals that had survived in zoos and breeding centres. These originated from twelve individuals, of which only seven (4 males and 3 females) were pure Lowland bisons (spp. *bonasus*). The first European bison was re-introduced into the wild in 1952 in the Białowieza forest, Poland (Cabon-Raczynska et al. 1987; Krasińska and Krasiński 2013).
Today, the number of animals exceeds 5,500 spread across most of Europe. More than 3,500 animals range freely or under semi-natural conditions in 9 different countries (Raczynski, Jan; Bolot 2014). Though the population of the European bison has been successfully restored from a historical bottleneck, the population is, until this day, at risk, as genetic diversity remains low (Tokarska et al. 2011; Karbowiak et al. 2014). The bisons in Almindingen therefore not only contribute to the shaping of the landscape, but also to secure the species.

Large herbivores (≥100 kg) are believed to have a profound effect on vegetation composition and structure and are argued to contribute to shaping the pre-agricultural landscape. Several large herbivores foraged the northern part of Europe in the early Holocene (Aaris-Sørensen 1998; Svenning 2002). The primeval forest contained habitats of closed forest, semi-closed woodlands, moors, grassland and meadows. However, the extent to which the open nature types were present is not agreed upon. The traditional view of the landscape covered by closed forest is challenged by claims that the vegetation would be opened by the maintenance of storms, fires and

the impact of large herbivores (Svenning 2002; Vera 2000; Bradshaw & Mitchell 1999; Mitchell 2005; Birks 2005).
The possible effects of large herbivores on present-day landscapes are limited, as just a few species remain in the wild. Today, only red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) are considered large, free ranging herbivores in Denmark (Aaris-Sørensen 1998), and most grazing is undertaken by domesticated animals, predominantly cattle (Buttenschøn 2007). Each grazer provides a unique set of characteristics that shape the environment of which they inhabit with a significant impact on structure, plant species composition and productivity by creating spatial and temporal heterogeneity and by modulating succession (Vera 2000; Hester et al. 2000; Gordon & Prins 2008; Mitchell & Kirby 1990; Huntly 1991; Hobbs 1996).
To try and resemble past ecosystem dynamics, novel contributions to Danish management practices include the reintroduction of past-natural large herbivores, such as the European bison.
As main mechanisms, large herbivores affect plant communities by selective feeding behaviour, trampling, deposition of urine and faeces, and zoochorous seed dispersal (Gordon & Prins 2008; Hester et al. 2000). For a European bison with a high body mass (males: 580-920kg, females: 320-640kg) their trampling has a profound impact on fracturing the vegetation. This increases the number of vegetation gaps that provide habitats for species' niches to occur by natural succession (Gordon & Prins 2008; Krasińska & Krasiński 2013). In addition, the bison's dust baths also contribute to gaps in the vegetation (Krasińska & Krasiński 2013).

Selective feeding of the animals can lead to changes in plant species composition. Preferences for dominant species and tall growth often have the greatest effect on species composition, as the competitive interactions between the plant species are reduced, which allows for greater coexistence (Gordon & Prins 2008). This gives room for more light-demanding species, upsurge mosaic structures, and subsequently creates favourable environments for increased biodiversity.
The feeding behaviour is believed to be not only controlled by preference, but also influenced by inter- and intraspecific competition, plant community structure, and plant defences (Gordon & Prins 2008; Danell et al. 2006; Hester et al. 2006). Likewise, supplementary feeding (Kowalczyk et al. 2011; Krasińska et al. 2000) and thread avoidance (Brandtberg & Dabelsteen 2013; Brown et al. 2012) effect behavioural patterns, and subsequently the foraging patterns (Krasińska & Krasiński 2013).

Studies that map the food preferences of the European bison have been collected in the Polish part of the Białowieza forest (Borowski & Kossak 1972; Kaminski et al. 2010; Kowalczyk et al. 2011; Gębczyńska et al. 1991), the Belarusian part (Korochkina 1969) and central Caucasus Mountains, Russia (Kaz´min & Smirnov 1992).
In the Białowieza Forest, the bisons selectively feed on *Rubus idaeus* and *Milium efusum* and, relative to their abundance, avoid *Anemone nemorosa, Ranunculus repens, Fraxinus excelsior, Tilia cordata,* and *Acer platanoides*. Several species were identified as main food sources (Appendix 1). However, these were not compared to their level of abundance in the studies of concern (Borowski & Kossak 1972; Kaminski et al. 2010; Kowalczyk et al. 2011; Gębczyńska et al. 1991; Korochkina 1969; Krasińska & Krasiński 2013)
The bison's main food sources in the Caucasus Mountains are recognized to be: *Festuca rubra, Rubus plicatus*, shrubs, and bark in the non-vegetative season (Kaz´min & Smirnov 1992). Though similarities between studies occur, food preferences differ between locations and could reflect local adaptation and dietary plasticity of the European bison.

Feeding plasticity is known from *B. bonasus*' close relative the American bison (*Bison bison*), and several other herbivores (Rayé et al. 2011; Suryawanshi et al. 2010; Waggoner & Hinkes 1986).
The ability to adjust to the forage available could be especially important for herbivores in sea-

sonal environments, and possibly contribute to their adaptability to new habitats.
Plasticity of the European bison is expressed in the assumed temporal variation towards a more wood dominated diet in the winter.
Bisons are ruminants, allowing an efficient absorption of energy from the available diet, but also prolonging the gastrointestinal transit time, when less digestible items are consumed. This constrains the amount of woody material in a ruminant diet (Janis 1976; Van Dybe, G.M.; Brockington, N.R.; Szocs 1980; Breymeyer, A. I. ; Van Dyne 1980). When compared to domestic cattle, the European bison digests lignin better which is suggested as an adaptation to woodland habitats (Kowalczyk et al. 1976). Although recognised as an intermediate feeder in winter, the European bison is generally considered a grazer (Krasińska & Krasiński 2013; Hofmann 1989; Gębczyńska et al. 1991; Jędrzejewska & Jędrzejewski 1998). However, the classification of herbivores into grazers, browsers, and intermediate-feeders often implies that herbivores shift their diet along a grazer/browser continuum in response to environmental factors (Hofmann 1989). The extent to which bison are believed to browse differ between studies. In the vegetative season, the documented contribution from browsing constitutes between 7-33% of their diet (Jędrzejewska & Jędrzejewski 1998; Wróblewski 1927; Kaminski et al. 2010; Borowski & Kossak 1972). Depending on the availability of herbaceous vegetation and extent of supplementary feeding, the diet consists of 16-79% woody material during the winter (Kaz´min & Smirnov 1992; Kowalczyk et al. 2011). In a recent study by R. Kowalczyk et al. (2011), the bison went from being predominantly browsing when not fed to browsing very little when heavily fed. The supplementary feed consisted of packed silage made of natural meadow vegetation, supplemented with hay, or open access to agricultural areas.
Since the reintroduction in Białowieza forest, the bisons have been winter fed as a part of the management practice (Krasińska & Krasiński 2013) which could possibly influence the results of studies on bisons' winter diet.

The browsing behaviour of the European bison is believed to inhibit succession of trees in forest gaps (Vera 2000; Kuijper et al. 2009). By browsing, stripping bark off trees and feeding on seedlings and saplings, the bison functions as a bioengineer and is considered a keystone species in natural grazing systems (Kuijper et al. 2010; Vera 2000). The extent to which the European bison browses will reflect its contribution to achieving a more open forest. The strong influence of supplementary feeding on the browsing behaviour has raised questions of the natural feeding habits of the bison.

Study objectives

The bisons in Almindingen are watched carefully and the effect of their activities on the area is closely monitored.
Floristic registrations are conducted every other year to monitor any developments (Jønsson 2014). However, vegetative analyses to describe the effects of herbivores' presence often suffer from a time lag (Kuijper et al. 2010). Based on dietary analysis, this study aimed to explore the niches of the European bison in Danish nature.
To my knowledge, studies on the bison's food preferences have not been conducted outside the aforementioned areas.

The Bornholm bisons have already shown different patterns of habitat selection (Møller Pedersen & Bech Stensgaard 2015; Brandtberg & Dabelsteen 2013), which is likely to affect food preferences. This study investigates if the local environment influences their choice of food, and

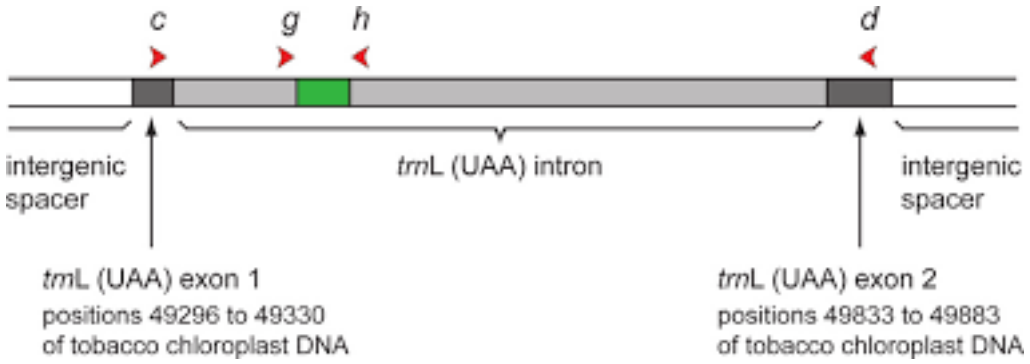


Figure 1. Position of the primers *c*, *d*, *g* and *h* on the chloroplast trnL (UAA) gene. The region amplified with primer *g* and *h* is indicated in green (Taberlet et al. 2007).

monitors possible annual variations in diet composition. Information on temporal variation and differential resources can improve our knowledge of the use of the bison in management practices and their impact on forest openness. The results of the study are discussed in context of vegetation dynamics and the potential of the European bison as a bioengineer in Danish nature. Finally, the applied method and its feasibility in dietary studies is evaluated.

Approach

Previous studies on the bison's diet have investigated foraging traces (e.g. bark stripping or bites on vegetation) (Borowski & Kossak 1972; Hofman-Kamińska & Kowalczyk 2012; Brender 2016), by direct observation (Borowski & Kossak 1972), microhistological fecal analysis (Waggoner & Hinkes 1986), macroscopic ruminal analysis ((Gębczyńska et al. 1991), and recently by DNA meta-barcoding analysis (Kowalczyk et al. 2011). Alternative methods could include sprouting of dung pads and stable isotope analysis of dungs, but to my knowledge this has not been applied for diet assessments of the European bison.

In this study, meta-barcoding Ilumnia Miseq-based second generation sequencing is employed, to identify food objects in fecal samples. This method has the advantages of being non-invasive, time efficient, and has the ability to process a large amount of data. The accuracy and complexity of the method makes it ideal for monitoring diet shifts (De Barba et al. 2014). To my knowledge, this is only the second time this method has been applied to perform diet analysis of the European bison. In this study, a fragment of the chloroplast trnL intron (Taberlet et al. 2007) is amplified for identification of plant prey. The short region (g-h) was selected for amplification, in respect of the degraded sample material.
The application of this region have proved a convenient tool for estimating herbivore diet (De Barba et al. 2014).

MATERIALS AND METHODS

Study area

The study area is located in Almindingen forest on the island of Bornholm, Denmark (55.1031-55.1208°N, 14.9317-14.96355°E). The enclosure covers 200 ha and fosters a species rich plant assemblage.

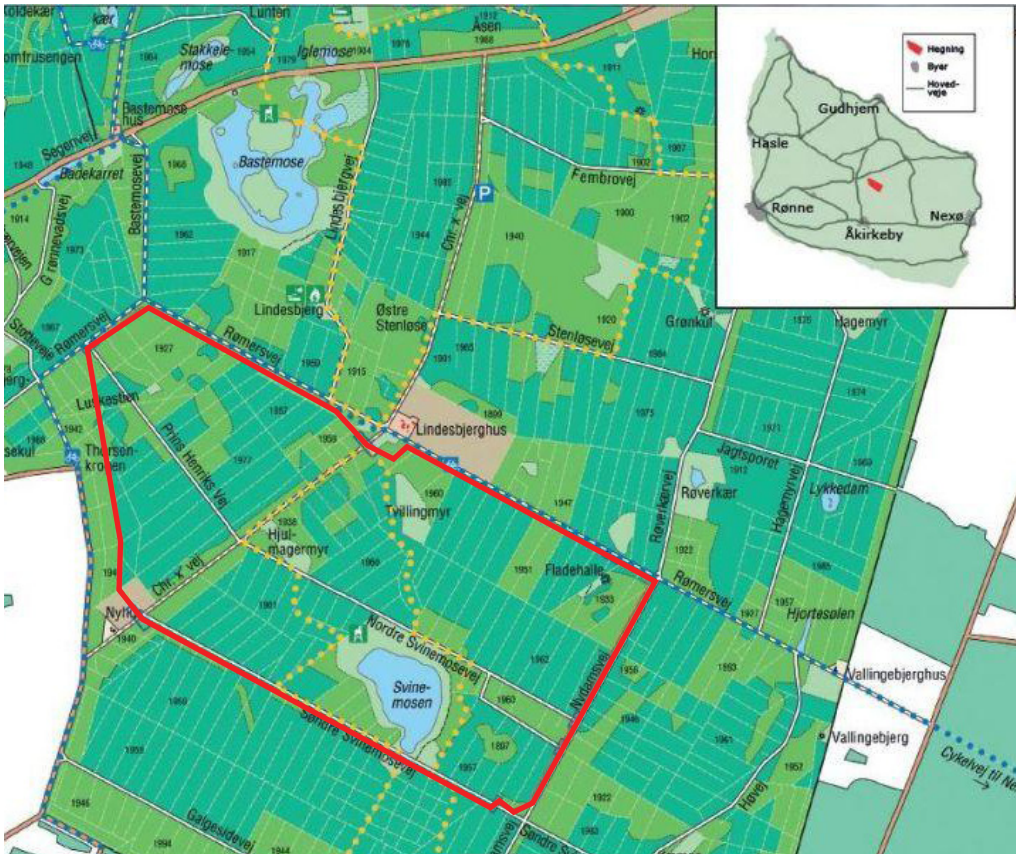


Figure 2. The enclosure in Almindingen. All roads are open for pedestrians and bikes. Smaller dirt paths are marked in yellow dots. Chr. X's vej is an unpaved road open for cars.
(Photo by NST 2015).

The forest within the bison-enclosure is characterized by large areas of even-aged stands, predominantly Norway spruce (*Picea abies*). Trees harvested in the forest are primarily Norway spruce, Oak (*Quercus* sp.) and Beech (*Fagus sylvatica*) (Orbitt 2015). The ground vegetation and regeneration in the forested areas is scarce and mostly appear in ruts and smaller glades (Jønsson 2014).

Table 1. Main habitat types in the fencing characterised by species dominance. Others include roads, lakes, mixed conifers and mixed broadleaves. Based on data from Jønsson (2014).

Vegetation characteristic	Area (ha)	Coverage (%)
Alnus glutinosa and *Betula* sp.	3.04	1.52
Fagus sylvatica	12.41	6.20
Quercus sp.	30.40	15.20
Picea abies	94.57	47.29
Clear-cut	17.64	8.82
Meadow	11.17	5.58
Calamagrostis epigejos	1.52	0.76
Other	29.27	14.63

The open areas mainly consist of newly exposed soils following clear-cuts. These areas are dominated by different species of bryophytes: *Rumex acetocella, Deschampsia flexuosa, Senecio sylvaticus* and to a lesser extent regeneration from the surrounding forest (NST Bornholm 2014).
The largest coherent area of meadow is placed centrally in the enclosure. The ground is moist and fosters a variety of species only sporadically found in the rest of the area. In the year of sampling (2015) a high abundance of orchids (Orchidaceae) was observed in this area.

The climate is temperate with clearly marked vegetative and non-vegetative season. The coldest month is February (mean temperature -0.3°C) and the warmest is August (16.7°C). The annual mean temperature is 7.9°C and the annual rainfall is 609mm/year (DMI 2016).
Almindingen is inhabited by two other ungulates: fallow deer (*Dama dama*) and roe deer (*Capreolus capreolus*), which are not inhibited by the bison-fence. There are no large predators on Bornholm (Orbitt 2015).
The area is open to the public and receives approx. 100,000 visitors per year, mainly in July and August (Marcussen 2015).

Full habitat exploration and successful breedings suggest that the animals thrive in the enclosure (Orbitt 2015; Møller Pedersen & Bech Stensgaard 2015). In 2015 (year of sampling) the population reached 17 animals in the middle of June. Due to a high number of new-born calves in 2015 public access was limited to guided tours from 20/06-21/07 in the year of sampling.
The animals are winterfeed when availability of food is considered insufficient. Winterfeeding did not occur before end of sampling 2015. Lick stones have been placed in the fencing to provide salts and minerals for the animals (Orbitt 2015). The bison's water from natural resource's and have so far received no medical care at Bornholm.
In August (2015) two adults and one calf suffered from liver fluke (*Fasciola hepatica*) attack. Parasitic attack is not uncommon for the European bison, and the liver fluke is found in prevalent 44-50% and 100% in previous studies (Karbowiak et al. 2014). These attacks should not affect the results.

Sampling protocol

Fresh dung samples were collected from June-December 2015. Sampling was conducted over 3-6 consecutive days in the middle of each month. The sampling regime was chosen in order to ensure data was temporally independent, and to investigate temporal variations in diet composition. On a given day, the bisons were followed from dawn and prior to dusk, until dungs were collected or nightfall.
Degradation of dung was minimized by collecting shortly after observing defecation, or by judging dung freshness by dung temperature, appearance and colonisation by flies.
The external dung surface was excluded to limit potential contamination, and the sample collected using the interior of a plastic bag. One sample was composed by mixing subsamples taken from different parts of a single dung pile. Samples were immediately placed in a cooling bag for the duration of no more than 5 hours and subsequently frozen at -18 °C. When returned to the laboratory, the samples were stored at -80 °C until DNA extraction. In total, 133 faecal samples were collected from the bisons.

95 samples were selectively chosen for extraction. The samples were selected to limit pseudoreplication both spatial (i.e. collection site within the bison enclosure) and temporal (i.e. within sampling week), though adult bisons will only defecate once per day (Krasińska & Krasiński 2013). All samples that by appearance possibly originated from calves or ill individuals were excluded, as they might express different preferences due to an altered physical state.

Laboratory procedure

Extraction
All extractions, and initial PCR steps were conducted at Department of Geosciences and Natural Resource Management, Frederiksberg.
Three extraction kits were tested based on methods from similar studies or kits designed for fecal extraction (De Barba et al. 2014; Kowalczyk et al. 2011). The quality of the final eluates were evaluated on NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, USA) and post amplification on agarose (1.5%) gel electrophoresis in different dilutions. The kit that retained the best quality of DNA was selected for further optimization (see; *Supplementary methods*).
Samples were transferred by disposable spatula to 1,5 ml safe-lock Eppendorf tubes. Subsamples taken from one sample constituted the 0.0326-0.0344 g used for each extraction. All extractions were performed using DNeasy Plant Mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol for wet material. The protocol was further optimized to accommodate high carbohydrate content and other contaminants, as well as to effectively lyse the cells. The TissueLyser procedure was modified by an additional freezing period of samples for 90 sec in liquid nitrogen in order to acchieve sufficient brittleness of the material subsequent mechanical disruption.
Final eluate was composed of 2x50 µl buffer AE centrifuged through the collection membrane. This modification was made to increase overall DNA concentration.
All extractions were preformed over a three week period and completed with a blank control extraction without sample to monitor possible contamination. Final eluate was stored at -18 C until amplification.

PCR amplification with target primers

Gradients of MgCl2 concentration, annealing temperature and modifications of the thermal cycle were tested to determine when amplification was most sufficient (see: supplementary material). The primer pair trnL-g (5´-GGGCAATCCTGAGCCAA-3´)/ trnL-h (5´-CCATTGAGTCTCTGCACCTATC-3´) is specific for a 10-143 bp sequence of chloroplast DNA, used as target region (Taberlet et al. 2007). Primers were Illumnia-tagged on the 5' to prepare samples for addition of Dual indices and Illumina adapters.

Amplifications were carried out in volumes of 25 µl using the Promega GoTaq DNA Polymerase kit. The manufacturer's protocol was followed adding 0,35 µl of Illumina tagged primer trnL g and h (10 M) (Taberlet et al. 2007), 2 µl Template DNA, 5 µl of Colourless GoTaq Reaction Buffer but with the addition of 2 µl MgCl2 and 5 µl Q-solution (Qiagen). All samples were conducted using an Applied Biosystem (Carlsbad, CA, USA) PCR system 2700 under the following conditions; initial denaturation 3 min at 95 °C, 35-40 cycles denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s and a final extension at 72 °C for 5 min.
For each round of extraction processed a sample with DNase/RNase free water was included as a blank control.

Representative samples from the months that did not give positive results were tested in dilutions of 1:1, 1:2, 1:5 and 1:10 (see: supplementary material)
PCR was verified with electrophoresis of 5 µl of product in 1.5% agarose in 0.5 TBE-buffer run at 100V for up to 60 minutes, and subsequently samples was stored at 4 °C. Samples with successful amplification and the least dimer were favoured in the selection of the final samples. All PCR reactions were duplicated and pooled afterwards. Only month that provided at least 10 verified samples/month were allowed for further analysis (June (12), July (13), August (14)). In total 40 samples, including one control extraction was sent to Section of Biotechnology (AAU, Aalborg, Denmark) for further processing.

PCR Clean-up, index and sequensing

The PCR-products were purified with Agencourt AMPure XP beads (Beckmann Coulter, CA, USA). The manufacturer's protocol was followed with a dry time included and the following exceptions: Sample/bead solution ratio was 4:5 (instead of 5:9) and 80% ethanol was used (instead of 70 %). Volume of elution buffer was increased to 52.5 µl to ensure sufficient contact with the beads.

Dual indices and Illumina adapters were attached using the Nextera XT Index Kit (Illumina, USA) following the manufacturer's protocol. A second clean-up step with Ampure XP was conducted. Deviations from the protocol were as described, only sample/bead solution ratio was 5:5.6 and elution buffer 27.5 µl. Twenty-five µl of the final eluate was transferred for library quantification. Representative subsamples were on a High Sensitivity D1000 Screentape to verify quality and size of the products (see Supplementary methods). Libraries were finally quantified and concentrations measured. All libraries were adjusted to equimolar concentrations before they were pooled.
The final pool was sequenced on a MiSeq (Illumina, USA), using a MiSeq reagent kit v3 (Illumina, USA). Final library loading concentration was 20 pM. A 20% PhiX library was included to serve as control for the low diversity libraries.

Sequence analysis and taxon assignation

All data was imported into NEBC Bio-linus (Field et al. 2006) and quality trimmed with NGS toolkit v. 2.3.3. (Patel & Jain 2012) for PERL programming. This included removal of sequences with ambiguous nucleotides, removal of residual sequencing adaptors and a minimum quality phred score of 30%. The dataset was screened for sequences with an occurrence < 2. Sequences were separated based on the unique indices introduced during the sample preparation. For each sample, reads with similar stretches of base pairs were locally clustered. The OUT-table was generated with QIIME (Caporaso et al. 2010).

Sequence duplicates were identified and data merged in the individual samples with an Excel VBA macro. Four sequences was detected in the extraction negative control with an occurrence of reads >14. As a consequence and to exclude sequences that were likely to occur from amplification errors or chimeras, sequences with occurrence lower then 20 were removed. Taxon assignation was archived using the Genbank reference database and their neucleotide BLAST tool. Each blast was evaluated and refined using local vegetation data (NST Bornholm 2014; Mossberg et al. 2007). The data set was then trimmed for non-plant taxon and taxon with no kinship to Bornholm at family level (Appendix 1). A quality trim was included identifying all sequence with a ID-score < 0.98 as "unknown". All taxonomic duplicates were merged, sequence differences regardless.

All supplementary data for the taxon are derived from Den Nye Nordiske Flora (Mossberg et al. 2007).

Statistical analysis

To offset the influence of differences in degradation rates, age and plant material all data models are based on presence/absence data (Nichols et al. 2016).

A multiple correspondence analysis (MCA) was conducted which allowed me to investigate the influence of several qualitative parameters. The geometrical presentation was set to evaluate samples (rows) in relation to the present/absence data of the 64 species detected (columns). The matrix created was then presented in multiple graphical displays (dimensions) ordering from most to least explicative. Only the two most explicative dimensions are displayed in the figures in the result section, the x-axis corresponding to the most explicative.

The parameters tested in this were; date of sampling, habitat of sampling, months of sampling and date of extraction.

The MCA was performed in R v. 2.3.4 (R Development Core Team 2011) with FactoMineR v. 1.33 (Lê et al. 2008).

Following setting was applied; Active variables; species, Supplementary variables: date of collection, date of extraction, habitat of collection, month of sampling and the Continuous Supplementary variable; Sample ID.

The bison's selection for graminoid vegetation was calculated using Ivlev's electivity index (modified by Jacobs 1974); $D = (r-p)/(r+p-2pr)$, where r is the proportion of a given graminoid in the samples among total graminoid identifications in samples, and p is the proportion of a given graminoid present in the enclosure. D range from 1 (strong positive selection) to -1 (strong negative selection), with 0 corresponding to random selection. Presence/absence data from Data from NST Bornholm (2014) was used for calculation of species relative abundance (n=1000), and calibrated according to habitat occurrence (Jønsson 2014).

RESULTS

Table 2. List of diet objects identified within B. Bonasus dungs (n=39). Functional groups are identified according to Mossberg et al, 2014. Identification refined using local vegetation data are highlighted in bold.

Functional group	Family	Sequence identification	No. of dungs in which found	Sequence similarity (%)*
Graminoids	Cyperaceae	*Carex* sp.	1	100
	Juncaceae	*Juncus* sp.	4	100
		Luzula sp.	1	100
	Poaceae	*Calamagrostis* sp.	39	100
		Dactylis glomerata	34	100
		Deschampsia flexuosa	39	100
		Glyceria sp.	2	100
		Phragmites australis	36	100
		Poaceae	20	99/100
	Sparganiaceae	*Sparganium* sp.	6	100
	Typhaceae	*Typha* sp.	20	100
Herbs	Apiaceae	Apiaceae	1	99
	Asteraceae	Asteraceae	33	99/100
	Boraginaceae	*Myosotis* sp.	1	100
	Caryophyllaceae	*Stellaria* sp.	1	100
	Dryopteridaceae	***Athyrium filix-femina***	6	100
	Euphorbiaceae	Euphorbiaceae	6	98/99
	Fabaceae	*Lathyrus pratensis*	36	100
		Lotus corniculatus	25	100
		***Lotus* sp.**	5	100
		Trifolium sp.	38	99/100
	Lamiaceae	Lamiaceae	1	100
		Mentha sp.	1	100
	Lythraceae	***Lythrum* sp.**	1	99
	Onagraceae	*Chamerion augusti-folium*	17	100
		Epilobium sp.	2	100
		Onagraceae	17	100
	Orchidaceae	*Epipactis* sp.	1	100
	Oxalidaceae	***Oxalis acetosella***	29	100
	Plantaginaceae	*Plantago* sp.	26	100
		Veronica chamaedrys	3	100
	Polygonaceae	*Persicaria* sp.	10	100

(Table 2 - continued)

Functional group	Family	Sequence identification	No. of dungs in which found	Sequence similarity (%)*
		Rumex sp.	22	100
	Primulaceae	*Lysimachia* sp.	39	98/100
	Ranunculales	*Ranunculus* sp.	3	100
	Rosaceae	*Alchemilla* sp.	5	100
		Filipendula ulmaria	28	99/100
		Filipendula vulgaris	12	98
		Potentilla anserina	21	100
		Potentilla sp.	4	98/99/100
	Rubiaceae	*Galium* sp.	2	100
	Scrophulariaceae	*Melampyrum* sp.	9	99
	Solanaceae	Solanaceae	1	100
	Urticaceae	*Urtica* sp.	1	99
	Violaceae	*Viola* sp.	2	100
Shrubs	Caprifoliaceae	*Lonicera* sp.	9	100
	Ericaceae	*Vaccinium* sp.	6	100
	Grossulariaceae	*Ribes* sp.	11	99/100
	Rosaceae	*Rubus idaeus*	39	100
Trees	Adoxaceae	*Sambucus* sp.	8	100
		Viburnum opulus	1	100
	Betulaceae	*Alnus* sp.	29	98/100
		Betula sp.	35	98/100
		Betulaceae	1	99
	Fagaceae	***Fagus sylvatica***	11	99/100
		Quercus sp.	39	100
	Oleaceae	Oleaceae	5	100
	Pinaceae	*Abies* sp.	1	100
		Picea sp.	34	99
	Rosaceae	*Crataegus* sp.	32	100
	Salicaceae	*Populus* sp.	2	100
		Salix sp.	38	100
	Sapindaceae	*Acer* sp.	38	100
Undefined	Rosaceae	Rosaceae	1	98

**More than one Sequence similarity represents the different similarity scores from merged sequences post filtering (Supporting information 3).*

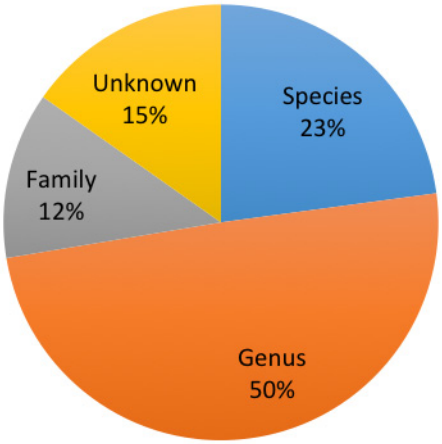


Figure 3. Proportion of sequences (n= 105) identified to a given taxonomic level using Genbanks BLAST tool and local vegetation data. Unknown represents all sequences with an ID-score < 0.98.

DNA from fecal samples of the European bison was successfully extracted with three different kits (Supplementary methods, Table 4). Initial quality of PCR products differed a lot between the different kits (Supplementary Methods, Figure 8, Figure 9).
Amplification of the trnL g-h region was successfully obtained in 56 (58.3%) of the 96 faecal samples selected for this study (Supporting information 2). Thirty-nine of the samples meet the criteria for further amplification described in Materials and methods. Of these 12 (30.8%) was collected in June, 13 (33.3%) in July and 14 (35.9%) in August.

The next-generation sequencing produced 1,876,321 reads. After the filtering processes 1,281,983 sequences remained, corresponding to 105 unique sequences. The database did not provide a positive match in 16 cases (ID-score < 0.98; i.e. unknown) (Supporting information 3). Of the remaining, 24 were identified to species level, 52 to genus level and 13 to family level (Figure 3). When similar taxonomic assignation was merged the residual 64 were distributed as follows; species 16 (25%), genus 39 (61%) and family 9 (12%).
Eighty-nine unique sequences constituted the final 64 identifications. Seven sequences were refined using local vegetation data (highlighted in bold, Table 2). The reference database provided a 100% match for 66 (74.2%) of the sequences. Fifteen (16.8%) were accepted with a 99% sequence similarity and the remaining 8 (9%) with a 98% match. Identifications ranged broadly on family level and derived from 36 different families.

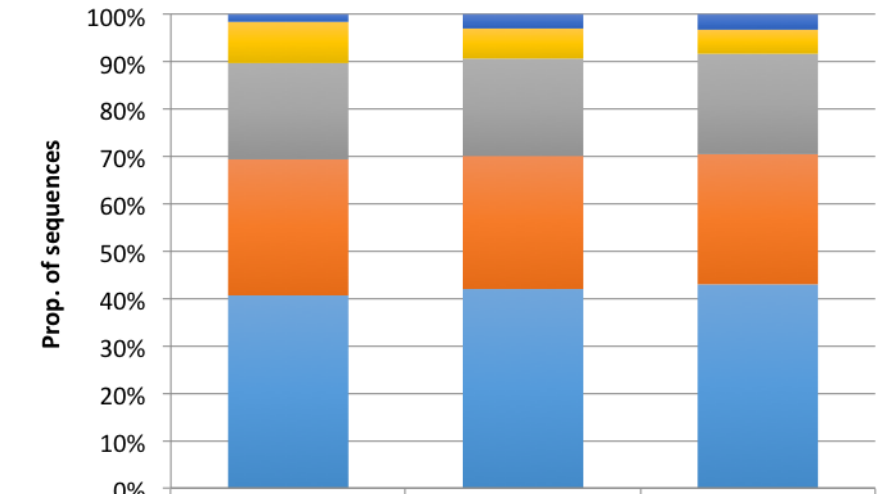
Table 3. List of families only registered in the Meta-barcoding analysis or the vegetation analysis conducted in the enclosure 2014.

	Family	No. of samples in which found	Presence in sample (%)
Fecal sample analysis using Meta-barcoding (n:39)	Apiaceae	1	2.6
	Orchidaceae	1	2.6
	Lythraceae	1	2.6
	Solanaceae	1	2.6
	Euphorbiaceae	6	15.4
	Sparganiaceae	6	15.4
	Grossulariaceae	11	28.2
Vegetation analysis using 5m circles (n:1000)*	Taxaceae	1	0.1
	Ruscaceae	3	0.3
	Menyanthaceae	8	0.8
	Brassicaceae	26	2.6
	Equisetaceae	112	11.2

** (NST Bornholm 2014)*

The mean of diet subjects per faecal sample was 25.3 (SD ± 3.7) and ranged between 15 and 32. DNA analysis successfully identified 14 taxon that have not been identified in the vegetation registration from 2012 and 2014. On family level Meta-barcoding revealed 7 new families in the enclosure. Some families found in relatively high abundance in the enclosure were not discovered in the genetic analysis. The trnL region for all taxon registered within these species were present in the Genbank database with the expectance of Ruscaceae (Polygonatum multiflorum).

The highest sum of sequence identifications was detected in August (335). The distribution on samples reviled August (23.9) to contain a lower mean of sequences/sample when compared to June (25.8) and July (25.5).



	June	July	August
Undefined	5	10	11
Graminoids	27	21	17
Shrubs	63	68	71
Trees	89	93	92
Herbs	126	139	144

Figure 4. Proportion of sequences identified to functional group in each sample, separated by collection month (June: n=12, July: n=13, August: n=14). Undefined represent identifications where sequences could not be assigned to a single group due to their taxonomic level. The functional groups are identified according to Mossberg et al., 2014.

All functional groups were found in the three months sequenced (Figure 4). A likelihood ratio test was performed to revel whether the months differed in proportions of different functional groups. This was done to see if the diet of the European bison shifted toward more woody material throughout the seasons. The test reviled no difference in the distribution of functional groups (P: 0.99).

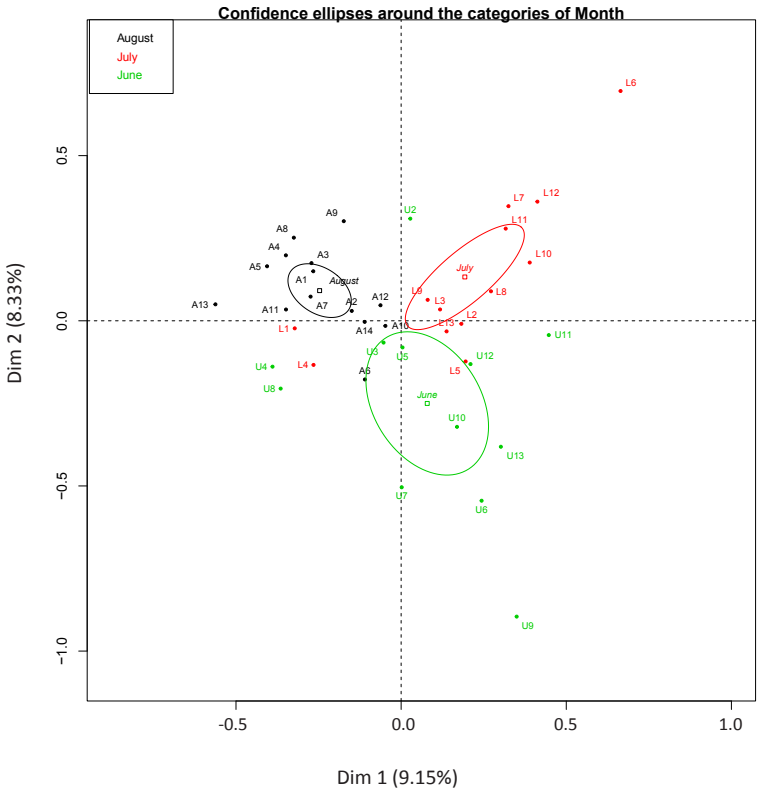


Figure 5. Comparison of content in samples between months on the first factor plane of the MCA. U represent samples from June, L samples from July and A samples from August. Circles represent the confidence ellipse (equivalent to a confidence interval) related to a particular month. First factor plane explains 17.8% of the total percentage of variance.

A multiple correspondence analysis (MCA) found a significant difference between the compositions of taxon in samples between months (Figure 5). Fluctuations of abundance of different species or genera are displayed in Figure 6, expressing switch between resources in the vegetative season.

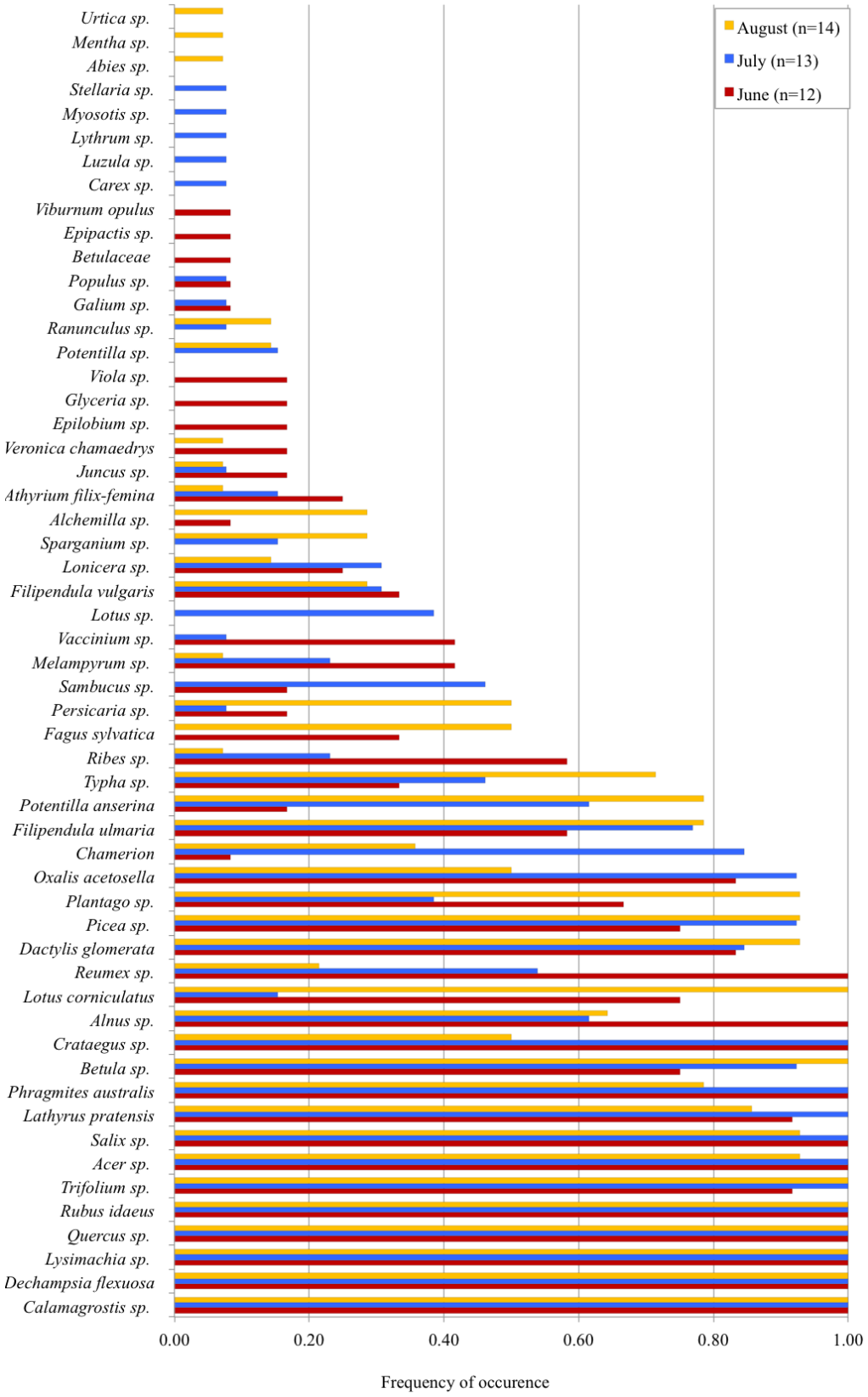


Figure 6. Rate of occurrence of species and genus's identified in dung samples from European bison separated by collection month.

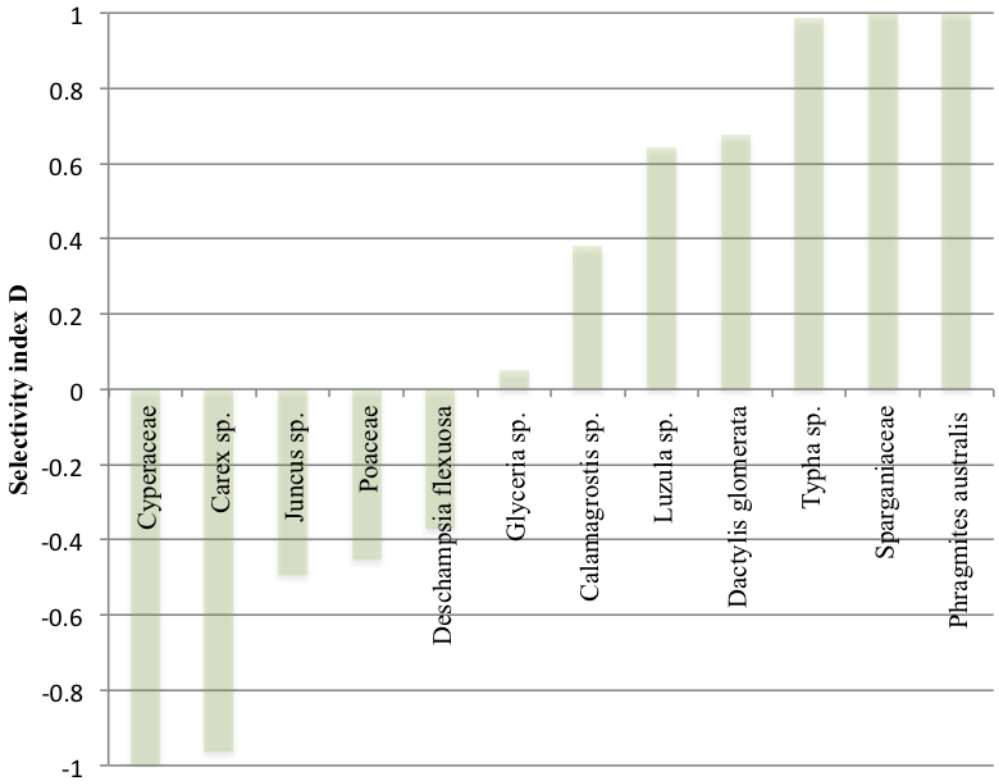


Figure 7. Ivlev's electivity index, modified by Jacobs (1974) for graminoids. One indicates a strong positive selection and -1 a strong negative selection. Zero indicates random selection (For details see Supporting information 4).

To investigate if the bison selectively feed on particular species, this study compared the relative fecal content of graminoids taxon to their relative abundance in the enclosure (Figure 7).
Three species were only registered in one dataset (i.e. meta-barcoding or vegetation analysis) and consequently expressed as selectivity very strong (1) or low (-1).

DISCUSSION

Woody bison

Data for this study was successfully obtained from samples collected from June to August 2015. The diet composition of the European bison is believed to shift towards winter and become more browse dominated (Krasińska & Krasiński 2013; Akimov et al. 2009; Gębczyńska et al. 1991; Cabon-Raczynska et al. 1987). Seasonal diet-shifts are known from other ungulates (Cederlund & Nystrøm 1981; Rayé et al. 2011; Suryawanshi et al. 2010) and express a convenient plasticity to a fluctuating availability of resources.
Yet for the European bison this have not been confirmed in macroscopical rumen content analysis by Gębczyńska (1991). As this study did not succeed in amplifying DNA from winter month samples it was unable to confirm this previous stated assumption. However the temporal development in successful amplifications between months (Supporting information 2) is likely to indicate a diet shift, possibly towards more lignified material. The phenolic compounds connected to lignin metabolism inhibit amplification and result in low quality DNA (Rachmayanti et al. 2009). This supports the diet-shift-hypothesis as the gradual decline in extraction quality could be explained by an increased contribution of woody substances.
The seasonal change reflects either a change in availability of preferred forage or a seasonal change in the quality of material (Suryawanshi et al. 2010). However a diet shift or its motivation cannot be confirmed without further analysis. Nonetheless this study detected an unknown factor gradually effecting the amplification as the vegetative season halts.

In the sequenced samples, the monthly contribution of trees and shrubs constituted 48.6 - 51.9% (Figure 4).
Compared with previous studies this is the largest proportion ever, to be documented in the vegetative season.
The result challenges the general assumption of the European bison as a grazer, but could also be an adaptation to the available food resources at Bornholm. When herbivores are moved to new locations, the animals gradually adapt to the new resources which occasionally express vast variations from items consumed in their previous habitat (Illius, A. W.; Gordon 1993).
However the use of different methodological approaches, could also explain inconsistencies between this and previous studies. Earlier studies assessing the diet composition relied on rumen content analysis (Gębczyńska et al. 1991), direct observation (Borowski & Kossak 1972), microhistological fecal analysis (Waggoner & Hinkes 1986) or investigating foraging traces (Brender 2016; Jønsson 2014; Borowski & Kossak 1972; Hofman-Kamińska & Kowalczyk 2012). The study objectives or the limited techniques available at the time of these studies did not foster data fit for quantitative analysis. Meta-barcoding has the advantage of being objective, taxonomically precise and able to process huge amounts of data. With the accuracy of the method, this study may be the first to document the true diet composition of the European bison in the vegetative season.

A meta-barcoding approach has previously been used to study the diet composition of the bison. Kowalczyk et al. (2011) used meta-barcoding to determine to what extent supplementary winter-feeding modified the diet composition. The methodology was very similar to the one in this study. Their results reviled that when herbaceous material was available, browsing decreased extensively.
During sampling for this study herbaceous vegetation was plenty abundant. Nevertheless, bison receiving any form of supplementary feeding in winter (Kowalczyk et al. 2011), expressed a lower abundance of trees and shrubs in their diet compared to results in this study. The preference for the herbaceous feed in winter may reflect seasonal change in the quality of material to

browse, thus the supplementary feed represents a food resource being both energy-maximizing and time-minimizing (Bergman et al. 2001). When compared to the bison that relied on natural resources in winter the contribution of trees and shrubs exceeded the findings in this study. This supports the diet-shift hypothesis, even though the contribution from trees and shrubs may be larger in the vegetative season than previously assumed.
An interesting point in comparing the results is that Kowalczyk et al. (2011) relied on the relative read abundance (RRA) whereas this study use presence/absence data. However, the RRA of shrubs and trees from this study express an even greater contribution (88.1-88.2%) to the diet. Nonetheless, the use of RRA in fecal analysis is currently undocumented, and thus values are incomparable.

The comparison of the two studies is substantiated by the assumption that differences between sites do not affect the bison's intake. However, the bison in Almindingen have already been documented to spend more time in coniferous habitat in the vegetative season, compared to the free ranging herds in Poland. This is presumed to be a sign of threat avoidance (Brandtberg & Dabelsteen 2013; Jønsson 2014). Although bison habitat selection predominantly is determined by food preferences (Tobias et al. 2011), threat avoidance is known to have an impact on foraging patterns (Stankowich 2008; Brown et al. 2012). There are no large predators on Bornholm, yet bison can express anti-predator behaviour in response to human activity (Brown et al. 2012; Kerley, Kowalczyk, and Cromsigt 2012). The high number of visitors in the summer months may cause the bison to forage in refuge in the forest, thus increasing the addition of trees and shrubs to their diet. However, the bison enclosure was closed 30 days prior to and throughout the whole sampling during July, where the bison were exposed to reduced human disturbances. This was not reflected in the relative contribution of trees and shrubs (Figure 4) and suggests that the bison foraging patterns are not disturbed by human activity.
Moreover, the trees and shrubs identified in this study are predominantly deciduous (Table 2). This does not support the theory of increased use of coniferous habitats to affect the contribution.
As far as this study was able to investigate I attribute the relative contribution to rely on local preference or inadequate methods in previous studies.

Use of habitats

This study was able to document a significant difference in taxon (i.e. species, genera and families identified Table 2) composition in the bison diet between months (Figure 5). A study of habitat preferences for the Bornholm bison also express short-term variation in the vegetative season (Møller Pedersen & Bech Stensgaard 2015).
As food preferences are the main factor in determining habitat use (Krasiñska et al. 2000; Larter & Gates 1994), these are likely to be connected. Plants in seasonal environments have a high degree of temporal variation in their chemical composition, as plant nutrient contents are highest during the early stages of growth (Mattson Jr. 1980; J. Gordon & H.T. Prins 2008)
Differences in peak of the flowering phase of communities can contribute to short-term variability in habitat preferences and explain the fluctuations of both habitat and food preferences detected for the Bornholm bison in the vegetative season. A similar pattern in habitat selection has been observed by Daleszczyk (2007) in the Białowieza forest where the bison showed a tendency to follow flowering peaks in forest undergrowth.
The short-term fluctuations in the bison diet have not been documented before. However other ungulates express similar variability as a response to seasonal vegetation dynamics (Rayé et al. 2011; Putman et al. 1987). The detection of short-term variability in food preferences underlines

the importance of collecting data over an extended temporal period.

The fluctuations in abundance of the different identifications between months (Figure 5) were evaluated in relation to flowering peaks (Mossberg et al. 2007) and occurrence in habitats (NST Bornholm 2014; Jønsson 2014).
No systematic relation between the species taken and their flowering peaks was identified. Data of flowering peaks relied on standardised data. Local data of flowering peaks in the sampling periods may have presented a pattern.
However, I did find that the plants most frequently found in the samples, were most abundant in the open habitats or light deciduous forest, suggesting these habitats as main foraging sites.
The European bison's preference for foraging in open land is supported by previous studies (Cabon-Raczynska et al. 1987; Daleszczyk 2007; Krasiñska et al. 2000).
Further the diet analysis revealed several species that were almost solely registered in the meadow (i.e. *Lysimachia* sp., *Trifolium* sp., *Persicaria* sp., *Epilobium* sp., *Glyceria* sp., *Plantago* sp., *Potentilla anserine*, *Typha* sp.). This is consistent with the Bornholm bison's preference for the open habitats in Almindingen (Jønsson 2014), although it could also be an effect of the species rich habitat, likely to be overestimated due to the use of presence/absence data.
More specific, the study of their habitat preferences declared clear-cut as their preferred habitat (Jønsson 2014). A tendency in preference for particular clear-cut areas were found among polish herds (Kuijper et al. 2009), and are indicated in and this study where two plants primarily registered in the clear-cut area was found in high abundance (*Salix* sp., *Lathyrus pratensis*) (NST Bornholm 2014). Several grazers are known to exploit the better quality resources (Bjørneraas et al. 2012; Hester et al. 2000; Codron et al. 2007), and the materials from such woody species in June-August samples are more likely to present sprouts and buds, rather than heavy lignified material as bark and branches. Natural regeneration is normally high following clear-cuts (Bobiec et al. 2000; Runkle 1981).
However, clear-cut areas in Almindingen expressed relatively low recruitment (Jønsson 2014). This could be explained by intense browsing by the bison, and possibly account for the increased contribution of trees and shrubs in the diet analysis (Figure 4).
Roe deer and fallow deer, also present in the enclosure, follow the same tendency for the newly exposed soils (Kuijper et al. 2009) and their presence in the enclosure could account for the low recruitment. In particular fallow deer are known to have a feeding strategy similar to the European bison (Hofmann 1989; Kamler, J., Dvorak, J., Kamlerova 2003), but is only present in very low numbers on Bornholm.
Regardless, the high contribution of trees and shrubs in the bison's diet will reflect their assistance in the creation of a more open forest. A preference for clear-cut areas in particular could increase the potential impact of bisons in managed forest. Furthermore, this adds to the thriving debate of the shape of pre-agricultural landscapes (Svenning 2002; Vera 2000; Bradshaw & Mitchell 1999; Mitchell 2005; Birks 2005) and supports the theory of large herbivores' contribution to the maintenance of open forest gaps (Vera 2000).
However the aforementioned biases on species rich communities on presence/absence data affect the validation of the open areas as main foraging sites and the assumption are to be considered carefully.
Conversely, species poor communities can be underestimated. The increased time spent for the Bornholm bison in the habitats dominated by conifers, could be due to a local food preference, overlooked as an effect of the low diversity this habitat holds. The few species found in high abundance in the coniferous forests *Deschampsia flexuosa*, *Oxalis acetosella* and *Picea* sp. (NST Bornholm 2014) were found in 39, 29 and 34 of the samples, respectively (Table 2). However, the dominating species of the forest floor are generally found in high abundance in the enclosure

(NST Bornholm 2014), and it seems reasonable to propose that the data does not provide evidence for the coniferous forests being a preferred foraging habitat. Negative selection of single species (i.e. *Oxalis acetosella*) in a tight mosaic is limited by the bison's craniodental morphology (Krasińska & Krasiński 2013; Van Dybe, Brockington and Szocs 1980; Hofmann 1989). They forage broadly on the herbaceous layer including several plant as secondary import in their diet (Gordon & Prins 2008). Plants diversity is low in the coniferous habitats and even short-term grazing in the area would presumably include the aforementioned species. As meta-barcoding is a highly sensitive tool even small amount would be revealed in the analysis. In support of this Jønsson (2014) did not find evidence of the conifers as a preferred foraging site either, due to the low amount of bark stripping registered at the site. Previous studies have revealed that bison's use of a habitat is affected by climatic factors such as precipitation and temperature (Daleszczyk 2007). Other ungulates are known to use the conifers as cool ruminating sites, when temperature rises (Gordon & Prins 2008). This corresponds with the increased use of the conifers in the summer season. However habitat abundance could hold some of the explanation, although does not account for the temporal variation.

Food preferences and management practises

Previous studies revealed that 80 % of the plants consumed by the European bison in Poland are present in Denmark (Kunstmann 2003). However not all of them are registered in the enclosure (Jønsson 2014). This study was able to identify 6 species and 25 genera that have not been registered in previous diet analyses, and confirm the consumption of 24 plants (Appendix 1). As an animal inhabits a new habitat they are likely to search for food items similar to their previous habitat (Illius and Gordon 1993). The new species registered and not being present in Poland are an evident adaptation to the local environment.
Some of the species found in high abundance (≥20 samples) (Table 2) are of particular importance in management practices, as they contribute to scrub encroachment of open areas. The open vegetation types associated with the traditional cultural landscape are at risk when management is stopped and secondary succession is allowed (Svenning 2002). *Salix* sp. and *Betula* sp. are pioneer species adapted to light exposed environments, and constitute a potential threat to these areas. Similarly, *Rubus idaeus* and *Crataegus* sp. provide shelter and work as nurseries for the establishment of woodland (Bakker et al. 2004; Vera 2000). This is the first time *Crataegus* sp. is identified in bison diet, however potential browsing marks from bison were previously registered on the plant within the enclosure (Jønsson 2014).
The other species have previously been identified to contribute substantially to the diet of the European bison (Krasińska & Krasiński 2013). A preference for *R. idaeus* was identified on two previous occasions and indicated in this study by the high frequency of this species in the sample (Table 2). Preferences for browse material appear to be similar for the European bison and domestic cattle (Buttenschøn, 2007, this study). However, domestic cattle rarely feet on *R. plicatus* (and presumably neither *R. idaeus*) (Buttenschøn 2007), and predominantly feed on grasses (Van Dybe, Brockington and Szocs 1980). According to the findings in this study, the European bison should be acknowledged as a far more effective scrubland regulator than domestic cattle.

Graminoid preferences of the European bison have only been explored to a limited extent (Appendix 1) although this would yield valuable information that could benefit the prediction of their effect on grassland. This study identified three new species of graminoids in the bison diet (Table 2). Surprisingly, data revealed a preference for several 'rough' species/genera (*Typha* sp., *Calamagrotis* sp., *Phragmites australis*) of graminoids and a negative selection of the 'softer' species *Deschampsia flexuosa* (Figure 7). Softer grasses are most often negatively affected by

grazing as they constitute a rich diet easy to digest. Conversely, negative selection for the rough grasses favours them in selection and contributes to an increase in their abundance, which potentially will reduce diversity by damaging those species that already suffer a competitive disadvantage (Sternberg et al. 2000; Huntly 1991; Olff & Ritchie 1998; Rosenthal & Kotanen 1994). *Calamagrostis* spp. is considered 'rough' in this assumption eventhough *C. canescens* represents a softer species within the genus. Due to the presence of *C. canescens* on Bornholm, it could not be excluded from the taxon assignation, yet, it has not been registered in the enclosure (NST Bornholm 2014). Relative to the rough species *C. epigejos* which is dominating the grassland vegetation in the enclosure, it is not likely to be found in high abundance.
The strong election for *Typha* sp. and *Phragmites australis* could be an effect of limited registration (Supporting information 4). Both species are connected to wet habitat (Ellenberg et al. 1992) where none of the survey circles are placed (Jønsson 2014). In fact, no registrations of *P. australis* have been made within the enclosure naturally assigning it a high index score (D = 1).
Though it would be considered incorrect to evaluate on an index (D), when the genus is only present in one dataset, *P. australis* was found in 92% of the samples in the diet analysis an could be considered a main food source. Preferences for these species would suggest a so far unknown niche exploration of wet habitats for the European bison. The rough species are infrequently consumed by domestic grazers (Buttenschøn 2007) and thus the findings in this study promote the European bison as an effective new addition of livestock in management practices.
These results should be explored further by applying a more detailed vegetation analysis for mapping the coverage of individual graminoid species.

Evaluation of method

In this study 105 unique sequences were successfully identified. When compared to other studies (De Barba et al. 2014; Kartzinel et al. 2015; Quéméré et al. 2013; Rayé et al. 2011) using the trnL approach this study yielded a slightly lower taxonomic resolution, predominantly on species level (Figure 3). This could be explained by the conservative approach in taxonomic assignation applied in this study. When perfectly matched in the reference database, Quéméré et al. (2013) included ambiguous results to the given taxonomic level. Sequences in this study were assigned to highest common taxonomic level. Another factor that increases the taxonomic resolution in these studies is the parallel build of a reference database of possible prey. Other studies (De Barba et al. 2014; Rayé et al. 2011) complemented the trnL approach by sequencing additional markers for plants of the families Asteraceae, Cyperaceae, Poaceae and Rosaceae, and successfully increased taxonomic resolution. This study showed high resolution for Rosaceae, but low for both Asteraceae and Poaceae (Table 2).

Higher resolution would contribute to a greater understanding of the preferred food sources. Species within the same family or genus can differ on several parameters that can effect selection. Furthermore a higher distribution of taxon identified to species level would allow for the application of Ellenberg values. These could contribute to the identification of plant communities, preferred feeding habitats and a greater understanding of the bison's dietary plasticity.
Compared to non-genetic studies this study identified less species. This was to be expected as these studies have a broader temporal range and included multiple seasons. The short-term temporal variation reported in this study (Figure 5 and Figure 6) highlight the influence of sampling period. Late or early growth or temporal preferences for some plants could exclude species presence in dung during the sampling period of this study. Furthermore, spatial differences between sampling sites as species availability and size of foraging area could explain the greater diversity in the aforementioned studies.

Temporal differences could likewise explain differences between the vegetation analysis and diet analysis (Table 3).
Through the novel approach of meta-barcoding this study successfully identified several species, that were not found in the vegetation survey (2012 & 2014). This reveals this methods ability to identify low abundant species, and register changes in the vegetation as new species colonise.
A prerequisite to any control measures on non-native species is the ability to rapidly and accurately identify the putative threatening species. In a broader management perspective detection on low abundant species at an early stage help not only to permit an early stage evaluation of practises but also to identify undesired species.

An evaluation of the extraction protocol revealed a possible bias towards an overestimation of woody material.
When samples were transferred for extraction larger plant fragments in the dungs were avoided. However, smaller fragments may lead to an overestimation of these materials when brittled and disrupted. As lignified materials are poorly digested (Drożdż et al. 2003) they are more likely to occur in fragments and subsequently express a higher abundance. However only small amounts of DNA are present in lignified material (Rachmayanti et al. 2009), reducing the effect of this error. Several laboratory biases are limited by the use of presence/absence data, this one included. However to exclude this possible bias in future studies in which the use of RRA is substantiated, I recommend the use of sieved material for extraction.

Limitations of the data

In this study we used presence/absence date from the MySeq output, and not the relative read abundance (RRA). This was done in respect to the lack of research of the gastrointestinal environments effect on the biomaterials consumed. An important aspect of using any method to investigate diet preferences is to question whether or not the proportion of food items identified accurately reflects the proportion of food items consumed.

The inability of DNA meta-barcoding to provide quantitative results has been observed in a range of studies (Kraaijeveld et al. 2015; Pompanon et al. 2012; Ji et al. 2013) however, with the appliance of correction factors possible (Thomas et al. 2016). Fecal samples content are unknown, troubling the appliance of correction factors. Further, are the materials highly decomposed and have passed through a possibly selective digestion system. The quantitative consummation can be biased by laboratory procedures (i.e. variation in optimum amplification on family level (De Barba et al. 2014)), but possibly also by plant tissue differences and variation in the digestion efficiency. Digestibility of substances are known to differ in digestibility not only between species but also temporal (Cederlund & Nystr6m 1981; Kowalczyk et al. 1976; Drożdż et al. 2003) and thereby hold a potential bias. A resent study by Nichols, Åkesson, and Kjellander (2016) revealed a positive relationship between the amount of biomass identified macroscopically and the RRA obtained. However, when using presence/absence data showed a stronger resemblance to the macroscopical analysis. In support of this study DNA meta-barcoding and staple isotopes was compared to measure the quantitative consumption of ungulate diet (Kartzinel et al. 2015). Studies found that the two methods were highly comparable but did not account for quantitative information prior to consumption. Yet, staple isotopes in fecal samples have been found to reflect the actual consumption of various biomaterials (Salvarina et al. 2013). While these studies speak in favor of the use of RRA, the experiments did not reflect the gastrointestinal effect on fecal samples in this study. Whereas Nichols et al. (2016) used rumen content that was only

affected by the first step of the interior digestion (Gordon and Prins 2008), the stable isotopes were applied on bats with short gut-passage time (Salvarina et al. 2013) and a highly different digestion system.

Although there is a growing body of evidence that the RRA reflects the actual consumption, it is difficult to know the strengths and weaknesses of the method without controlled experimental studies.
As this method gain momentum in terms of diet assessment, more knowledge on the topic (of how the digestion system affect the true value of items consumed) would increase the power of the method (and benefit all future studies where this method was applied).

Concluding remarks

The European Bison was introduced to Almindingen forest as a bioengineer that would create favourable environment for increased biodiversity.
Based on results from this study the bison in the enclosure would contribute to a boost in species richness for the species connected to open woodland and in the areas dominated by the monoculturous *Calamagrostis epigejos*. The positive election for this grass could be considered an important niche partitioning of the bison, favouring other species in the succession.
Likewise, the bison's assistance in modulating more open woodland is reflected in the preference for foraging in open habitats and in the increased proportion of browsing.

Furthermore, this study found evidence for a feeding plasticity, both temporal and spatial, for the European bison. The fluctuating species composition found in their diet and the bison's adaptability to the local vegetation in Almindingen suggests a that a broader range of habitats might be suitable for introduction.
The unusual high proportion of woody substances which was detected in the bison diet raise the question if the findings can be explained solely as a local adaptation or if the results moreover should give the European bison a more intermediate position in the browser-grazer continuum thus reflecting a global misconception of the bison as a grazer.

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SUPPLEMENTARY MATERIALS & APPENDICES

SUPPLEMENTARY MATERIALS

Supplementary methods

In this study I used Meta-barcoding to identify plant prey of the European bison at Bornholm. The method is a novel approach that allows DNA based identification from high-throughput DNA sequencing, in this case on environmental DNA (e-DNA). E-DNA defined as "genetic material obtained directly from environmental samples (soil, sediment, water, etc.) without any obvious signs of biological source material (Thomsen & Willerslev 2015)" can contain a myriad of DNA from different organisms. DNA of interest can be extracted and amplified by using specific primers identifying the group of interest (Thomsen & Willerslev 2014).
In this study the trnL (UAA) intron in the chloroplast were used to identify plant DNA from fecal samples (Figure 1). The region is sufficiently variable to differentiate between taxon, yet conserved enough to limit the variations within species (Taberlet et al. 2007). Considering the size of the whole trnL (254–767 bp) it is not optimal for highly degraded material as fecal samples, as longer fragments are likely to breakdown during decomposition and unable identification. However a smaller part of this region meets the requirements for fecal analysis as it is both shorter and contains secondary structures (i.e. the P6 loop) making it less vulnerable to degrade (Taberlet et al. 2007). This region (trnL g-h) have proved as a valuable tool when assessing the diet through fecal samples (De Barba et al. 2014) and were used in this study.
Three kits were selected for extraction. The following sections covers details of the most important decisions made on the way to achieving a successful laboratory protocol.

Nanodrop test of final eluate of three different kits.

Following extraction the eluates were tested on Nanodrop. The test can reveal if the extractions were successful and enable evaluation of the purity of the product. Nanodrop is a spectrophotometer that by addition of 1 µl can specify sample content the based on absorbance on different wavelength. The general plateau of absorbance for nucleic acid is at 260 nm. Ratios (i.e. 260/280 and 260/230) are used as indications for the purity of the sample. However this covers all DNA extracted from the e-DNA. Protein and phenol are two contaminants that absorb strongly at 280 nm. A 260/280 ratio < 2 are considered impure and may indicate the presence of these compounds. Several other phenols along with carbohydrates absorb at 230 nm. The 260/230 ratio is used as a secondary measure of sample purity and range between 2.0-2.2 for pure samples.

When samples was amplified and tested on agarose gel following the procedure described in Materials and Methods the result came out negative. However the extraction was validated on Nanodrop (Table 4), and allowed further investigation of the unsuccessful amplification. The Nanodrop detected a high abundance of contaminant compared to DNA at wavelength corresponding to both carbohydrates, phenolic compounds and protein.
Extraction with Blood and Tissue expressed a higher yield then extraction with the other kits.
Based on the results from the Nanodrop samples were diluted to eliminate the effect of the contaminants.

First successful PCR

Table 4. Nanodrop test of eluate from three different kits. Each kit was tested in two extractions.

Sample ID	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Type
PowerFecal-1	18.6	ng/µl	0.37	0.25	1.50	0.67	DNA
PowerFecal-2	22.7	ng/µl	0.45	0.30	1.51	0.70	DNA
Blood & Tissue-1	63.0	ng/µl	1.26	0.87	1.45	0.53	DNA
Blood & Tissue-2	61.8	ng/µl	1.24	0.84	1.47	0.54	DNA
Plant Mini-1	28.3	ng/µl	0.57	0.46	1.22	0.52	DNA
Plant Mini-2	36.7	ng/µl	0.73	0.65	1.12	0.45	DNA

The dilution of the samples resulted in a successful binding between the primers and the target region for Blood & Tissue samples 1:10 (E & G) and to a lesser extend for Plant mini 1:10 (I & K) (Figure 8). Based on this result I only proceeded with these two extraction kits.
New extractions were conducted with Blood & Tissue and Plant mini including the adjustments described in the Extraction protocol based on the high contaminants registered in nanodrop-test.
The new samples expressed good yield and resulted in a successful binding between the primers and the target region, regardless the dilution for Plant mini (E-H) (Figure 9). As Blood & Tissue samples were successful only when diluted, this could be a sign of contamination. This was later confirmed with Nanodrop.

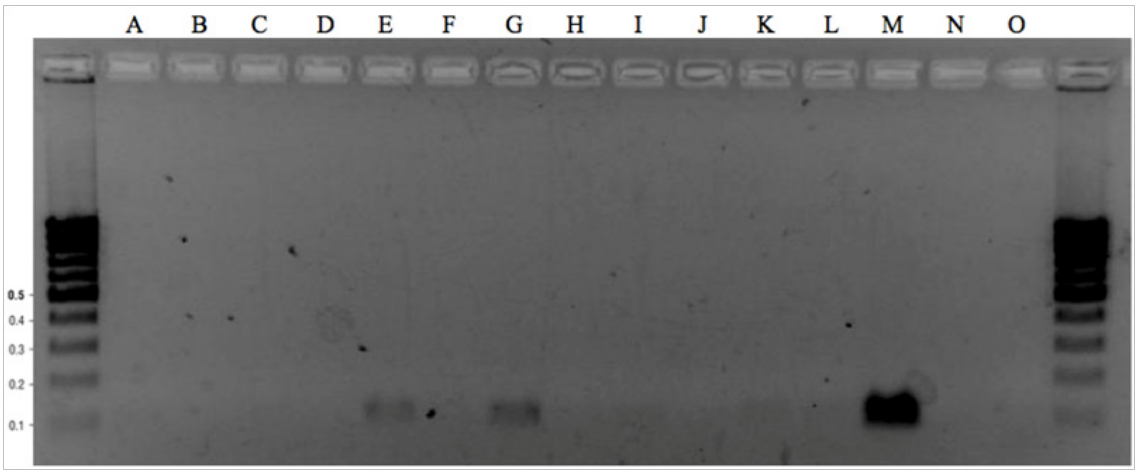


Figure 8. PCR product from three different kits were loaded on the gel. All samples were loaded in two dilutions (1:10, 1:100) of the same sample, followed by a second eluate extracted with the same kit. PowerFecal (A-D), Blood & Tissue (E-H), Plant mini (I-L), Positive control (M), Negative control (N), empty (O). 2-log latter availed in both ends of the gel.

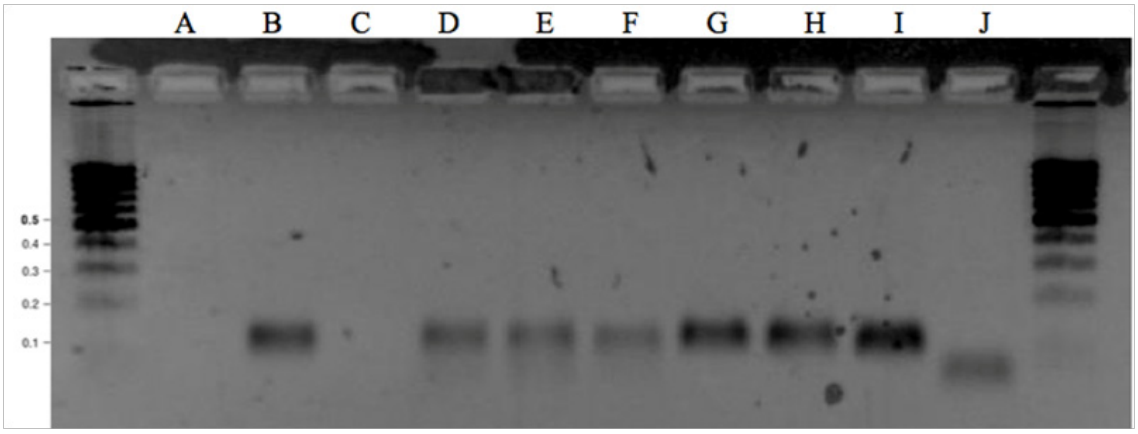


Figure 9. PCR product from two different kits were loaded on to the gel. All samples were loaded in two dilutions (1:1, 1:10), followed by a second eluate extracted with the same kit. Blood & Tissue (A-D), Plant mini (E-H), Positive control (I), Negative control (J). 2-log latter availed in both ends of the gel.

Optimizing PCR

Based on the success in amplification with Plant mini I processed all following extraction with this kit. However, when the selected method was applied with tagged-primers, no bands were detected. Tagged primers can be less specific then untagged primers and stressed the need to optimize the PCR.

Annealing temperature

During annealing the primers bind to the single-stranded DNA-template. If the annealing temperature is too high or too low primers are unable to bind. Moreover the annealing temperature affects the PCR efficiency and specificity.
I tested a thermo gradient to revel the optimum annealing temperature.

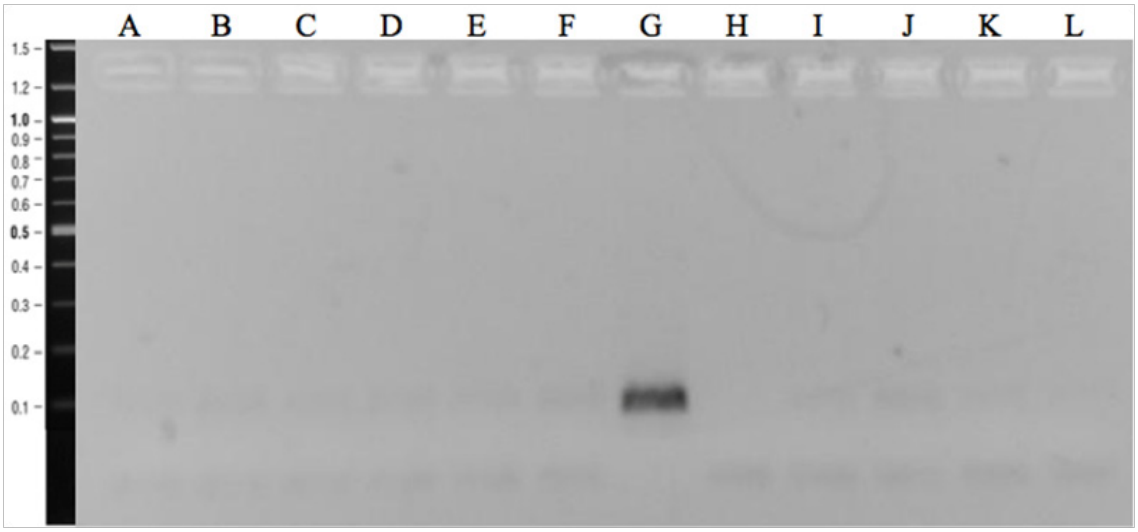


Figure 10. Two samples were exposed to different annealing temperatures. Positive and negative controls were exposed to annealing temperatures of 55,1°C (G and H respectively). Following annealing temperatures was set for; A-B: 51.6°C, C-D: 53.2°C, E-H: 55.1°C, I-J: 56.7°C and K-L: 57.6°C. 2-log latter availed in both ends of the gel.

The positive control were loaded at 55.1°C due to previous success at this temperature (Figure 10). Though the amplification was only weekly confirmed amplification only succeeded at annealing temperatures 55.1°C (E, F) and 56.7 °C (I, J). Too low annealing temperature can result in such non-specific priming and too high in no product. As I evaluated the product slightly stronger for E and F and continued with this annealing temperature.

Mg++

Iincreased concentration of Mg++ upsurges Taq activity but at the expense of specificity. I tested a Mg++ gradient with the addition of a 25 mM MgCl2 solution and adjustment of DNase-free water accordingly.

MgCl2 had a profound effect on the amplification increasing the successful amplification of the product 100-200 bp long (Figure 1). Increase in MgCl2 was positively correlated with quantity of the products, however negatively correlated with the specificity of the Taq. An addition of 2 µl MgCl2 were selected due to a conservative approach to minimize errors due to increased of Mg++ concentration. All future amplifications were carried out with this concentration

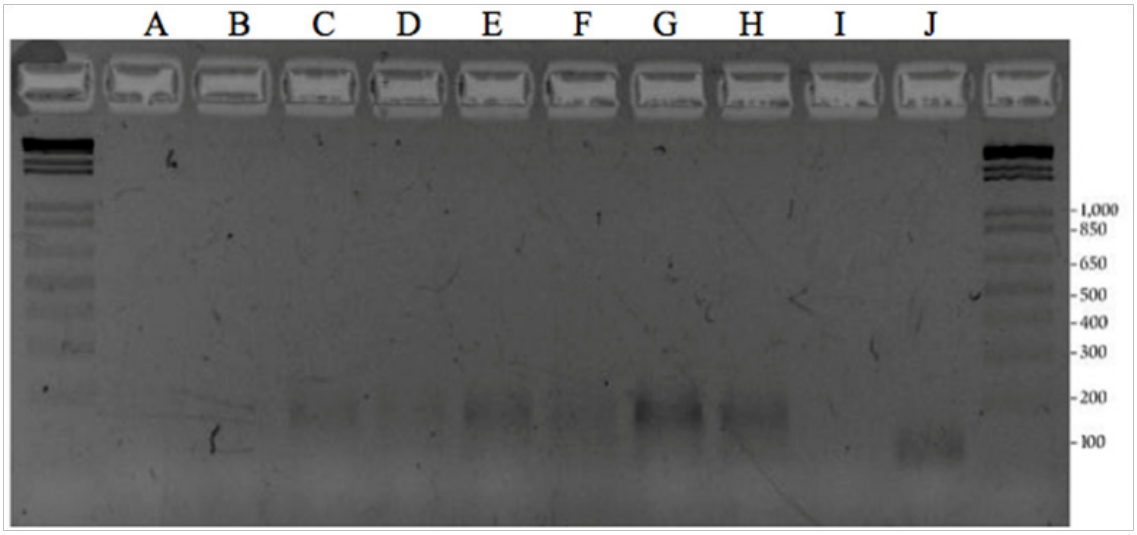


Figure 11. Two samples of good and reasonable quality respectively, were tested to a MgCl2 gradient at 40 cycles. The better sample was loaded on the gel prior to the poorer. The addition of 1 µl MgCl2 (A, B), 1.5 µl MgCl2, (C, D), 2 µl MgCl2, (E, F) or 2.5 µl MgCl2 (G, H) were added with water reduced accordingly. Samples were loaded on the gel followed by a negative (I) and a positive control (J). 1-kB latter availed in both ends of the gel.

Dilution

Amplification of samples from the months in autumn and winter did not succeed with the optimized protocol.

Due to the phenolic compounds in lignin and the well known seasonal diet shift for several herbivores towards being more wood dominated in winter I suspected an increase in inhibitors. Representative samples from the months that did not give positive results were tested in a dilution gradiant to follow the effect.

The samples were first run 35 cycles. As the method is highly sensitive keeping the number of cycles low limits the risk of amplifying contaminants (Pegard et al. 2009). However the amplification were unsuccessful at 35 cycles and increased to 40 cycles (Figure 12).

Samples that resulted in a positive amplification were from August (F, K, P), September (G, L) and October (H). The best results were achieved with a 1:2 dilution (F-H). All samples from August to December were tested in dilution 1:2 (Supporting information 2).

Figure 12. A representatives for the month August-December was loaded in order in dilutions 1:1 (A-E), 1:2 (F-J), 1:5 (K-O), 1:10 (P-T), Positive control (U) and negative control (V). All samples were run 40 cycles. 1-kB latter availed in both ends of the gel. 1-kB latter availed in both ends of the gel.

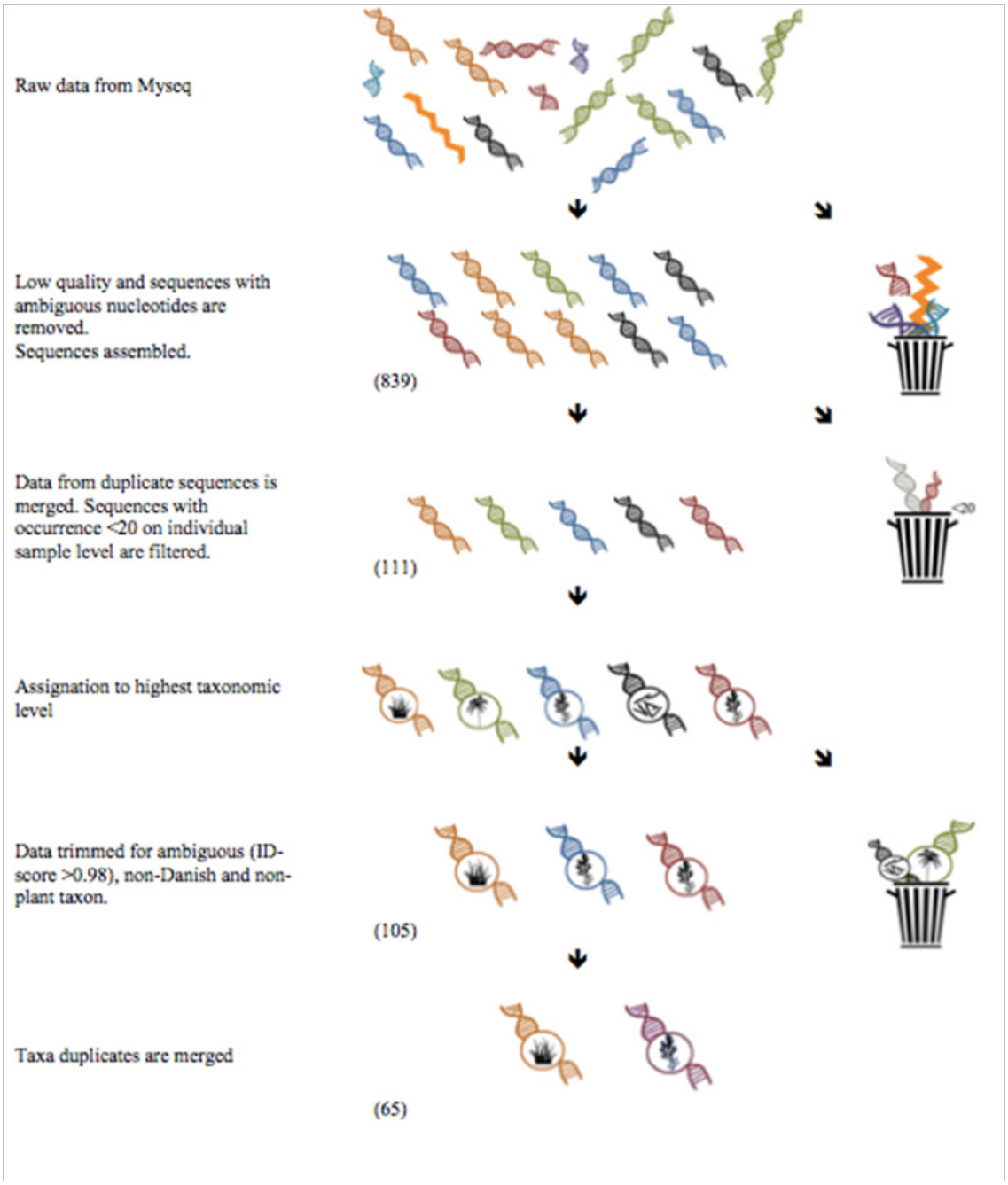
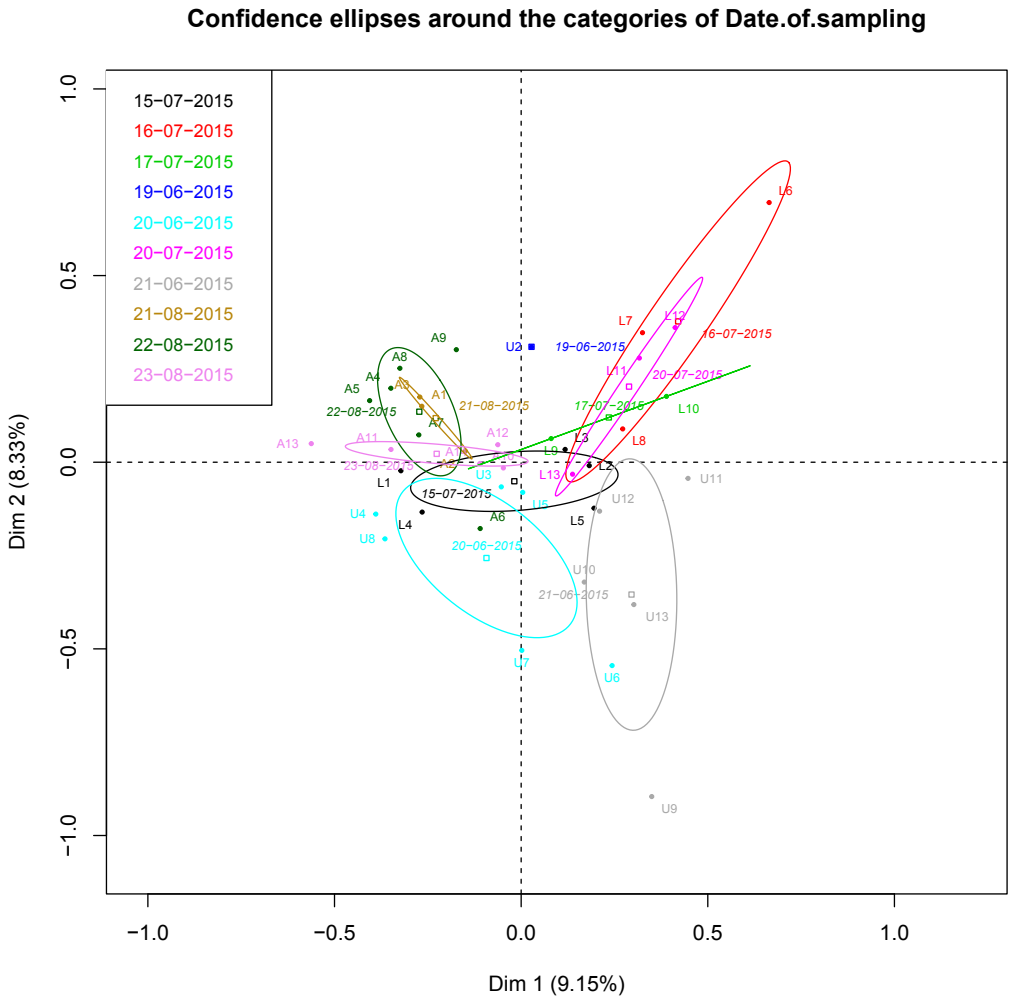


Figure 13. Flowchart summarizing the filtering and data analysis applied in this study. Quantity of OUT's left after each filtering process is listed under the illustration of concern.

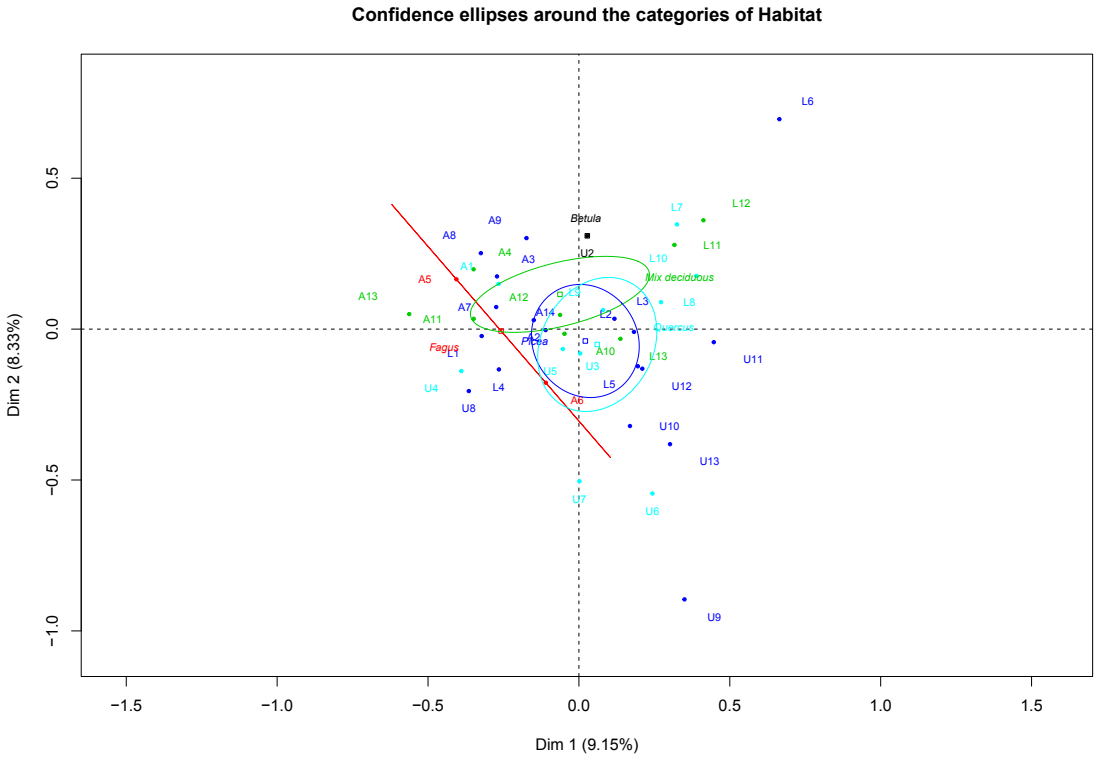
SUPPLEMENTARY FIGURES



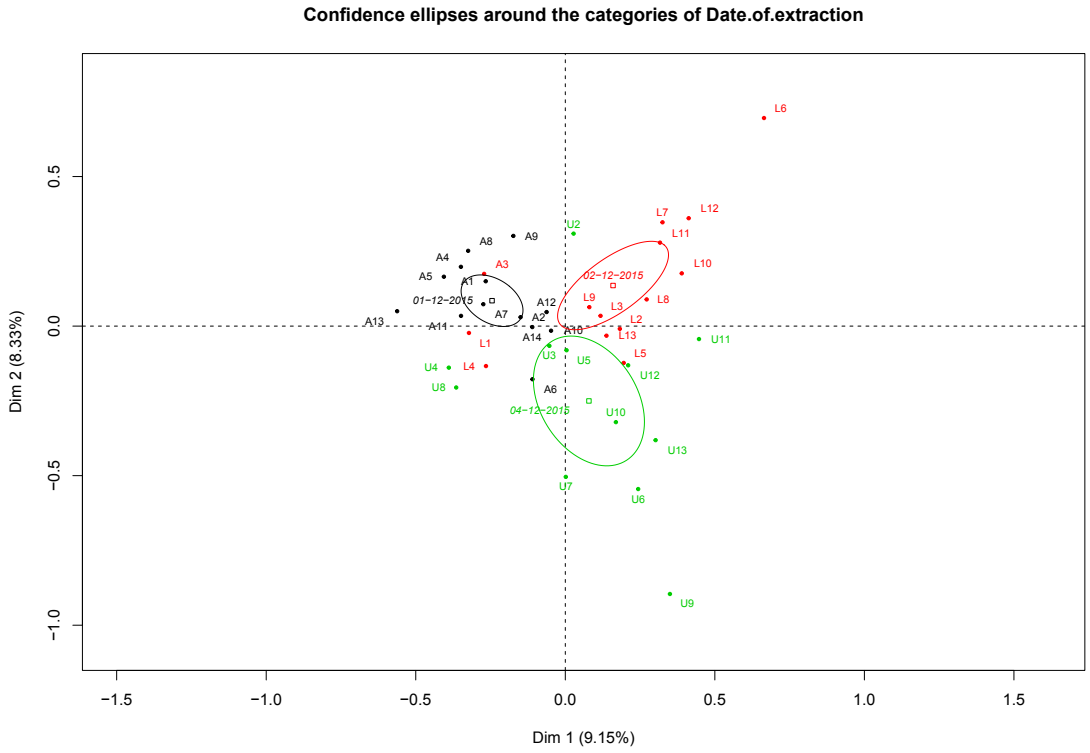
Supplementary figure 1. Comparison of diet objects in samples based on sampling date. Graphic illustration from the MCA displays the first factor plane explaining 17.8% of the variance in the dataset. U represent samples from June, L samples from July and A samples from August (for details on the individual samples see (Supporting information 1).

To evaluate sampling protocol the dates of sampling were tested as supplementary variables in a MCA. Dates of August and July were all clustered in the month they relate to (Supplementary figure 1) and showed no significant difference. Samples from 15-jul-15, and to some extend 17-jul-15 appear to be more similar to August samples then the other sampling dates of July. The three sampling dates of June are all significantly different from one another.
The results supports the theory of variations between month (Figure 5) to rely on not only be an effect of pseudo replication. The temporal spread in sampling are relatively short, and an incomplete replacement of the material of the gastrointestinal content could affect the samples to express greater similarity. However, samples from sampling dates in June are significantly different. Samples size (n:12) and the number of adults (13) does however not exclude this theory.

An evaluation of the sampling protocol regarding sampling habitat reviled no significant difference, thus samples was not contaminated when sampling.



Supplementary figure 2. Comparison of diet objects in samples based on sampling habitat. Graphic illustration from the MCA displays the first factor plane explaining 17.8% of the variance in the dataset. U represent samples from June, L samples from July and A samples from August (for details on the individual samples see (Supporting information 1).



Supplementary figure 3. Comparison of diet objects in samples based on extraction date. Graphic illustration from the MCA displays the first factor plane explaining 17.8% of the variance in the dataset. U represent samples from June, L samples from July and A samples from August (for details on the individual samples see Supporting information 1).

Evaluation of extraction protocol reviled that samples extracted in different dates were significantly different. However extraction date and month of sampling included the same samples. The extraction control contained only few low abundant (>14) sequences and all data was filtered subsequently for sequences <20, thus extraction date should have no influence on the samples. Due to an error during extraction sample ID A3 was extracted with samples from July.
I further investigated the properties of this sample and found that it had a greater similarity to the samples from August then samples from July. Though the extraction date is most likely not to have affected the samples, this result emphasises the importance of a solid laboratory protocol securing independent variables.

SUPPORTING INFORMATION

Supporting information 1. Laboratory schedule for selection and information of samples. Habitat of selection refers to tree dominance at the location of sampling. Samples that meet the criteria post amplification and are included in this study are highlighted in italic. Sampling ID *EK* represents the extraction control.

Sample ID	Sampling ID	Month of collection	Date of extraction	Date of sampling	Time of sampling	Habitat of sampling
U1	Jun 1	June	4-Dec-15	19-Jun-15	afternoon	Picea
U2	Jun 2					Betula
U3	Jun 3			20-Jun-15	afternoon	Quercus
U4	Jun 4					
U5	Jun 5					
U6	Jun 6					
U7	Jun 7					
U8	Jun 8			20-Jun-15	evening	Picea
U9	Jun 9			21-Jun-15	morning	Picea
U10	Jun 10					
U11	Jun 11					
U12	Jun 12					
U13	Jun 13					
L1	Jul 1	July	2-Dec-15	15-Jul-15	morning	Picea
L2	Jul 2					
L3	Jul 3					
L4	Jul 7			15-Jul-15	noon	Picea
L5	Jul 8					
L6	Jul 9			16-Jul-15	noon	Picea
L7	Jul 10					Quercus
L8	Jul 11					
L9	Jul 12			17-Jul-15	morning	Quercus
L10	Jul 13					
L11	Jul 14			20-Jul-15	morning	Mix deciduous
L12	Jul 15					
L13	Jul 16					
A1	Aug 1	August	1-Dec-15	21-Aug-15	afternoon	Quercus
A2	Aug 2					Picea
A3	Aug 3		2-Dec-15			
A4	Aug 4			22-Aug-15	morning	Mix deciduous
A5	Aug 5					Fagus
A6	Aug 6					

(Supporting infromation 1 - continued)

Sample ID	Sampling ID	Month of collection	Date of extraction	Date of sampling	Time of sampling	Habitat of sampling
A7	Aug 9			22-Aug-15	afternoon	Picea
A8	Aug 11					
A9	Aug 12					
A10	Aug 14			23-Aug-15	morning	Mix deciduous
A11	Aug 15					
A12	Aug 16					
A13	Aug 19			23-Aug-15	afternoon	Mix deciduous
A14	Aug 20					Picea
S1	Sep 3	September	4-Dec-15	14-Sep-15	afternoon	Picea
S2	Sep 4					
S3	Sep 5					
S4	Sep 7			14-Sep-15	evening	Picea
S5	Sep 8					
S6	Sep 9			15-Sep-15	morning	Clear-cut
S7	Sep 10					
S8	Sep 11					
S9	Sep 14			15-Sep-15	afternoon	Fagus
S10	Sep 15					clear-cut
S11	Sep 16			16-Sep-15	noon	Picea
S12	Sep 17					
S13	Sep 18					
S14	Sep 20					
O1	Okt 1	October	1-Dec-15	10-Oct-15	afternoon	Picea
O2	Okt 3					
O3	Okt 4					
O4	Okt 6			11-Oct-15	morning	Clear-cut
O5	Okt 7					
O6	Okt 10			11-Oct-15	afternoon	Clear-cut
O7	Okt 11					
O8	Okt 12			12-Oct-15	noon	Clear-cut
O9	Okt 13					
O10	Okt 14					
O11	Okt 18			14-Oct-15	morning	Mix deciduous
O12	Okt 19					
O13	Okt 21			14-Oct-15	afternoon	Clear-cut

(Supporting infromation 1 continued)

Sample ID	Sampling ID	Month of collection	Date of extraction	Date of sampling	Time of sampling	Habitat of sampling
O14	Okt 22					
N1	Nov 1	November	30-Nov-15	15-Nov-15	afternoon	Picea
N2	Nov 2					
N3	Nov 3					
N4	Nov 6			16-Nov-15	morning	Clear-cut
N5	Nov 7					
N6	Nov 8			16-Nov-15	afternoon	Mix deciduous
N7	Nov 9					
N8	Nov 10					
N9	Nov 13			18-Nov-15	morning	-
N10	Nov 14					
N11	Nov 15					
N12	Nov 18			18-Nov-15	evening	Clear-cut
N13	Nov 19					
N14	Nov 20					
D1	Dec 1	December	17-Dec-15	13-Dec-15	afternoon	Fagus
D2	Dec 2					Picea
D3	Dec 3					Quercus
D4	Dec 4			14-Dec-15	morning	Mix deciduous
D5	Dec 5					
D6	Dec 6					
D7	Dec 7			14-Dec-15	noon	Mix deciduous
D8	Dec 8					
D9	Dec 9			16-Dec-15	morning	Clear-cut
D10	Dec 10			16-Dec-15	noon	Picea
D11	Dec 11					
D12	Dec 12			16-Dec-15	afternoon	Picea
D13	Dec 13					
D14	Dec 14					
EK			17-Dec-15	-	-	-

Supporting information 2. Succesrate in PCR amplifications arranged by month (June - December). Numbers express the quality of the PCR-product based on gel electrophoresis where -:no product, 1: weak product, 2: medium product and 3; strong product. Numbers marked in red present the samples that were pooled and sent for further processing.

June.

Cycles	40	35	35
Dilution	1:1	1:1	1:1
Sample ID	A	B	C
U1	-	-	-
U2	1	2	1
U3	2	2	3
U4	2	3	3
U5	2	3	3
U6	1	1	2
U7	1	1	2
U8	2	3	3
U9	1	1	2
U10	3	3	3
U11	2	1	2
U12	2	2	2
U13	2	1	1
EK	-		

July.

Cycles	40	35	35
Dilution	1:1	1:1	1:1
Sample ID	A	B	C
L1	3	2	3
L2	3	2	3
L3	3	1	2
L4	3	1	2
L5	2	1	2
L6	3	2	3
L7	3	2	3
L8	3	2	3
L9	3	2	3
L10	3	1	3
L11	2	1	2
L12	2	1	2
L13	3	1	2
EK	-		

August.

Cycles	40	35	40	40
Dilution	1:1	1:1	1:2	1:2
Sample ID	A	B	C	D
A1	-	1	1	1
A2	1	1	1	2
A3	1	1	2	1
A4	1	1	1	1
A5	1	1	2	2
A6	2	1	2	2
A7	1	-	2	3
A8	1	-	2	1
A9	-	1	1	1
A10	1	1	1	1
A11	1	-	2	2
A12	1	-	2	2
A13	1	2	3	3
A14	2	1	1	1

September.

Cycles	40	35	40
Dilution	1:1	1:1	1:2
Sample ID	A	B	C
S1	1	-	1
S2	-	-	-
S3	-	-	1
S4	1	-	-
S5	-	-	-
S6	2	-	1
S7	3	-	2
S8	3	-	3
S9	2	-	2
S10	1	-	-
S11	1	-	-
S12	-	-	-
S13	1	-	-
S14	-	-	-

October.

Cycles	40	35	40
Dilution	1:1	1:1	1:2
Sample ID	A	B	C
O1	-	-	-
O2	-	-	-
O3	-	-	-
O4	1	-	-
O5	1	-	-
O6	-	-	-
O7	1	-	-
O8	-	-	-
O9	-	-	-
O10	-	-	-
O11	-	-	-
O12	1	-	-
O13	1	-	-
O14	-	-	-

November.

Cycles	40	40
Dilution	1:1	1:2
Sample ID	A	B
N1	-	-
N2	-	-
N3	-	-
N4	-	-
N5	-	-
N6	-	-
N7	-	-
N8	-	-
N9	-	-
N10	-	-
N11	-	-
N12	-	-
N13	-	-
N14	-	-

December.

Cycles	40	40
Dilution	1:1	1:2
Sample ID	A	B
D1	-	-
D2	-	-
D3	2	3
D4	-	-
D5	-	-
D6	-	-
D7	-	-
D8	-	-
D9	-	-
D10	-	-
D11	-	-
D12	-	-
D13	-	-
D14	-	-

Supporting information 3. Taxon assignation to all unique sequences identified (105). Before taxonomic assignation, sequence duplicate were merged and assigned to lowest common OUT ID. Sequence details can be found in Appendix 1 on the given OUT ID. Sequence identifications that did not meet the criteria described in Methods and Materials are highlighted in bold.

OTU ID	ID-score	Sequence identification	Sum reads
denovo2	1	*Potentilla anserina*	25,455
denovo9	0.99	*Fagus sylvatica*	108
denovo10	1	*Trifolium* sp.	5,772
denovo14	0.99	*Picea* sp.	6,314
denovo15	1	*Salix* sp.	97,624
denovo17	0.93	*Acer* sp.	181
denovo18	1	*Persicaria* sp.	3,210
denovo19	1	*Asteraceae*	2,368
denovo25	1	*Lotus* sp.	1,964
denovo27	0.96	Rosaceae	72
denovo28	1	*Rumex* sp.	7,721
denovo30	1	*Ribes* sp.	764
denovo31	1	*Plantago* sp.	6,119
denovo40	1	*Betula* sp.	288
denovo45	0.99	Euphorbiaceae	181
denovo51	1	*Fagus sylvatica*	23
denovo52	0.98	*Betula* sp.	1,028
denovo60	1	*Persea americana*	1,840
denovo61	0.96	Fabaceae	23
denovo64	0.98	*Filipendula vulgaris*	1,670
denovo68	1	*Quercus* sp.	13,4518
denovo69	1	*Calamagrostis* sp.	10,559
denovo70	0.98	*Lysimachia* sp.	23
denovo77	1	*Rubus idaeus*	563,410
denovo85	1	*Rubus idaeus*	1,523
denovo90	1	Solanaceae	21
denovo93	1	*Chamerion augusti-folium*	929
denovo95	0.94	*Betula* sp.	23
denovo105	1	E. Coli	541
denovo108	1	*Galium* sp.	63
denovo112	1	*Athyrium filix-femina*	297

(Supporting infromation 3 - continued)

OTU ID	ID-score	Sequence identification	Sum reads
denovo120	1	*Acer* sp.	42,420
denovo122	0.94	*Acer* sp.	493
denovo128	1	*Carex* sp.	26
denovo129	1	*Juncus* sp.	188
denovo130	1	*Lysimachia* sp.	44
denovo138	1	*Lathyrus pratensis*	5,636
denovo142	1	*Epilobium* sp.	398
denovo143	1	Poaceae	686
denovo148	1	*Fagus sylvatica*	38
denovo151	1	*Oxalis acetosella*	2,321
denovo152	1	*Lotus corniculatus*	7,486
denovo156	1	*Sparganium* sp.	384
denovo159	0.99	Asteraceae	2,639
denovo162	1	*Typha* sp.	2,266
denovo168	1	*Veronica chamaedrys*	136
denovo173	1	Onagraceae	2,012
denovo177	1	*Trifolium* sp.	7,036
denovo180	1	*Deschampsia flexuosa*	34,057
denovo185	1	*Betula* sp.	102,093
denovo198	0.98	*Alnus* sp.	2,819
denovo206	1	*Phragmites australis*	6,823
denovo212	1	E. *Coli*	5,696
denovo219	1	*Alnus* sp.	67,595
denovo227	1	*Alchemilla* sp.	258
denovo233	1	*Populus* sp.	46
denovo235	1	E. *Coli*	331
denovo237	1	*Salix* sp.	118
denovo238	1	*Sambucus* sp.	527
denovo242	0.99	*Melampyrum* sp.	1,941
denovo249	1	*Dactylis glomerata*	3,431
denovo256	0.91	*Acer* sp.	27
denovo261	0.94	Unknown plant	182
denovo268	0.99	*Trifolium* sp.	22
denovo269	0.94	*Alnus* sp.	29
denovo286	1	*Crataegus* sp.	11,649
denovo291	1	*Fagus sylvatica*	1,639

(Supporting infromation 3 - continued)

OTU ID	ID-score	Sequence identification	Sum reads
denovo302	1	Oleaceae	188
denovo317	1	E. *Coli*	497
denovo321	0.98	*Potentilla* sp.	62
denovo345	0.98	*Betula* sp.	3,135
denovo348	1	*Vaccinium* sp.	244
denovo361	1	*Quercus* sp.	76
denovo366	0.93	Fabaceae	20
denovo375	1	*Filipendula ulmaria*	6,831
denovo376	1	Lamiaceae	22
denovo382	1	*Ranunculus* sp.	157
denovo400	1	*Lysimachia* sp.	86,062
denovo421	0.99	*Filipendula Ulmaria*	949
denovo422	0.99	*Ribes* sp.	557
denovo430	1	*Stellaria* sp.	33
denovo433	1	*Potentilla* sp.	1,553
denovo434	1	*Viola* sp.	47
denovo437	0.99	*Lythrum* sp.	29
denovo464	0.99	*Urtica* sp.	55
denovo467	0.95	Rosaceae	100
denovo470	0.99	Betulaceae	102
denovo499	1	*Fagus sylvatica*	95
denovo500	1	*Lonicera* sp.	535
denovo523	0.99	Apiaceae	21
denovo535	1	Asteraceae	395
denovo536	0.99	*Potentilla* sp.	64
denovo562	1	*Glyceria* sp.	45
denovo563	0.99	*Fagus sylvatica*	326
denovo564	1	*Mentha* sp.	25
denovo567	1	*Potentilla anserina*	97
denovo572	1	*Luzula* sp.	22
denovo585	1	E. *Coli*	87
denovo595	0.95	Unknown Plant	20
denovo645	0.98	Euphorbiaceae	21
denovo646	0.99	Poaceae	103
denovo656	0.94	Rosaceae	27
denovo660	0.98	Rosaceae	23

(Supporting infromation 3 - continued)

OTU ID	ID-score	Sequence identification	Sum reads
denovo665	1	*Viburnum opulus*	29
denovo725	1	*Abies* sp.	20
denovo750	0.94	Primulaceae	21
denovo761	0.96	Rosaceae	27
denovo774	0.94	*Acer* sp.	40
denovo790	1	*Myosotis* sp.	30
denovo810	0.93	*Populus* sp.	38
denovo818	1	*Epipactis* sp.	21

Supporting information 4. Ivlev's electivity index on Graminoids. Vegetation analysis based on vegetation survey data from NST Bornholm (2014) and habitat distribution data from Jønsson (2014).

	Veg. data 2014		Fecal samples		
	Relative abundance	**Proportion (*100)**	**No. of dungs in which present**	**Proportion (*100)**	**Index D**
Cyperaceae	279.1	0.02	0	0.00	-1.00
Carex sp.	2858.5	0.21	1	0.00	-0.96
Juncus sp.	770.4	0.06	4	0.02	-0.49
Poaceae	3099.5	0.23	20	0.10	-0.45
Deschampsia flexuosa	4696.7	0.34	39	0.19	-0.37
Glyceria sp.	122.8	0.01	2	0.01	0.05
Calamagrostis sp.	1332.4	0.10	39	0.19	0.38
Luzula sp.	14.8	0.00	1	0.00	0.64
Dactylis glomerata	516.3	0.04	34	0.17	0.68
Typha sp.	11.2	0.00	20	0.10	0.99
Sparganiaceae	0.0	0.00	6	0.03	1.00
Phragmites australis	0.0	0.00	36	0.18	1.00

APPENDICES

Appendix 1. Species that have been documented in previous studies (Borowski & Kossak 1972; Kaminski et al. 2010; Kowalczyk et al. 2011; Gębczyńska et al. 1991; Korochkina 1969; Krasińska & Krasiński 2013; Pucek et al. 2004) as part of the bison diet in relation to their occurrence in previous studies at Bornholm and this present study. Brackets in Academic name specify the taxa found in this study when it differs from the previous findings in previous studies. Update of Jønsson (2014).

	Academic name	Number of dungs in witch found (n =39) (this study)	Registered in the enclosure (NST Bornholm 2012; 2014)	Registered with bite in Almindingen (Jønsson 2014)
Trees	*Acer platanoides*		-	-
	Acer pseudoplatanus (*Acer* sp.)	38	X	X
	Alnus glutinosa (*Alnus* sp.)	29	X	X
	Betula pubescens (*Betula* sp.)	35	X	X
	Carpinus Betulus		X	X
	Fraxinus excelsior		X	X
	Picea abies (*Picea* sp.)	34	X	X
	Pinus sylvestris		-	-
	Populus tremula		-	-
	Quercus robur (*Quercus* sp.)	39	X	X
	Salix sp.	38	-	X
	Tilia cordata		-	X
	Ulmus sp.		-	X
Shrubs	*Calluna vulgaris*		X	-
	Corylus avellana		-	-
	Euonymus verrucosa		-	-
	Frangula alnus		-	-
	Ledum palustre		-	-
	Rubus ideaus	39	X	X
	Sorbus aucuparia		X	X
	Vaccinium myrtillus		-	-
	Vaccinium uliginosum (*Vaccinum* sp.)	6	-	-
	Vaccinium vitis-idaea		-	-
	Vibirnum opulus	1	-	-

(Appendix 1 - continued)

	Academic name	Number of dungs in witch found (n =39) (this study)	Registered in the enclosure (NST Bornholm 2012; 2014)	Registered with bite in Almind-ingen (Jønsson 2014)
Herbs	*Actaea spicata*		-	-
	Aegopodium podagraria		-	-
	Ajuga reptans		-	-
	Anemone nemorosa		X	-
	Artemisia vulgaris		-	-
	Asarum europaeum		-	-
	Asperula odorata		-	-
	Beta vulgaris sp.		-	-
	Brassica sp.		-	-
	Capsella bursa-pastoris		-	-
	Cardamine amara		-	-
	Chenopodium sp.		-	-
	Chrysosplenium alternifolium		-	-
	Circaea lutetiana		-	-
	Cirsium oleraceum		-	-
	Dentaria bulbifera		-	-
	Epilobium alsinifolium (*Epiolium* sp.)	2	-	-
	Ficaria verna		-	-
	Filipendula ulmaria	28	X	-
	Fragraria vesca		-	-
	Galeobdolon luteum		-	-
	Galium sp.	2	X	-
	Genista tinctoria		-	-
	Geranium Robertianum		-	-
	Geum rivale		X	X
	Geum urbanum		-	-
	Hepatica nobilis		-	-
	Lathraea squamaria		-	-
	Lathyrus pratensis	36	X	-
	Leontodon autumnalis		-	-
	Lotus corniculatus	25	-	-
	Majanthemum bifolium		-	-
	Mercurialis perennis		-	-
	Mycelis muralis		-	-
	Oxalis acetosella	29	X	-
	Paris quadrifolia		-	-

(Appendix 1 - continued)

	Academic name	Number of dungs in witch found (n =39) (this study)	Registered in the enclosure (NST Bornholm 2012; 2014)	Registered with bite in Almind-ingen (Jønsson 2014)
Herbs	*Pirola* sp.		-	-
	Plantago sp.	26	-	-
	Potentilla anserina	21	-	-
	Primula officinalis		-	-
	Ranunculus lanuginosus		-	-
	Ranunculus repens (*Ranunculus* sp.)	3	X	-
	Rumex sp.	22	X	-
	Solanum tuberosum		-	-
	Sonchus arvensis		-	-
	Stachys sylvatica		X	-
	Stellaria holostea (*Stellaria* sp.)	1	X	-
	Stellaria media		X	-
	Stellaria nemorum		-	-
	Urtica dioica (*Urtica* sp.)	1	X	X
	Valeriana officinalis		-	-
	Veronica chamaedrys	3	X	-
	Vicia sp.		X	-
Graminoids	*Calamagrostis arundinaceae* (*Calamagrostis* sp.)	39	X	X
	Carex hirta (*Carex* sp.)	1	X	-
	Carex sylvatica		X	-
	Millium efusum			

APPENDIX 2

Original data can be sent on request
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