Busted by the Bite

Molecular Evidence of Cryptic Foraging Behaviors in Large Herbivores

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Doctoral Thesis
Swedish University of Agricultural Sciences
Umeå 2013

Acta Universitatis agriculturae Sueciae
2013:86

Cover: A moose getting busted by the bite
(Art: Shannon Hobbs)

ISSN 1652-6880
ISBN (print version) 978-91-576-7910-9
ISBN (electronic version) 978-91-576-7911-6
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Print: Arkitektkopia AB, Umeå 2013

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Abstract

Large herbivores are charismatic species known to engineer ecosystems through a variety of effects. Conflicts can sometimes arise when these effects are undesirable. However, without detailed knowledge on herbivore selectivity for landscapes, patches and plants, these positive and negative effects remain difficult to predict. Such species and sex-specific selectivity have inherent evolutionary and ecological mechanisms. In order to study such mechanisms it is important to study the partitioning of resources at multiple scales. Most studies have looked at large-scale resource partitioning (such as movement patterns) but fewer study the fine-scale levels of selectivity such as the individual bites taken by herbivores. This level of detail is, however, important because it is essentially the direct mechanism through which ecosystem effects are manifested.

Specifically for the browsing herbivore guild, such fine-scale studies have largely been impractical due to forested habitats which limit direct observation of behaviors. DNA-based diagnostics are becoming more and more popular within ecology as they provide vital data to answer certain questions. In this thesis I developed two versions of a method to differentiate between five species of large herbivore browsers using trace amounts of environmental DNA left at browsed twigs. The first version uses a traditional amplification method for identifying the species of browsers and the second uses an advanced and more sensitive method for identifying the species and sex of browsers.

Using environmental DNA, I determined species-specific browsing patterns across three studies. I found overall that traditional methods for attributing browsing at the species level tend to be misleading. In one study I show that although one species may be blamed for forest plantation damages, DNA evidence showed a partitioning between three herbivore species. I also document the partitioning of plant parts among different sized ungulates and show that overlap in browsing heights and bite diameters is much larger than previously assumed. In another study I experimentally verified the selectivities of free-ranging herbivores for three species of trees. This thesis thus not only develops new molecular ecological tools but also provides new insights into resource partitioning and hence the ecosystem effects of herbivores.

Keywords: Cervid, Deer, eDNA, Niche, Scandinavia, SNP, Sweden

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Dedication

To my sister Elizabeth

If I have seen further it is by standing on the shoulders of giants
Isaac Newton

Cat ions are paws-itive.
Unknown

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Nichols RV, Königsson H, Danell K, Spong G (2012). Browsed twig environmental DNA: diagnostic PCR to identify ungulate species. *Molecular Ecology Resources* 12(6), 983-989.

II Nichols RV, Spong G. An eDNA-based SNP assay for ungulate species and sex identification (manuscript).

III Nichols RV, Spong G. Ungulate browsing on conifers during summer (manuscript).

IV Nichols RV, Cromsigt JPGM, Spong G. Tree species selection by temperate ungulates (manuscript).

V Nichols RV, Cromsigt JPGM, Spong G. Nested browsing heights and bite diameters in a temperate ungulate assemblage (manuscript).

Paper I is reproduced with the permission of the publishers.

The contribution of Ruth V. Nichols to the papers included in this thesis was as follows:

I Nichols designed and performed the necessary lab work and experiments and was mainly responsible for the writing.

II Nichols was mainly responsible for designing and executing lab work as well as writing the manuscript.

III Nichols and Spong analysed the data jointly and Nichols was mainly responsible for the writing.

IV Nichols was mainly responsible for the experimental design, field and lab work as well as the analyses and writing the manuscript.

V Nichols was mainly responsible for the sampling design, field and lab work as well as the analyses and writing the manuscript.

Abbreviations

DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
PCR	Polymerase chain reaction
SNP	Single nucleotide polymorphism

1 Introduction

Since the discovery of the structure of DNA (Watson & Crick, 1953) and the invention of technology for DNA amplification, numerous biological fields have grown substantially. Ecology has been one of the last major fields in biology to catch on to this genetics wave. This has led to the creation of a new branch of science called Molecular Ecology. As a field, it has multiplied substantially, and new journals have been created to further its growth.

Molecular methods in ecology allow for detailed studies of the genetic structures of populations, and help us to understand which species are at risk of extinction. Furthermore, advances in the sensitivities of DNA extraction, amplification and sequencing allow us to take a peek into our past to examine extinct species using ancient DNA (aDNA) from fossils. In addition, logistically difficult questions within ecology are more feasible to study using DNA-based technologies. Modern day aquatic and terrestrial species compositions are now being investigated using environmental DNA (eDNA) where no other method allowed such studies (Taberlet *et al.*, 2012). Some of the first studies using eDNA were in environments where rare and non-culturable bacteria where discovered (Schloss & Handelsman, 2003; Rondon *et al.*, 2000; Ogram *et al.*, 1987). Newer eDNA applications study the composition of fish species within marine and freshwater systems (Foote *et al.*, 2012; Ficetola *et al.*, 2008) and terrestrial species diversity is studied using 'dirt' DNA (Andersen *et al.*, 2012; Bienert *et al.*, 2012). Using DNA barcoding it is also possible to investigate the contents of animal waste to determine which species were included in their diet (Pompanon *et al.*, 2012; Symondson, 2002).

In addition to studying species presence and absence, ecologists are also interested in animal behaviors and how these behaviors affect large scale ecological processes. Sometimes these behaviors are difficult to study using traditional methods, thus warranting the development of new methods. This

thesis documents the development and first applications of a new eDNA-based method for studying the cryptic foraging behaviors of mammalian herbivores.

1.1 Herbivore Foraging and Ecosystem Effects

Worldwide, conflicts with large herbivores are increasing due to expanding populations and lack of predators (Cote *et al.*, 2004). Through their interactions with plants, large herbivores can dramatically influence ecosystems and cause substantial economic losses to crops and forestry plantations (Martin *et al.*, 2010). However, large herbivores are charismatic species which most people want to conserve. Moreover, some of their effects can be considered as positive. By directly consuming plants, large herbivores affect plant regrowth patterns (McNaughton, 1983) which over time can result in more productive habitats such as grazing lawns (McNaughton, 1985). By consuming plants and defecating waste, herbivores also directly affect nutrient cycling (McNaughton *et al.*, 1988). These direct effects cascade down to affect insect, mammal and bird communities as well (Martin *et al.*, 2011; Suominen, 1999). Thus, large herbivores are known as 'ecosystem engineers' (Cote *et al.*, 2004; Persson, 2003; Suominen, 1999; Jones *et al.*, 1994).

The mechanisms behind these ecosystem effects depend on the selectivity of herbivores for landscapes, patches, plants and plant parts (Hobbs, 1996; Senft *et al.*, 1987). Over time such preferences can change the successional state of a forest to trees that are less preferred as forage by herbivores (Hobbs, 1996). Thus, to predict the effects of herbivores, we must understand herbivore selectivity. For example, ungulates often select woody plants with varying concentrations of tannins that decrease food digestibility and are toxic in large quantities (Bryant *et al.*, 1991). Many species of ungulates have tannin-binding salivary proteins to deal with such plant defenses (Clauss *et al.*, 2005). Thus we see the results of plant-animal coevolution in an ungulate's selectivity for certain plants over others and over time their effect on the landscape.

1.2 Resource Partitioning

Herbivore selectivity for landscapes, patches, plants and plant parts is determined by the evolutionary context behind sympatric species interactions. Sympatric species have the potential to overlap in the types of resources that they are adapted to exploit (Schoener, 1968). When resources are limiting, this overlap in resource use may lead to competition, which over time may result in the extinction of one or more species (Putman, 1986) or evolution into discrete patterns of resource use (Grant, 1986).

Resource partitioning can be studied at multiple scales such as space, habitat and diet (Schoener, 1974). This thesis will mainly address dietary resource partitioning. For ungulates, most of the literature on dietary partitioning resides within the grazing guild, or those ungulates whose primary diets are grasses high in lignin and cellulose (Hofmann, 1989). The predominant theory about resource partitioning in grazers suggests that these ungulates forage differentially based on body mass-driven tolerances for food quality. Allometry states that metabolic rates decline disproportionally with body mass (Kleiber, 1933), while the length of the digestive tract is proportional to body mass (Demment & Van Soest, 1985). In other words, body mass increases the tolerance and absolute intake of lower quality grasses high in lignin through increased digesta retention times (Jarman, 1974; Bell, 1970). For example, longer grasses tend to have higher lignin concentrations than shorter grasses and selection for grass height has been shown to be related to herbivore body mass (Vesey-Fitzgerald, 1960). These theories about allometry and body mass-driven tolerances have also been suggested for browsing ungulates, but there tends to be a lack of empirical studies within the browsing guild.

1.3 Sexual Segregation

Males and females of the same species also have the potential to overlap in resource use which may over time result in disparate resource use patterns and hence downstream ecosystem effects. Sexual segregation, or the differential use of space, habitat and diet among males and females, has commonly been noted in large herbivores, and some of the first hypotheses to explain it were put forward by Darwin (Darwin, 1871). Some researchers consider sexual segregation to be akin to studying males and females as separate species segregating along a niche axis (Bowyer, 2004; Barboza & Bowyer, 2000). In fact, the same allometric relationships used to predict dietary partitioning between species also may apply to predict dietary sexual segregation (Illius & Gordon, 1987). Male ungulates tend to be larger than females (Owen-Smith, 1992) and thus have proportionally larger rumens, making them more adapted to high fiber diets. Comparatively, females are smaller and have smaller rumens that cannot digest high fiber foods as thoroughly, and when females are lactating, the demand for foods high in nutrients increases dramatically (Bowyer, 2004). These digestive-centered hypotheses generate many testable predictions about sexual segregation. For example, some studies have shown that males tend to take larger diameter bites (containing more fiber) than females (Spaeth *et al.*, 2004; Ginnett & Demment, 1997; Hjeljord *et al.*, 1982).

In addition, by compiling the results of multiple studies, Mysterud (2000) found overall that the frequency of sexual segregation increased with the degree of sexual size dimorphism in browsing ungulates.

1.4 Temperate Browsers

Within browsing ungulates, the ability to study resource partitioning between species and sex at such fine scales is limited without direct observation. Commonly used methods to study browsing include radio-tracking, pellet counts, microhistology, and tracks in soft ground or snow (Schemnitz, 1980). All these methods are useful yet they do not yield data on fine-scale resource partitioning (i.e. where browsing actually occurs). Some studies do examine actual browsing points, but nearly all such studies attribute the browsing to one species (typically the one which is the most predominant) due to the difficulties involved in differentiating browsing damages between species and sexes. However, multispecies systems are very common and management plans should understand and account for the different effects of the different ungulate species as well as sexes. Thus, in this thesis I developed DNA-based tools to understand and predict the species and sex specific effects of browsing.

1.5 Objectives

The aim of this thesis was to develop and apply a new DNA-based method that distinguishes browsing between different species. I address the following questions:

1. Can the species and sex of ungulates be reliably identified using DNA left on browsed twigs? (Papers I and II)

2. How can the sensitivity of previous methods be improved upon using single nucleotide polymorphisms (SNPs)? (Paper II)

3. Which ungulate species was primarily responsible for conifer plantation damages? (Paper III)

4. Do temperate ungulates partition their use of certain tree species? (Paper IV)

5. How do temperate ungulates partition browsing height and bite diameter? Is this related to body mass? (Paper V)

2 Materials and Methods

2.1 Study Species

Sweden has five species of ungulates which fall under the family Cervidae, or the "deer family". Moose (*Alces alces*) is the largest, weighing on average 434.5 kg (Pérez-Barbería & Gordon, 2001). Red deer (*Cervus elaphus*) is the second largest and weighs approximately 162.6 kg. Fallow deer (*Cervus dama)* and roe deer (*Capreolus capreolus*) are the smallest of these species, weighing in at 56.3 kg and 23.8 kg respectively. Roe deer exhibit marginal levels of sexual size dimorphism (Gaillard *et al.*, 1993) compared to fallow deer, red deer and moose which are known to have significant levels of sexual size dimorphism (Thirgood, 1996; Miquelle *et al.*, 1992; Clutton-Brock & Guinness, 1982). Reindeer (*Rangifer tarandus*) also occur in Sweden, but not in the areas that I studied, so although they are included in our species identification kit (Paper II), I will not go into detail about them here. Wild boars (*Sus scrufa*) are also ungulates which occur in Sweden, but they are not considered to be in the same foraging guild as cervid species and thus are not included in this thesis.

Moose and roe deer are known to eat woody plants and shrubs extensively and fall farther toward the browser end of the grazer-browser spectrum than the other two species (Hofmann, 1989). Red deer and fallow deer are both considered to be intermediate feeders, but fallow deer are known to graze more often than red deer (Obidziński *et al.*, 2013; Hofmann, 1989).This knowledge thus yields predictions for how these species will partition browse in the forest, but no studies have directly attributed bite marks at plants among these species. In fact, moose are most often the species blamed for browsing damages within forest plantations despite other ungulates being present (Hörnberg, 2001; Harkonen, 1998; Heikkila, 1991). Using the method described below it is now

possible to perform much more detailed studies on resource partitioning patterns and the browsing impacts of these different ungulate species.

2.2 Method Description & Development

2.2.1 Bite DNA Collection & Extraction

When ungulates bite twigs, the bite marks are often clearly identifiable (Figure 1), but they may be easier to identify under certain conditions than others (Paper IV). For all studies, browsed bites were collected by clipping the top 1.5-2 cm of each twig into a centrifuge tube filled with phosphate-buffered saline solution (Paper I). During the developmental stages, I tested multiple commercial extraction protocols to find one that was both the most effective and the most practical for use on a large number of samples (Paper I).

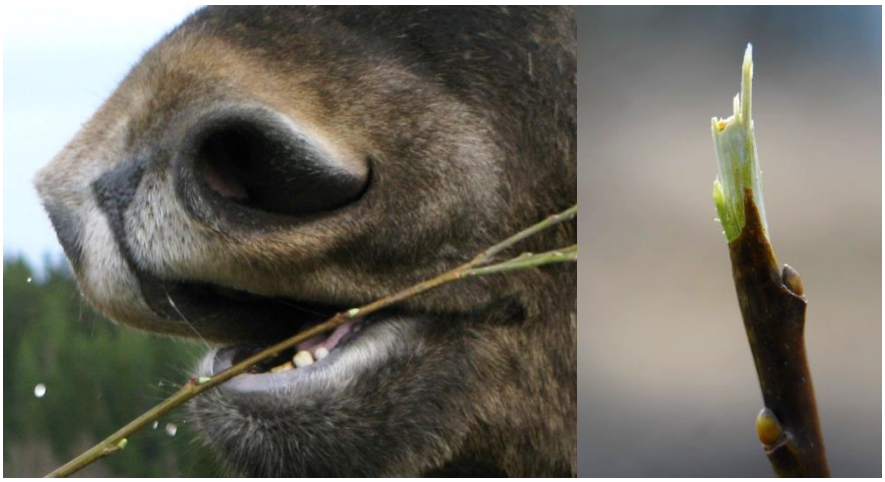

Figure 1. A moose biting a twig and depositing saliva (left-hand picture). A freshly browsed twig (right-hand picture) which can be collected for bite DNA analyses. Photo credit: Kjell Danell.

2.2.2 Fragment Based PCR

The most well known DNA technology is perhaps the Polymerase Chain Reaction (PCR) because it makes undetectable amounts of DNA detectable in a relatively short time period (Mullis & Faloona, 1987). It is not, however, without its disadvantages (Altshuler, 2006). Often in order for it to be useful, a process of optimization is required wherein the optimal chemical concentrations need to be found. Thus, I tested many commercial enzyme kits with differing concentrations of chemicals to find the most optimal PCR

conditions (Paper I). Using the optimized protocol, Figure 2 shows a typical result for identifying moose from bite DNA.

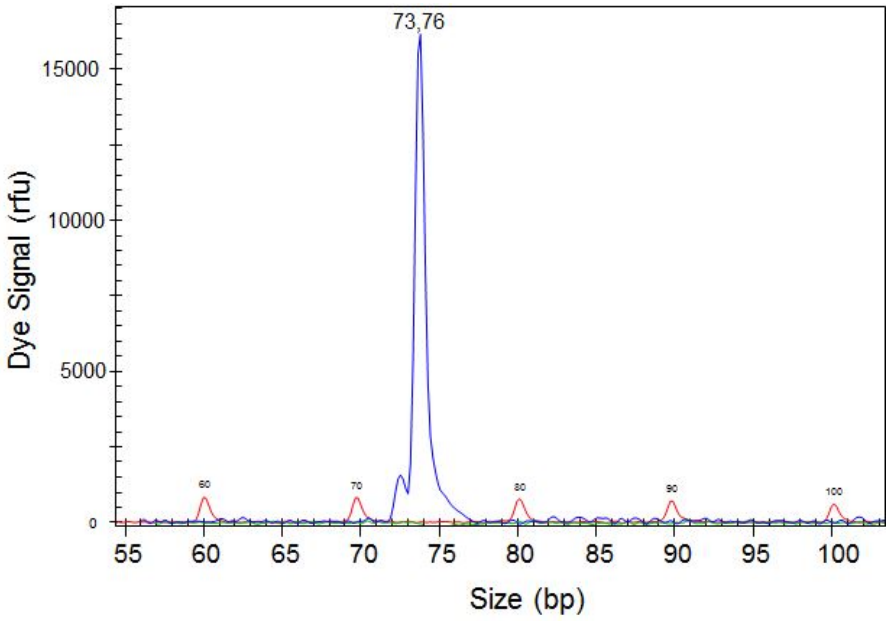

Figure 2. An electropherogram showing the correct fragment length and flourophore for moose (74 bp with a blue color). X-axis is fragment size in base pairs. Y-axis is dye signal in relative fluorescent units.

2.2.3 SNP Array

Single nucleotide polymorphisms (SNPs) are molecular markers that have been gaining in popularity because they may potentially improve on the shortcomings of past molecular markers (such as microsatellites and other fragment based analyses). In particular, SNPs are the marker of choice for use in forensic studies due to their increased senstitivies and affinity for degraded DNA (Borsting *et al.*, 2013; Sobrino *et al.*, 2005). Thus, I sought to develop SNPs for use with bite DNA. To develop species-specific SNPs I used mitochondrial sequences published in the National Center for Biotechnology Information (NCBI) and to find sex-specific SNPs I sequenced portions of the X and Y-chromosome versions of the Amelogenin gene (Paper II). After aligning sequences I manually identified SNP that were conserved across multiple species and designed SNPtype assays using Fluidigm®'s design

criteria. We tested these SNPs with bite DNA using a Fluidigm® 96.96 Dynamic Integrated Fluidic Circuit Array. Based on those results, I present a new way to genotype bite DNA samples using SNPs (Figure 3).

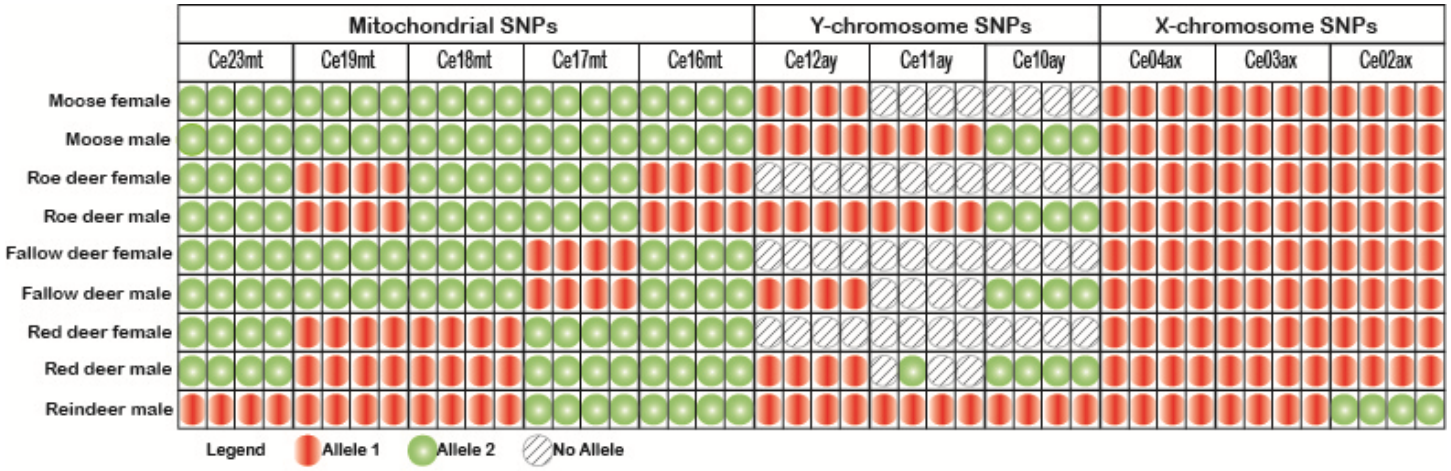

Figure 3. SNP genotyping guide for species and sex identification of ungulates. Some SNPs were found to not be useful for sexing certain species. Ce12ay shows allele 1 for both moose males and females whereas roe deer, fallow deer and red deer females show no alleles. Ce11ay shows allele 2 once for a red deer male out of 4 replicates, indicating that this SNP is not reliable for use in red deer although it appears to work well for moose and roe deer males. No female reindeer samples were available so we do not yet know how the pattern will look for differentiating male from female reindeer samples.

2.3 Applications: Field Studies

This thesis used both an experimental and descriptive approach for determining species-specific foraging dynamics.

2.3.1 Field Cafeteria Test

Classical cafeteria tests are used to identify animal preferences for certain plant species. However, they are often only practical in single-species systems or when performed in captivity. When they are done in the field, researchers generally assume them to be visited by the predominant species in the area (Jia *et al.*, 1995). Here, I improved on such studies by differentiating the bites made by different ungulate species in a field cafeteria trial using three species of trees (Paper IV). This study was performed in the winter. I measured browsing height, bite diameter at point of browsing and snow depth around stations.

2.3.2 Natural Browsing

In two other applications of this new method I collected samples of browsing on standing trees. This was done in one case to simply determine the species of ungulate primarily responsible for damages on conifers in plantation stands (Paper III). Forest owners had seen an increase in the red deer population as well as increased damages, thus they believed the red deer were primarily responsible. Thus, bite DNA samples were collected and sent to the lab by these forest plantation owners. In another case, I investigated fine-scale resource partitioning patterns among coexisting deer species (Paper V). These samples were collected in the autumn. I used a grid system to determine sampling locations (detailed in Paper V). At sampling locations I collected samples of browsed bites and recorded the following data: tree species on which browsing occurred, browsing height and bite diameter at point of browsing.

3 Results and Discussion

3.1 Bite DNA Extraction

While testing commercial DNA extraction protocols (Paper I), I experienced a trade-off between practicality and effectiveness. In order to process many samples (hundreds) I found that automated (robotic) protocols were the most practical as they could dramatically reduce the potential for pipetting errors and save time. However, these robotic protocols were found to be less effective (with a 50-60% success rate) than the manual extraction protocol (which had a 75% success rate). In favor of practicality, I used the robotic extraction protocols in the majority of the samples. However, technology is always advancing which allows for method improvement. Using the SNP-based approach I found that sample success rates surpassed those of the manual kit, compared to the fragment-based approach. Using freshly collected browse samples that were extracted with the second robotic extraction protocol found in Paper I, I saw an increased success rate when using the SNP array (Paper II) over the fragment based approach. Seven out of eight (87.5%) were successfully genotyped at the species level and the remaining sample gave an ambiguous result where it could have been a moose or a red deer. This success rate is superior to the success rate found for samples extracted using the same robotic protocol and the fragment analysis protocol, which was 50% (Paper I). I also tested the SNP assay on field-collected samples that were run two years ago using the fragment based approach. I found that 56% of those samples did not work when using the SNP-based approach. However, storing DNA samples at low concentrations and subjecting them to multiple freeze-thaw cycles is known to cause increased rates of fragmentation (Lahiri & Schnabel, 1993). Thus all genotyping analyses should be done directly after DNA extraction to avoid such degradation effects.

3.2 Sample Viability

In Paper I, I conducted a time series experiment to determine the effect of time on sample amplification success (Figure 4). I found that the proportion of amplifiable samples decreased over time. More than half amplified up to 10 weeks after browsing, and a small proportion still amplified up to 24 weeks after browsing. Using the new SNP-based approach, this window of detectability will most likely widen. It is remarkable that bite DNA can remain viable for so long under environmental stressors. In comparison, water eDNA was found to be viable for less than 4 weeks (Dejean *et al.*, 2011). It might be that when the animals chew on twigs, they deposit DNA underneath woody fibers which may protect the DNA from degradation via UV light and loss via precipitation.

I also found that the amplification success rates were different across studies. In the field cafeteria study (Paper IV), the success rate was surprisingly low (31%), but I attributed this to freeze-thaw cycles that may have caused buds to drop from the presented branches making them appear to be bites when they actually were not. In comparison, I had higher amplification success rates (75% and 54%) for all the natural browsing samples which were not collected during times when it was freezing and thawing (Papers III and V). In addition, the success rate of the conifer samples (80%) was higher than that of the natural deciduous samples (54%), which may reflect that it was much easier to correctly identify conifer bites over deciduous tree bites. However, these conifer samples were also extracted with the manual extraction kit which showed a higher success rate than both robotic extraction protocols used for the samples in Papers IV and V.

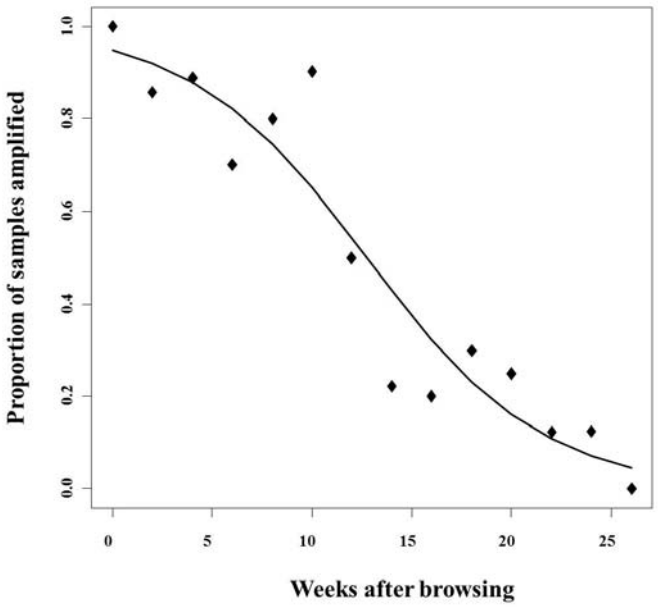

Figure 4. Amplification success over time using known-browser samples from captive moose, red deer and roe deer. Modeled using a GLM with a binomial error distribution (Z_{13}=-5.884, P < 0.0001). DNA was extracted using the DNA Investigator kit and it was amplified using the protocols found in Paper I.

3.3 Species Versus Sex

The types of diagnostics required for determining species versus sex from an unknown DNA sample are vastly different. The reason is that species level identification can be done using mitochondrial DNA which has a greater copy number than nuclear DNA which is required for sex identification. Thus, even after increasing the sensitivity of diagnostics using SNPs (Paper II), I still had much greater success in determining the species over the sex of the ungulates. Inherent in this problem is the issue of allelic dropout, which I define below.

Accounting for dropout is very important for genotyping studies because ignoring it can introduce vast errors. For example, consider a gene: it has two different versions (called alleles). Individuals have two copies of these alleles so they can either have two of the same version (homozygote) or one of each version (heterozygote). If for a heterozygote individual, one allele is subject to drop-out, while the other is not, that individual will be incorrectly identified as a homozygote. In criminal forensics, such errors can put an innocent into

prison. Thus, steps are taken to avoid such mistakes such as creating redundancies in genotype matching and consensus genotypes.

Allelic dropout occurs either via allele absence or PCR failure. Allele absence can be a true result (i.e., it was not there in the first place) or it can be due to degraded or low quantity DNA. PCR failure can happen via a number of mechanisms, but some basic ones are the presence of PCR inhibitors and pipetting errors (Altshuler, 2006). Classical microsatellite methods have attempted to deal with these problems using multiple-tube approaches (Taberlet *et al.*, 1996) where reproducibility in results is key. However, SNPs are not subject to the same types of errors as microsatellites, thus new genotyping criteria that account for SNP dropout need to be made. The field of molecular ecology seems to be lagging behind in this, but criminal forensics has made advances on this front due to a much greater demand for true and accurate results (Borsting *et al.*, 2013). However, SNP genotyping criteria are often lab specific. Genotyping error rates are specific to the assays, chemicals and machines used in labs.

In my study (Paper II), I did not use allelic differences to sex eDNA samples. Rather I used the presence of the Y-chromosome (using 2-3 SNPs) to sex males and its absence to sex females. Thus, I needed to estimate how often dropout might occur in these eDNA samples and use that number to estimate how confident I could be that I had correctly sexed a female. Without known-sex bite DNA samples I could not empirically determine dropout rates for the Y-chromosome. However, every individual has at least one X-chromosome so all tested field samples were used to find its average dropout rate. Using this empirically determined dropout rate I modeled the probability of also observing Y-chromosome dropout (Paper II). Thus, when using 2 Y-SNPs (as in for moose, fallow deer and red deer) and 4 replicates (8 individual reactions or markers per sample), the probability that the all Y-markers would dropout was 0.077. When using 3 Y-SNPs (for roe deer) and 4 replicates (12 reactions or markers), the probability that all Y-markers would dropout was 0.022. Thus if I accepted an error rate of 2.2-7.7%, I could sex female samples simply by the presence of 1 X-marker (and 0 Y-markers).

However, as in forensic diagnostics, the level of accuracy required may need to be higher, and this is dependent on the study and question at hand. Increasing the number of replicates and the strictness of criteria for genotyping decreases error rates. Thus, I also examined increasingly strict genotyping criteria by using 2-3 X-markers (and 0 Y-markers) to reliably sex female samples and 2 Y-markers for males. Depending on the criteria used, I was able to sex 34-57% of browsed twig samples (Paper II). Nonetheless, further testing is required using known browser samples to empirically determine Y-

chromosome drop-out rates and thus which criteria are appropriate. In addition, with further experimentation and SNP development, identification of the individual that browsed will also be possible.

3.4 Browsing Habits

In one of the first method applications, I performed a short case study on browsing damages (Paper III). Here, the primary goal was to determine which species of ungulate were responsible for increased browsing damages on conifer species in Sweden. Despite the assumption by forest landowners that the main species responsible was red deer, I show that it was in fact primarily caused by moose (Table 1). Thus, a decision without these results may have caused these forest landowners to make a prejudiced action against the red deer which were only secondarily responsible.

In the field cafeteria test, I found that all species tended to prefer deciduous over coniferous species. This confirms previous studies (Bergman *et al.*, 2005; Bergström & Hjeljord, 1987; Elliott & Loudon, 1987) and may have implications for plant defense guilds (Paper IV). I.e. All ungulates tended to avoid Scots pine when it occurred amongst more palatable species, but without an appropriate pine-only control, the idea that palatable species provide protection for Scots pine remains untested. I also observed a preference by moose and roe deer for goat willow over aspen which was not present for red deer (Paper IV). In our natural browsing study we did not explicitly test for tree species preferences because we did not take relative abundance data. However, in general all species overlapped in their use of most plant species (Table 1). In addition, across all studies we found very few bites from fallow deer, yet this is to be expected since fallow deer are considered to graze more often than they browse (Obidziński *et al.*, 2013; Hofmann, 1989).

Before bite DNA, many assumptions were made about the foraging habits of these large herbivores. Specifically for these species, it has often been assumed that the larger species concentrate their foraging efforts on the apical portions of plants (Seaton *et al.*, 2011; Vehviläinen & Koricheva, 2006). In fact, this assumption has been used to differentiate between herbivore bites in some experiments (Vehviläinen & Koricheva, 2006). However, I show both in the experimental (Paper IV) and descriptive (Paper V) data that even the larger species foraged close to the ground. I found bites for moose and red deer down to 20 and 16 cm respectively (Paper V). Moose in particular might need to bend down on their forelegs to reach these levels. Nonetheless, a blanket assumption that large species do not forage close to the ground is inaccurate. In

addition, the smallest species (roe deer) selected bite diameters that were larger than those that have been reported for this species (Shipley *et al.*, 1999). The largest diameter found for roe deer in my cafeteria trial was 10.2 mm and the mean (±s.d.) diameter was 4.9 ± 2.0 mm. In comparison, Shipley *et al.* (1999) modeled and reported mean bite diameters (of captive roe deer) which were approximately 1-3 mm on a variety of tree species. In comparison, the mean diameter for roe deer browsing on standing trees in the autumn was 2.0 ±0.8 mm (Paper V). Thus, the assumptions made using captive roe deer did hold for free-ranging roe deer during the autumn, but during winter I found that wild roe deer selected much larger diameters.

Table 1. *Numbers of bites (and percentage of diet) found for each tree species across all studies*

	Moose	Red deer	Fallow deer	Roe deer
P. sylvestris[1]	95 (66)	33 (69)	3 (100)	0
P. abies[1]	48 (34)	15 (31)	0	0
P. tremula[2]	10 (30)	25 (47)	2 (50)	17 (31)
S. caprea[2]	21 (64)	26 (49)	2 (50)	35 (65)
P. sylvestris[2]	2 (6)	2 (4)	0	2 (4)
A. glutinosa[3]	46 (11)	11 (13)	2 (13)	3 (7)
B. pendula[3]	130 (31)	31 (36)	0	12 (27)
B. pubescens[3]	63 (15)	15 (17)	3 (20)	12 (27)
C. avellana[3]	7 (2)	1 (1)	1 (7)	0
C. betulus[3]	0	1 (1)	0	0
F. alnus[3]	22 (5)	2 (2)	0	1 (2)
M. gala[3]	0	2 (2)	0	1 (2)
P. spinosa[3]	6 (1)	1 (1)	0	0
P. tremula[3]	42 (10)	4 (5)	1 (7)	4 (9)
Prunus spp.[3]	2 (<1)	1 (1)	1 (7)	0
Q. robur[3]	0	0	2 (13)	0
S.aucuparia	22 (5)	5 (6)	0	2 (4)
Salix spp.[3]	80 (19)	13 (15)	5 (33)	10 (22)

1 Conifer plantation samples
2 Cafeteria trial samples
3 Natural browsing samples

3.5 Snow & Selectivity

By comparing browsing heights between Papers IV and V (Figure 5) I found that the presence of snow influenced the amount of foraging height

stratification between ungulate species. Without a snow cover I found that browsing height increased with ungulate body mass ($F=42$, $DF=1$, $R^2=0.07$, $P < 0.0001$, slope=1.05) and mean browsing heights were significantly different for the following species pairs: moose/roe deer, moose/red deer and red deer/roe deer (Paper V). However, when there was a snow cover, I found no differences in mean browsing heights between species (Paper IV) and I found no effect of body mass on browsing height ($F=0.38$, $DF=1$, $R^2=0.003$, $P=0.54$). I also analyzed the total variances in browsing heights as proxies for niche breadth. Without snow, I found the variances in browsing heights to be significantly different across species (Paper V), whereas they were not significantly different when there was snow (Brown-Forsythe test: $F=0.63$, $DF=3$, $P=0.59$). These results suggest that the presence of snow may dramatically reduce the amount of resource partitioning between these species along the browsing height axis.

In comparison to browsing height, the presence of snow appeared to increase overall browsing diameters for all ungulate species (Figure 6). Although mean bite diameters were not different across species in either Paper IV or V, on a per species basis, mean bite diameters were greater in the snow than without (moose: $F=130.3$, $DF=1$, $P < 0.0001$; red deer: $F=119.5$, $DF=1$, $P < 0.0001$; fallow deer: $F=6.8$, $DF=1$, $P=0.02$; roe deer: $F=83.3$, $DF=1$, $P < 0.0001$). In addition, the variations in bite diameters were greater with snow than without (moose: $F=53.7$, $DF=1$, $P < 0.0001$; red deer: $F=44.8$, $DF=1$, $P < 0.0001$; fallow deer: $F=4.6$, $DF=1$, $P < 0.05$; roe deer: $F=18.9$, $DF=1$, $P < 0.001$). Thus, all species were more selective for smaller bite diameters in the autumn (Paper V) compared to the winter when there was a snow cover and the animals were more likely to be nutritionally stressed (Paper IV).

Taking these results together, we can infer that seasonality and the presence of snow may reduce ungulate selectivity for certain plant parts and thus increase overlap between these species. This has also been observed in a North American system where ungulates showed increased overlap in plant species consumption during severe winters compared to mild winters (Jenkins & Wright, 1988; Jenkins & Wright, 1987). I.e. Deeper snow increased levels of overlap and potential for competition, which is what I also observed across my two studies.

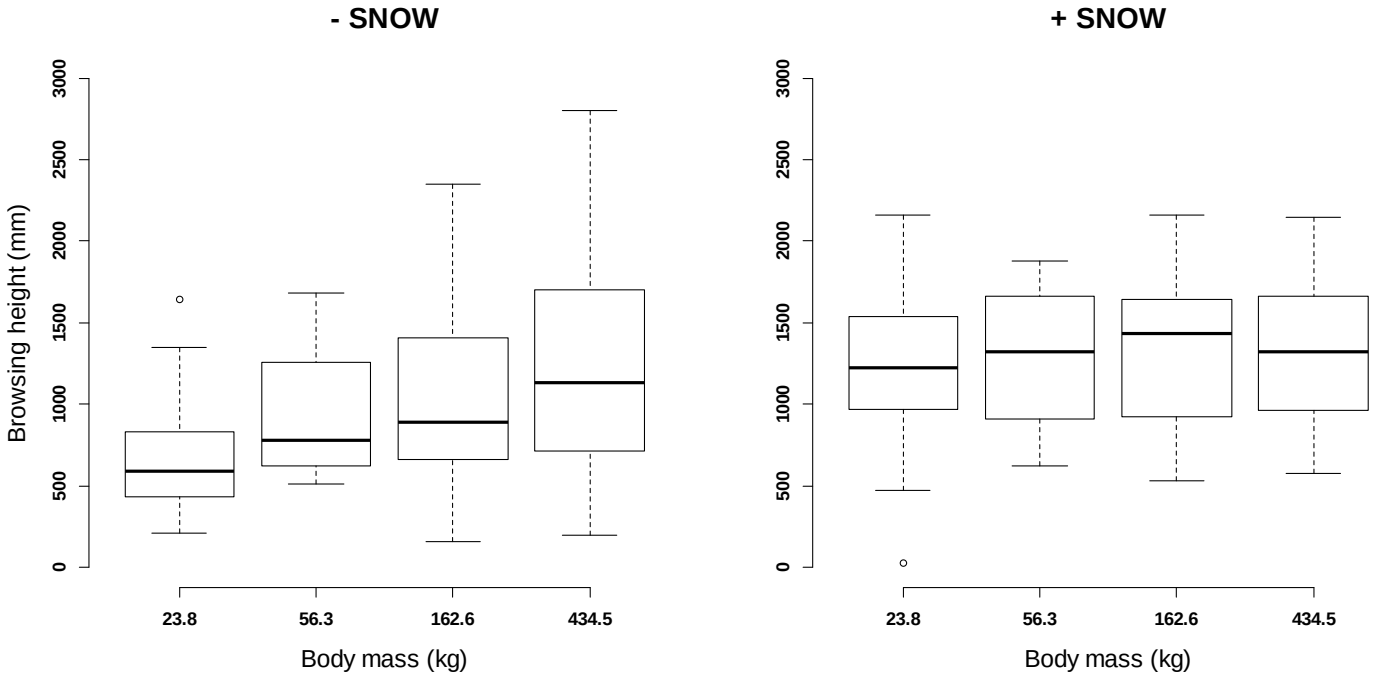

Figure 5. Browsing height distributions in order of increasing body mass: roe deer, fallow deer, red deer and moose (Pérez-Barbería & Gordon, 2001) with and without snow cover.

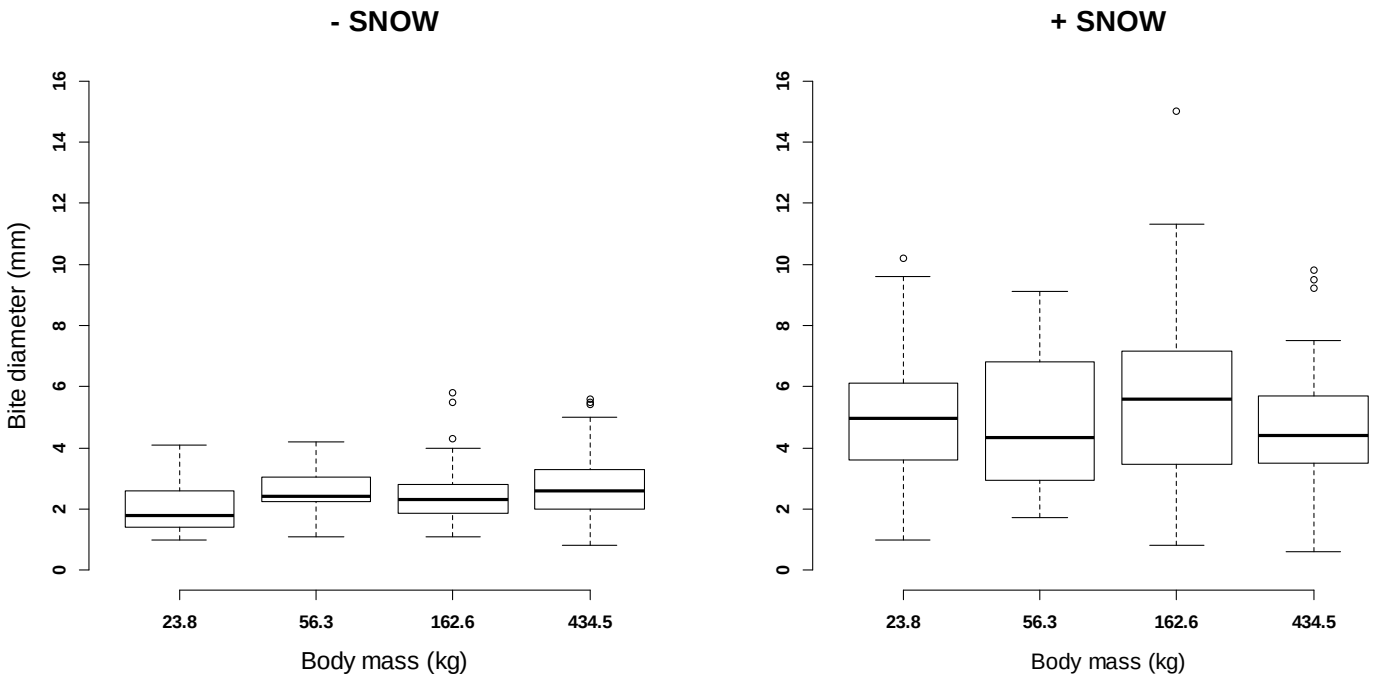

Figure 6. Bite diameter distributions in order of increasing body mass (Pérez-Barbería & Gordon, 2001) with and without snow cover.

4 Conclusions – Future Perspectives

Research into the fine-scale foraging preferences of browsing ungulates has largely been lacking. This thesis provides a new method for such studies and also provides some of the first case studies documenting the use of this method.

As noted, traditional methods for attributing browsing damages to the correct ungulate species may be misleading. Thus, by using the new method I present in this thesis, forest managers can directly identify the responsible ungulate species and take appropriate actions to mitigate their negative effects on forest plantations. For example, the effect of tree species composition on Scots pine damages is widely debated, where some studies show decreased damages due to the neighboring species (Hörnberg, 2001) whereas others show the opposite effect (Harkonen *et al.*, 2008). These conflicting results may be a direct result of attributing all herbivore damages to a single herbivore species when in fact herbivore species differ in their selectivity for plants and plant communities. Thus, to better predict the effects of plant neighborhoods and hence future damages, field-based cafeteria trials can now be done where multiple herbivore species can be differentiated.

In addition, future research can be done on the different browsing patterns for males versus females. Namely, sexual segregation along the dietary axis can be investigated using bite DNA. For example, bite diameter and foraging height stratification based on sex can also be studied using bite DNA.

The success of my SNP assay shows that there is enough nuclear DNA present in bite DNA samples for reliable amplification and hence individual identification. Through individual identification, studies on individual browsers will be made possible where we can potentially study behavioral trade-offs, such as the effect of risk on foraging behaviors (Creel & Christianson, 2008; Creel *et al.*, 2005). In addition, population size and genetic diversity measures might be made possible using these non-invasive DNA samples.

Thus, this thesis provides a powerful new tool within the ecological-genetics toolkit for identifying species, sex, and (in the future) individual-specific browsing patterns. With the knowledge gleaned from such studies, we will be able to better predict and manage the vast effects of large herbivores on ecosystem processes.

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Acknowledgements

First of all, I would like to thank all my teachers and mentors who have helped me along the journey of my scientific career. I want to thank my mom and dad for buying the ‘dragon genetics game’ that my sister and I played on the computer when we were young. That was my first exposure to the concept of genes. I’d like to thank my high school Biotechnology teacher, Mrs. Moriarty, for getting me excited about gene technology and getting me involved in my first real lab experience at UC Davis. I would like to especially thank all the members of the Rice Lab whom I worked with at UC Santa Barbara: Bill Rice, Andrew Stewart, Urban Friberg, Ali Pischedda, Tristan Long, and Paige Miller.

Many people helped me throughout my project. Of course, the most important was my main supervisor, Göran Spong, who advised and supported me the whole way through. I also want to thank Joris Cromsigt for his ideas and guidance through the last half of my PhD time. I would also like to thank Kjell Danell for good advice on both my thesis chapters and life in Sweden in general. I also want to thank Helena Königsson for her vital aid in the laboratory. The members of my lab group (Anita Norman, Ellinor Sahlén and Ida-Maria Blåhed), my former office-mate (Jon Andersson) and all the other PhD students that have been so awesome and so much fun to have around, so thanks to them!

The friends that I have made here in Sweden have been my support since I live so far from home. I would like to thank them for that (there’s too many to mention all by name).

Finally, I want to thank my sister, Elizabeth, my mom, Linda, and my dad, Steve, for all the love and encouragement they gave me growing up and all that they continue to give me from the other side of the world.