THE COST OF ROAMING FREE: ASSESSING THE EFFECTS OF PLANT SECONDARY METABOLITES ON DIET SELECTION AND NUTRITIONAL CONDITION IN A FREE-RANGING GENERALIST HERBIVORE.

by

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DEDICATION

For my family, especially my grandfather who will always be my greatest inspiration, and my mother who has always supported me in everything I have done.

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ABSTRACT

Large vertebrate herbivores have a wide variety of browsing options available. However, most plants contain a suite of plant secondary metabolites (PSMs) that can have toxic effects when ingested. Herbivores must therefore make dietary choices that minimize the potentially harmful effects of PSM ingestion and maximize the use of available nutrients and protein. During winter months, in northern latitudes, climatological factors restrict browsing options and many populations of herbivores must subsist primarily on forage that is relatively poor in nutritional quality and high in PSMs. Many species of herbivores have developed a suite of behavioral and physiological adaptations to cope with increased PSM exposure, including selective foraging and increased detoxification efficiency of potentially harmful PSMs. However, detoxification of PSMs may be energetically costly, exacerbating the effects of winter nutritional deficits, which in turn further decreases nutritional condition of free-ranging populations. As a result, PSMs may directly influence diet selection and winter nutritional condition of free-ranging herbivores. We used moose (*Alces alces*) on Isle Royale National Park, Michigan, to test how PSMs influence the diet selection, rates of intake and the physiological consequences of diet selection in a large mammalian herbivore during winter. We identified browsing patches and bite marks on balsam fir (*Abies balsamia*) trees and examined the PSM chemical profile and protein content of browsed and unbrowsed trees. We found that both diet selection and rate of intake by moose was directly influenced by PSMs. However, environmental conditions (e.g. snow depth) were the primary governing factor of intake rate. We also examined the effect of PSMs on nutritional condition of island moose using urine collected from snow. We quantified the concentrations of three metabolites found in urine: Glucuronic Acid (GA) as a biomarker of PSM exposure, Urea Nitrogen (UN) as a biomarker of overall nutritional condition, and Creatinine (C) to correct for individual sample dilution from snow volume or relative hydration of individual animals. We found that UN:C was positively correlated with increased GA:C, indicating higher PSM exposure which may reduce nutritional condition of moose. Furthermore, we found that increased ingestion of monoterpenes, a primary class of PSMs in fir, also directly correlated with decreased nutritional condition of moose. By employing techniques that can be applied generally to test the consequences of PSMs on nutritional ecology of herbivores, this research contributes to our understanding of herbivore foraging ecology and the potential nutritional consequences of “bottom-up” regulation of natural populations of herbivores by plants. The methods detailed here-in are applicable to testing consequences of PSMs in multiple species and habitat types. Specifically, measuring GA from urine collected snow provides managers and wildlife scientists with a simple, effective, non-invasive, and relatively inexpensive means by which to monitor the link between diet quality, behavioral and physiological adaptations to PSMs, and the nutritional consequences of diet quality. Finally, increased ability to utilize biomarkers of diet quality and nutritional condition may considerably contribute to the understanding of the health and population dynamics of difficult to manage or economically important herbivores.

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LIST OF ABBREVIATIONS

PSM	Plant Secondary Metabolite
AUC	Area Under the Curve (on chromatogram)
GA	Glucuronic Acid
UN	Urea Nitrogen
C	Creatinine
GAE	Gallic Acid Equivalent
TAE	Tannic Acid Equivalent
AICc	Akaike’s Information Criterion, with sample size adjustment
DW	Dry Weight
WW	Wet Weight
EO	Essential Oils

GENERAL INTRODUCTION

Advances in the theoretical framework of population ecology have led to a long-standing debate as to the forces that regulate herbivore populations. Some population ecologists argue that predation (i.e., top-down process) is the principle means by which herbivore populations are regulated. This perspective is largely supported by numerous empirical studies on predator-prey interactions and density dependent population oscillations (Hairston et al. 1960; Rosenzweig and MacArthur 1963; Tanner 1975; Erlinge et al. 1984; Messier 1994; Sinclair and Pech 1996; Pople et al. 2000; Jansen 2001). Counter to this, other researchers have proposed that relative abundance of primary producers (i.e., bottom-up process) regulates herbivore populations, and by extension, obligate predator populations (Coe et al. 1976; White 1978; Bach 1980; Newsome et al. 1989; Báez et al. 2006). Modern theoretical approaches to population ecology hold that a combination of top-down and bottom-up processes work synergistically to regulate herbivore populations (Menge 2000; Denno et al. 2005; Elmhagen and Rushton 2007; Laundré et al. 2014). However, not all systems are created equal; thus, the relative strengths of trophic control interactions may be system or species specific and subject to spatial and temporal variation (Hunter and Price 1992; Sæther 1997; Gripenberg and Roslin 2007; Hopcraft et al. 2010). Generalists living in highly connected systems may suffer high predation rates, leading to a greater top-down effect. Conversely, dietary specialists or herbivores living in predator-free systems are highly susceptible to resource limitations, and thus, may experience greater bottom-up effects.

For the purpose of this thesis, we focused on a generalist herbivore living in a natural system with minimal predation. In such a system, both quantity and quality of available forage may be tantamount to herbivore fitness. In the absence of top-down controls, populations can be expected to increase, thereby leading to increased competition for preferred resources among conspecifics. An antagonistic effect in response to resource availability can also be expected, potentially altering the availability and distribution of resources at the landscape scale. Vertebrate herbivores are seldom resource limited in terms of forage availability. However, they are often highly selective of the plants that they consume, favoring plants higher in available protein and lower in defensive plant secondary metabolites (PSMs) (Stolter 2008; Frye et al. 2013; Ulappa et al. 2014; Fauchald et al. 2017). Moreover, herbivores can accelerate or decelerate ecosystem processes, thereby leading to changes in nutrient cycling (Bezemer and van Dam 2005; Olofsson 2006) which can influence changes in plant morphology and nutritional quality as well as plant community dynamics including composition and distribution (Agrawal 1998; Stolter 2008; de Jager et al. 2009; Ulappa et al. 2014; Ricca et al. 2016). Clearly, forage quality and the responses of herbivores to forage quality is important when considering population dynamics of herbivores and plants.

To address how plant quality influences herbivore populations, we investigated diet selection by a large generalist herbivore and assessed the consequences of ingested PSMs on their nutritional condition. We conducted this research using the tri-trophic wolf-moose-balsam fir system on Isle Royale National Park, Michigan USA (Appendix A). Isle Royale has been studied continuously for more than 50 years as a model single predator-single prey system, leading to great advancements in the understanding of predator/prey relationships (Vucetich et al. 2011). With the current wolf population on Isle Royale at a critical low with only two known individuals and no known breeding pairs at the time of this writing (Peterson and Vucetich 2016), predation is almost non-existent on the island, leaving the moose population without population controls exerted by an apex predator. With moose being the only large herbivore on the island, the decline in wolf population provides a unique opportunity to study the effects of forage quality on the diet selection behavior and relative nutritional condition of a large generalist herbivore without the confounding effects of predation.

In the first chapter, we investigated behavioral adaptations and plant-scale foraging decisions made by moose. PSMs can vary both among trees and patches of trees of the same species (Thompson et al. 1989), and they can influence the foraging decisions of herbivores (Frye et al. 2013; Forbey et al. 2013; Ulappa et al. 2014; Fauchald et al. 2017) including moose (Stolter 2008). We hypothesized that PSMs would negatively influence diet selection of island moose. To test this hypothesis, we used a paired sample design (browsed vs. unbrowsed trees) spanning a period of three years to examine dietary factors (i.e. monoterpenes, total phenolics, total tannins, and crude protein) influencing diet selection and intake rate of moose. We focused solely on balsam fir (*Abies balsamea*) because it is the principle winter forage for moose on Isle Royale (Parikh et al. 2016). We also examined how winter environmental conditions (e.g., snow depth) can affect intake rates. We found that island moose foraged selectively with regard to PSM concentration which, along with snow depth, also influenced intake rate. We found that a single monoterpene, camphene, was the strongest predictor of moose browsing, with moose selectively browsing on trees that had higher concentrations of camphene. Intake rate, as determined by bite diameter of stems browsed by moose, was slightly inversely correlated with camphene concentration and strongly positively correlated with snow depth.

In the second chapter, we investigated how behavioral and physiological adaptations in response to PSMs influence the nutritional condition of moose. We used urine samples from individual moose to isolate and quantify glucuronic acid (GA) as a biomarker of PSM exposure. Controlled feeding studies in herbivores have shown a positive relationship between ingestion of PSMs and GA excretion (Jakubas et al. 1993; Guglielmo et al. 1996; Servello and Schneider 2000; Sorensen et al. 2005). We measured urea nitrogen (UN) as a biomarker of overall nutritional condition. In mammals urea nitrogen (UN) is generally a byproduct of protein breakdown (Weiner et al. 2014). When individuals are not meeting protein needs through dietary intake, they breakdown endogenous proteins to make up the balance, resulting in an increase in UN excretion and a decrease in nutritional condition (Moen and DeIgiudice 1997; Fouillet et al. 2008). Finally, we measured creatinine (C) levels in urine samples to correct for individual sample dilution from snow volume or relative animal hydration. Creatinine is produced from the breakdown of phosphocreatine in skeletal muscle at a relatively constant rate based on the lean muscle mass, thereby making it useful to correct for dilution effects (DelGiudice 1995; Doi et al. 2009). These physiological biomarkers were used to examine relationships between GA:C and UN:C within and across 17 years of field data.

We predicted that increased GA:C concentrations would be positively correlated with increased UN:C values and that increased PSM exposure would be positively correlated with both GA:C and UN:C values. To test these hypotheses, we used moose urine collected from snow and chemical profiles of browsed balsam fir trees as identified by fresh browsing marks. Positive inter and intra-annual correlations were observed between GA:C and UN:C across 17 of the 18 years of collected samples. Additionally the relative amount and rate of monoterpenes (a class of PSMs) consumed by moose was significantly correlated with decreased nutritional condition in island moose.

Results detailed in this thesis provide further understanding of the effects of bottom-up regulation of browsing and nutritional consequences for large generalist herbivores. This research provides a simple, quantitative, relatively inexpensive, and non-invasive, methodology for monitoring how diet quality influences the nutritional condition of wild herbivores. These methods are not species specific and thus can be used to monitor the nutritional condition of a variety of avian and mammalian herbivores. Furthermore, this research informs basic science and applied management practices for ungulate population biology as well as for species that are limited in distribution, difficult to manage, economically important, or of conservation concern.

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CHAPTER ONE: PLANT SECONDARY METABOLITES DRIVE DIET SELECTION AND INTAKE RATE BY MOOSE

Abstract

Free-ranging vertebrate herbivores must make dietary choices that balance the ingestion of necessary nutrients while minimizing exposure to potentially harmful plant secondary metabolites (PSMs). During winter months, food availability becomes highly restricted for some herbivores, making it difficult to avoid plants high in PSMs. During winter, moose (*Alces alces*) living on Isle Royale, Michigan are restricted to foraging on evergreen trees that are relatively high in PSMs and low in principle nutrients. Specifically, balsam fir (*Abies balsamea*) is a chemically defended conifer that is the principle food source in winter for moose living on Isle Royale. We evaluated the dietary quality of winter balsam fir in both eastern and western regions of Isle Royale as well as the chemical and physical parameters governing diet selection and intake rate for island moose. We identified foraging patches of moose and collected representative samples from browsed and unbrowsed trees within each patch. We analyzed the chemical characteristic of each browsed and unbrowsed tree by quantifying total and individual monoterpenes, total tannins, total phenolics, and crude protein. Crude protein and bornyl acetate differed between east and west regions of Isle Royale but neither influenced diet selection. A single monoterpene, camphene, was the strongest predictor of moose browsing, with moose selectively browsing on trees that had higher concentrations of

camphene. Intake rate, as determined by bite diameter of stems browsed by moose, was slightly inversely correlated with camphene concentration and strongly positively correlated with snow depth. These results indicate that both PSMs and environmental conditions influence foraging behavior of a large generalist herbivore.

Introduction

Vertebrate herbivores consume a wide variety of plant species that vary in concentrations of nutrients and plant secondary metabolites (PSMs). Spatial and temporal variation in PSMs is influenced by many factors including environmental stress (Kopsell and Kopsell, 2008), light quality and availability (Li and Kubota 2009), soil fertility (Ormeño et al. 2008), invading pathogens (Glazebrook 2005) and direct effects of herbivory (Dicke and van Loon 2000). Herbivores make foraging decisions at multiple spatial scales, ranging from stem selection at the plant scale to patch selection at the landscape scale (de Jager et al. 2009; Frye et al. 2013). Damage by herbivores also has the potential to influence plant chemistry (Ulappa et al. 2014) and communities (Stolter 2008). For example, white spruce (*Picea glauca*) has been shown to increase the production of camphor in response to browsing by snowshoe hares (*Lepus americanus*) (Sharam and Turkington 2009). Similarly, browsing by moose (*Alces alces*) on two species of willow (*Salex phylicifolia* and *S. myrsinifolia)* resulted in the induction of certain phenolic compounds which were subsequently selected against by moose during return visits to these same plants (Stolter et al. 2005).

It is widely accepted that PSMs function principally as deterrents against herbivory. Two classes of PSMs, phenolic and monoterpenes, are among the most studied with regard to how PSMs influence herbivory (Acamovic and Brooker 2005; Kumbaşlı et al. 2011). Monoterpenes are found in the essential oils of the leaves and fruits of many plant species. They have no known nutritional value and often function as chemoattractants or chemorepellents (McGarvey and Croteau 1995; Crowell 1999). Most monoterpenes are volatile compounds that can be released by the plant as a result of herbivore damage (Dudareva et al. 2004), which may function as chemical communication to undamaged conspecific plants to upregulate production of PSMs (Shiojiri et al. 2015; Karban et al. 2016). Many studies indicate that monoterpenes are used as a direct defense against both vertebrate and invertebrate herbivores (Gong and Zhang 2014; Hansen et al. 2016). Tannins are phenolic compounds that can bind to dietary protein (Degabriel et al. 2008; Moore et al. 2010). Many plants contain a suite of simple (hydrolysable) or complex tannins which can inhibit digestion of proteins, and some tannins have been shown to exhibit chronic hepatotoxic effects (Reed 1995). However, not all phenolic compounds are protein-binding tannins. Non-tannin phenolic compounds may be linked to dietary health benefits including antioxidant (Rice-Evans et al. 1997; Medina 2011) and anti-carcinogenic (Adlercreutz et al. 2000; Owen et al. 2000; Touillaud et al. 2007; Patterson 2011) properties.

Direct toxicity or reduced digestibility resulting from the ingestion of PSMs could result in major metabolic (Kohl et al. 2015), energetic (Sorensen et al. 2005), or fitness costs (Iason 2005; Degabriel et al. 2009) to herbivore consumers. Thus, selection is expected to favor herbivores that mitigate the deleterious effects of PSMs while still meeting nutrient requirements and maintaining a positive net energy balance. To achieve these outcomes, suites of behavioral and physiological adaptations are often used by herbivores. Behavioral responses to PSMs may include avoidance of plants containing high levels of PSMs, selection of specific PSMs, or diet mixing (Freeland and Janzen 1974; McArthur et al. 1991). In terms of avoidance, many studies have documented free-ranging animals selecting the highest quality forage available. For example, free-ranging Greater sage-grouse (*Centrocercus urophasianus*) selected plants, patches, and species of plants to browse with lower PSMs than those that were abundantly available but not selected by grouse (Frye et al. 2013). By contrast, in some cases herbivores select for, rather than avoid, specific PSMs. For example, some avian herbivores select food items higher in UV-reflecting PSMs (Siitari et al. 2002; Rajchard 2009). Diet mixing, whereby foragers consume different food types to avoid excessive exposure to any one PSM or combination of PSMs, is likely the most common behavioral adaptation employed by herbivores (Provenza et al. 2003; Wiggins et al. 2005). However, it is important to note that behavioral responses are not mutually exclusive and likely work in concert with other adaptive mechanisms to regulate exposure to PSMs (Torregrossa and Dearing 2009).

Feeding studies with captive animals can often inform research on the foraging behavior of wild herbivores. For example, these studies often provide researchers with valuable information about forage preferences in heterogeneous habitats (Hjältén et al. 2004), sensitivities to PSMs (Baker et al. 2005), and metabolic efficiencies (Servello and Schneider 2000; Sorensen et al. 2005; Shipley et al. 2012) in species of interest. Captive studies can also reveal how diet quality affects body condition and reproduction for vertebrate herbivores (Pehrson and Lindlöf 1984; Servello and Schneider 2000; Parker 2003). However, while captive studies may help explain certain elements of foraging behavior, information obtained from captive feeding trials does not necessarily correlate with observable foraging behaviors in wild populations (Forbey et al. 2013). Wild herbivores are generally faced with more complex foraging choices than are their captive counterparts (Moore et al. 2010). For example, animals in wild populations must make foraging decisions that balance the need for nutrients with the need for protection from predators (Searle et al. 2008; McArthur et al. 2014), the elements (Parker et al. 1999), and competition with other herbivores (Beck and Peek 2005). These interactions often result in animals selecting habitats with decreased forage quality in favor of the protection it provides. For example, wild ungulates select for denser, more mature forest stands with decreased movement relative to open habitats during times of increased winter snowfall (Dussault et al. 2005; Gaudry et al. 2015). Additionally, elk (*Cervus elaphus*) select lower quality and more open habitat following wolf reintroduction to Yellowstone National Park (Hernández and Laundré 2005; Mao et al. 2005).

In most natural ecosystems, the abundance of forage options available to herbivores, combined with interactions between habitat structure and predation risk, make it difficult to assess the effects of PSMs on wild populations. In the present study, we used the balsam fir (*Abies balsamea*) – moose (*Alces alces*) system in Isle Royale National Park, Michigan in central North America (Appendix A) to investigate how nutritional and chemical quality of plants shape diet selection by a large free-ranging generalist herbivore. This system presents an ideal opportunity to test how PSMs influence diet selection because balsam fir is the primary source of winter browse for moose on Isle Royale (Montgomery et al. 2013) and it exhibits inter-patch variation in protein and PSM concentrations (Thompson et al. 1989). This variation in dietary composition allowed us to assess whether island moose select diet based on nutrient and chemical quality. Additionally, low wolf densities during our study period (two known individuals on Isle Royale with no known breeding pairs at the time of this writing; Peterson and Vucetich 2016) minimized the influence of predation on moose foraging behavior, effectively increasing our ability to investigate the role of PSMs while limiting the confounding influences of anti-predator behaviors. Finally, because our experimental design made use of snow to track individual animals to foraging areas, we were able to investigate how snow depth influenced the foraging behavior of moose. Few, if any, ecosystems can off these exclusive parameters for a natural study of wild herbivores. Thus, conditions on Isle Royale provided a unique opportunity to test whether nutritional quality and chemical defenses of balsam fir influence diet selection and feeding rate by a wild population of moose. We used this study system to test the following predictions regarding foraging decisions by moose:

1. Given distinct geological differences between east and west regions (Appendix A), we predicted that nutrient and PSM concentrations of balsam fir will be significantly different between the east and west regions of the island.
2. Browsed balsam fir would have lower concentrations of PSMs and higher % crude protein than unbrowsed balsam fir.
3. Intake rate of balsam fir would be negatively correlated with concentrations PSM in browsed plants but would generally increase as snow accumulation increases.

Methods

To test these predictions, we compared the concentrations of nine individual monoterpenes, total monoterpenes, total phenolics, total tannins, and crude protein in balsam fir trees browsed and unbrowsed by moose during winter months. We also measured bite diameter and snow depth within foraging patches. Trees were analyzed as paired samples with browsed and unbrowsed identified in each foraging patch.

Sample Collection

All samples were collected on Isle Royale between late January and early February in of each of the three study years (2013, 2014, and 2015). Each year, samples were collected from browsed balsam fir trees on both east and west regions of Isle Royale (Fig 1.1).

Dietary selection of specific fir trees by moose in the field was documented by identifying bite marks exclusive to moose. We used these data to assess dietary parameters that influence both selection and intake rate of balsam fir. Because moose are the only large herbivore on Ilse Royale that consume balsam fir, we were able to use bite mark data to determine intake rate and diet selection without physically observing moose browsing behavior. For samples collected during 2014 and 2015, the diameter of browsed twigs at the site of observed bite marks on each tree were measured with calipers to the nearest millimeter (mm), these measurements were not taken during sample year 2013. Diameter measurements acquired in 2014 and 2015 were used as a proxy to estimate intake rate of balsam fir (Risenhoover 1987, Fig 1.2). We sampled unbrowsed lateral stems on the same whorl as the browsed stems for later analysis of the chemical content of browsed trees (note: *A. balsamea* typically grows one new whorl of lateral branches concentrically around the main trunk each year; Silvertown and Dodd 1999; Fig 1.3). We clipped these samples at the point on the stem that corresponded to the same diameter as the observed bite mark. We also collected samples from the nearest non-browsed tree that was located within reach of the moose (approximately 1m) with respect to the tracks in the snow. Samples collected from unbrowsed trees were collected from the same whorl and at the same bite diameter as the bite marks on the paired browsed trees. We collected paired samples from three browsed and three unbrowsed fir trees within each distinct foraging patch (i.e., an area along the track of the moose where observable browse marks were identified on a minimum of three individual trees). Additionally, snow depth (cm) was measured at the base of each tree sampled. Areas where moose tracks moved straight through a patch of trees without signs of active browsing, or where moose browsed only one or two trees, were not included. Samples from browsed and unbrowsed fir trees were placed in individual plastic bags and stored at -20^{0}C for later analysis. Twigs from the browsed and unbrowsed trees represent paired samples (n=3 pairs/patch) that were used to determine selectivity.

Sample Preparation

Clippings from browsed and unbrowsed fir trees were frozen using liquid nitrogen and ground with a mortar and pestle to an average particle size of ~ 2 mm. A subsample (100mg wet weight, WW) of each ground sample was placed in a 20mL glass head-space vial with a crimp style cap for analysis of monoterpenes. Subsamples of each ground sample were placed in separate 1.5mL micro-centrifuge tubes for analysis of total phenolics (50mg WW) and tannins (200mg WW). Approximately 2-4g WW of each ground sample was dried for three days at 69^{o}C in a Thermo-Scientific, Heratherm oven. Dried samples were re-weighed to obtain sample dry weights. These samples were then sent to Dairy One Laboratories (Ithaca, New York, USA) for analysis of available nitrogen content via combustion method AOAC 990.03.

Chemical Analysis of Samples

Monoterpenes in balsam fir were measured using headspace gas chromatography. Concentrations were measured with an Agilent 7694 headspace auto-sampler coupled with an Agilent 6890N gas chromatograph (GC). Headspace vials containing 100mg WW of each ground sample were heated for a period of 20 min at 100°C to achieve vial equilibrium. One mL of headspace gas was then injected into a J&W DB-5 capillary column (30m x 250µm x 0.25µm). We identified monoterpenes via co-chromatography with known standards, and quantified the area under the curve (AUC) of major monoterpenes, which we defined as those comprising >1% total AUC and present in >70% of all samples (Fig 1.4). Detailed operating parameters for the GC headspace sampler are provided in Appendix B.

Total phenolics in plant samples were analyzed by eluting 50mg WW of each ground sample with 1.0mL of HPLC grade methanol. Eluted samples were placed in a sonicating water bath and sonicated for a period of 3 min, two times at 25°C with a 2 min break between sonications to minimize heating. Samples were centrifuged at 13,000g for a period of 5 min. The supernatant was filtered through glass wool into a 1.5mL amber vial. Samples were analyzed for total phenolics following procedures from Ainsworth and Gillespie (2007). Briefly, 20µL subsamples were diluted to a total volume of 100µL (1:5 factorial dilution) with HPLC grade methanol. Samples were prepared in triplicate in a 96-well plate with 20µL diluted sample extract. Each sample was reacted with 100µL 10% Folin-Ciocalteu reagent (Sigma 47742; Sigma-Aldrich, St. Louis, Missouri, USA) and 80µL 7.5% sodium carbonate solution. All reagents were thoroughly mixed inside the plate wells. Plates were then wrapped in aluminum foil and incubated at room temperature for a period of 120 min. Plates were read on a Biotek SynergyMX multi-mode micro-plate spectrophotometer at 765nm. Each sample was paired with a negative control and ran with a range (0 – 1.0 mg/ml) of known standards of Gallic acid (Cat #5995-86-8, Fisher Scientific, Waltham Massachusetts, USA).

Total tannin content was analyzed using radial diffusion methods detailed in Hagerman (1987). Each ground sample (200mg WW) was eluted in 1.0mL of 70% acetone, placed in a sonicating water bath and sonicated for a period of 10 min at 25^{o}C, centrifuged at 13,000g for a period of 5 min and filtered through glass wool into a 1.5mL micro-centrifuge tube. Bovine serum albumin (100mg, BSA, fraction V, fatty acid-free, Cat #126575 Millpore Corp. Billerica, Massachusetts) was dissolved in an agarose gel solution mixed in a 50mM acetic acid and 60µM ascorbic acid buffer (pH ca. 5.0). Protein-agar solution was poured into a 245 x 245mm petri tray and stored at 4^{o}C until the gel was hardened. A 4mm cork borer was used to create 4mm wells in the hardened agar gel spaced ≈25mm apart. Four 8.0µL aliquots of each sample were added to wells. Plates were covered and placed in a Thermo-Scientific Heratherm oven at 30^{o}C for 96 hours. Concentration of tannins per sample was calculated from precipitate rings that formed in the gel as tannin compounds bound to the BSA protein. Precipitate rings were measured horizontally and vertically to the nearest millimeter. Horizontal and vertical diameters were averaged together for a mean diameter for each precipitate ring and the area was determined. Samples were analyzed with standards of known concentrations (0.5 g/mL to 10 g/mL) of Tannic acid (Sigma 403040; Sigma-Aldrich, St. Louis, Missouri, USA).

Statistical Analyses

Spatial Variation in Quality of Fir

To assess spatial variation in dietary parameters, we conducted Wilcoxon rank sum tests with continuity correction using mean, patch level, values to compare each dietary (total monoterpenes, each of nine individual monoterpenes, number of different monoterpenes, total phenolics, total tannins, % crude protein), structural (snow depth), and behavioral (bite diameter) variables between east and west regions of Isle Royale. We used Kruskal-Wallis ranked sum tests to assess temporal variation in these same dietary parameters across all three years of the study.

Diet Selection

To test which dietary variables best predicted diet selection, we first conducted a multivariate analysis (JMP Pro version 12.1.2) with all dietary covariates (i.e., total monoterpenes, each of nine individual monoterpenes, total phenolics, total tannins, and % crude protein). Collinear variables (i.e., $|r| > 0.7$, Appendix C) were removed from the analysis. Specifically, if unknown compound were correlated with known compounds, the unknown compound was removed from further analysis. Moreover, if two or more unknown compounds were correlated, the compound with the greatest impact on plant chemistry based on available literature was retained and the others were removed from further analysis (Fig C.1). For all remaining non-correlated covariates, we compiled a candidate model set by fitting individual conditional logit (clogit) models to identify factors associated with diet selection. For each clogit model, the browsing condition of each sampled tree in each foraging patch (i.e., browsed or unbrowsed) was treated as the binary response while nutrient and PSM covariates were treated as continuous predictors.

Each clogit model was then subjected to univariate analysis with $\alpha > 0.5$ considered to be non-predictive of selection. Models with $\alpha > 0.5$ were removed from the candidate model set. Remaining models were evaluated using Akaike's Information Criterion values with sample size bias adjustment (AICc) to determine which covariates best predicted diet selection.

Bite Diameter

Using an analysis similar to the one described above, we compiled a candidate model set to evaluate predictors of bite diameter as a proxy for intake rate, with an additional structural covariate of snow depth. Generalized Linear Models (GLM) were fit to each of the covariates that was not removed due to multi-collinearity. Each of these models was subjected to univariate analysis at $\alpha > 0.5$ to remove nonpredictive covariates from the candidate model set. Following univariate analysis, remaining models were evaluated by AICc to determine which covariates best predicted bite diameter. Bite diameter measurements were not recorded during the winter of 2013, so this year was not included in analyses.

For both selection and bite diameter candidate sets, models with AICc values higher than that of the null (intercept only) were removed from further consideration. Models with AICc values lower than that of the null yet greater than 2 AICc units of difference from the top model (ΔAICc) were deemed to not carry enough model weight (w_i) to be sufficiently predictive and were removed from further consideration. Models with AICc values lower than that of the null and less than 2 ΔAICc from the top model were considered competing models and were included in a second round of model selection in which all possible combinations of competing model covariates were considered. Unconditional variance estimates were used to estimate 85% and 95% confidence intervals for estimated use coefficients for top models following second round model selection. Statistical analyses were conducted in R version 3.2.2 (R Foundation for Statistical Computing 2015).

Threshold of Selection

We explored the selection threshold for the top model that best predicted diet selection using a Generalized Additive Model (GAM) and smoothing parameters to determine the mean concentration of the patch (average concentration of browsed and unbrowsed) at which selection occurred for the top covariate. Selection was determined by the difference of top covariates between browsed and unbrowsed plants within each foraging patch. Positive differences (higher values in browsed plants vs. unbrowsed) indicated selection in favor of that covariate, whereas negative differences indicated avoidance of that covariate. Difference values equal to zero indicated of lack of selection for that covariate. The model was plotted with 95% Bayesian confidence intervals using the package {mgcv} in R.

Results

Spatial Variation in Quality of Fir

There was no difference between east and west regions of the island for the majority of covariates except bornyl acetate and % crude protein. Both these parameters were significantly higher in the western region compared to the eastern region (Table 1.1), but neither of these covariates predicted browsing. There was considerable temporal variation in the majority of covariates with a general trend for largest bite diameter, greatest snow depth and highest concentrations of monoterpens in 2014, highest % crude protein and total phenolics in 2013, and highest concentration of tannins in 2015 (Table 1.2). Detailed spatial comparisons by sample year for each covariate are shown in Appendix D (Table D.1).

Diet Selection

Browsed and unbrowsed trees were similar in overall composition of monoterpenes (Fig 1.4), but varied with respect to a single monoterpene, camphene. Concentrations of camphene were significantly higher in browsed fir trees compared to their paired unbrowsed counterparts (Fig 1.5). Total monoterpenes, total phenolics, total tannins, and % crude protein did not differ significantly between browsing states (Fig 1.6).

Following univariate analysis of clogit models prior to model selection, camphene and bornyl acetate emerged as significant parameters explaining browsing state ($p = 0.009$, CI = 1.005 – 1.04; $p = 0.048$, CI = 1.00 – 1.062, respectively; Table 1.3). Island-wide, camphene alone accounted for 71% of the total weight of the candidate model set and fit the data better than the null model (AICc = 90.84, $w_i = 0.71$, table 1.4). Camphene was the strongest predictor of diet selection for moose on Isle Royale at both east and west ends of the island (Appendix E, Tables E.1 - E.3). Bornyl acetate emerged as the second model of the candidate set (AICc = 94.47, $w_i = 0.12$, Table 1.4). However, model performance for bornyl acetate was greater than 3 ΔAICc values from the top model, accounted for only 12% of the total weight and had a narrow range at 95% confidence that included 1.00 on the lower boundary. For these reasons, bornyl acetate was determined to not be sufficiently reliable as a predictor of browsing selection, and model averaging was not used. Following first round AICc model selection, camphene was the only top model that remained. Analysis of estimated use coefficients indicated that odds of plant selection increased by 2.2% for each unit increase in camphene (AUC/mg DW, Table 1.5).

Bite Diameter

Univariate analysis of Generalized Linear Models (GLM) identified three covariates with a significant influence on bite diameter: camphene, bornyl acetate, and snow depth (Table 1.6). Using diameter-weight-length equations specific to *A. balsamea* on Isle Royale (Risenhoover, 1987, Appendix F), we were able to extrapolate intake rate of island moose based on bite diameter of browsed twigs on browsed trees (Fig 1.2). In general, larger bite diameters equate to larger rates of dry matter intake (Appendix F, Fig F.1). Following first round AICc model selection, camphene again emerged as the top predictor of bite diameter (AICc = 98.33, w_i = 0.38, Table 1.7). Two competing models were also supported: snow depth (AICc = 99.32, w_i = 0.23) and bornyl acetate (AICc = 99.58, w_i = 0.20, Table 1.7). Second round model selection of all possible combinations of the top variables resulted in an additive model that contained snow depth and camphene as the best predictors of bite diameter (AICc = 91.81, w_i = 0.65, Table 1.8), and the only model with an AICc value lower than that of the null. Analysis of use with respect to balsam fir browsing indicated that bite diameter was positively associated with snow depth and negatively associated with camphene. Bite diameter increased by 1.2% for each unit increase in snow depth (cm) and decreased by 0.7% for each unit increase in camphene (AUC/mg DW, Table 1.5, Fig 1.7).

Threshold of selection

A Generalized Additive Model (GAM) was used to explore the threshold of selection for the best-performing model, camphene. This model showed no selection for camphene when mean concentration of camphene in the patch was below 80 AUC/mg DW. As camphene concentrations increased in the patch above ≈95 AUC/mg DW, selection for plants by moose steadily increased (Fig 1.8).

Discussion

Overall, results are consistent with our predictions that diet quality influences diet selection and intake rate by moose, with one major exception. We predicted that moose would select to browse on fir trees with lower concentrations of PSMs and higher % crude protein compared to unbrowsed trees. In contrast to this prediction, our results showed that moose at Isle Royale browsed preferentially on fir trees containing higher concentrations of camphene (Fig 1.5, 1.8). We consider two possible explanations for this result that are not necessarily mutually exclusive. First, camphene may be beneficial, or at least non-harmful to moose. Camphene is a common hydrocarbon monoterpene found in the essential oils (EO) of many plants and it is generally accepted that EO toxicity is rarely the result of a single component, rather it is the result of a summation of all EO constituents (Bekele and Hassanali 2001; Miresmailli et al. 2006). Studies investigating toxicity of individual components of many EO are generally limited to invertebrate herbivores; relatively few focus on vertebrates. In studies of invertebrate herbivores, camphene was shown to be among the least toxic EO terpenoids (Cook and Hain 1988; Abdelgaleil et al. 2009). Camphene-specific effects on vertebrate herbivores are poorly understood. However, camphene does not have deleterious effect on the activity of digestive enzymes in avian herbivores (Kohl et al. 2015). Moreover, in dose dependent trials, camphene increased insulin response and decreased liver damage associated with diet in mice (Kim et al. 2014). Additionally, many monoterpenes may serve as chemo-attractants to some species (Yan et al. 2005; Brilli et al. 2009). It is possible that browsed trees are producing higher amounts of camphene due to other ecological interactions (Holopainen and Gershenzon 2010; Iason et al. 2011) and that moose, through some as yet unidentified physiological pathway, are attracted to these trees because of the heightened camphene production.

An alternative explanation for higher levels of camphene in browsed trees relative to unbrowsed trees is that it represents a response by fir to browsing. It is well documented that certain PSMs, including many volatile monoterpenes, are induced in response to both biotic and abiotic stressors. For example; herbivory (Dicke and van Loon 2000), pathogens (Glazebrook 2005), light and temperature (Peñuelas and Llusià 2002; Li and Kubota 2009), drought (Turtola et al. 2003), and competition for resources (Ormeño et al. 2007) can cause induced defenses in plants. Anecdotal field observations during sample collection also suggest that browsed trees are more likely to be repeatedly browsed in subsequent visits by moose than unbrowsed trees. However, in a post-hoc analysis of our data, we found no statistical relationship between camphene concentrations and browsing history intensity (Regression, $F_{1,68} = 0.41$, $p = 0.52$). Therefore, it does not necessarily follow that camphene is induced in these trees as a result of repeated browsing by moose. Nebeker et al. (1995) demonstrated that stressed lodgepole pine trees (*Pinus contorta*) expressed higher concentrations of certain monoterpenes, including camphene. Browsed fir trees could be increasing camphene production due to an unrelated environmental stressor, and the moose are being attracted to those trees through the heightened camphene production.

Results also indicated that although moose browsed preferentially with respect to camphene, intake rates as indicated by bite diameter, decreased as camphene concentrations increased. These results indicate that moose are selecting for relatively higher concentrations of camphene, which in turn is associated with smaller bite diameters, and therefore, lower intake rates. Lower intake rates of plants with higher camphene may require a longer foraging period to reach satiation which is inconsistent with evidence that herbivores maximize fitness gains while minimizing other risks (Brown and Kotler 2004; Searle et al. 2008; McArthur et al. 2014; Nobler 2016). Island moose subsist primarily, but not exclusively, on balsam fir during winter months (Parikh et al. 2016). Moose may select for smaller bites of higher camphene because of diet mixing. Herbivores often use a mixed diet to help mitigate the cumulative effects of any one PSM or suite of PSMs (Provenza et al. 2003; Wiggins et al. 2005; Torregrossa and Dearing 2009). Moose may be selecting fir trees higher in camphene because it helps regulate the effects of ingested PSMs from other browse sources (e.g., cedar) that were not analyzed in this study. Additionally, many constituents of plant EO provide beneficial anti-microbial activity in ruminants (Benchaar and Greathead 2011). These benefits include decreased methane production, decreased bloating, and increased digestion efficiencies. However, toxicity issues associated with EO and their constituents remain. Moose may be selecting for camphene because it helps mitigate the effects of certain deleterious gut microbial activity, thereby allowing them to consume more biomass of chemically defended forage, but may be selecting for smaller bites due to camphene's cooccurrence with other harmful PSMs. In our data, camphene was strongly correlated with an unknown monoterpene (unknown 12.08, r = 0.977, Appendix Fig C.1). It is possible that this unknown compound lacks any dietary benefit and is instead associated more directly with EO toxicity. Conversely, camphene itself may have beneficial effects at low doses, yet detrimental effects at high doses. To the best of our knowledge there is no direct evidence linking camphene to toxicity or digestive efficiency in ruminants.

Bite diameter, and therefore intake rate, was positively correlated with snow depth in that larger bite diameters were found in areas of deeper snow. Winter conditions with deep snow may subject herbivores to greater risk of predation and increased energy demands of movement. Under these energetically costly conditions, herbivores must still balance the need for nutrition and protection from predators with the resources they have available. Our results show that intake rates of moose increased as snow depth increased, indicating a shift to a more efficient foraging strategy (Tadesse and Kotler 2013). Although greater bite diameter indicates increased rate of intake, we do not know the relative effect of larger bite diameters on digestive efficiency. Larger bite diameters increase the amount of difficult to digest fibrous material taken in, thereby lowering the principle nutrients per bite (Shipley et al. 1999). Herbivores are expected to adopt patch foraging strategies that maximize nutrient intake while simultaneously addressing the energetic requirements of other activities (Searle et al. 2005; Shipley 2010). The increased energetic demands of movement in deep snow may cause individual moose to consume larger bites, despite those bites being relatively less nutritious. Dussault et al. (2005) reported that during periods of heavy snow fall, moose selected habitats that provided shelter from snow and decreased movement within those shelters. It is possible that island moose elect to move less in deep snow and remain in a foraging patch longer than they would in light snow conditions. Under these conditions, forage resources would become increasingly limited the longer the animal remained in that patch. As a result, moose remaining in foraging patches would have to use more of the available browse, resulting in larger bite diameters (Vivas and Saether 1987; Shipley 2010; Rea et al. 2015). It is also possible that increased bite diameters are not a function of depleting resources and are, in fact, a function of intake rate resulting from an increased need to maintain thermal regulation. Indeed, one possible source of error in our data is that it did not include temperature as a possible predictor of intake rate. Thus, our data may not fully address the metabolic energy requirements that dictate foraging behavior of wintering moose. Direct observation studies are needed to further our understanding of the relationship between resource availability, climatological factors, and intake rate by moose.

Some research suggests that male moose in particular, select habitat based on dietary energy requirements rather than protection (Main 2008; Bjørneraas et al. 2011). In such cases digestible energy (DE) may be the most important dietary factor (Parker et al. 1999; Cook et al. 2004) which may be limited or completely outweighed by energetic costs of detoxifying co-occurring PSMs (Dearing et al. 2005; Sorensen et al. 2005). Therefore, herbivores are expected to select forage options highest in energy and lowest in PSMs. Parikh et al. (2016) reported that Northern white cedar (*Thuja occidentalis*), another abundant evergreen on both east and west ends of Isle Royale, contained more DE than balsam fir. Yet, island moose browse preferentially on fir trees (Montgomery et al. 2013; Parikh et al. 2016). It is possible that cedar contains higher concentrations of

PSMs than balsam fir, thereby negating the energetic benefit of increased DE that cedar contains. Neither Parikh et al. (2016) nor the present study examined the PSM concentrations in white cedar on Isle Royale. Future studies concerning winter dietary selection of moose on Isle Royale should include a chemical profile of white cedar or other dietary components in addition to balsam fir.

We found significant annual variation in dietary quality with respect to the majority of our tested dietary variables (Table D.1). However, our samples were collected during the same season each year. Variation between seasons within a single year may be more severe and significant to survival. Northern ungulates experience seasonal variation in energetic metabolic requirements (Wam and Hjeljord 2010) and quality of summer forage is believed to be more importantly linked to winter survival than the forage available during winter (Cook et al. 2004; McArt et al. 2009; van Beest et al. 2010; Wam and Hjeljord 2010). However, that does not negate the importance of winter forage quality. If female winter nutritional condition is not sufficient to accommodate the energetic demands of fetal gestation and lactation, reproductive complications such as low birth weight (Adams 2005), weakened immune system (Treanor 2012), congenital birth defects (Legbo et al. 2010) and reproductive failure (Loudon et al. 1983), may arise. If winter dietary quality is not sufficient to maintain relatively healthy body conditions, fitness costs may result, potentially influencing population dynamics.

We have shown that PSMs influence the foraging selection behavior and intake rates of a large generalist herbivore. These results can inform further research into ungulate nutritional ecology and habitat selection. Additionally, our results may have important implications for the management practices of geographically isolated populations. To fully understand population dynamics of a prey species, we must understand seasonal behavioral adaptations that include diet and habitat selection in addition to the more broadly studied consumptive and non-consumptive consequences of predators. As top-down regulation by predators on the island continues to diminish, regulation of the Isle Royale moose population is likely to become increasingly governed by availability and quality of forage. Understanding and monitoring these dietary characteristics will become necessary for the successful and continued maintenance of both moose and fir populations.

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Table 1.1 Mean concentrations ($\bar{x}$), Wilcoxon rank sum test (W), significance (p-value), and estimated difference value (est, with 95% confidence intervals, CI) for plant secondary metabolites (total, individual, and number of monoterpenes >1% total AUC and present in >70% samples (AUC/mg DW), total phenolics (mgGAE/g DW), total tannins (µM TAE/g DW)), nutrient content (% crude protein), snow depth (cm), and bite diameter (mm) in balsam fir trees (*Abies balsamia*) at patches browsed by moose (*Alces alces*) at both east and west ends of Isle Royale National Park, Michigan, USA during winter 2013, 2014, and 2015.

Parameter	East ($\bar{x}$)	West ($\bar{x}$)	W	p-value	est (95% CI)
Total Monoterpenes	1105.6	1095.70	5176	0.699	13.073 (-57.217 - 78.373)
Unknown 12.08[1]	13.07	13.32	4849	0.686	-0.223 (-1.193 - 0.767)
α-pinene	106.73	105.13	5237	0.593	2.133 (-6.03 - 9.72)
Camphene	89.22	89.92	4918	0.813	-0.542 (-6.79 - 5.71)
β-pinene	553.1	538.60	5339	0.434	16.382 (-24.54 - 58.10)
Myrcene	17.33	16.77	5521	0.221	0.870 (-0.47 - 2.14)
R-limonene	164.61	170.01	4977	0.925	-0.988 (-15.16 - 13.45)
Terpinolene	9.63	9.43	5269	0.540	0.309 (-0.69 - 1.32)
Borneol[2]	46.56	41.32	5435	0.309	2.274 (-2.059 - 6.689)
***Bornyl Acetate**	**36.77**	**48.18**	**3713**	**0.002**	**-9.325 (-15.607 - -3.594)**
Number of compounds[3]	8.69	8.60	5228.4	0.584	4.8e-05 (-1.807 - 4.834)
Total Phenolics[4]	29.41	28.99	4952	0.877	-0.181 (-3.14 - 2.98)
Total Tannins[4]	5.64	5.79	4965	0.658	0.136 (-0.55 - 0.81)
***Crude Protein**	**8.87**	**9.42**	**3321.5**	**<0.000**	**-0.625 (-0.89 - -0.34)**
Snow Depth	55.23	54.07	5504	-0.764	2.999(-1.99 - 7.00)
Bite Diameter[5]	2.94	2.73	728.4	0.143	0.199 (-0.067 - 0.466)

***Significant difference between island regions α = 0.05 in bold**

[1] Unidentifiable monoterpene, numbers following "unknown" refer to retention time (min) in the chromatogram resulting from gas chromatography.

[2] Borneol concentrations were measured but were excluded from analysis due to inconsistency in repeat samples and lack of adequate control standards.

[3] Total number of monoterpenes with retention times < 24 minutes, > 1% total AUC, and present in > 70% of samples.

[4] Total phenolics and tannins, concentrations in mg Gallic Acid Equivalence (GAE, phenolics) or µM Tannic Acid Equivalence (TAE, tannins)/g DW.

[5] Bite diameter, diameter of the twig (mm) at the observed bite mark by moose on browsed trees. Bite diameter data was not collected during sample year 2013.

Table 1.2 Mean concentrations for each of three years (2013, 2014, and 2015) ($\bar{x}$), chi square value (χ2), and p-value for plant secondary metabolites (total, individual and number of monoterpenes >1% total AUC and present in >70% samples (AUC/mg DW), total phenolics (mgGAE/g DW), total tannins (µM TAE/g DW)), nutrient content (% crude protein), snow depth (cm), and bite diameter (mm) in balsam fir trees at patches browsed by moose at Isle Royale National Park, Michigan, USA during winter. Chi square and significance values results from nonparametric univariate comparison (Kruskal-Wallace rank sum test) tests are shown for each parameter compared between sample years indicating a high degree of temporal variation in PSM and nutrient concentrations.

Parameter	2013 ($\bar{x}$)	2014 ($\bar{x}$)	2015 ($\bar{x}$)	χ^2	p-value
***Total Monoterpenes**	**1033.15**	**1211.12**	**1036.6**	**21.60**	**<0.001**
Unknown 12.08[1]	13.39	13.60	12.47	3.42	0.181
***α-pinene**	**108.20**	**112.55**	**96.2**	**11.49**	**0.003**
Camphene	88.11	94.63	84.83	5.82	0.055
***β-pinene**	**528.97**	**614.39**	**483.81**	**22.74**	**<0.001**
***Myrcene**	**16.13**	**19.58**	**15.03**	**35.54**	**<0.001**
***R-limonene**	**149.50**	**193.96**	**151.81**	**19.67**	**<0.001**
Terpinolene	9.55	9.81	9.2	1.61	0.447
***Borneol[2]**	**22.41**	**44.59**	**65.09**	**73.01**	**<0.001**
***Bornyl Acetate**	**42.09**	**34.84**	**49.6**	**13.29**	**0.001**
***Number of compounds[3]**	**8.63**	**8.85**	**8.44**	**6.31**	**0.043**
***Total Phenolics[4]**	**46.08**	**19.13**	**24.89**	**134.88**	**<0.001**
***Total Tannins[4]**	**3.33**	**5.89**	**7.81**	**107.4**	**<0.001**
***% Crude Protein**	**10.01**	**8.81**	**8.59**	**58.87**	**<0.001**
***Snow Depth**	**40.29**	**69.76**	**50.84**	**145.19**	**<0.001**
***Bite Diameter[5]**	**na**	**3.01**	**2.65**	**7.20**	**0.007**

***Significant difference between island regions $\alpha = 0.05$ in bold**

[1] Unidentifiable monoterpene, numbers following "unknown" refer to retention time (min) in the chromatogram resulting from gas chromatography.

[2] Borneol concentrations were measured but were excluded from analysis due to inconsistency in repeat samples and lack of adequate control standards.

[3] Total number of monoterpenes with retention times < 24 minutes, > 1% total AUC, and present in > 70% of samples.

[4] Total phenolics and tannins, concentrations in mg Gallic Acid Equivalence (GAE, phenolics) or µM Tannic Acid equivalence (TAE, tannins)/g DW

[5] Bite diameter, diameter of the twig (mm) at the observed bite mark by moose on browsed trees. Bite Diameter data was not collected during sample year 2013.

Table 1.3 Z-score (z), p-value and 95% confidence interval (CI) for univariate analysis of conditional logistic predictors against binary response of browsed/unbrowsed balsam fir trees at Isle Royale National Park, Michigan USA. Significant predictors based on univariate analysis are shown in bold, predictor variables with $p > 0.5$ were considered "non-predictive" and removed from further analysis.

Predictor Category	Predictor Variable	*z*	p-value	95% CI
PSM	***Camphene**	**2.576**	**0.009**	**1.005 - 1.04**
	***Bornyl Acetate**	**1.98**	**0.048**	**1.00 - 1.062**
	β-pinene	1.403	0.16	0.999 - 1.004
	Number of Compounds[1]	1.3	0.194	0.869 - 2.002
	R-limonene	-0.662	0.508	0.994 - 1.003
	Terpinolene	0.281	0.778	0.930 - 1.101
	Total Tannins[2]	-0.819	0.413	0.784 - 1.105
	Total Phenolics[2]	-0.026	0.979	0.934 - 1.069
Nutrient	Crude Protein	-0.012	0.991	0.462 - 2.143

*** Significant predictor of browsing based on univariate analysis at $\alpha = 0.05$.**

[1] Total number of monoterpenes with retention times < 24 minutes, > 1% total AUC, and present in > 70% of samples.

[2] Total phenolics and tannins, concentrations in mg Gallic Acid Equivalence (GAE, phenolics) or µM Tannic Acid Equivalence (TAE, tannins)/g DW.

Table 1.4 **Model components, log likelihood (LL), number of parameters (K), Akaike's Information Criterion with sample size bias-adjustment (AICc), change in AICc from the top model (Δ AICc), and model weight (w_i) for the model selection used for the browsing of balsam fir patches used by Moose at Isle Royale National Park, Michigan, USA during winter 2013, 2014, and 2015. Conditional logit models were used with plant browse condition (browsed/unbrowsed) as the binary response for each model. Top models, with < 2 Δ AICc from the top model and with an AICc value lower than the null model, are shown in bold.**

Predictor category	Model	Log Likelihood (LL)	Number of parameters (K)	AIC_c	ΔAIC_c	Akaike weight (w_i)
PSM	**Camphene**[1]	**-44.41**	**1**	**90.84**	**0.00**	**0.71**
	Bornyl Acetate[1]	-46.23	1	94.47	3.63	0.12
	Tannins[2]	-46.79	1	95.59	4.76	0.07
	β-pinene[1]	-47.38	1	96.77	5.94	0.04
	NULL	-48.52	0	97.04	6.20	0.03
	Number of Compounds[3]	-47.64	1	97.29	6.45	0.03
	R-limonene[1]	-48.3	1	98.61	7.78	0.01

[1] Monoterpene compounds, AUC/mgDW > 1% total AUC and present in >70% of samples, and with univariate analysis $p < 0.5$

[2] Total tannins, µM Tannic Acid Equivilence (TAE)/g DW

[3] Total number of monoterpenes with retention times < 24 minutes, > 1% total AUC, and present in > 70% of samples.

Table 1.5 Confidence intervals (85% CI) for estimated use coefficients (Est) predicting tree use by moose foraging on balsam fir. Confidence intervals that do not overlap 1.0 for Conditional Logit (clogit) models and confidence intervals that do not overlap 0.0 for Generalized Linear models (glm) are shown, indicating parameters that predict odds of use reliably. Parameters shown are top models from AICc model selection (models with < 2 Δ AICc from the top model, with AICc values lower than the null model). Predictor variables are listed in order of increasing AICc value.

Analysis	Model Type	Predictor Variable	Est	85% CI
Diet Selection	Conditional Logit	Camphene	1.022	1.01 - 1.035
Bite Diameter	Generalized Linear	Camphene	-0.007	-0.011 - -0.004
		Snow Depth	0.012	0.005 - 0.018

Table 1.6 t-value (t), p-value and 95% confidence intervals (CI) for univariate analysis of generalized linear models of bite diameter as described by predictor variables of plant secondary metabolites (individual and number of monoterpenes >1% total AUC and present in >70% samples (AUC/mg DW), total phenolics (mgGAE/g DW), total tannins (µM TAE/g DW)), nutrient content, and environmental properties of balsam fir trees at Isle Royale National Park, Michigan USA. Significant predictors based on univariate analysis are shown in bold, predictor variables with p>0.5 were considered "non-predictive" and removed from further analysis.

Predictor Category	Predictor Variable	*t*	p-value	95% CI
PSM	***Camphene**	**-2.726**	**0.008**	**-0.012 - -0.002**
	***Bornyl Acetate**	**-2.469**	**0.016**	**-0.01 - -0.001**
	Number of Compounds[1]	-1.90	0.062	-0.140 - 0.694
	Terpinolene	-1.462	0.149	-0.052 - 0.007
	β-pinene	-0.39	0.698	-0.001 - 0.001
	Total Tannins[2]	-0.369	0.713	-0.064 - 0.043
	R-limonene	-0.088	0.93	-0.002 - 0.002
	Total Phenolics[2]	-0.469	0.641	-0.024 - 0.015
Nutrient	Crude Protein	-401	0.69	-0.180 - 0.119
Environmental	***Snow Depth**	**2.523**	**0.014**	**0.003 - 0.021**
	Region	-1.736	0.087	-0.459 - 0.028

*** Significant predictor of bite diameter based on univariate analysis at α = 0.05.**

[1] Total number of monoterpenes with retention times < 24 minutes, > 1% total AUC, and present in > 70% of samples.

[2] Total phenolics and tannins, concentrations in mg Gallic Acid Equivalence (GAE, phenolics) or µM Tannic Acid Equivalence (TAE, tannins)/g DW.

Table 1.7 Model components, log likelihood (LL), number of parameters (K), Akaike's Information Criterion with sample size bias-adjustment (AICc), change in AICc from the top model (Δ AICc), and model weight (w_i) for the model selection used for determining bite diameter of balsam fir trees used by moose at Isle Royale National Park, Michigan, USA during winter 2014, and 2015. Generalized linear models were used with the diameter of the browsed twig at the observed browsing site (bite diameter (mm)) as the response variable for each model. Top models, with < 2 Δ AICc from the top model and with an AICc value lower than the null model, are shown in bold.

Predictor category	Model	Log Likelihood (LL)	Number of parameters (K)	AIC_c	ΔAIC_c	Akaike weight (w_i)
PSM	**Camphene[1]**	**-45.97**	**3**	**98.33**	**0.00**	**0.38**
	Snow Depth[2]	**-46.47**	**3**	**99.32**	**1.00**	**0.23**
	Bornyl Acetate[1]	**-46.60**	**3**	**99.58**	**1.25**	**0.20**
	Number of Compounds[3]	-47.79	3	101.96	3.63	0.08
	Region[2]	-48.08	3	102.54	4.22	0.05
	NULL	-49.60	2	103.39	5.06	0.03
	Terpinolene[1]	-48.52	3	103.41	5.09	0.03

[1] Monoterpene compounds, AUC/mgDW > 1% total AUC and present in >70% of samples, and with univariate analysis $p < 0.5$

[2] Environmental property: snow depth (cm) at tree and region (east versus west) of foraging site.

[3] Total number of monoterpenes with retention times < 24 minutes, > 1% total AUC, and present in > 70% of samples.

Table 1.8 Model components, log likelihood (LL), number of parameters (K), Akaike's Information Criterion with sample size bias-adjustment (AICc), change in AICc from the top model (Δ AICc), and model weight (w_i) for second round model selection of the generalized linear models used for determining bite diameter of balsam fir trees browsed by moose at Isle Royale National Park, Michigan, USA during winter 2014, and 2015. Diameter of the browsed twig at the observed browsing site (bite size (mm)) was the response for each model. Top models, with < 2 Δ AICc from the top model and with an AICc value lower than the null model, are shown in bold. Models described are combinations of top model variables from first round model selection.

Model	Log Likelihood (LL)	Number of parameters (K)	AIC_c	ΔAIC_c	Akaike weight (w_i)
Camphene[1] + Snow Depth[2]	**-41.58**	4	**91.81**	**0.00**	**0.65**
NULL	-41.42	5	93.82	2.01	0.24
Snow Depth[2] + Borneal Acetate[1]	-44.48	4	97.61	5.80	0.04
Camphene[1]	-45.97	3	98.33	6.52	0.02
Camphene[1] + Bornyl Acetate[1]	-44.95	4	98.55	6.75	0.02
Snow Depth[2]	-46.47	3	99.32	7.52	0.02
Bornyl Acetate[1]	-46.60	3	99.58	7.77	0.01

[1] Monoterpene compounds, AUC/mgDW > 1% total AUC and present in >70% of samples, and with univariate analysis $p < 0.5$

[2] Snow Depth at foraging site (cm).

FIGURES

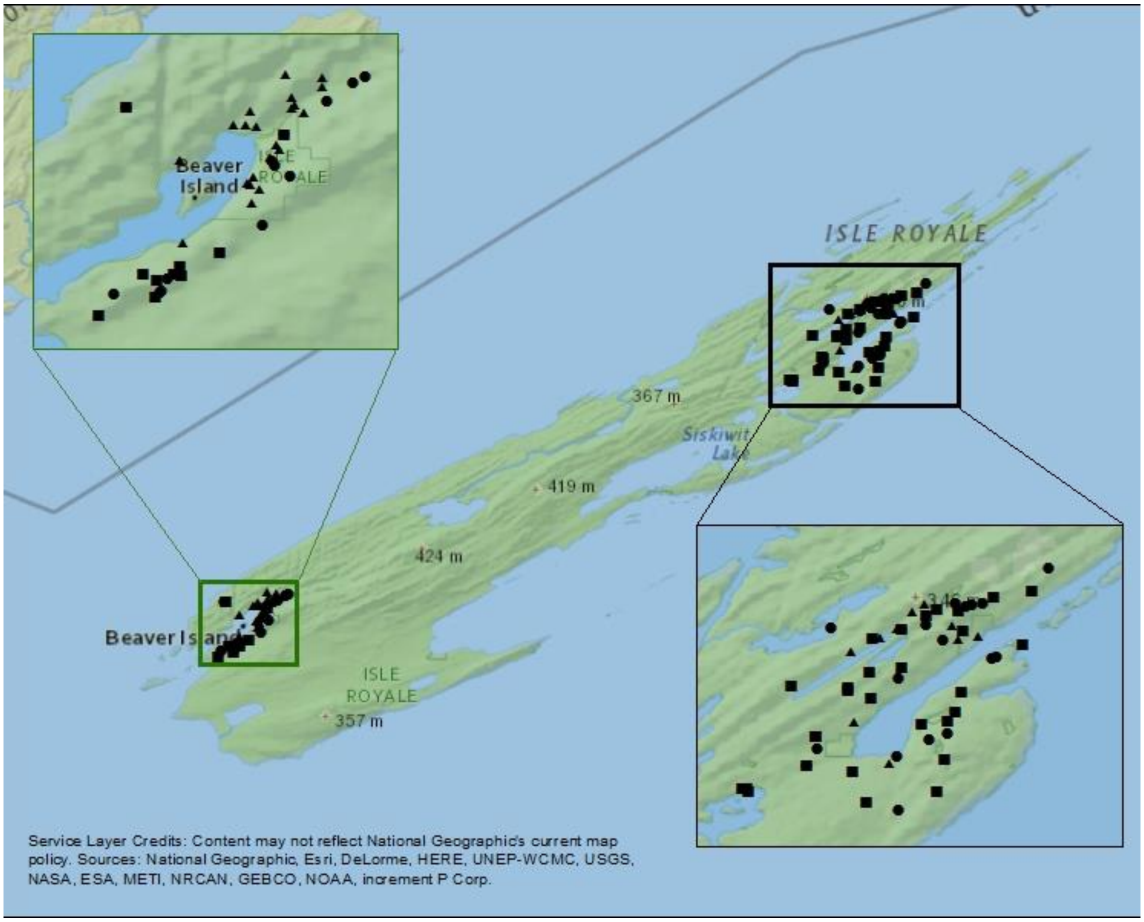


Fig 1.1 **Aerial view of Isle Royale National Park, Michigan, USA indicating location of patches browsed by moose during late January and early February where plants were sampled during 2013 (circles, n = 31), 2014 (squares, n = 39), and 2015 (triangles, n = 32) from east and west regions.**

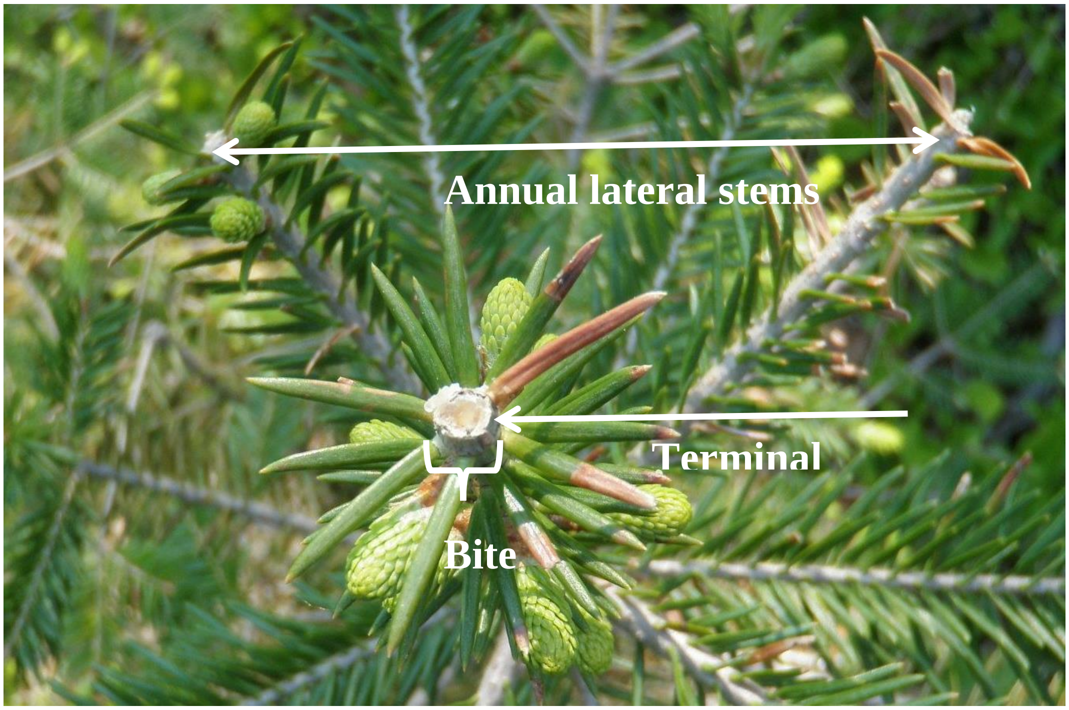


Fig 1.2 Bite marks used to determine bite diameter on terminal and lateral stems indicating moose browsing on an immature balsam fir tree (*Abies balsamea*) at Isle Royale National Park, Michigan, USA. Photo credit J. S. Forbey.

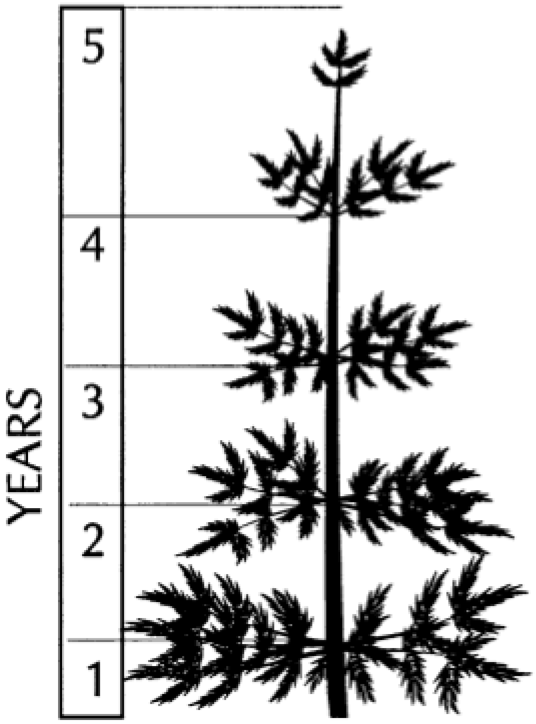
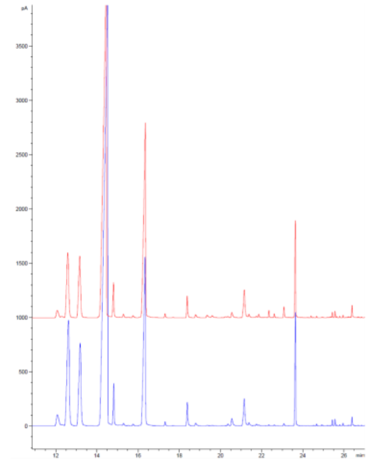


Fig 1.3 **Balsam fir grows a new whorl of branches each year (1= oldest, 5=newest). Browsed and respective non-browsed samples were collected from the same whorl as observed browse marks. Samples from browsed terminal stem (top stem in year 5) were collected from the previous year's whorl if multiple stems were not present. Figure from woodlandstewardship.org, courtesy of University of Minnesota Extension.**



#	Retention time	Compound
1	12.08	unknown
2	12.6	α-pinene
3	13.19	camphene
4	14.41	β-pinene
5	14.77	myrcene
6	16.24	(+)-limonene
7	18.4	terpinolene
8	21.1	borneol
9	23.6	bornyl acetate

Fig 1.4 Representative monoterpene profiles of browsed (blue, bottom) and unbrowsed (red, top) balsam fir trees collected from foraging patches used by moose at Isle Royale National Park, Michigan, USA during winter. Peaks show individual compounds, with the height of the peak (AUC) indicating relative concentration of the compound at a given retention time. Identity of peaks were determined with co-chromatography using a standard cocktail containing each of the known compounds.

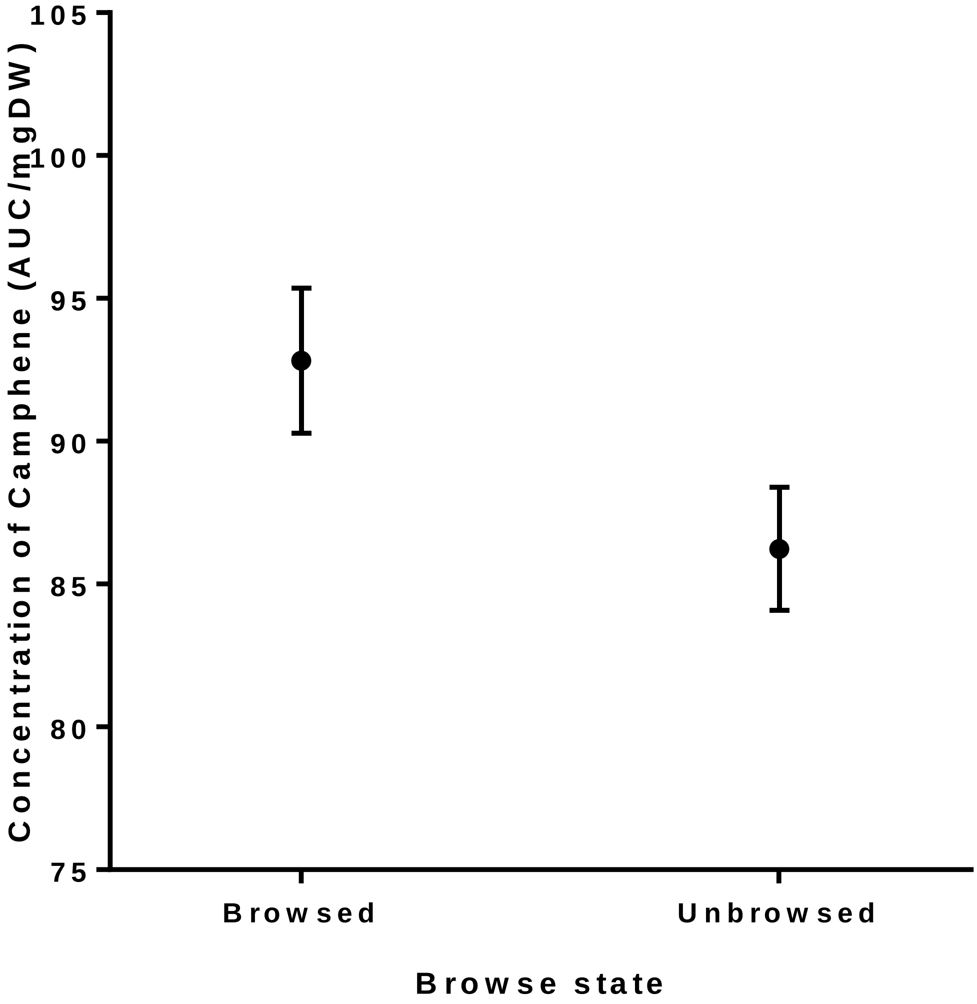


Fig 1.5 Mean ± SEM of camphene concentrations (AUC/mgDW) for browsed and unbrowsed balsam fir within foraging patches used by moose at Isle Royale National Park, Michigan, USA. Camphene explained 71% of diet selection by moose (Conditional Logit model; AICc = 90.84, model weight (w_i) = 0.71)

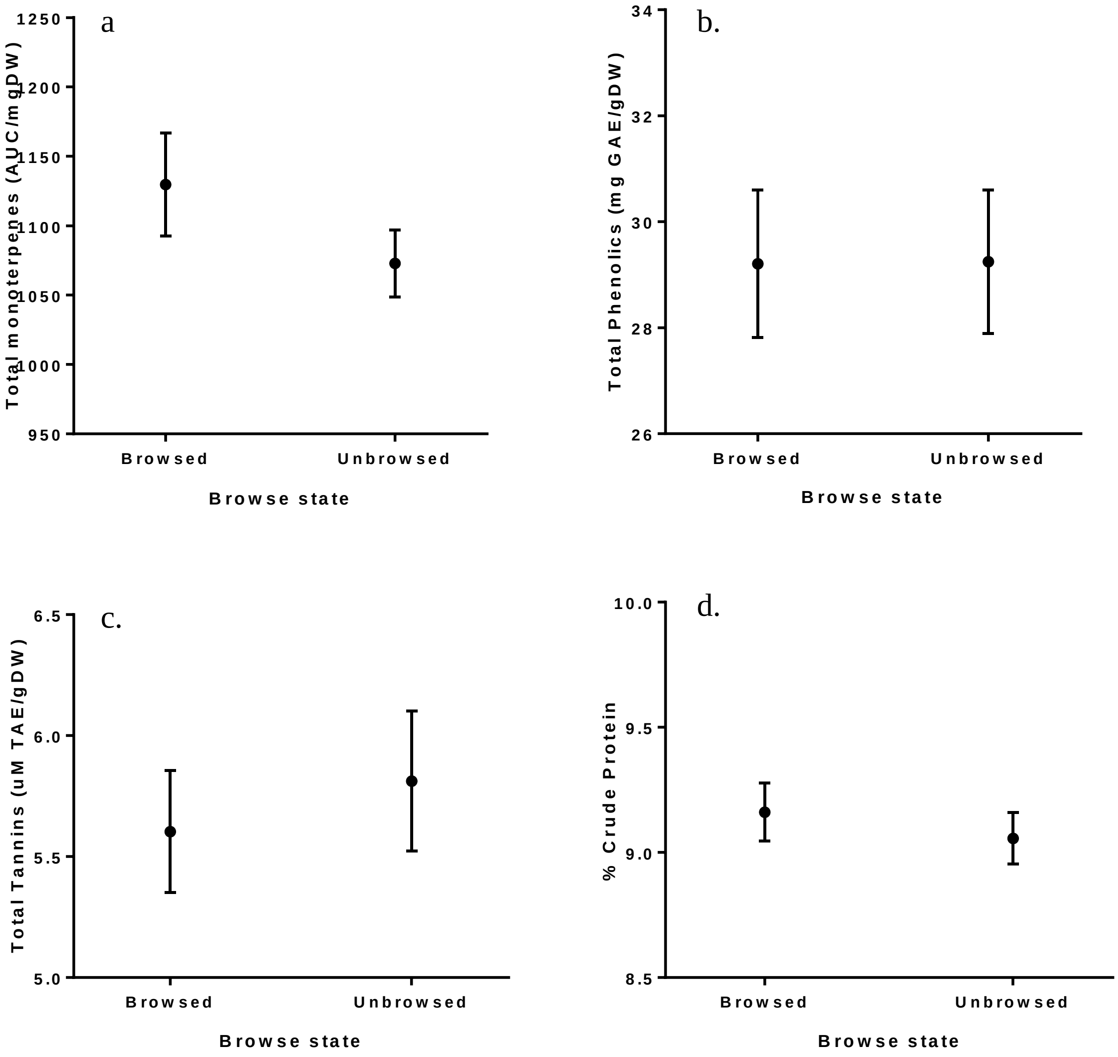


Fig 1.6 **Mean ± SEM of dietary characteristics for browsed and unbrowsed balsam fir within foraging patches used by moose at Isle Royale National Park, Michigan, USA. Samples were collected in winter 2013, 2014, and 2015 (n = 101). Dietary characteristics include: (a) total monoterpene concentrations (AUC/ 100 µg dry weight [DW]), (b) total phenolic concentration (mg GAE/g DW), (c) Total Tannin concentrations (uM TAE/g DW), (d) crude protein (%).**

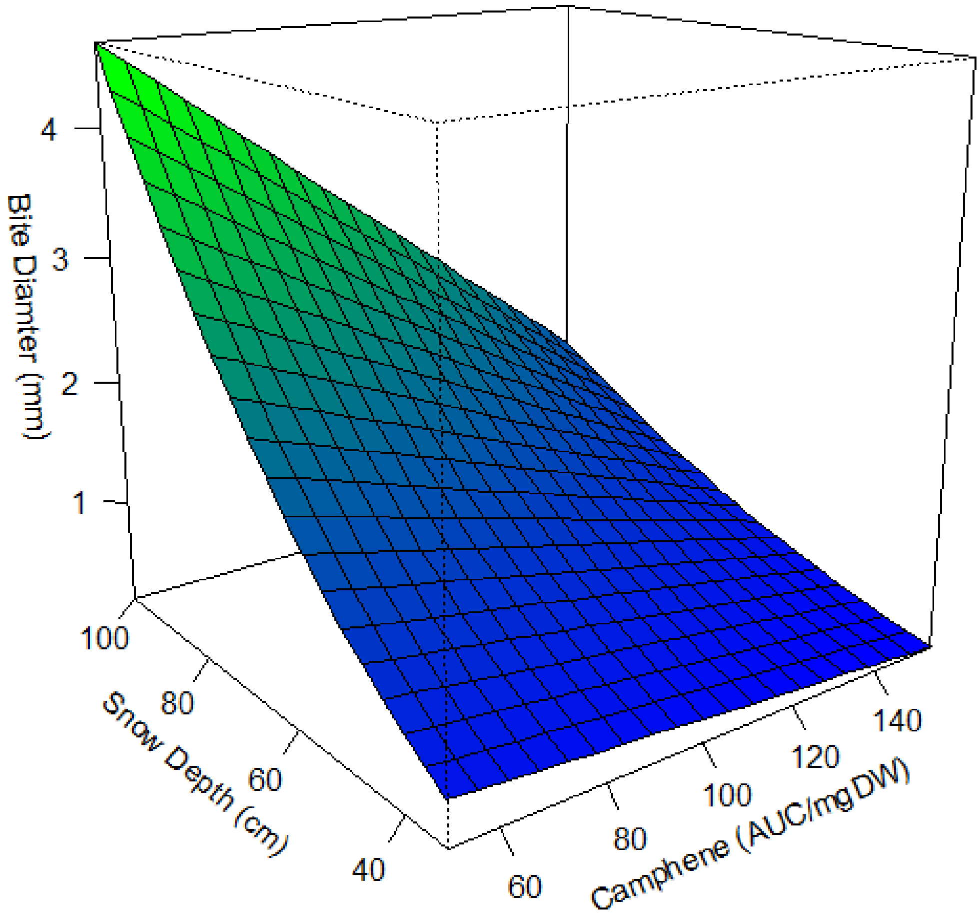


Fig 1.7 **Response surface depicting the additive effects of increasing camphene (AUC/mg DW) and snow depth (cm) on bite diameter (mm) of balsam fir trees browsed by moose during winter at Isle Royale National Park, Michigan, USA. Response surface was generated using top covariates in model selection.**

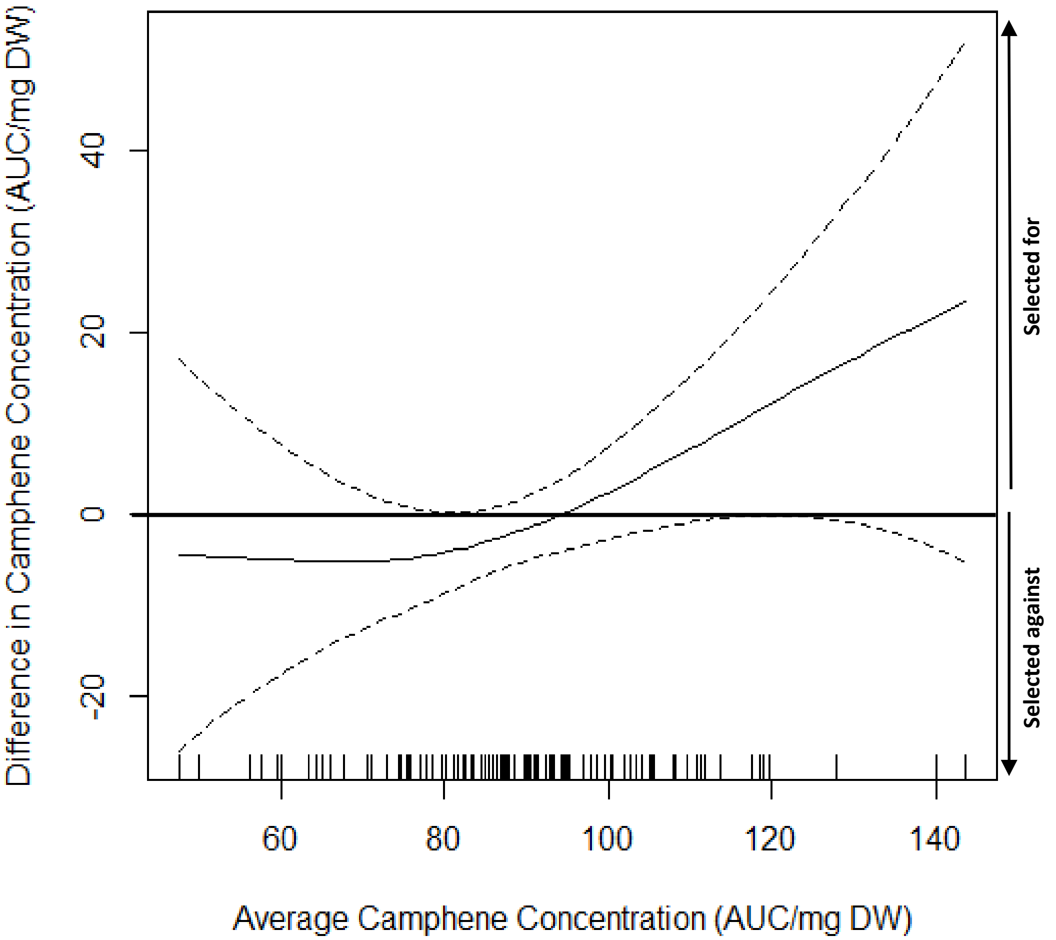


Fig 1.8 **Difference in concentrations of camphene (AUC/mg DW) between paired browsed and non-browsed balsam fir samples, represented by tick marks along the inside of the x-axis (n = 101) from Isle Royale National Park, Michigan, USA as a function of mean concentration of camphene (AUC/mg DW) in that patch. Samples included patches browsed by moose from three years: 2013 (n=31), 2014 (n=38), and 2015 (n=32). Values above zero theoretically indicate selection for camphene, and indicate higher camphene concentrations in browsed plants than non-browsed, while values below zero indicate selection against and have lower camphene concentrations in browsed plants. The dark horizontal line at 0.0 on the y-axis indicates where no selection occurs. The solid black line represents the smoothed fit for the generalized additive model, and dashed lines represent 95% confidence intervals derived from the model.**

CHAPTER TWO: PLANT SECONDARY METABOLITES INFLUENCE THE NUTRITIONAL CONDITION OF A FREE-RANGING MAMMALIAN HERBIVORE

Abstract

Free-ranging vertebrate herbivores consume a wide variety of potentially harmful plant secondary metabolites (PSMs). Herbivores use both behavioral (diet selection, altered intake rates, diet mixing) and physiological (regulated absorption and detoxification) adaptations to limit the negative consequences of PSMs. Because these adaptive responses can be energetically expensive, the extent to which behavioral and physiological responses to PSMs are used may influence the nutritional condition and ultimately fitness of herbivores. We used a free-ranging population of moose (*Alces alces*) on Isle Royale National Park, Michigan to determine how behavioral and physiological responses to dietary PSMs influenced the nutritional condition of overwintering moose. We identified bite marks on the dominant forage of moose in winter (balsam fir, *Abies balsamea*) and quantified total monoterpene and total phenolics of fir consumed by moose. To evaluate behavioral adaptations, we accounted for PSMs in fir that had been browsed (selection), bite diameter (estimate of intake rate), and diet mixing (the proportion of fir consumed using histological analysis of fecal pellets) to calculate the proportional intake rate of PSMs consumed by moose from fir. To evaluate both behavioral and physiological adaptations, we quantified glucuronic acid to creatinine ratios (GA:C) from snow-collected urine samples spanning 17 consecutive years. GA is a metabolite excreted in urine that represents the amount of PSM consumed,

absorbed, and detoxified via the glucuronidation pathway which is the major detoxification pathway for vertebrates. The same urine samples were used to quantify urea nitrogen to creatinine ratios (UN:C) as a biomarker of nutritional condition for individual moose. We found that GA:C, which accounts for both behavioral and physiological responses to dietary PSMs, was a better predictor of nutritional condition of moose than accounting only for behavioral responses to PSMs (proportional intake rate of PSMs). Further, we found that high detoxification via the GA pathway was inversely correlated with nutritional condition within and among the 17 years investigated. These results indicate that GA:C, which captures both behavioral and physiological adaptations for PSMs, is dynamically linked to the nutritional condition of free-ranging vertebrate herbivores over time. The likely explanation for that linkage is that GA:C captures both the behavioral and physiological energetic costs of foraging, beyond the PSM concentrations of a single plant.

Introduction

Foraging decisions made by free-ranging herbivores can have important consequences on their nutritional condition, and ultimately fitness (Harder and Kirkpatrick, 1994). Specifically, nutritional condition can, in part, explain the growth and reproduction of individual animals and populations. In general, reduced nutritional condition of animals can compromise individual and population level fitness factors including fecundity, recruitment and reproduction (Cook et al. 2004; Adams 2005; Parker et al. 2009; Legbo et al. 2010; Wam and Hjeljord 2010). While mechanisms explaining this relationship are limited, poor nutritional condition can lead to acute hepatic consequences (Sansinanea et al. 2000) leading directly to impaired immunity and increased susceptibility to parasites and disease (Lafferty and Gerber 2002; Demas et al. 2011; Jolles et al. 2015), and ultimately compromise population growth (Cook et al. 2004; Parker et al. 2009).

Animals can maximize nutritional condition through behavioral strategies that maximize the amount and quality of food consumed. Although plants provide relatively high biomass of food, many plant species contain a suite of potentially toxic chemical defenses known as plant secondary metabolites (PSMs) that limit intake by herbivores. PSMs can compromise a diversity of physiological functions (Iason 2005) such as protein absorption and nitrogen assimilation (McArthur et al. 1991; Iason 2005; Degabriel et al. 2008; Moore et al. 2010), and enzyme function (Forbey et al. 2011; Kohl et al. 2015). The physiological consequences of ingesting PSMs may explain compromised protein turnover (Au et al. 2013), energy budgets (Guglielmo et al. 1996; Sorensen et al. 2005b), and reproductive success (Brittas 1988; DeGabriel et al. 2009).

To minimize systemic concentrations of potentially harmful PSMs, herbivores rely on adaptive behavioral (see chapter 1) and physiological (Dearing et al. 2005; McLean and Duncan 2006) mechanisms. Behavioral adaptations include diet selection (Stolter et al. 2005; Jones et al. 2006; Frye et al. 2013; Ulappa et al. 2014; Chapter 1), altered intake rates (Sorensen et al. 2005a; McLean et al. 2008; Torregrossa and Dearing 2009), and diet mixing (Wiggins et al. 2005). However, these strategies may compromise net energy and nutrient balance (Searle et al. 2007). Moreover, habitat conditions can limit the extent to which herbivores can be selective, maximize intake rates, mix diets and by extension, influence nutritional condition. For example, increased snow depths during winter months can increase an individual’s energy expenditure as it searches for suitable forage (Parker et al. 1999), or it can restrict an individual’s movement, thereby, restricting access to high quality forage (DelGiudice 1995). Such dietary restrictions have the potential to further reduce net energy balance and thus reduce the nutritional condition of herbivores.

Since it is not possible for herbivores to completely avoid the consumption of PSMs through behavioral adaptations (Foley et al. 1999; Provenza et al. 2003), several physiological adaptations help herbivores minimize systemic concentrations of ingested and absorbed PSMs. For example, conjugation of PSMs with glucuronic acid (GA) via the uridine 5'-diphospho-glucuronosyltransferase (UGT) enzyme represents a major detoxification pathway in vertebrate herbivores that facilitates excretion of ingested and absorbed PSMs (Ghosal et al. 2004, Servello and Schneider 2000). GA is produced in the liver through the enzymatic oxidization of hepatic glucose, resulting in a highly soluble sugar-acid complex that can be excreted (Steimer 1993). The excretion of GA-conjugated metabolites results in a direct net loss of hepatic glucose and endogenous energy reserves. Increased excretion of GA is energetically expensive and was positively correlated with a greater intake of PSMs in herbivores in captive feeding trials (Hewitt and Kirkpatrick 1997, Guglielmo et al. 1996, Sorensen et al. 2005b). Thus, GA can be viewed as a biomarker that captures both behavioral (the amount of PSMs consumed) and physiological (the amount of PSMs absorbed and detoxified, at an energetic cost) adaptations. The energetic cost of GA conjugation could force a positive feedback loop, with regard to nutritional condition during winter dietary restriction, effectively increasing the herbivore’s energetic deficit. Such feedbacks would lead to an overall negative energy balance and further exacerbate a decline in nutritional condition. As dietary restriction increases in winter and herbivores are forced to consume less varied or more chemically defended forage, GA production is expected to increase and nutritional condition is expected to decline. Additionally, the glucuronidation pathway is substrate limited, relying on the availability of hepatic glucose for GA production (Cassaret et al. 2013). Thus, reduced energy reserves could limit GA production, and by extension, limit detoxification capacity forcing individuals to reduce their intake rates.

The vast majority of the research into the energetic or nutritional consequences of ingested PSMs has employed the use of captive feeding studies (Reichardt et al. 1984; Servello and Schneider 2000; Mangione et al. 2001; Sorensen et al. 2005b; Au et al. 2013; Clauss et al. 2013; Ulappa et al. 2014). Few studies have focused on vertebrate herbivores in the wild (DeGabriel et al. 2009), likely because of the difficulty associated with measuring the intake and detoxification of PSMs by individual foragers, and linking these parameters to nutritional condition and population level effects.

One approach to determining the link between the behavioral and physiological consequences of PSMs in the diet and nutritional condition of free-ranging herbivores is to quantify GA in the urine along with two other chemical metabolites, urea nitrogen (UN) and creatinine (C). Urine samples can be obtained non-invasively from urine soaked snow left at foraging patches by mammalian herbivores (DelGiudice 1995; Moen and DelGuidice 1997). UN represents the largest pool of free nitrogen in the mammalian body and its production is directly related to changes in catabolism of dietary and endogenous proteins (Weiner et al. 2014). During periods of relatively low protein intake, UN is recycled through hydrolysis to make up the deficiency in the protein balance (Fouillet et al. 2008). Therefore, the effectiveness of the urea recycling pathway is time limited. As UN levels in the blood serum diminish, recycling urea through hydrolysis becomes increasingly ineffective. To make up the balance of this nitrogen deficiency, animals often increase catabolic rates of endogenous protein, resulting in increased concentrations of UN in urine and decreased nutritional condition (Moen and DelGiudice 1997).

To standardize the relative amount of GA and UN excreted by animals, creatinine (C) is measured in the same urine sample. Creatinine is the end result of creatine phosphate metabolism in skeletal muscles (Doi et al. 2009) and is excreted as a waste product exclusively in urine (Casey and Greenhaff 2000) at a relatively constant daily rate based on lean muscle mass (Moen and DelGiudice 1997). By evaluating the ratio of GA or UN to C, the processes of detoxification and nitrogen turnover relative to body mass can be estimated. Determining the ratio of GA and UN to C simultaneously corrects for urine dilution factors associated with snow or relative hydration of individual animals (DelGiudice 1995). The ratio of GA:C in urine can provide insights into PSM exposure and UN:C ratios can provide insights into the nutritional condition of the same animal. Delguidice (1995) reported that UN:C ratios >3.5 in ungulates are indicative of accelerated catabolism of endogenous protein and are associated with starvation and substantially reduced nutritional condition. Furthermore, prolonged low UN:C ratios detected in wild populations may be indicative of enhanced renal reabsorption of UN, indicating chronic nutritional restriction which may be a precursor to the dangerously high ratios (>3.5) (Delgiudice et al. 1990).

Our primary objective in the present study was to examine the links between behavioral responses to PSMs, energetic costs of detoxification, and nutritional condition in a free-ranging herbivore. Specifically, we investigated the relationship between GA:C and UN:C relative to the proportional intake rate of PSMs using the balsam fir (*Abies balsamea*)–moose (*Alces alces*) system in Isle Royale National Park, Michigan in central North America (Appendix A). Isle Royale was ideal for this study because predation has already been characterized in this system and moose are the only large herbivores present on the island browsing on balsam fir trees (Vucetich and Peterson 2004). Additionally, there is little to no migration or emigration and moose are generally solitary during winter so urine samples collected during winter are from individual moose. Moreover, evaluation of fir trees browsed during winter offers an effective method for evaluating concentrations of PSMs selected and ingested by moose. By measuring bite diameters on browsed fir trees, we could estimate the biomass intake rate (Risenhoover, 1987, Fig F.1) and by extension, the relative intake rate of PSMs from fir. Further, using fecal micro-histology of moose pellets, we can determine relative proportions of known PSMs from a specific plant component in an animal's diet. Finally, metabolites that are biomarkers of detoxification (glucuronic acid, GA), nutritional condition (urea nitrogen, UN) and relative metabolism (creatinine, C) can be quantified in urine deposited by individual moose at foraging patches where PSMs from selected fir, intake rate of fir and proportion of fir consumed are also measured. To our knowledge, this represents the first study to evaluate the relative importance of PSMs in plants at foraging patches, behavioral (selection, rate of intake and proportional intake of a specific plant species) and physiological adaptations to PSMs (GA) in explaining the nutritional condition (UN) in free-ranging vertebrate herbivores. We used this system to test the hypothesis that nutritional condition is more dependent on the behavioral and physiological responses to

PSMs by herbivores than the quality of available forage or considering behavioral responses alone. Based on our hypothesis, we made the following predictions:

1. GA:C in urine, which accounts for both behavioral and physiological responses to PSMs, will better predict nutritional condition of moose (UN:C ratios) than the concentrations of PSMs in fir or accounting for behavioral responses alone.
2. GA:C in urine will predict nutritional condition of moose (UN:C ratios) within and among years.
3. Variation in GA:C in urine of moose is a function of relative intake of PSMs in fir.

Methods

To test these predictions, we analyzed samples collected from balsam fir trees browsed by moose for total concentrations of two classes of PSMs: monoterpenes and phenolics. We used bite diameter at the site of a browsed fir twig to determine intake rate (Appendix F) and we used fecal microhistology to determine proportion of fir in diet. To determine the general reliance on glucuronidation pathways and nutritional condition of overwintering moose, we analyzed urine samples collected from snow for total GA as a biomarker of toxin exposure, UN content as a biomarker of nutritional condition, and C as an internal standard to account for dilution effects of snow and relative hydration of individual animals.

Sample Collection.

All balsam fir, fecal and urine samples were collected from both east and west ends on Isle Royale National Park located in the northwest portion of Lake Superior, North America (47°50′ N, 89°00′ W; Appendix A, Fig A.1). Fir samples were collected between late January and early February annually from 2013 to 2015. Urine samples were collected annually between late January and early February 1994 to 2015 (Appendix G). We located and followed single moose tracks until urine and fecal samples in the snow along the tracks were observed and collected. Tracks with multiple animals were avoided to ensure that each sample was collected from a single individual. Urine samples were collected from snow by collecting all the urine soaked snow (≈ 50 cm^3) in 50 mL Falcon tubes. Urine deposits that penetrated the snow cover to the bare ground beneath were not collected. In the laboratory, urine samples were thawed, mixed and transferred to plastic 15mL conical tubes and stored at -20° for later analysis.

Samples from browsed balsam fir trees from were collected when tracks of moose led researchers to a foraging patch. A foraging patch is defined as an area along the track of the moose where the moose demonstrated active browsing on at least three trees. Areas where moose browsed only one tree or areas where the track moved straight through a patch of trees without signs of active browsing were not included as foraging patches. We located recent browse marks on fir trees along the path of moose tracks within a foraging patch. For samples collected during 2014 and 2015, twig diameter at the site of the observed bite marks on each tree was measured with calipers to the nearest millimeter. Typically, the trunk and lateral branches of *A. balsamea* grow one new whorl concentrically around the main trunk each year (Silvertown and Dodd 1999; Chapter 1, Fig 1.2). Un-browsed lateral stems on the same whorl as the browsed stems were measured with calipers to the same diameter as the observed browse mark and samples were taken at that point. Samples were collected from three browsed fir trees within each foraging patch along the tracks of the moose. Samples from browsed fir trees were stored in sealed Zip Lock bags at -20^0 for later analysis.

Preparation of Samples

Clippings from browsed plants were frozen using liquid nitrogen and were ground using a mortar and pestle to an average particle size of ~ 2 mm. A subsample (100mg wet weight, WW) of each ground sample was placed in a 20mL glass head-space vial with a crimp style cap for analysis of monoterpenes. Subsamples of each ground sample were placed in separate 1.5mL micro-centrifuge tubes for analysis of total phenolics (50mg WW). Approximately 2-4g of each ground sample was dried for three days at 69^{o}C in a Thermo-Scientific, Heratherm oven. Dried samples were sent to Dairy One Laboratories, Ithaca, New York, USA for analysis of available nitrogen content via combustion (method AOAC 990.03). Urine samples were thawed and filtered through glass wool into 2.0mL aliquots for analysis of glucuronic acid (GA), urea nitrogen (UN), and creatinine (C).

Chemical Analysis of Samples.

Monoterpenes in balsam fir were measured using headspace gas chromatography. Concentrations were measured with an Agilent 7694 headspace auto-sampler coupled with an Agilent 6890N gas chromatograph (GC). Headspace vials containing 100mg WW of each ground sample were heated for a period of 20 min at 100^{o}C to achieve vial equilibrium. Headspace gas (1mL) was then injected into a J&W DB-5 capillary column (30m x 250µm x 0.25µm). We identified monoterpene content via co-chromatography with known standards and quantified the area under the curve (AUC) for total monoterpenes present in browsed plants. Detailed operating conditions for the headspace auto-sampler can be found in Appendix B.

Total phenolics in plant samples were analyzed by eluting 50mg of each ground sample with 1.0mL of HPLC grade methanol. Eluted samples were placed in a sonicating water bath and sonicated for a period of 3 min, two times at 25°C with a 2 min break between sonications to minimize heat. Samples were centrifuged at 13,000g for a period of 5 min. The supernatant was filtered through glass wool into a 1.5mL amber vial. Samples were analyzed for total phenolics following procedures from Ainsworth and Gillespie (2007). Briefly, 20µL subsamples were diluted to a total volume of 100µL (1:5 dilution) with HPLC grade methanol. Samples were prepared in triplicate in a 96-well plate with 20µL diluted sample extract. Each sample was reacted with 100µL 10% Folin-Ciocalteu reagent (Sigma 47742; Sigma-Aldrich, St. Louis, Missouri, USA) and 80µL 7.5% sodium carbonate solution. All reagents were thoroughly mixed inside the plate wells by repeatedly drawing and expelling well contents with a multi-channel pipette. Completed 96-well plates were then wrapped in aluminum foil and incubated at room temperature for a period of 120 min. Plates were read on a Biotek SynergyMX multi-mode micro-plate spectrophotometer at 765nm. Each sample was paired with a negative control and ran with a range of known standards of Gallic acid (Cat #5995-86-8, Fisher Scientific, Waltham Massachusetts, USA).

Urine samples spanning 17 years (between 1994 and 2015, Appendix G) were analyzed for UN and total C by Wolff Laboratories, Inc., Bloomington, Minnesota, USA, utilizing spectrophotometric procedures and reagents provided by JAS Diagnostics, Inc. Miami, Florida (Cat #BUN2.S01, urea nitrogen; Cat# CRE2.S01, creatinine). UN and C in urine samples were read on a Cobas Mira spectrophotometer at 510nm and 340nm, respectively. Urine samples were analyzed for total GA following procedures modified from Blumenkrantz and Asboe-Hansen (1973). Briefly, 20µL of thawed urine was diluted to a total volume of 2.0mL in 0.01M borax buffer (pH ca. 9.5, 1:100 dilution). Each diluted sample (333µL) was reacted for 5 min on a heating block set to 100^{o}C with 2.0mL concentrated sulfuric acid (A300-212, Fisher Scientific, Waltham, Massachusetts, USA) and then treated with 12.5mM sodium tetraborate decahydrate. Once removed from heat, samples were placed in an ice bath and allowed to cool to room temperature. Once samples had cooled, they were oxidized for a period of 10 min with 50µL of 0.15% 3-Phenylphenol (Sigma 262250, Sigma-Aldrich, St. Louis, Missouri, USA) dissolved in 100mL 0.5% sodium hydroxide solution. Each sample reaction was conducted in duplicate and 200µL of each reaction was transferred to a 96-well plate for spectrophotometric analysis at 520nm on a Biotek SynergyMX multi-mode micro-plate spectrophotometer. Each sample was paired with a negative control and analysis of samples was repeated if the coefficient of variation between replicates exceeded 5%. Samples were analyzed with standards of known concentrations of D-Glucuronic acid (Sigma G-5269; Sigma-Aldrich, St. Louis, Missouri, USA) ranging from 0.97 mg/dL (50µM) to 6.7 mg/dL (350µM). GA and UN values were taken as ratios to C values to standardize for dilution factors related to snow volume and animal hydration.

Statistical Analysis.

While our data set spanned multiple years, 2014 represented the only year for which we had data to calculate intake rate of PSMs (from bite diameter), proportion of PSMs from fir consumed (from microhistology), and proportional intake rate of PSMs in fir consumed. Intake rate (IR) of monoterpenes (AUC/gDW/min) and total phenolics (mgGAE/gDW/min) from fir was calculated as the product of the overall IR (g/min), as determined from the diameter of the observed bite mark on fir browsed by moose (Appendix F), and the measured concentration of monoterpenes (AUC/gDW) or phenolics (mgGAE/gDW) in fir samples. Proportion of PSMs from fir consumed (%) was calculated as the product of proportion fir in diet estimated from microhistological analysis of fecal pellets (Parikh et al 2016) and measured concentration of monoterpenes (AUC/gDW) or phenolics (mgGAE/gDW) in fir samples. Proportional IR of PSMs in fir was calculated as the product of the proportion of fir consumed (%) and calculated IR of monoterpenes (AUC/gDW/min) or total phenolics (mgGAE/gDW/min) consumed.

To assess the potential additive effects of monoterpenes and phenolics on nutritional condition, we normalized both total monoterpene and total phenolics data to fit a scale between 0 and 1 using the following equation:

$$z_i = \frac{x_i - \min(x)}{\max(x) - \min(x)}$$

where z represents the normalized value and x represents either monoterpenes or phenolics. Normalized values of monoterpenes and phenolics were then added together for each sample and were further multiplied by calculated IR and proportion of fir in diet to determine IR of total normalized PSMs, proportion of total normalized PSM in diet, and proportional IR of total normalized PSMs using procedures described above.

To test the relative influence of PSMs alone, behavioral adaptations to PSMs alone (IR, proportion, and proportional IR of PSMs), and combined behavioral and physiological adaptations to PSMs (GA:C) on nutritional condition of moose (UN:C ratios) within a year (2014), we used Akaike’s

Information Criterion values with sample size bias adjustment (AICc) of Generalized Linear Models (GLM). We compiled a candidate model set comprised of total PSMs (monoterpenes and phenolics), IR of fir, proportion of fir in diet, and proportional IR for each of three PSM variables (monoterpenes, phenolics, and total normalized PSMs) to evaluate predictors of nutritional condition. Only models with AICc values lower than that of the null (intercept only) and less than 2 AICc units of difference from the top model (ΔAICc) were deemed to carry enough model weight (w_i) to be sufficiently predictive (Table 2.1). All models not fitting this description were removed from further consideration.

To test if GA:C in urine predicted nutritional condition of moose (UN:C ratios) within and among years, we used least squares regression analysis to investigate the relationship between AIC top models and UN:C by plotting individual GA:C for all moose during 2013, 2014, and 2015. Additionally, we assessed the long term, intra-annual relationship between GA:C and UN:C by regressing the full 17-year data set (n > 35 / year) using average annual values of GA:C and UN:C.

To test if intake of PSMs in fir explains variation in GA:C values in urine of moose, we used regression analysis to examine relationship between total monoterpenes, total phenolics, and normalized total PSMs and the excretion of GA:C. Regression analysis was conducted using calculated IR of total PSMs, proportion of total dietary PSMs, and proportional IR of total PSMs.

Sample sets for each year were checked for statistical outliers using a Grubbs Outlier Test. Data points with Grubb's significance ($\alpha = 0.05$) were removed resulting in three samples that were excluded from the final analysis. All statistical analyses were carried out using Prism version 7.02, Graphpad Software, San Diego, California, USA and R version 3.2.2 (R Foundation for Statistical Computing 2015).

Results

When assessing how PSM concentration in fir, behavioral adaptations (e.g., selection, intake rate, and diet mixing), and combined behavioral and physiological adaptations to PSMs (GA:C) predict nutritional condition using GLM AICc selection, GA:C emerged as the strongest predictor of UN:C (Table 2.1). GA:C accounted for 91% of the total weight of the candidate model set and fit the data better than the null model (AICc = 85.17, $w_i = 0.91$). There were no competing models in the candidate set. Proportional IR of monoterpenes (%AUC/gDW/min) emerged as the second-best model for the data but was > 2 ΔAICc from the top model and accounted for only 7% of total weight.

There was a significantly positive relationship between GA:C and UN:C within individual moose within each of three years: 2013 ($Y = 0.02x + 1.62$; $F_{1,39} = 4.558$, $p = 0.04$, $R^2 = 0.105$, 95% CI = 0.001 – 0.039) 2014 ($Y = 0.076x + 0.706$; $F_{1,35} = 28.19$, $p < 0.001$, $R^2 = 0.45$, 95% CI = 0.047 – 0.105), and 2015 ($Y = 0.053x + 0.31$; $F_{1,25} = 25.43$, $p < 0.001$, $R^2 = 0.504$, 95% CI = 0.032 – 0.075; Fig 2.1). The positive relationship between GA:C and UN:C was similar between east and west regions of the island (Appendix G). We observed inter-annual variation in both GA:C and UN:C (Fig 2.2) and a significant positive relationship between average annual GA:C and UN:C (Appendix G, Table G.2) over the entire 17-year data set (Y = 0.07x - 0.24; $F_{1,15}$ = 18.57, p = 0.0006, R^2 = 0.553, 95% CI = 0.035 – 0.104; Fig 2.3). While proportional IR of monoterpenes did not fit the definition of a competing model using AICc, there was a significant positive relationship between this variable and UN:C over the data set available in 2014 (Y = 0.018x + 2.56; $F_{1,30}$ = 15.41, p = 0.0005, R^2 = 0.34, 95% CI = 0.008 – 0.027) indicating a reduced nutritional condition associated with greater intake rate of monoterpenes (Fig 2.4).

There was no evidence that intake of PSMs explained GA:C in urine of moose. None of the variables examined (total monoterpenes, total phenolics, and normalized total PSMs in browsed trees, intake rate (IR) of total PSMs, proportion of total PSMs, and proportional IR of total PSMs) were significantly correlated with the excretion GA:C in moose urine (Appendix H).

Discussion

Our results show that increased intake of PSMs negatively influence nutritional condition of over-wintering moose, as indicated by elevated GA:C and UN:C levels in urine. Moreover, our ability to model the nutritional condition of moose improved when the predictor variable included both behavioral (intake rate and proportion of plant in diet) and physiological (absorption and detoxification) responses to PSMs (GA:C). Not only was GA:C a stronger predictor of UN:C than other parameters, it was a consistent and reliable predictor of nutritional condition in a free ranging generalist herbivore both within and across years. These results indicate that the behavioral and physiological processes associated with consuming, absorbing and detoxifying PSMs via the glucuronidation pathway may negatively affect the nutritional condition of herbivores.

Energetic expense associated with glucuronidation can potentiate a positive feedback loop in the energetic deficit associated with nutritional restriction. As length of the restricted period (e.g., duration of winter) increases, so does the energetic cost of detoxification, exacerbating the lower nutritional condition of moose over time. The present study marks the first study that we are aware of linking diet quality, physiology, and animal behavioral traits to nutritional outcomes in a wild vertebrate herbivore.

GA:C explained 55% of the intra-annual variation in the nutritional condition of wild moose (Fig 2.3). However, counter to our prediction, intake of PSMs in fir was not significantly correlated with GA:C excretion (Appendix H, Fig H.2). A positive relationship between higher intake of PSMs and increased GA production has been well documented in controlled feeding trials for a variety of vertebrate herbivores (Guglielmo et al. 1996; Hewitt and Kirkpatrick 1997; Servello and Schneider 2000; Sorensen et al. 2005b). However, these studies measured acute response to the introduction of a single PSM or PSMs from a single plant species to captive animals that were previously maintained on diets lacking PSMs. In contrast, our study measured PSMs and GA in wild herbivores in a natural system where concentrations of PSMs from a variety of plants are a consistent dietary constituent and animals are physiologically acclimated to PSMs. Conjugation with GA is not the only metabolic detoxification pathway used for the clearance of potentially harmful PSMs and theory suggests that different classes of PSMs are detoxified through distinct metabolic pathways which we did not assess (Dearing and Cork 1999). For example, wild herbivores may rely more on the induction of other substrate limited detoxification pathways such as sulfoxidation or amino acid conjugation (Casarett et al. 2013), metabolism from specialized gut microbiota (Kohl 2012; Forbey et al. 2013), or regulated absorption (Appendix J) to potentially reduce reliance on GA relative PSM intake.

Lack of relationship between intake of PSMs in fir and GA:C may reflect behavioral adaptations that are not considered in captive studies. For example, herbivores rarely subsist on a single forage type. Even under winter conditions, when forage options are limited, some diet mixing still occurs in the majority of plant/herbivore systems (Provenza et al. 2003). For example, while balsam fir is the dominant forage available and consumed by island moose during winter (Vucetich and Peterson 2004; Montgomery et al. 2013; Parikh et al. 2016) and proportional intake of crude protein (%) from balsam fir demonstrated a significantly positive correlation with UN:C values (Appendix I), fir is not the only forage consumed. White cedar (*Thuja accidentallis*) and a variety of dormant deciduous twigs are also consumed, but to a lesser extent (Parikh et al. 2016). In our study, we identified and quantified PSMs from balsam fir as the principle forage of moose but did not investigate the contribution of PSMs from white cedar which can comprise 27% of the diet of island moose. PSMs from balsam fir are only a subset of chemicals consumed by moose and excreted via the glucuronidation pathway. For example, Servello and Schneider (2000) reported that cedar significantly increased urinary GA:C concentrations relative to deciduous diets but balsam fir increased concentrations of GA:C to a greater extent than cedar consumption in white-tailed deer.

With balsam fir being the largest component of winter diet for moose (Parikh et al. 2016), it is reasonable to assume that GA concentrations should correlate with the proportion and rate of PSMs in fir consumed by moose. Our lack of findings with regard to this prediction may best be explained by considering the time of year that the samples were collected. In our study, samples were collected between late January and early March each year, well into the dormant period for deciduous trees. By this time moose had already been feeding almost exclusively on conifers, primarily balsam fir, for more than two full months. It is possible that within that period urinary GA:C levels could have reached an average concentration based on an average daily intake of PSMs. This conclusion is supported by the basic premise of metabolic theory which states that the rate of a metabolic process is a function of mass, temperature, and amounts of raw materials put into the system (Marquet et al. 2004). With relatively consistent daily temperatures during the sample period and relatively consistent daily average intake of PSMs from balsam fir, changes in moose mass may happen too slowly to reflect changes in GA:C concentration. Therefore, given our experimental design, it stands to reason that a relationship between GA:C and PSM concentration consisting of slight variations in average daily intake of PSMs may not be detected within a winter season.

An alternative, but not mutually exclusive, explanation is that GA:C is influenced by more than intake of PSMs (Ghosal et al. 2004). Glucuronidation is also responsible for the excretion of endogenous stress hormones (Steimer 1993) and hepatic bilirubin (Kamisako et al. 2000). Moreover, GA production can be stimulated by the presence of parasitic endobiotics (Ritter 2000). Free-ranging herbivores experience a myriad of complex ecological interactions that cannot be simulated in controlled experiments where the tight relationships between PSM intake and GA have been demonstrated (Guglielmo et al. 1996; Hewitt and Kirkpatrick 1997; Servello and Schneider 2000; Sorensen et al. 2005b). As a result, findings that link GA:C to PSMs in controlled experiments where stress and parasites are held constant may not be transferable to wild populations. Moose on Isle Royale are currently facing increased intraspecific competition for resources due to lack of top-down population controls (Wilmers et al. 2006) and a potentially depleting food resource (Peterson 1999), as well as tick infestations (DeIgiudice et al. 1997). These factors likely contribute to individual GA:C concentrations and confound the ability to detect relationships between GA:C and ingested PSMs in fir.

It is widely recognized in the field of wildlife ecology that access to suitable forage is an important factor in herbivore habitat selection and population growth. Studies investigating habitat selection and bottom-up regulation of herbivores generally focus on forage availability (Cook et al. 2004; Stephens 2008; van Beest et al. 2010; Wam and Hjeljord 2010; Bjørneraas et al. 2011) and quality (Villalba et al. 2002; Provenza et al. 2003; Wiggins et al. 2005; Parker et al. 2009; Frye et al. 2013; Kohl et al. 2015). The presence of PSMs in natural browse and their influence on herbivory is not a new concept in the study of plant herbivore interactions. However, a direct link between PSMs and herbivore nutritional condition has yet to be established in wild populations. Captive feeding trials have previously been the primary means for assessing the link between PSMs, the excretion of GA and nutritional consequences (e.g., UN, energy budgets, Servello and Schneider 2000; Sorensen et al. 2005b; Shipley et al. 2012; Saltz and White 1991a, b). In the present study we have filled this gap, linking behavioral and physiological responses to diet quality (PSMs) to the nutritional condition of a free-ranging herbivore. In so doing, we have taken the first step in linking consequences of PSM ingestion to individual and population level fitness parameters. We have demonstrated that the process of detoxification through conjugation with GA predicts nutritional condition as evident by increased UN:C under winter conditions. We demonstrated this effect both within years (Fig 2.1) and among years (Fig 2.3). Quantification of GA:C is non-invasive, easy, and relatively inexpensive means for predicting the nutritional condition of individuals and populations over time and space. Moreover, our results suggest that GA:C represents a more informative parameter to assess the consequences of PSMs on herbivores, than quantifying PSMs in forage alone because it accounts for behavioral and physiological responses to PSMs. As such, variance in GA:C over time may be a useful indicator of the variable amount of PSM consumed and detoxified per unit time, which represents an important aspect of understanding PSM-mediated plant-herbivore dynamics (Liu et al. 2008; Feng et al. 2009). Because GA:C is dynamically linked to the nutritional condition of moose over time (Parikh et al. 2016, Fig 2.2), it may also help explain population densities relative to predation, parasites and other factors. Monitoring the potential costs of detoxification of ingested PSMs and nutritional condition through the collection and analysis of urine samples can provide managers with novel data to make informed decisions regarding habitat and population management practices and help identify populations in greatest need of management. Difficult to manage species or species of critical concern may benefit, at the population level, from an understanding of the physiological effects of poor diet quality on nutritional condition over time.

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Tables

Table 2.1 Model components of Generalized Linear Models; log likelihood (LL), number of parameters (K), Akaike's Information Criterion with sample size bias-adjustment (AICc), change in AICc from the top model (Δ AICc), and model weight (w_i) for the model selection used for determining predictor of UN:C in moose (*Alces alces*) at Isle Royale National Park, Michigan, USA during winter 2014.

Model	Log Likelihood (LL)	Number of parameters (K)	AIC_c	ΔAIC_c	Akaike weight (w_i)
GA:C[1]	-39.16	3	85.17	0.00	0.91
Proportional IR of Monoterpenes[2]	-41.77	3	90.39	5.22	0.07
IR of Monoterpenes	-43.96	3	94.77	9.60	0.01
Proportion of Monoterpenes	-44.84	3	96.53	11.36	0.00
Total normalized IR of PSMs[3]	-45.48	3	97.81	12.64	0.00
Total normalized proportion of PSMs	-45.60	3	98.06	12.89	0.00
Total normalized proportional IR of PSMs	-45.72	3	98.29	13.12	0.00
Proportional IR of Phenolics	-45.83	3	98.52	13.25	0.00
Proportion of Phenolics	-46.40	3	99.65	14.48	0.00
IR of Phenolics	-46.53	3	99.92	14.75	0.00
NULL	-48.40	2	101.21	16.04	0.00
Total Normalized PSM[3]	-47.73	3	102.32	17.15	0.00
Total Phenolics	-48.03	3	102.91	17.74	0.00
Total Monoterpenes	-48.25	3	103.36	18.19	0.00

[1] Glucuronic Acid (GA) to Creatinine (C) ratio present in moose urine samples.

[2] Proportional intake rate (IR) – calculated as the rate of intake multiplied by the proportion of fir present in the diet.

[3] Total normalized PSMs – calculated by normalizing monoterpenes and phenolics to a 0 -1 scale. Normalized values were added together for total PSM value.

Figures

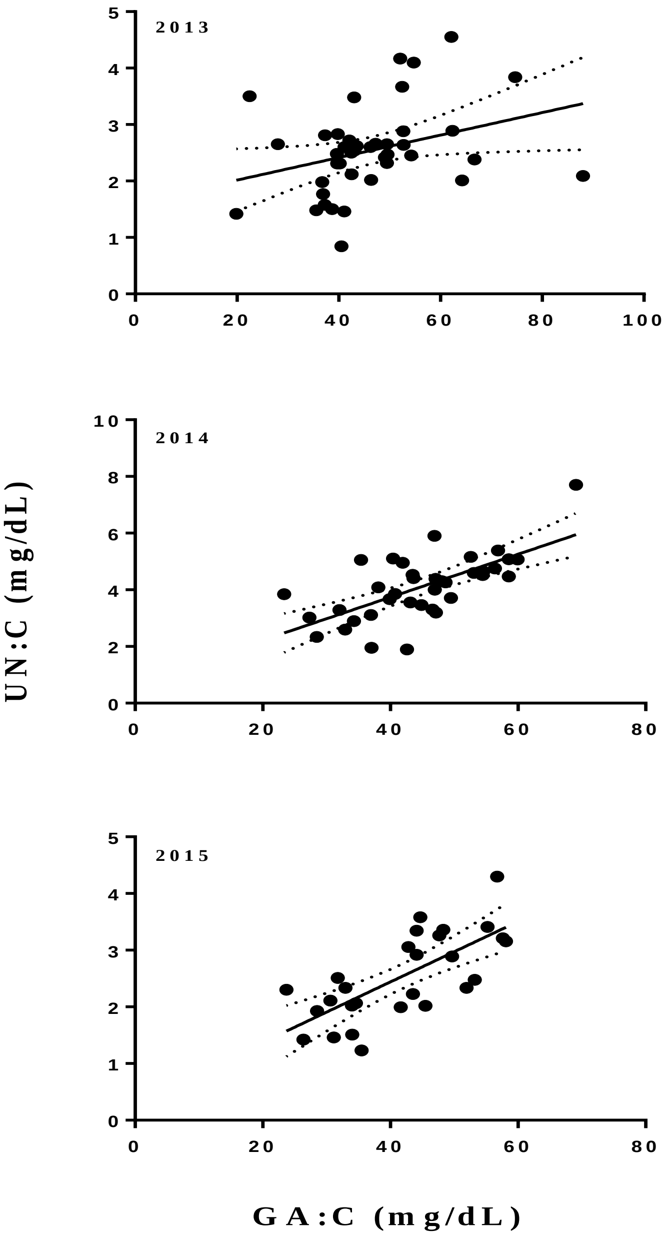

Fig 2.1 Linear regression between GA:C and UN:C from moose urine at Isle Royale National Park, Michigan, USA for three years; 2013 (Y=0.02x + 1.62; $F_{1,39} = 4.558$, $p = 0.04$, $R^2 = 0.105$, 95% CI = 0.001 – 0.039), 2014 (Y=0.76x + 0.706; $F_{1,35} = 28.19$, $p < 0.001$, $R^2 = 0.45$, 95% CI = 0.047 – 0.105), and 2015 (Y=0.053x + 0.31; $F_{1,25} = 25.43$, $p < 0.001$, $R^2 = 0.504$, 95% CI = 0.032 – 0.075). Solid line represents the least squares regression with dashed lines representing 95% confidence intervals.

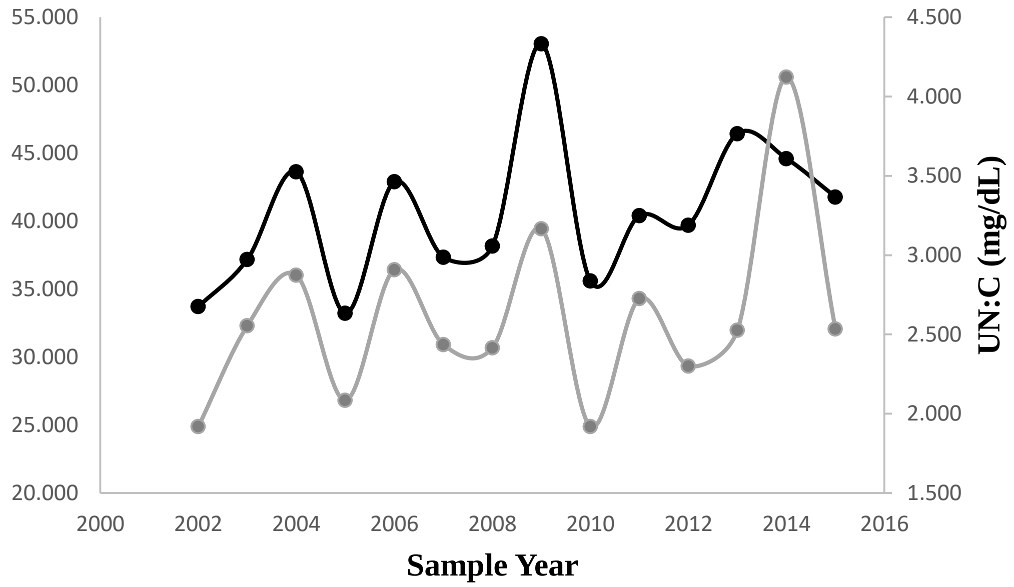

Fig 2.2 Intra-annual variation in urinary GA:C (black) and UN:C (grey) for moose on Isle Royale National Park from 2002 - 2015. Points represent average values for each sample year. Only continuous data was used, years with missing or anomalous data (1994, 1997, and 2001) are not included.

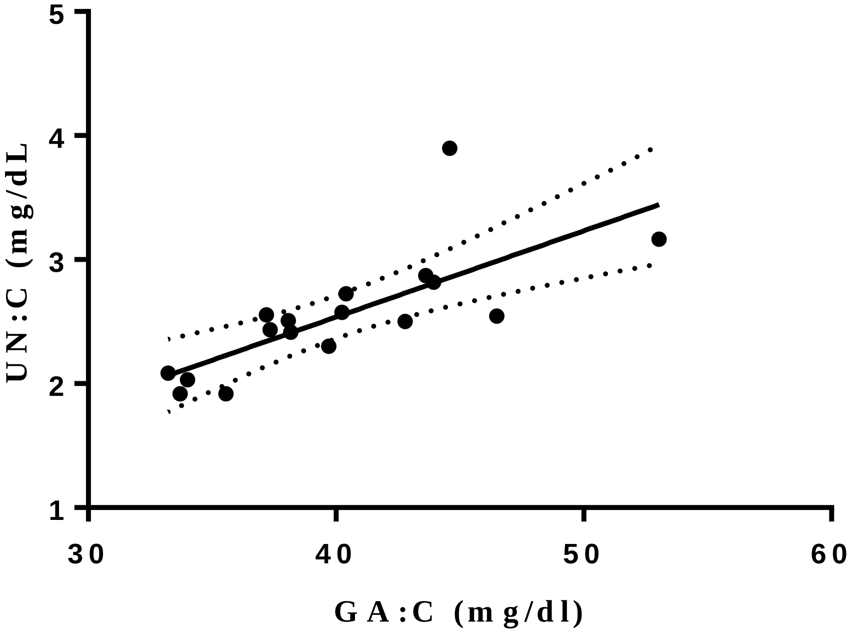

Fig 2.3 17-year relationship between UN:C and GA:C of urine from free-ranging moose on Isle Royal National Park from 2002 - 2015. Points represent annual averages of all samples from individual moose (Y=0.069x – 0.244; F1,15 = 18.57, p = 0.0006, R2 = 0.553, 95% CI = 0.035 – 0.104). Solid line represents the least squares regression between the two variables with dashed lines representing the 95% confidence intervals.

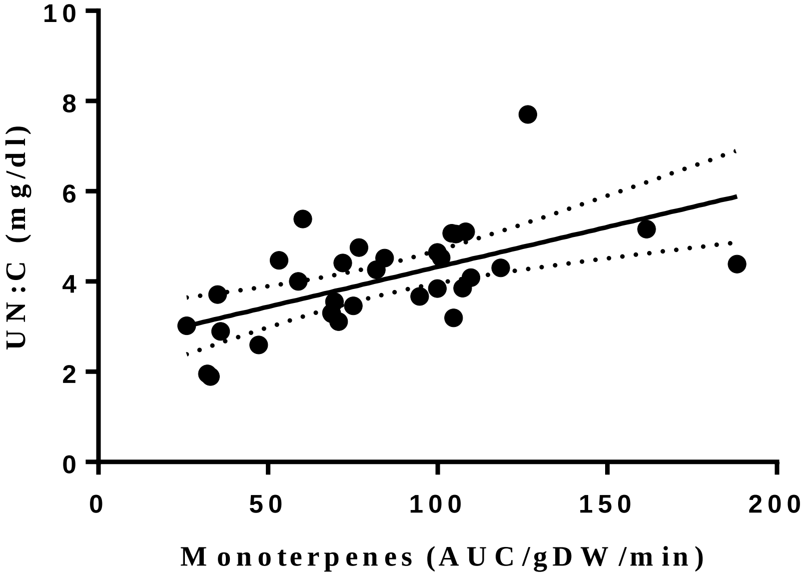

Fig 2.4 Relationship between the proportional IR of monoterpenes (AUC/gDW/min) and UN:C (mg/dL) for the 2014 data set (n = 40, Y=0.018x + 2.56; $F_{1,30}$ = 15.41, p = 0.0005, R2 = 0.34, 95% CI = 0.008 – 0.027). Solid line represents least squares regression while the dotted lines represent 95% confidence intervals.

MANAGEMENT IMPLICATIONS

Isle Royale National Park

Herbivores comprise the bulk of economically important game species in most states. As such, understanding the factors that regulate their populations is of critical importance for many local and regional revenue streams. Herbivores can have important impacts on plant communities and ecosystems which can in turn have important impacts on ecosystem function. Diet selection and intense browsing by large herbivores can alter landscape vegetative communities, diversity, and structure by inducing changes in plant and soil chemical characteristics (Singer and Schoenecker 2003; Stolter 2008; Ulappa et al. 2014). Many large herbivores function as keystone species in forested systems (Kowalczyk et al. 2011). In systems containing large herbivores with negligible predation, herbivores play an additional role of ecosystem engineers with the potential to change ecosystem function (Olofsson et al. 2004; Olofsson 2006; Post and Pedersen 2008). For example, Ricca et al. (2016) showed that predator free herbivory in the Aleutian Islands, Alaska resulted in a dramatic decrease in forage plant community composition and a shift in ecosystem processes.

Few natural systems contain large herbivore populations that are not, at least in part, regulated by predation. Isle Royale National Park has had a long-standing history as one of very few single predator-single prey system, and has been a model for the study of predator-prey interactions. With the near total loss of the wolf population on the island in recent years, moose population regulation may be increasingly governed by forage resources. Given these conditions, researchers have an opportunity to assess the effect moose, with limited predation, will have on plants and also the role that plant availability and quality will have in influencing moose populations. We have demonstrated that plants can influence the behavior and physiology of moose in ways that could alter their population dynamics through nutritional deficits. We used urinary metabolites, specifically glucuronic acid (GA) as a biomarker for exposure to plant chemical defenses, as well as urea nitrogen (UN) as a biomarker of their overall nutritional condition to investigate the effects of PSMs on winter nutritional condition of moose. We found strong correlations between GA and UN for 17 of 18 years. These results suggest that energetic demands increase as intake of PSMs increase, which in turn adds to the nutritional stress of moose. This positive feedback loop ultimately could result in continually decreasing nutritional condition in herbivores. Population GA levels follow the same general trends as population size of the moose on Isle Royale (Fig 3.1) suggesting that interspecific competition for resources could further increase energetic demands. In other words, as herbivore populations increase in size, it may become increasingly difficult for herbivores to locate nutritious forage, thereby increasing their reliance on nutritionally poor forage options and perpetuating the effects of nutritional restriction. This trend has serious implications for population biology in systems where resources are limited or predation is minimal.

In 1996, lack of predation pressure allowed moose populations to reach an all-time high leading to a near total loss of winter forage resources and a dramatic population crash in moose due to starvation (Peterson 1999; Fig 3.2). Current trends in moose population growth place tremendous pressure on balsam fir stands on the island which have not fully recovered from the 1996 over-population of moose. If moose populations continue to grow at their current rate, a near total extirpation of browsable balsam fir may be imminent. As such, we would expect to see annual increases in GA and UN concentrations with potential increases in PSM concentrations in remaining fir trees. Isle Royale is designated as a protected National Park, and as such, generates large amounts of revenue through tourism. Leaving the moose to their fate and assuming that bottom-up, density dependent forces will directly regulate moose populations may be misguided. Unchecked population growth could have disastrous consequences for the moose, fir, and the island ecosystem, which could negatively influence revenue generated through anthropogenic use. A stable equilibrium between herbivores and their food source is unlikely to exist in systems without predation (Sæther 1997; Terborgh et al. 2001). It is more likely that density dependence will regulate populations in a delayed fashion, leading to large carrying capacity overshoots followed by high instances of winter die-offs. Such delayed density dependence could severely alter the islands vegetative communities and significantly reduce its carrying capacity for moose. History has repeatedly shown that large herbivore over-abundance can have severe consequences for trophic cascades and ecological processes (Ripple and Beschta 2005; Griggs et al. 2006). Author and philosopher George Santayana is credited with coining the phrase “Those who cannot learn from history are doomed to repeat it”. The ecosystem at Isle Royale may be irreversibly facing just such a fate if steps to manage the moose populations are not taken. Either by thinning moose numbers through direct anthropogenic interference or through re-introduction of wolves, direct management of the Isle Royale moose population is needed to preserve current ecosystem function.

Other Systems

We demonstrated the utility of urinary GA and UN as biomarkers PSM exposure and winter nutritional condition in a single species system. However, the use of these biomarkers could provide a novel mechanism by which to monitor overwintering nutritional status for many plant-herbivore systems. Climate change is exposing herbivores to additional ecological pressures that include loss of habitat and invasion of non-native and domestic species (Thomas et al. 2004; George et al. 2008). Climate change is also allowing many herbivores to expand historical home ranges into areas where they previously did not occur, while simultaneously restricting ranges of certain niche species (Parmesan 2006). Range expansion can lead to increased interspecific conflict and competition for resources as well as interspecific disease transmission. Declines in moose populations along the southern periphery of their range have been correlated with overlapping ranges of high populations of white-tailed deer (Lankester 2010). Moose declines in this region are commonly attributed to warmer winters which allow increased deer presence and the spread of *Parelaphostrongylus tenuis,* a meningeal worm carried by white-tailed deer that is pathogenic to moose (Rempel 2011). Other effects of climate change on herbivores include increased PSMs and decreased tolerance to PSMs (Dearing et al. 2008) as well as increased abundance of and decreased resistance to parasites (Gray et al. 2009; Molnár et al. 2013). All of these could be detected in excretion of GA (see Chapter 2), effectively increasing potential energetic deficits. In the case of herbivores, quantification of urinary GA and UN may provide managers with an "early warning" physiological metric of populations that may be in trouble, regardless of the health their habitat.

Wildlife managers currently collect and maintain a litany of data on herbivore population dynamics including population size, distribution, birth rates, death rates, and many more. Much of this data is gathered through direct observation of animals as well as a variety of mark and recapture techniques. These data are useful in determining management successes, failures, and future directions, but collection of such data is time and labor intensive. What is missing is an easily obtained physiological metric pertaining directly to animal health as it relates to their environment. Urinary metabolites discussed herein provide this metric at a minimal cost to management agencies. Methods discussed in this thesis are simple, non-invasive, relatively inexpensive, and applicable to a wide range of avian and mammalian herbivores. Analysis of urinary GA and UN (or uric acid in avian herbivores) has the potential to provide a rapid, single-point reference pertaining directly to the physiological and nutritional condition of free-ranging populations that links to their diet, energy budget and nutritional status. We recommend that field observation or mark and recapture methods of mammalian herbivores be adjusted to allow for the collection of urine when possible for the analysis of GA and UN metabolites. Laboratory diagnostics procedures could easily be enacted at minimal cost, yet produce great benefits to wildlife managers interested in monitoring and maintaining the health of wildlife populations and entire trophic systems.

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Figures

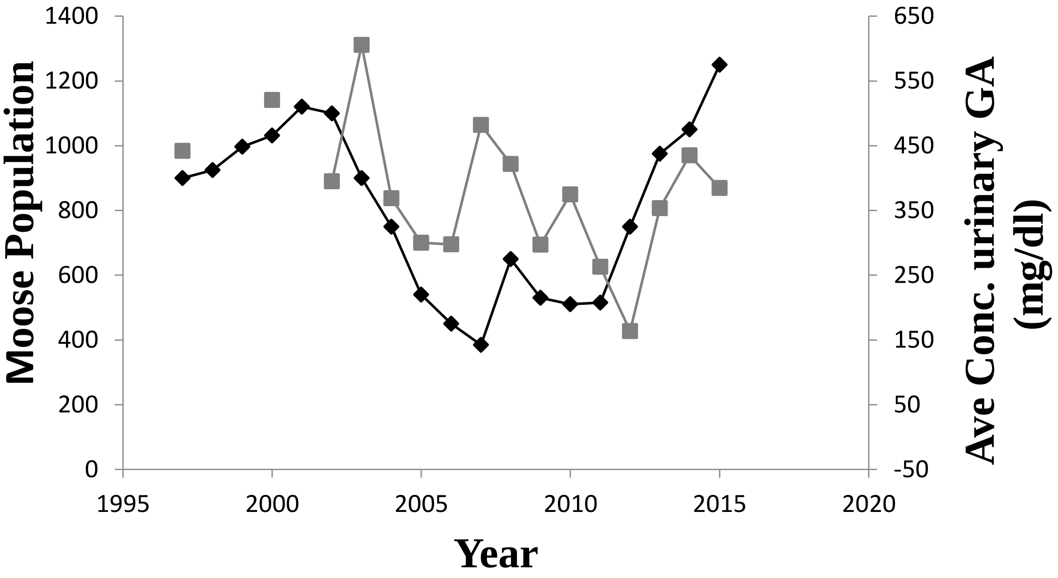

Fig 3.1 **Population trends of Isle Royale moose between 1995 and 2015 (diamonds, black line) and average urinary glucuronic acid (GA) concentration (squares, grey line). Broken line representing GA between 1995 and 2002 is due to missing annual values resulting from sample contamination or loss.**

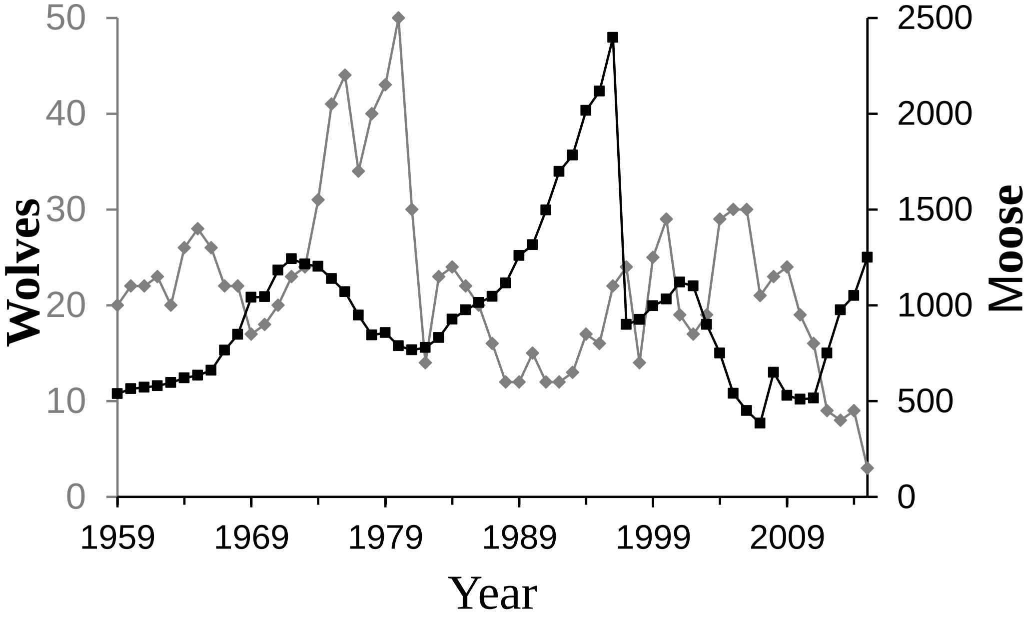

Fig 3.2 **Populations of wolves (diamonds, grey line) and moose (squares, black line) on Isle Royale National Park, Michigan U.S.A between 1959 and 2015. Figure courtesy of J.A. Vucetich, Michigan Technological University, used with permission.**

Appendix A

Study system: Isle Royale National Park

Study system: Isle Royale National Park

We met the objectives outlined in this thesis by investigating moose population dynamics in a wolf-moose-balsam fir system on Isle Royale National Park, Michigan, USA. A critically distinctive feature of this research was the ability to test behavioral and physiological responses to diet quality and measure those physiological consequences over time in a system where predation has already been characterized. Isle Royale is a 544 km^2 island situated in the north-western region of Lake Superior, North America (47°50'N, 89°00'W) (Fig A.1). It is well known as one of the only known single predator-single prey ecological systems and has been studied continuously in that capacity for over 50 years. The island is populated by a population of moose (*Alces alces*) and grey wolves (*Canus lupus*). There is no direct evidence indicating how moose arrived on Isle Royale (Vucetich et al. 2011). They either swam the short distance from the mainland or were introduced by people in the early 20th century (Scarpino 2011), with recent evidence supporting the introduction hypothesis (Scarpino et al. 2015). Wolves arrived by crossing an ice bridge that formed during a particularly cold winter between 1948 and 1950 (Vucetich et al. 2011). These populations are isolated from mainland populations with little to no immigration or emigration and hunting is strictly prohibited (Vucetich and Peterson 2004). Island climate is characterized by warm summers (≈50° – 75° F) and cold, wet winters (≈-6° – 21° F). Winter snowfall ranges from moderate (≈29.6 cm) to severe (≈72.4 cm) with an average summer precipitation of ≈9.02 cm/month (Parikh et al. 2016).

The island sits at the boundary between boreal and northern hardwood forests with large components of both biomes and there are distinct differences in vegetation and soil morphology between the east and west ends of the island (Sanders and Grochowski 2013). Receding Pleistocene glaciers deposited till on the western end of the island allowing for deeper, nutrient rich soils while glacial scouring exposed bedrock on the eastern end resulting in shallow, nutrient poor soils (Montgomery et al. 2014). Principle forage for moose on Isle Royale consists of balsam fir (*Abies balsamea*), white spruce (*Picea glauca*) and cedar (*Thuja accidentalis*) with balsam fir being the most important source of winter forage (Vucetich and Peterson 2004; Montgomery et al. 2013). A variety of deciduous trees and shrubs are also available including American mountain ash (*Sorbus Americana*), red osier dogwood (*Cornus stolonifera*), paper birch (*Betula papyrifera*) as well as *Populus spp* and *Acer spp*. (Montgomery et al. 2013; Parikh et al. 2016). Differences in soil morphology and content between the east and west ends of Isle Royale have led to important differences in the distribution of key browsing species. The western end is characterized by northern deciduous forest and dominated by a greater proportion of cedar trees. In the Eastern region, boreal forests types dominate with thick stands of mature balsam fir (DeIgiudice et al. 1997; Sanders and Grochowski 2013). Balsam fir is the principle source of winter browse for moose. Fir occurs across the island, but is found in much lower densities in the west end than in the east (DeIgiudice et al. 1997).

Isle Royale’s wolf-moose system provides a unique opportunity to study the effects of plant secondary metabolites on the population dynamics of prey species in general and ungulates in particular. Moose are the only large ungulate and prey species on the island. Hunting and vehicular traffic are prohibited leaving only predation and starvation as the leading causes of moose mortality. Moose leave browsing marks on selected trees that are easily identified and winter browse options are limited to only a few coniferous and deciduous species for which PSMs are easily tested. With wolf numbers on the island in critical decline, understanding the effects of diet selection and nutritional condition are important factors to understanding how diet quality influences moose population dynamics of moose. Conclusions of this research can have far reaching impacts into other predator-prey systems. Understanding the effects of PSMs on population dynamics of herbivores could aid in understanding food chain and food web stability where predation varies considerably.

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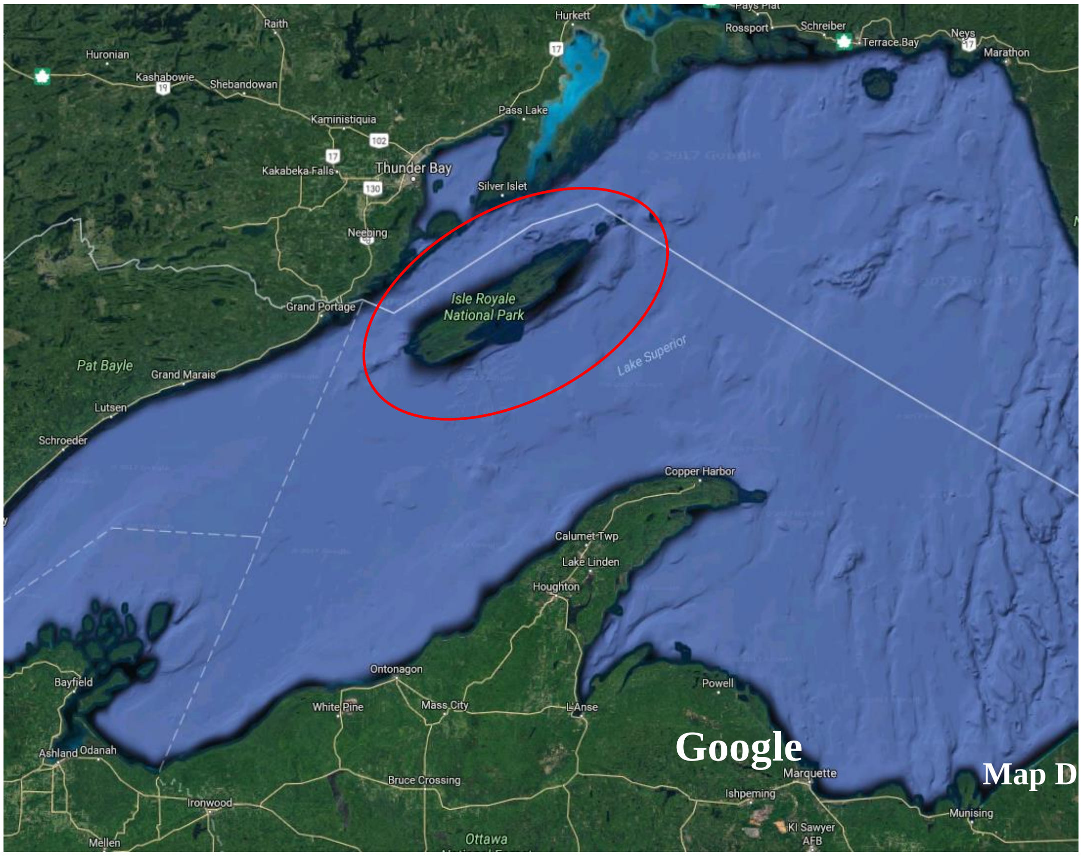

Fig A.1 Isle Royale; a 544 km2 island situated in the north-western region of Lake Superior, North America (47^{o} 50'N, 89^{o} 00'W). 22km from Ontario, Canada and 25km from Grand Portage, Minnesota.

Appendix B

GC Headspace autosampler parameters during monoterpene quantification analysis of balsam fir (*Abies balsamea*)

GC Headspace autosampler parameters during monoterpene quantification analysis of balsam fir (*Abies balsamea*)

Volatile monoterpenes were quantified using an Agilent 7694 headspace auto-sampler in tandem with an Agilent 6890N gas chromatograph. Ground sub-samples (100mg) of both browsed and unbrowsed balsam fir trees were placed in separate 20mL glass headspace vials. For each sample, 1mL of headspace gas inside the vial was injected into a J&W DB-5 capillary column (30m x 250µm x 0.25µm) following a 20 min vial equalization time.

Operating Parameters for the headspace auto-sampler were:

- Temperatures
    - oven temperature at 100°C
    - loop temperature at 110°C
    - transfer line temperature at 120°C
- Times
    - Vial equilibrium time = 20 minutes
    - Pressurization time = 0.20 minutes
    - Loop fill time = 0.50 minutes
    - Loop equilibrium time = 0.20 minutes
    - Injection time = 0.50 minutes
- Vial Shaking Parameters = OFF

Operating conditions for the Gas Chromatograph were:

- Splitless injector at 250^{o}C
- Flame ionization detector at 300^{o}C
- Oven temperature

  - 40^{o}C for a period of two (2) minutes.
  - Increase at a rate of 3^{o}C/min to 60^{o}C.
  - Increase at a rate of 5^{o}C/min to 120^{o}C.
  - Increase at a rate of 20^{o}C/min to 300^{o}C.
  - Held steady at 300^{o}C for a period of seven (7) minutes.
- Inlet pressure of 80KPa with a flow rate of 1.0mL/min.
- Make-up gas = Nitrogen
- Carrier gas = Helium

Retention times and peak areas of individual monoterpenes were calculated in HP ChemStation version B.01.00. Peak areas were calculated via integration of chromatogram curves. Compounds with peak areas greater than 1% of the total area and present in > 70% of samples were used in analysis.

Appendix C

Multivariate correlation matrix testing collinearity of dietary parameters in balsam fir trees

Multivariate correlation matrix testing collinearity of dietary parameters in balsam fir trees.

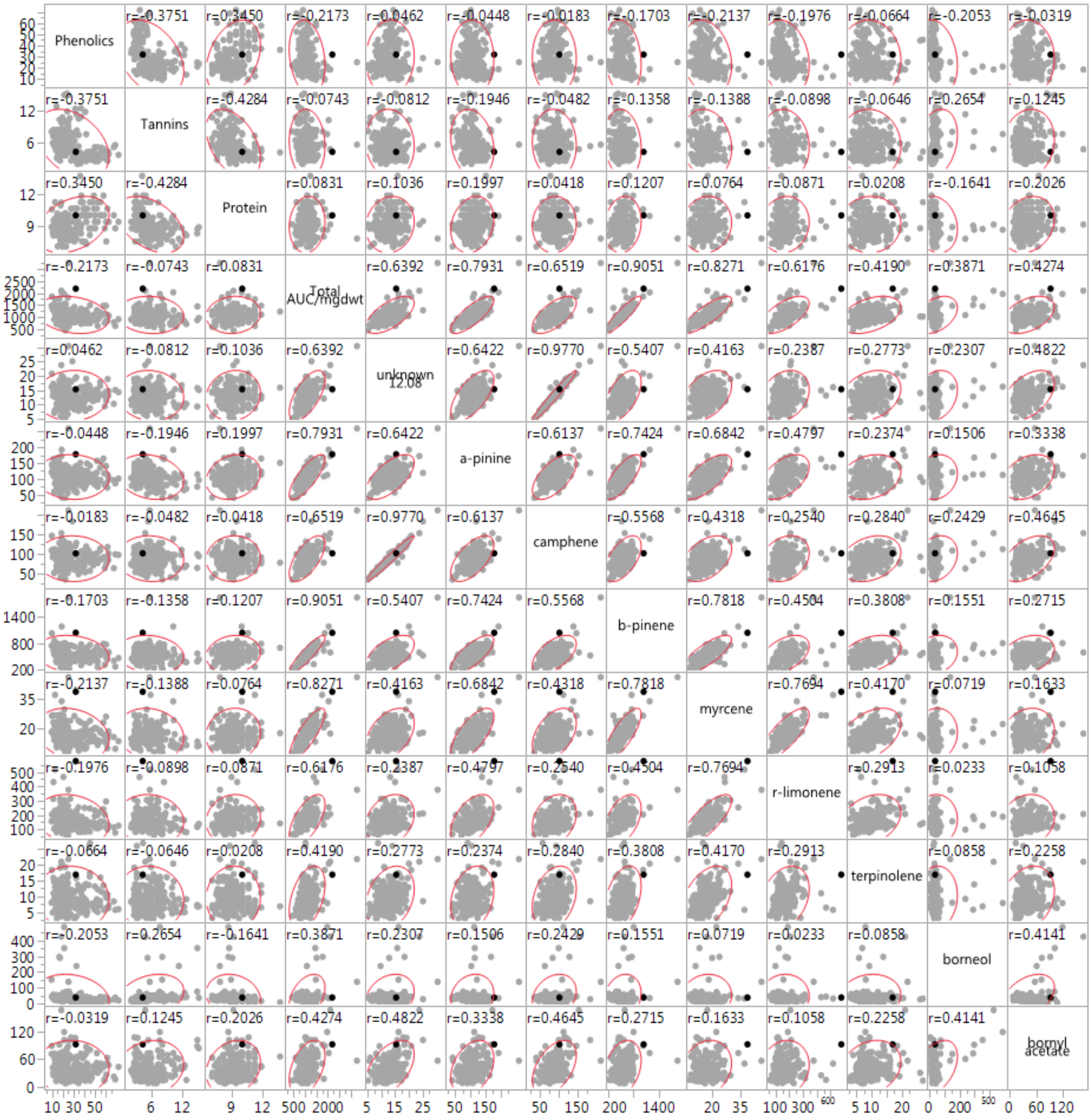

Fig C.1 Multivariate analysis of one unknown and eight known monoterpenes, total monoterpenes, total tannins, total phenolics, and % crude protein. Variables with r > 0.7 were considered to be strongly correlated and in violation of multicollinearity requirements.

* Alpha-pinene and myrcene were removed because they both correlate with beta-pinene (r = 0.7424 and r = 0.7818 respectively) and myrcene is also correlated with r-limonene (r = 0.7694). Individually, beta-pinene and r-limonene comprise significantly higher proportions of total AUC than do alpha-pinene and myrcene combined. This proportional difference is consistent across the entire sample set. Therefore, beta-pinene and r-limonene were retained for further analysis. Unknown with a retention time of 12.08 min was strongly correlated with camphene (r = 0.9770). Since this peak at 12.08 min was the only unknown monoterpene > 1% total AUC and present in > 70% of samples and it is correlated with a known terpene (camphene), the unknown compound was removed from further analysis.

**Total AUC was omitted from analysis because it is correlated with (and driven by) a number of individual chemicals. We selected individual monoterpenes because we know animals respond to individual chemicals, not just the sum of all parts (Stolter et al. 2005; Frye et al. 2013).

This multivariate analysis represents and “island-wide” analysis. Multivariate analyses with respect to island region (east versus west) as well as with respect to year were also conducted. All correlations remained consistent across all analysis models indicating that PSM correlations were governed at the plant level scale rather than spatial or temporal scales.

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Appendix D

East and west regional differences in dietary and environmental parameters by sample year.

Table D.1 **Mean concentrations ± SEM of plant secondary metabolites (total and individual monoterpenes >1% total AUC and present in >70% samples (AUC/mg DW), total phenolics (mg GAE/g DW), total tannins (µM TAE/g DW), nutrient content (% crude protein), snow depth (cm), and bite diameter (mm) in balsam fir trees (*Abies balsamia*) at patches browsed by moose (*Alces alces*) at East and West ends of Isle Royale National Park, Michigan, USA during winter 2013, 2014, and 2015.**

	2013		2014		2015	
Parameter	East ($\bar{x}$)	West ($\bar{x}$)	East ($\bar{x}$)	West ($\bar{x}$)	East ($\bar{x}$)	West ($\bar{x}$)
Total Monoterpenes	1034.69 ± 34.21	1031.03 ± 36.27	1174.26 ± 48.22	1314.81 ± 68.86	1046.63 ± 72.03	1031.34 ± 50.58
Unknown 12.08[1]	12.91 ± 0.40	14.05 ± 0.58	13.49 ± 0.58	13.91 ± 0.83	12.25 ± 0.69	12.58 ± 0.58
α-pinene	111.08 ± 3.77	104.22 ± 4.77	109.21 ± 4.35	121.91 ± 6.76	93.32 ± 6.21	97.71 ± 4.30
Camphene	85.05 ± 2.65	92.34 ± 3.52	94.11 ± 3.98	96.10 ± 5.64	83.62 ± 4.46	85.47 ± 3.62
β-pinene	535.03 ± 20.80	520.57 ± 23.14	593.20 ± 28.72	673.72 ± 44.74	480.67 ± 28.41	485.44 ± 25.12
Myrcene	16.40 ± 0.64	15.76 ± 0.81	18.90 ± 0.72	21.52 ± 1.33	14.86 ± 0.92	15.12 ± 0.88
R-limonene	151.16 ± 8.34	147.20 ± 8.18	180.85 ± 10.04	230.67 ± 20.81	145.28 ± 8.88	155.24 ± 12.81
Terpinolene	9.62 ± 0.72	9.45 ± 0.88	10.01 ± 0.51	9.26 ± 1.01	8.65 ± 0.84	9.48 ± 0.64
Borneol[2]	22.86 ± 1.49	21.77 ± 1.87	47.99 ± 5.40	35.09 ± 2.46	81.71 ± 24.02	56.38 ± 11.87
Bornyl Acetate	37.18 ± 3.06	48.88 ± 4.84	32.74 ± 2.60	40.71 ± 4.75	46.36 ± 5.20	51.30 ± 4.70
Number of compounds[3]	8.64 ± 0.16	8.61 ± 0.18	8.87 ± 0.11	8.80 ± 0.17	8.00 ± 0.21	8.50 ± 0.16
Total phenolics[4]	46.59 ± 1.87	45.38 ± 1.98	19.99 ± 0.70	16.70 ± 0.94	25.25 ± 1.60	24.71 ± 0.84
Total Tannins[4]	3.55 ± 0.15	3.01 ± 0.13	5.98 ± 0.23	5.56 ± 0.59	8.20 ± 0.54	7.60 ± 0.46
Crude Protein	9.98 ± 0.21	10.05 ± 0.19	8.41 ± 0.09	9.91 ± 0.15	8.20 ± 0.14	8.80 ± 0.08
Snow Depth	38.0 ± 1.32	43.46 ± 1.33	67.46 ± 0.86	76.20 ± 2.51	52.27 ± 1.86	50.09 ± 1.37
Bite Diameter[5]	na	na	3.06 ± 0.07	2.87 ± 0.19	2.62 ± 0.13	2.66 ± 0.12

[1] Unidentifiable monoterpene, numbers following "unknown" refer to retention time (min) in the chromatogram resulting from gas chromatography

[2] Borneol concentrations were measured but were excluded from analysis due to inconsistency in repeat samples and lack of adequate control standards.

[3] Total number of monoterpenes with retention times < 24 minutes, > 1% total AUC, and present in > 70% of samples.

[4] Total phenolics and tannins, concentrations in mg Gallic Acid Equivalence (GAE, phenolics) or µM Tannic Acid Equivalence (TAE, tannins)/g DW

[5] Bite diameter, diameter of the twig (mm) at the observed bite mark by moose on browsed trees. Not collected during sample year 2013.

Appendix E.

AICc model selection by region to determine region specific effects of dietary parameters on browsing selection by moose.

AICc model selection by region to determine region specific effects of dietary parameters on browsing selection by moose.

East and west regions of Isle Royale are characterized by distinct differences in soil morphology and soil nutrient composition (Appendix A). It is proposed that these differences in soil morphology further influence difference in dominant vegetation and plant community composition between the two regions (Fox-Dobbs et al. 2007). It is likely that these differences lead to differences in wind and light exposure which can directly result in bio-geochemical and nutrient cycling differences between the two regions (Li et al. 2007; Li and Kubota 2009). Concurrently, difference in nutrient cycling could directly affect PSM production and relative concentrations (Coley et al. 1985) between east and west fir trees. Indeed, our data showed significant difference between the two regions with respect to bornyl acetate and crude protein, with higher concentrations in the east (Table 1.1). It is reasonable to conclude that differences in PSM production and concentration could, in turn, result in differing selection criteria for moose browsing on the east and west ends of the island. We sought to determine if region specific behavior was a factor in moose diet selection.

Following univariate analysis of clogit models, six covariates remained in the candidate model set as possible indicators of diet selection; camphene, beta-pinene, bornyl acetate, r-limonene, tannins, and total number of compounds. Sub-setting the data by region and conducting individual AIC analysis on each region resulted in camphene as the top model for both regions. Beta-pinene resulted as a competing model (< 2 ΔAICc from the top model) in the eastern region (Table E.1), while tannins and bornyl acetate emerged as competing models in the western region (Table E.2). All competing models from both regional analyses were included in a second-round model selection in which all possible combinations of the four covariates were considered. Camphene again emerged as the top model candidate with an additive model containing camphene + tannins as the only competing model (Table E.3). For conditional logistic models, confidence intervals that cross 1.0 are considered to be non-predictive. Analysis of odds ratios and confidence intervals for simple models containing camphene and tannins showed that camphene reliably predicted browsing (exp.coef = 1.0224, 95% conf = 1.005 – 1.04), while tannins did not (exp.coef = 0.9306, 95% conf = 0.7836 – 1.105). Odds ratio analysis of the additive model containing both covariates showed that tannin performance within that model remained unpredictive (exp.coef = 0.991, CI = 0.8228 – 1.1931) indicating that camphene was the driving factor in model weight and performance. Furthermore, model averaging of the simple model containing only camphene and the additive model containing camphene + tannins had no effect on the model estimate (est = 0.02) of the top (camphene only) model.

An additive model containing camphene + beta-pinene was < 2 ΔAICc from the top, fitting the criteria of a competing model, however, it only accounted or 9% of the weight of the candidate set. Given the results of the odds ratio analysis of the additive model containing tannins, the additive model containing beta-pinene was not considered for further analysis. These results indicate that camphene is a reliable “island wide” and region specific predictor of browsing selection by moose.

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Table E.1 Model components, log likelihood (LL), number of parameters (K), Akaike's Information Criterion with sample size bias-adjustment (AICc), change in AICc from the top model (Δ AICc), and model weight (w_i) for the conditional logit selection models used for the browsing of balsam fir (*Abies balsamia*) patches used by moose (*Alces alces*) in the eastern region of Isle Royale National Park, Michigan, USA during winter 2013, 2014, and 2015. Plant use (browsed/unbrowsed) was the binary response for each model. Top models, with < 2 Δ AICc from the top model and with an AICc value lower than the null model, are shown in bold.

Predictor category	Model	Log Likelihood (LL)	Number of parameters (K)	AIC_c	ΔAIC_c	Akaike weight (w_i)
PSM	**Camphene[1]**	**-24.89**	**1**	**51.82**	**0**	**0.40**
	β-pinene[1]	**-25.60**	**1**	**53.23**	**1.41**	**0.20**
	NULL	-27.03	0	54.07	2.25	0.13
	Bornyl Acetate[1]	-26.13	1	54.30	2.48	0.11
	Number of Compounds[2]	-26.71	1	55.45	3.63	0.06
	Tannins[3]	-26.98	1	55.99	4.17	0.05
	R-limonene[1]	-26.98	1	56.00	4.18	0.05

[1] Monoterpene compounds, AUC/mgDW > 1% total AUC and present in >70% of samples, and with univariate analysis $p < 0.5$.

[2] Total number of monoterpenes with retention times < 24 minutes, > 1% total AUC, and present in > 70% of samples.

[3] Total tannins, µM Tannic Acid Equivalence (TAE)/g DW

Table E.2 Model components, log likelihood (LL), number of parameters (K), Akaike's Information Criterion with sample size bias-adjustment (AICc), change in AICc from the top model (Δ AICc), and model weight (w_i) for the conditional logit selection models used for the browsing of balsam fir (*Abies balsamia*) patches used by moose (*Alces alces*) in the western region of Isle Royale National Park, Michigan, USA during winter 2013, 2014, and 2015. Plant use (browsed/unbrowsed) was the binary response for each model. Top models, with < 2 Δ AICc from the top model and with an AICc value lower than the null model, are shown in bold.

Predictor category	Model	Log Likelihood (LL)	Number of parameters (K)	AIC_c	ΔAIC_c	Akaike weight (w_i)
PSM	**Camphene**[1]	**-19.51**	**1**	**41.07**	**0**	**0.31**
	Tannins[2]	**-19.81**	**1**	**41.67**	**0.60**	**0.23**
	Bornyl Acetate[1]	**-20.09**	**1**	**42.23**	**1.16**	**0.17**
	NULL	-21.49	0	42.98	1.91	0.12
	Number of Compounds[3]	-20.88	1	43.81	2.74	0.08
	R-limonene[1]	-21.30	1	44.64	3.57	0.05
	β-pinene[1]	-21.37	1	44.80	3.73	0.05

[1] Monoterpene compounds, AUC/mgDW > 1% total AUC and present in >70% of samples, and with univariate analysis $p < 0.5$.

[2] Total tannins, µM Tannic Acid Equivalence (TAE)/g DW.

[3] Total number of monoterpenes with retention times < 24 minutes, > 1% total AUC, and present in > 70% of samples.

Table E.3 Model components, log likelihood (LL), number of parameters (K), Akaike's Information Criterion with sample size bias-adjustment (AICc), change in AICc from the top model (Δ AICc), and model weight (w_i) for second round model selection of competing conditional logit models used for the browsing of balsam fir (*Abies balsamia*) patches used by moose (*Alces alces*) at Isle Royale National Park, Michigan, USA during winter 2013, 2014, and 2015. Plant use (browsed/unbrowsed) was the binary response for each model. Top models, with < 2 Δ AICc from the top model and with an AICc value lower than the null

Model	Log Likelihood (LL)	Number of parameters (K)	AIC_c	ΔAIC_c	Akaike weight (w_i)
Camphene[1]	**-44.41**	**1**	**90.84**	**0.00**	**0.23**
Camphene + Tannins[2]	**-43.44**	**2**	**90.95**	**0.11**	**0.22**
Camphene + β-pinene[1]	**-44.31**	**2**	**92.68**	**1.86**	**0.09**
Camphene + Bornyl Acetate[1]	-44.39	2	92.83	2.00	0.08
Camphene + β-pinene + Tannins	-43.36	3	92.85	2.01	0.08
Camphene + Bornyl Acetate + Tannins	-43.43	3	92.98	2.15	0.08
Bornyl Acetate + Tannins	-45.12	2	94.30	3.47	0.04
Bornyl Acetate	-46.23	1	94.47	3.63	0.04
Camphene + β-pinene + Bornyl Acetate	-44.28	3	94.68	3.84	0.03
NULL	-43.34	4	94.90	4.06	0.03
Tannins	-46.79	1	95.59	4.76	0.02
β-pinene + Tannins	-45.95	2	95.96	5.12	0.02
β-pinene + Bornyl Acetate + Tannins	-44.97	3	96.06	5.22	0.02
β-pinene + Bornyl Acetate	-46.08	2	96.22	5.38	0.02
β-pinene	-47.38	1	96.77	5.94	0.01

[1] Monoterpene compounds, AUC/mgDW > 1% total AUC and present in >70% of samples, and with univariate analysis $p < 0.5$.

[2] Total tannins, µM Tannic Acid Equivilence (TAE)/g DW.

Appendix F

Estimating intake rate from bite diameter of balsam fir trees

Estimating intake rate from bite diameter of balsam fir trees

Intake rate was estimated from a diameter-length-weight regression equation, $Y=13.1374X^{2.0795}$, (Figure F.1) determined by Risenhoover (1987) for balsam fir trees on Isle Royale, Michigan, USA.

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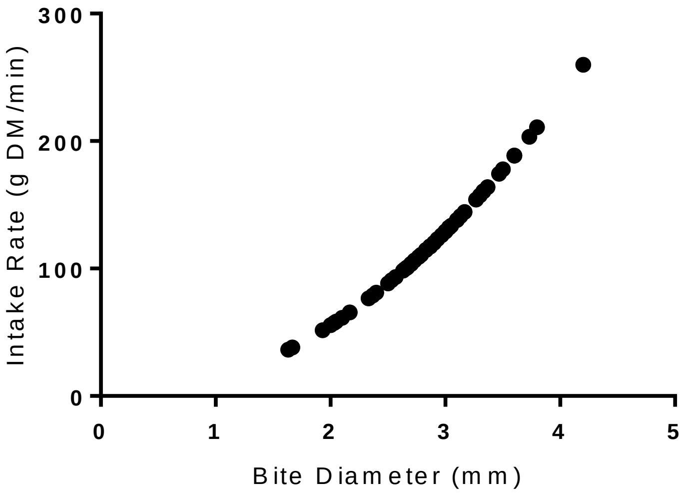

Fig F.1Intake rate as a function of bite diameter of balsam fir trees (*Abies balsamea*) browsed by moose on Isle Royale, Michigan, USA. Bite diameter measurements collected during sample years 2014 and 2015 (n=67).

Appendix G

Regional and island wide values of GA:C and UN:C for the 18-year sample set.

Regional and island wide values of GA:C and UN:C for the 18-year sample set.

Interannual variation in condition and PSM responses over time has important implications for the nutritional health and energy budgets of herbivores. To initially investigate interannual variation in these parameters, we collected urine samples, via urine soaked snow, annually during the months of February to March from 1994 to 2015. All samples were analyzed for urea nitrogen (UN) and creatinine (C) at Wolff Laboratories, Minneapolis Minnesota, USA. In general, there were more samples collected from the eastern region than from the west. Where possible, 20 randomly selected samples from each region (east/west) were tested for glucuronic acid (GA) at Boise State University (Table G.1). Samples from 1995, 1996, 1998, and 1999 were lost due to an undetected freezer failure during storage. This allowed samples from these years to warm up to room temperature for an unknown period of time, hence, they were not included in extended data set. All other years demonstrated a significantly positive relationship between GA:C and UN:C indicating that increased intake, absorption and detoxification of PSMs correlates with decreased nutritional condition, with the exception of sample year 2001 (Table G.2).

Samples collected during calendar year 2001 from the western region of Isle Royale routinely tested well below the average values for GA than all other years. GA values for samples collected from the eastern region during that year, as well as values for UN and C from both regions were all within average range of other year’s values and demonstrated the same positive relationship as all the other years in the 18-year sample set. When samples from the western region were added to the same analysis illustrating an “island-wide” analysis, that relationship was negated. Samples from the western region demonstrated similar relative UN and C concentrations as the eastern region. However, GA concentrations consistently (repeated chemical analysis of the same samples) returned results far below the average for other years. These mark the only samples in the entire extended data set where GA detection was so low as to be negligible in some samples. Due to these repeatedly demonstrated difference in GA concentration in this group of samples relative to the rest of the extended data set, sample year 2001 was deemed to be anomalous and removed from further consideration. Samples collected from east and west regions were stored separately after UN and C analysis and prior to GA analysis. It is possible that western samples became contaminated at some point during the interim.

The three-year sample set (2013 – 2015, Fig 2.1) was further analyzed with respect to region to determine if the relationship between GA:C and UN:C held constant for each region. East and West regions both demonstrated a positive correlation between GA:C and UN:C (Fig G.1).

Table G.1 **Mean ($\bar{x}$) and SEM of GA:C (mg/dL) and UN:C (mg/dL) values for each of 17 years of collected samples, n = 40 for each year. Values reflect the average values specific to east and west regions as well as across the entire island. Sequential gaps with respect to year between 1994 and 2000 are the result of freezer failure in which samples were lost. Sample year 2001 (bold) was removed from further analysis due to suspected alteration of samples from the west region which was an outlier relative to all other years.**

	GA:C west		UN:C west		GA:C East		UN:C East		GA:C Island		UN:C Island	
Year	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM
1994	27.125	1.878	1.954	0.221	40.895	2.120	2.109	0.172	34.010	1.780	2.031	0.139
1997	29.044	2.067	2.135	0.144	50.318	2.694	2.970	0.152	40.241	2.438	2.553	0.124
2000	33.733	2.890	2.145	0.128	42.432	2.239	2.871	0.141	38.083	1.934	2.508	0.111
2001	**20.088**	**2.630**	**2.630**	**0.168**	**36.571**	**1.361**	**2.710**	**0.143**	**28.330**	**1.969**	**2.670**	**0.109**
2002	28.246	1.293	1.712	0.118	39.181	2.684	2.127	0.198	33.713	1.701	1.919	0.118
2003	31.546	2.478	2.218	0.111	41.364	1.938	2.805	0.196	37.191	1.701	2.555	0.129
2004	30.227	1.306	2.426	0.195	57.020	5.568	3.320	0.207	43.624	3.545	2.873	0.157
2005	31.878	1.430	2.060	0.154	35.475	1.766	2.126	0.214	33.227	1.133	2.085	0.124
2006	45.568	5.409	2.928	0.327	40.218	2.937	2.890	0.268	42.893	3.025	2.909	0.208
2007	28.995	1.582	2.025	0.123	51.489	13.903	3.131	0.740	37.350	5.458	2.436	0.293
2008	34.276	1.855	2.216	0.156	46.279	2.223	2.835	0.102	38.169	1.708	2.416	0.119
2009	37.112	2.648	2.652	0.220	68.967	4.086	3.680	0.192	53.040	3.504	3.166	0.166
2010	37.457	2.101	1.968	0.163	33.748	2.197	1.872	0.155	35.602	1.532	1.920	1.532
2011	33.796	1.739	2.368	0.208	47.014	1.985	3.084	0.142	40.405	1.678	2.726	0.137
2012	37.530	2.458	2.194	0.354	41.889	2.209	2.408	0.173	39.709	1.668	2.301	0.196
2013	45.662	3.143	2.254	0.193	47.210	2.615	2.801	0.147	46.436	2.001	2.528	0.123
2014	40.216	2.529	3.446	0.314	46.060	2.121	4.349	0.200	44.599	1.744	4.123	0.179
2015	40.528	2.622	2.519	0.183	43.906	2.905	2.560	0.260	41.779	1.962	2.535	0.147

Table G.2 Regression statistics for GA:C (predictor) and UN:C (response) isolated from moose urine at Isle Royale National Park, Michigan USA for each year in the extend sample set. Sample year 2001(bold) was deemed anomalous and was removed from further analysis. All other years demonstrated a significantly positive relationship between GA:C and UN:C.

Year	n	F-stat	p	R^2_{Adj}	95% CI	eq
1994	39	6.55	0.015	0.127	0.006 - 0.049	y = 0.0274x + 1.0145
1997	38	16.64	< 0.001	0.297	0.014 - 0.043	y = 0.0287x + 1.4197
2000	40	18.67	< 0.001	0.312	0.017 - 0.048	y = 0.0328x + 1.2595
2001	**39**	**0.64**	**0.427**	**-0.009**	**-0.025 - 0.011**	**y = -0.0071x + 2.87**
2002	42	69.23	< 0.001	0.625	0.042 - 0.069	y = 0.0553x + 0.0545
2003	40	8.06	0.007	0.153	1.576 - 9.418	y = 0.0318x + 1.3719
2004	40	5.11	0.03	0.095	0.002 - 0.029	y = 0.0153x + 2.2064
2005	40	4.47	0.04	0.082	0.002 - 0.069	y = 0.0354x + 0.908
2006	38	14.28	< 0.001	0.264	0.017 - 0.056	y = 0.0361x + 1.3642
2007	35	215.17	< 0.001	0.863	0.043 - 0.057	y = 0.05x + 0.567
2008	37	15.01	< 0.001	0.28	0.018 - 0.058	y = 0.0383x + 0.9539
2009	40	20.16	< 0.001	0.329	0.021 - 0.056	y = 0.0279x + 1.689
2010	39	16.48	< 0.001	0.289	0.020 - 0.060	y = 0.0402x + 0.489
2011	40	14.86	< 0.001	0.262	0.021 - 0.066	y = 0.0432x + 0.9806
2012	40	52.76	< 0.001	0.57	0.090 - 0.159	y = 0.0894x + 1.248
2013	41	4.56	0.04	0.082	0.001 - 0.039	y = 0.0199x + 1.6183
2014	40	16.12	< 0.001	0.279	0.028 - 0.084	y = 0.0712x + 0.913
2015	44	20.17	< 0.001	0.308	0.029 - 0.075	y = 0.0518x + 0.286

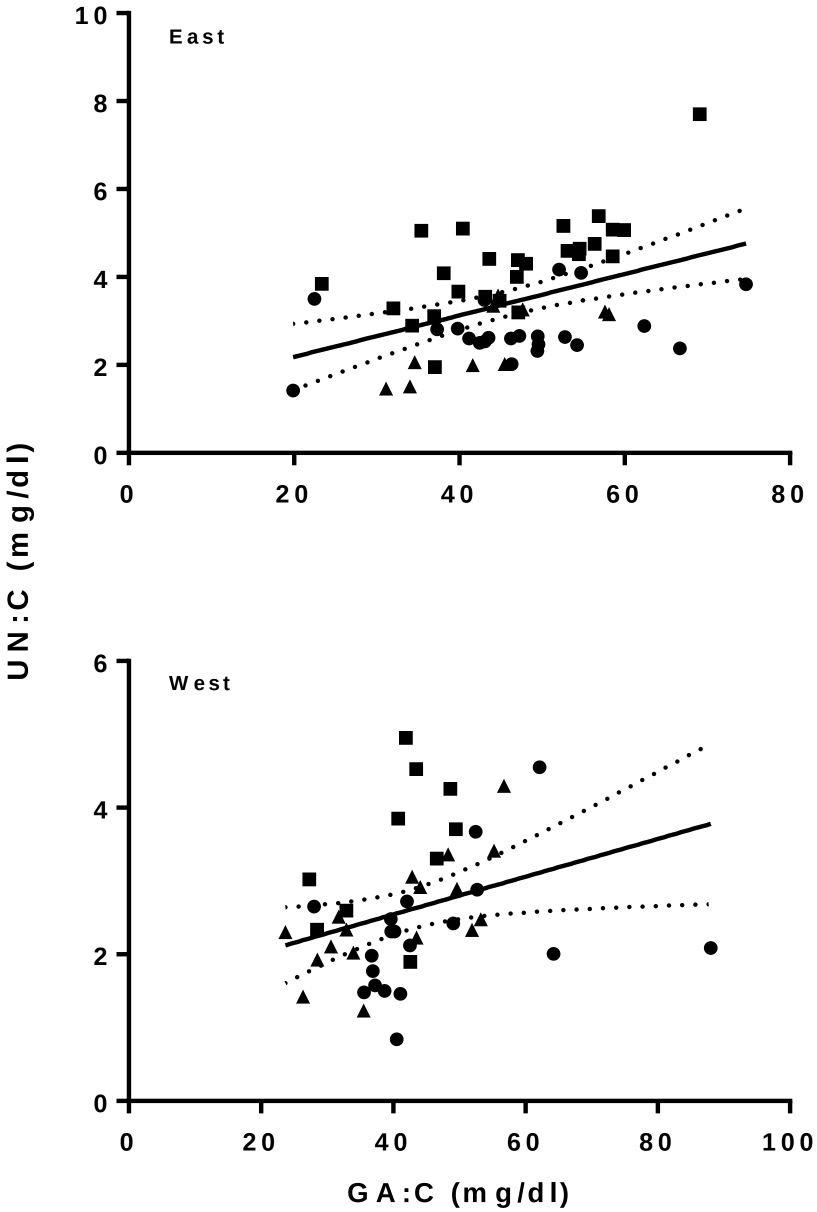

Fig G.1 **Linear regression of GA:C and UN:C for East (Y = 0.047x + 1.244) and West (Y = 0.026x + 1.515) regions of Isle Royale for the three-year sample set; 2013 (circles), 2014 (square), and 2015 (triangle). Solid line represents the least squares regression with dashed lines representing 95% confidence intervals. Both regions demonstrated a significantly positive relationship between GA:C and UN:C (east: $F_{1,56} = 12.78$, $p = 0.0007$, $R^2 = 0.19$; west: $F_{1,44} = 4.938$, $p = 0.031$, $R^2 = 0.10$).**

Appendix H

Influence of individual and normalized PSMs on GA:C and UN:C

Influence of individual and normalized PSMs on GA:C and UN:C

AICc model selection showed that the biomarker for toxin exposure, (glucuronic acid:creatine, GA:C), was the best predictor of nutritional condition (urea nitrogen: creatinine, UN:C) with proportional intake rate (IR) of monoterpenes being the second best predictor of nutritional condition (Chapter 2, Table 2.1). While not a competing model (> ΔAICc from top model), proportional IR of monoterpenes still resulted in a strong significantly positive relationship with UN:C. However, monoterpenes are not the only class of PSM produced by plants. Non-tannin phenolic compounds are another class produced by most plants, many of which are attributed to dietary health benefits, primarily through antioxidant activity (see chapter 1). In contrast, some phenolic compounds can form harmful intermediary products (Sakihama et al. 2002) which may have cytotoxic effects (Lapidot et al. 2002). As a result, these potentially harmful intermediary compounds may have additional influence on detoxification requirements and nutritional condition.

To assess the potential additive effects of phenolics on detoxification and nutritional condition, we normalized both total monoterpene and total phenolics data to fit a scale between 0 and 1 using the following equation.

$$z_i = \frac{x_i - \min(x)}{\max(x) - \min(x)}$$

These two normalized values were added together for a total normalized PSM value for each plant sample. AICc analysis (Chapter 2, Table 2.1) showed that proportional IR, which includes behavioral responses to PSMs, was a better predictor of UN:C for both monoterpene and phenolics than when we considered only individually. As a result, we focused solely on proportional IR when considering effects of three PSM classes (i.e. monoterpenes, phenolics, and total normalized PSM) on GA:C and UN:C and did not include total PSMs in browsed fir, proportion of fir in diet, or IR (from bite diameter) alone.

Proportional IR of all three categories of PSMs found in browsed fir trees; monoterpenes ($Y=0.018x + 2.56$; $F_{1,30} = 15.41$, $p = 0.0005$, $R^2 = 0.34$, 95% CI = 0.008 – 0.027), phenolics ($Y=0.0005x + 3.35$; $F_{1,30} = 5.22$, $p = 0.03$, $R^2 = 0.15$, 95% CI = 0.00005 – 0.0009) , and normalized total PSM ($Y=2.29x + 3.1$; $F_{1,30} = 5.41$, $p = 0.03$, $R^2 = 0.15$, 95% CI = 0.278 – 4.293) showed significantly positive relationships with UN:C (Fig H.1). Of the three, phenolics was the least significant, possibly due to the many health benefits known to be associated with non-tannin phenolics (Sakakibara et al. 2003; Mennen et al. 2005; Ainsworth and Gillespie 2007). Of the three variables tested, none demonstrated any relationship with GA:C levels; monoterpenes ($Y=0.077x + 35.34$; $F_{1,30} = 2.598$, $p = 0.117$, $R^2 = 0.08$, 95% CI = -0.021 – 0.174), total phenolics ($Y=0.001x + 43.4$; $F_{1,30} = 0.22$, $p = 0.64$, $R^2 = 0.007$, 95% CI = -0.003 – 0.005), normalized total PSM ($Y=14.8x + 38.71$; $F_{1,30} = 2.6$, $p = 0.118$, $R^2 = 0.08$, 95% CI = -3.96 – 33.55; Fig H.2). This trend is likely due to the many other ecological factors, beyond diet quality, that influence glucuronidation (see chapter 2).

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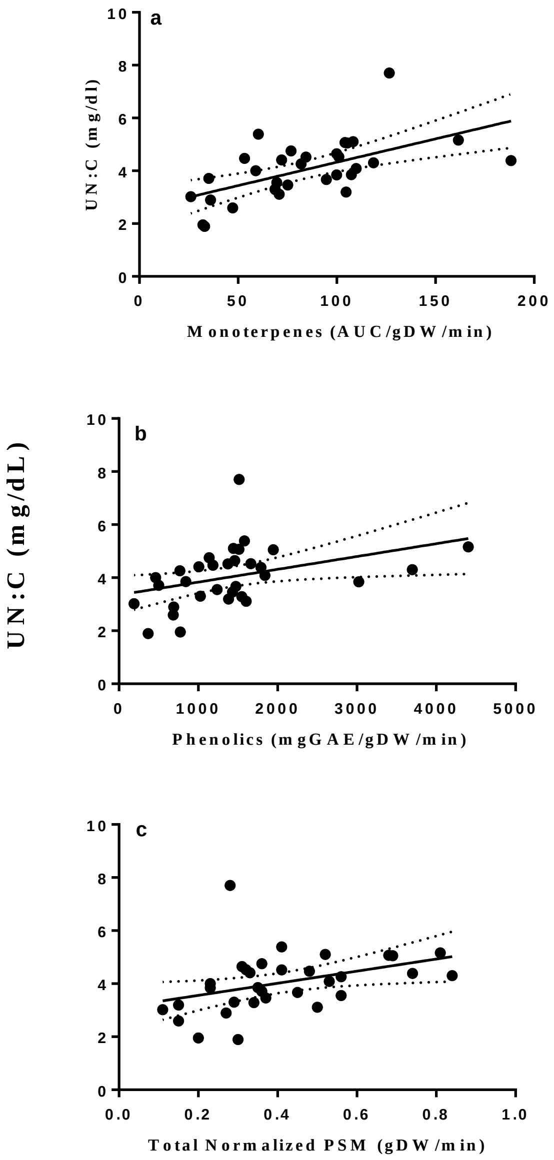

Fig H.1 Relationship between proportional IR of PSMs in balsam fir trees browsed by moose at Isle Royale National Park, Michigan USA during late winter, 2014 and urinary UN:C. a) Proportional IR of total monoterpenes (Y=0.018x+2.56; $F_{1,30}$ = 15.41, p = 0.0005, R^2 = 0.34, 95% CI = 0.008 – 0.027). b) Proportional IR of total phenolics (Y=0.0005x+3.35; $F_{1,30}$ = 5.22, p = 0.03, R^2 = 0.15, 95% CI = 0.00005 – 0.0009). c) Proportional IR of normalized total PSM (Y=2.29x+3.1; $F_{1,30}$ = 5.41, p = 0.03, R^2 = 0.15, 95% CI = 0.278 – 4.293). Solid lines represent least squares regression while dotted lines represent 95% confidence intervals.

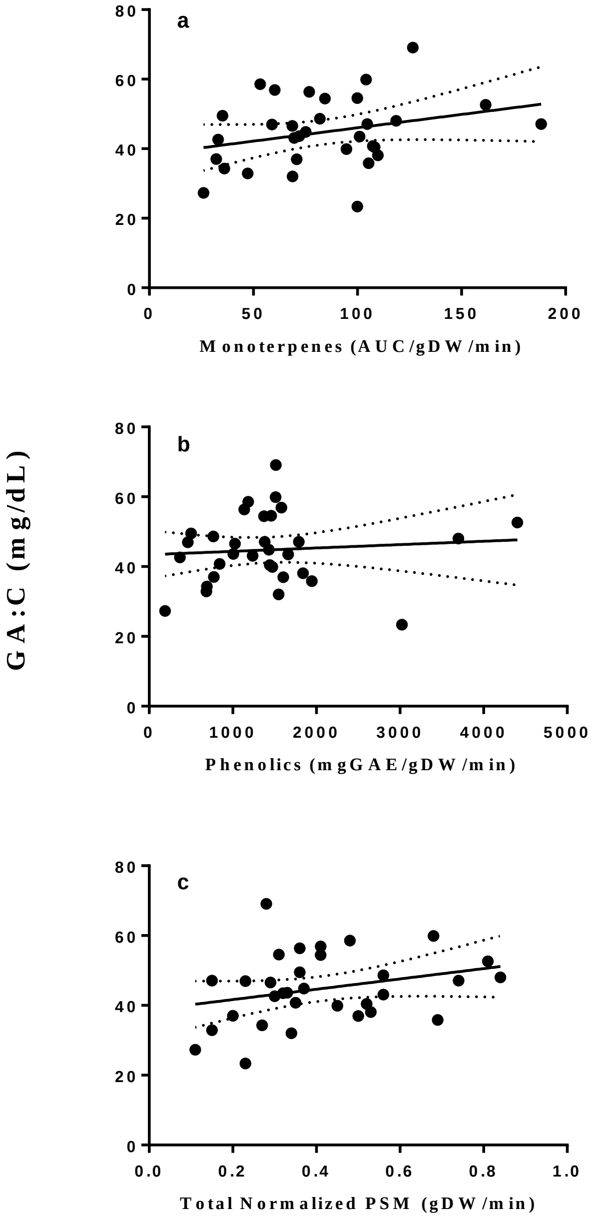

Fig H.2 Relationship between the proportional IR of PSMs from balsam fir trees browsed by moose at Isle Royale National Park, Michigan USA during late winter, 2014 and urinary GA:C. a) Proportional IR of total monoterpenes ($Y=0.077x+35.34$; $F_{1,30} = 2.598$, $p = 0.117$, $R^2 = 0.08$, 95% CI = -0.021 – 0.174). b) Proportional IR of total phenolics ($Y=0.001x+43.4$; $F_{1,30} = 0.22$, $p = 0.64$, $R^2 = 0.007$, 95% CI = -0.003 – 0.005). c) Proportional IR of normalized total PSM ($Y=14.8x+38.71$; $F_{1,30} = 2.6$, $p = 0.118$, $R^2 = 0.08$, 95% CI = -3.96 – 33.55). Solid lines represent least squares regression while dotted lines represent 95% confidence intervals.

Appendix I

Influence of Dietary Protein on Urinary Urea Nitrogen.

Influence of Dietary Protein on Urinary Urea Nitrogen.

Urea Nitrogen (UN) is the end product of protein catabolism in the mammalian body, it comprises the largest pool of free nitrogen in the body and its production is directly related to changes in catabolism of both dietary and endogenous proteins (Weiner et al. 2014). When utilizing UN:C ratios to determine nutritional condition of overwintering populations of wild herbivores, it is important to consider the effects of dietary protein as well as catabolized endogenous protein. To examined the effects of crude protein on nutritional condition we calculated the percent crude protein in browsed plants and regressed the proportional intake rate of crude protein with UN:C values. We predicted that proportional IR of crude protein would demonstrate a positive relationship with UN:C values but would not show any relationship with GA:C. We found that proportional IR of crude protein did have a significantly positive relationship with UN:C values (Y=0.19x + 2.92; $F_{1,31} = 7.761$, $p = 0.009$, $R^2 = 0.20$, 95% CI = 0.051 – 0.331; Fig I.1), and did not affect GA:C values (Y=0.75x + 39.6; $F_{1,31} = 1.084$, $p = 0.306$, $R^2 = 0.034$, 95% CI = -0.722 – 2.23) as predicted.

These results seem counterintuitive when viewed in the context that increased UN:C equates to decreased nutritional condition. However, it is important to consider that urinary UN levels are highly correlated with blood serum UN levels (Kohn et al. 2005), which are in turn governed by the catabolism of both dietary and endogenous proteins (DelGiudice 1995; Parker et al. 2005; Weiner et al. 2014). When considered in this context, increases in UN:C ratios attributed to increased protein intake can be seen as a healthy increase for overwintering animals. Conversely, increases in UN:C associated with increased consumption of PSMs can be seen as an increase in endogenous protein catabolism and hence, a reduction in nutritional condition. Ungulates voluntarily reduce intake during winter months yet still require a daily protein intake greater than 6.8% ± 0.8% for maintenance requirements (Timmermann and McNicol 1988; Sauvé and Côté 2007). Although balsam fir is the most nutritious forage option available for overwintering moose on Isle Royale (Parikh et al. 2016) its protein content (9.2% ± 0.11) is still barely sufficient to meet daily needs of browsing moose when considering potential voluntary reduction of intake associated with behavioral avoidance of PSMs. Protein assimilation may be further compromised by the presence of co-occurring PSMs which can inhibit digestive enzymes (Kohl et al. 2015), decrease protein digestibility (Mehansho et al. 1987; Foley and Moore 2005) and effect whole body protein turnover (Au et al. 2013). Increased protein consumption may help to mitigate endogenous effects of PSM metabolism. However, at present, we do not possess any method for differentiating UN produced from dietary protein from that produced from endogenous proteins.

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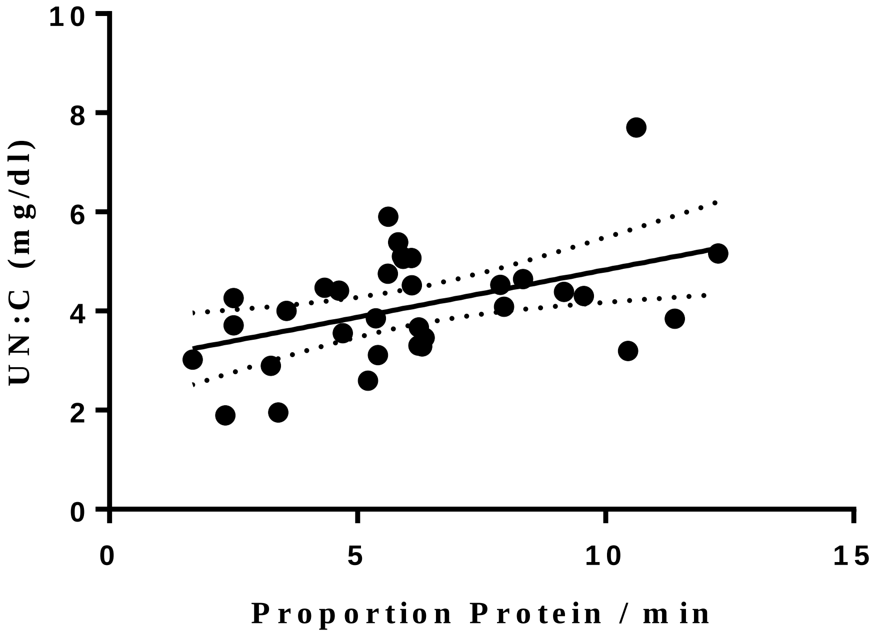

Fig I.1 Relationship between proportional intake of crude protein of balsam fir consumed by moose and UN:C values present in moose urine (Y=0.19x + 2.92; $F_{1,31}$ = 7.761, p = 0.009, R^2 = 0.20, 95% CI = 0.051 – 0.331). Solid lines represent the least squares regression while dashed lines represent the 95% confidence intervals.

Appendix J

Other Notable Observations

Other Notable Observations

Relative Absorption of Consumed Monoterpenes

Our results showed no correlation between ingested PSMs and GA:C output. One possible explanation is that not all ingested PSMs are absorbed into the blood stream. Absorbed PSMs relative to those consumed may be a more indicative metric of the effect of PSMs on energetic budgets and detoxification systems then PSM concentrations in browsed plants alone. Monoterpene profiles for balsam fir trees on Isle Royale have been characterized (Chapter 1, Fig 1.4) and their effects on the nutritional condition of moose have been shown (Chapter 2, Fig 2.2). To further understand the effects of monoterpenes on moose energetic budgets, and by extension, nutritional condition, it would be useful to know what proportion of ingested monoterpenes were absorbed and detoxified through the hepatic system. To test this, we collected moose pellets at each identified browsing patch and placed 0.5g of fresh fecal material in a 20mL headspace vial. Relative concentrations of monoterpenes excreted unchanged were measured using an Agilent 6890N gas chromatograph (GC), following specifications detailed in chapter 2. Results show that all major identified compounds present in the balsam fir samples were also present in the fecal pellets with the exception of one, borneol, which showed negligible concentrations in fecal pellets relative to plant concentrations (Fig J.1). Monoterpenes are lipophilic organic compounds and as such should be readily absorbed in the GI tract (Sorensen et al. 2006). However, some data suggests that monoterpenes are not easily absorbed through the intestinal tract, and those that are can take up to five days to be detoxified and cleared from the body (Köppel et al. 1981). Therefore, it is not useful for us to use these data as a metric for how much of the ingested monoterpenes were absorbed and subsequently detoxified.

Biomarkers of Physiological Processes

Urine Urea Nitrogen as an Index of Nutritional Condition

Historically, fecal nitrogen (FN) has been the primary means by which researchers established the nutritional condition of free-ranging herbivores. Fecal droppings from wild populations are readily available, provide a convenient and noninvasive sampling technique, and nitrogen content of droppings remain relatively constant for 2-3 weeks post-defecation regardless of environmental conditions (Jenks et al. 1990; Leslie et al. 2008). However, the use of FN as an indicator of nutritional condition has faced considerable criticism regarding its efficacy in studies involving wild herbivores. Hobbs (1987) and Leslie and Starkey (1987) both reported that ingestion of PSMs can elevate FN concentrations regardless of dietary nitrogen consumed. Protein binding activity of simple and complex tannins provides the simplest explanation of this elevated FN when herbivores consume plants. In the absence of tannin binding salivary proteins, tannins can bind insolubly with ingested plant proteins or digestive proteins, reducing overall protein assimilation and increasing nitrogen passage in the feces (Juntheikki 1996; Osborn and Ginnett 2001; Shimada 2006). Loss of dietary protein is greatly decreased in the presence of tannin binding salivary proteins (McArthur 1991). Even in the presence of salivary proteins, dietary protein can still be lost through tannin complexation if tannin concentrations are sufficiently high. In a controlled study, Osborn and Ginnett (2001) reported that tannin complexation significantly increased FN in white-tailed deer and Mould and Robbins (1981) demonstrated that that when plants containing high levels of secondary metabolites represent >33% of the diet of a captive elk, protein digestibility was decreased and FN increased. Leslie et al (2008) argue that with exception of certain extremes, herbivores rarely consume a diet made up of >33% of a single plant species high in PSMs. While this may be supported by diet mixing under summer forage condition, many herbivores are subjected to extreme dietary restriction during winter, often limited to a few forage options high in PSMs. Thus, the use of fecal nitrogen to determine nutritional condition of overwintering animals may not be useful and an alternative approach is needed. Quantification of UN:C from urine recovered from winter snow provides an alternative measure of nutritional condition without the effects of tannin complex inflation demonstrated in FN studies.

The use of UN:C ratios obtained from snow collected urine as an index for the nutritional condition has faced some criticism (Parker 2003). Säkkinen et al. (2001) argued that UN:C ratios have complex dynamics that are sensitive to both seasonal and dietary changes, and thus, are not a useful indicator of nutritional condition. White et al. (1995) argued that UN:C ratios are subject to variation among sex and age and are only useful when the sex and age of the animals are known and analysis is considered within that context. They further argued that even within the proper age-sex context, UN:C values are non-indicative of condition until >20% of fall fat reserves have been depleted. An additional limitation of UN:C utility for determining nutritional condition in wild herbivores is that samples collected from highly gregarious species could contain urine from multiple animals. Thus, the use of snow collected UN:C requires special conditions rarely present in natural systems to maintain any utility.

While we acknowledge the proposed limitations, the use of UN:C as an index of nutritional restriction has been validated in natural systems (Larter and Nagy 2000; Delgiudice et al. 2001). In our study, many of these issues are limited. First, winter diet of moose on Isle Royale has been clearly characterized and remains largely consistent. Age-sex variation was not considered in our study and could not only influence UN:C, but also GA:C. Green and Fisher (1981) showed that both glucuronidation efficiency and relative use of different detoxification pathways vary by both sex and age in rats. Sex and age variation in the expression UGT enzymes responsible for glucuronidation have also been demonstrated in pigs (Kojima and Degawa 2014) and humans (Liston et al. 2001) Despite potential variation in UN:C and GA:C relative to age and sex we still found robust relationships linking the potential consequences of PSMs (GA:C) to nutritional condition. However additional analysis of samples could help tease apart variation in this relationship attributed to age and sex. Second, samples were collected in late winter when fat reserves are expected to be critically depleted or nearing critical levels. Finally, island moose are non-gregarious and samples were only collected if they came from single animals, as evident by tracks in the snow.

Correlation of Glucuronic Acid and Urea Nitrogen with Creatinine

Creatinine (C) is the end result in creatine metabolism in skeletal muscles (Doi et al. 2009). Creatine is endogenously produced in the liver or gained through dietary intake and is stored in the skeletal muscles as phosphocreatine. Energy in the form of ATP for muscular contraction is obtained through anaerobic degradation of stored phosphocreatine resulting in the reduced form, creatinine, which is excreted from the body exclusively in urine (Casey and Greenhaff 2000) at a relatively constant daily rate

(Moen and DeIgiudice 1997). Because its production is governed by muscular activity, creatinine concentrations in urine are dependent upon muscle mass and relative physical activity (Brisco et al. 2013). Thus, concentrations of creatinine provide a good indication of the lean muscle mass of the animal in question and is strongly correlated with body weight (Baxmann et al. 2008). We found that both GA and UN were significantly positive correlated with C in snow recovered urine (Fig J.2). These results indicate that larger animals possess greater detoxification capacity (or greater intake of PSMs) and greater rates of urea production under conditions of nutritional restriction than do smaller conspecifics. During winter months, when dietary options are limited, smaller animals have less endogenous energy reserves, higher mass specific metabolic requirements, and may have difficulty locating sufficiently nutritious forage. These results may provide important insights to population ecology by identifying populations of greatest concern regarding winter nutritional maintenance. Additionally, these results may provide management insights for range restricted and/or economically important populations and may help inform supplemental winter feeding programs.

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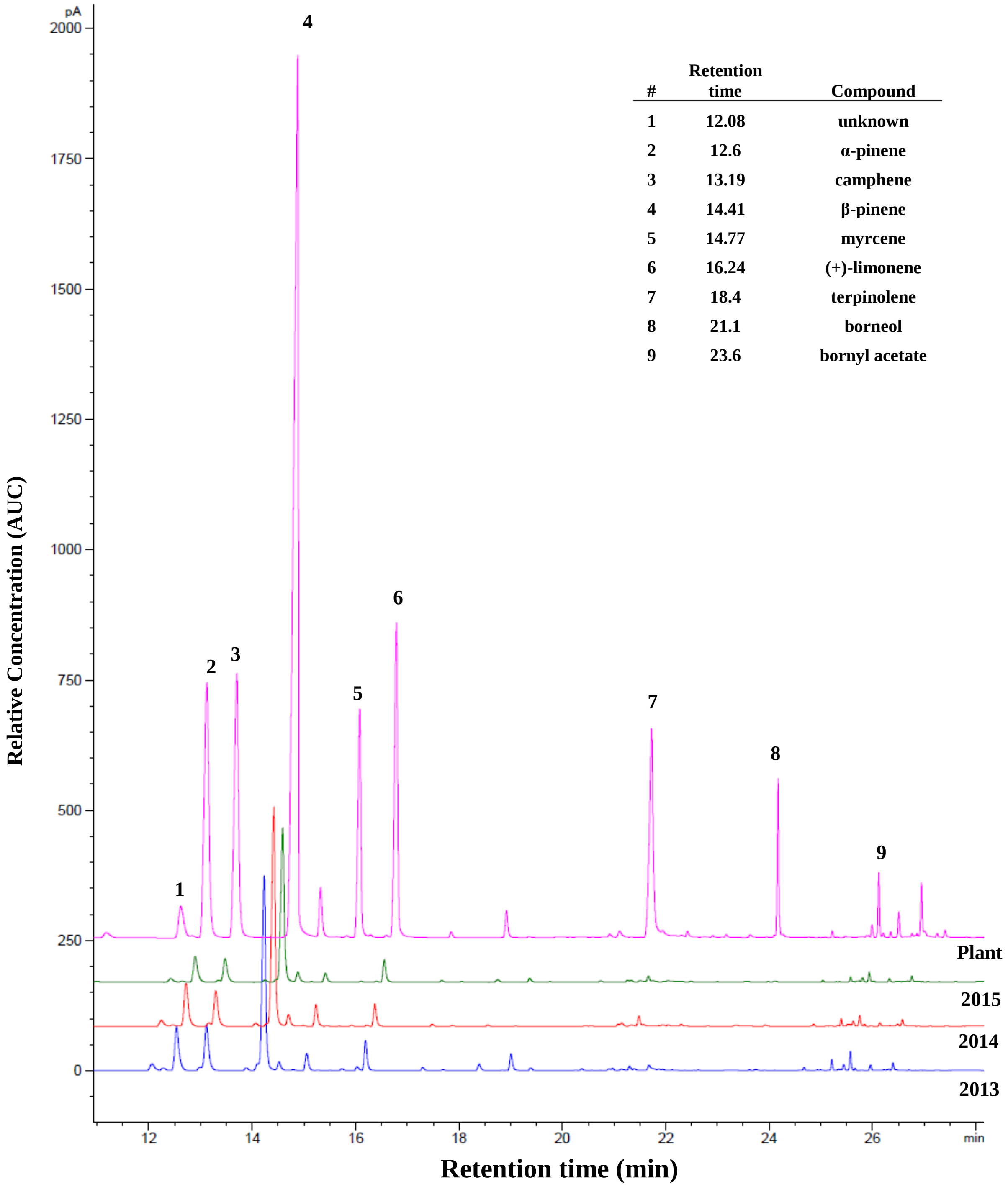

#	Retention time	Compound
1	12.08	unknown
2	12.6	α-pinene
3	13.19	camphene
4	14.41	β-pinene
5	14.77	myrcene
6	16.24	(+)-limonene
7	18.4	terpinolene
8	21.1	borneol
9	23.6	bornyl acetate

Fig J.1 Representative Monoterpene profile of balsam fir shown with monoterpene profiles of moose fecal pellets collected during 2013, 2014, and 2015 (each offset) winter field seasons at Isle Royale National Park, Michigan, USA. Peaks show individual compounds, with the height of the peak (AUC) indicating relative abundance of each identified compound.

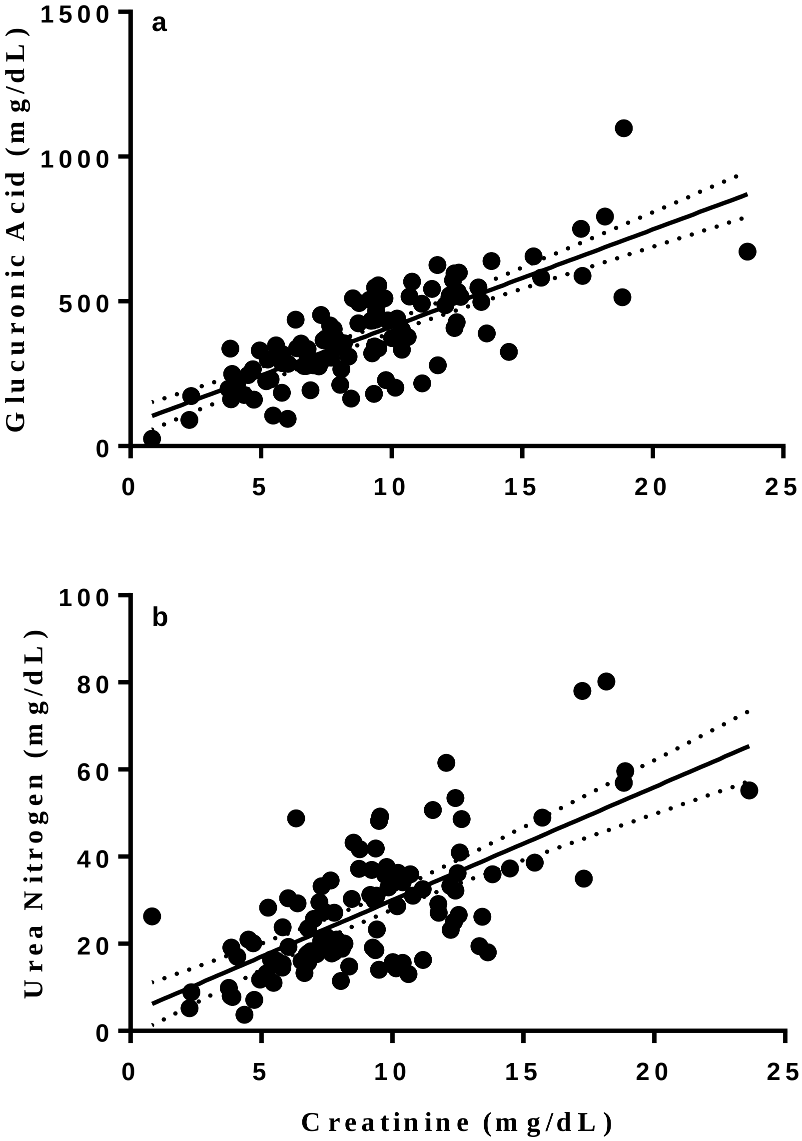

Fig J.2 **Relationship between (a) creatinine and glucuronic acid (Y = 33.58X + 76.63; $F_{1,102}$ = 165.5, $p < 0.001$, R2 = 0.619, 95% CI = 28.4 – 38.76) and (b) creatinine and urea nitrogen (Y = 2.595X + 4.053; $F_{1,102}$ = 92.23, $p < 0.001$, R^2 = 0.475, 95% CI = 2.059 – 3.131). Both relationships were significantly positively correlated with higher concentrations C correlating to higher concentrations of both GA and UN. Solid line represents least squares regression, dotted line represents 95% confidence intervals.**