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RESEARCH ARTICLE

Macro-nutritional balancing in a circumpolar boreal ruminant under winter conditions

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Abstract

1. Differences in botanical diet compositions among a large number of moose faecal samples collected during winter correlated with the nutritional differences identified in the same samples (Mantel-$r = 0.89$, $p = 0.001$), but the nutritional differences were significantly smaller ($p < 0.001$).
2. Nutritional geometry revealed that moose mixed Scots pine *Pinus sylvestris* and *Vaccinium* spp. as nutritionally complementary foods to reach a nutritional target resembling *Salix* spp. twigs, and selected for *Salix* spp. browse (Jacob's $D > 0$).
3. Available protein (AP) and total non-structural carbohydrates (TNC) were significantly correlated in observed diets but not in hypothetical diets based on food availability.
4. The level of Acetoacetate in moose serum (i.e. 'starvation') was weakly negatively associated with digestibility of diets ($p = 0.08$) and unrelated to increasing AP:TNC and AP:NDF ratios in diets ($p > 0.1$).
5. Our study is the first to demonstrate complementary feeding in free-ranging moose to attain a nutritional target that has previously been suggested in a feeding trial with captive moose. Our results add support to the hypothesis of nutritional balancing as a driver in the nutritional strategy of moose with implications for both the management of moose and food resources.

KEYWORDS
Alces alces, herbivory, nutritional ecology, nutritional geometry, ungulate

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1 | INTRODUCTION

Eating per se is easy. All animals do it, and its principal functions (e.g. the provision of energy and nutrients for body maintenance, thermoregulation, growth and reproduction) are well established. However, understanding specific food choices and their drivers has remained surprisingly elusive. The quality and quantity of what an animal consumes and when and where it eats directly affects its condition (Couturier et al., 2009; Felton et al., 2020) and survival (Verdolin, 2006), which, in turn, has implications for fitness (Ripple et al., 2001) and population dynamics (White, 1983).

The search for food is one of the strongest drivers of animal movement, from small-scale foraging bouts (Shaw, 2020) to large-scale, seasonal migrations (Merkle et al., 2016; van Moorter et al., 2021). While herbivores enjoy the advantage of their food being immobile, this does not necessarily imply that foraging is an easy task. Not only do herbivores have to choose from a plethora of different plant species, including potentially toxic ones, they also have to deal with such interrelated variables as the spatial distribution, biomass, morphology and bite size of prospective food plants (Parikh et al., 2017; Shipley et al., 1998; Spalinger & Hobbs, 1992; Wam & Hjeljord, 2010). Moreover, the chemical properties of different plant tissues may vary widely and interact, turning the apparent nutritional value of a given food type into a non-fixed parameter (Felton et al., 2016; Simpson & Raubenheimer, 2012). Herbivores also need to cope with non-dietary risks such as predation and weather (Hebblewhite & Merrill, 2011; Hoy et al., 2019), and most species are faced with the challenge of large seasonal differences in the properties and availability of their forage. Additionally, different herbivore species are specialized in different dietary 'niches' (e.g. European Cervidae, Spitzer et al. (2020)), but still have to fulfil their dietary and energetic needs.

Against the backdrop of such complexity, several theories have emerged to explain general patterns in the nutritional strategies of herbivores, including energy maximization (Belovsky, 1978; Schoener, 1971), protein maximization (Mattson, 1980), limitation of dietary fibre (Van Soest, 1994), regulation or limitation of minerals (Robbins, 1994), and regulation or avoidance of plant secondary metabolites (Freeland & Janzen, 1974). A recent review of food selection by northern cervids (Felton et al., 2018) confirmed these food constituents as important drivers of food selection, but, crucially, found little support for the traditional hypotheses of maximization or limitation of any single constituent.

Instead, it is becoming increasingly clear that foraging should be viewed as a dynamic process, which involves balancing multiple nutrients simultaneously (Simpson & Raubenheimer, 2012). Over the past decade, a growing body of evidence in support of this nutrient balancing hypothesis has emerged, spanning dozens of taxa from slime moulds (Dussutour et al., 2010) to primates (Felton et al., 2009; Rothman et al., 2011), and even humans (Raubenheimer & Simpson, 2016). The evidence suggests that animals, instead of having to individually learn the nutritional properties of potentially hundreds of food items and their intrinsic variabilities, have evolved nutrient-specific appetites that drive their foraging decisions towards attaining a target balance of a few key macronutrients (typically protein, carbohydrates and lipids). These appetites and their interactions are calibrated by natural selection to such an extent that the specific target is determined by the evolutionary, ecological and life-history circumstances of the species (Raubenheimer et al., 2022; Simpson & Raubenheimer, 2012, 2016).

Nutrients, however, generally do not occur as discrete units that an animal can freely choose from. Instead, 'food packages' arrive with every bite, representing varying combinations of nutrients and secondary metabolites (Felton et al., 2021). To achieve a desired target nutrient balance, animals need to regulate the proportions they eat of different food items, which contain nutrients in specific ratios (Felton et al., 2021; Parikh et al., 2017).

The Geometric Framework for Nutrition (GFN) is a modelling approach, which offers a means of studying the patterns and interactions between different nutrients or other diet constituents simultaneously (Simpson & Raubenheimer, 1993, 2012). The central principle of the GFN is that the nutritional requirements of an organism can be expressed graphically as either a point representing a given time period or a trajectory within an n-dimensional space, where each dimension represents a nutrient or other food constituent. One advantage of the GFN lies in that instead of focusing on the precise amount of nutrients in a specific food item, it offers a straightforward graphical way of evaluating patterns of balancing different nutrients. This makes the GFN especially suited for testing the nutrient balancing hypothesis (Simpson & Raubenheimer, 2012). The nutritional requirement (or 'intake target') can be reached if suitable foods are available. Different foods are represented as radials ('nutritional rails') in the food space, extending outwards from the origin of the graph with their slopes corresponding to the balance (i.e. the ratio) of nutrients in each food. Foods whose nutritional rails intersect with the intake target are considered nutritionally balanced as they allow an animal to move directly to its target. Nutritionally imbalanced foods do not intersect the intake target and constrain an animal to trading-off between eating too little of one nutrient against too much of another relative to the intake target, with possible costs in terms of health and fitness. Nutritionally, complementary foods are those whose nutritional rails fall on opposite sides of the intake target, thereby allowing an animal to reach its intake target by mixing the intake of such individually imbalanced foods. For a full description of the GFN, see Raubenheimer et al. (2009) and Simpson and Raubenheimer (2012).

Among the many species studied in the context of nutritional geometry, ruminants are currently greatly underrepresented (Raubenheimer et al., 2014). As far as we are aware, moose *Alces alces* is the only ruminant species to which the GFN has been applied to date. Once in a cafeteria experiment (Felton et al., 2016), and twice in studies investigating the diets of free-ranging populations in China (Ma et al., 2019) and southern Sweden (Felton et al., 2021). Contrary to most other organisms studied using the GFN, moose do not seem to leverage a protein intake target but rather keep non-protein macronutrients (NPM) constant, thereby resembling mountain gorillas in their patterns of macro-nutrient regulation (Raubenheimer et al., 2014).

To test several hypotheses related to nutritional balancing (Table 1), we used faecal DNA metabarcoding (Taberlet et al., 2018) and chemical analysis of forage plants to reconstruct the botanical and nutritional composition of moose winter diets along gradients of winter severity and plant species composition in central and northern Sweden (Figure 1). Moose are the largest member of the family Cervidae, are long-lived capital breeders, and as one of the most widespread circumboreal large mammals, a major driver of the functioning of boreal forests (Pastor et al., 1988). They are classified as strict browsers (sensu Hofmann, 1989) that have evolved a distinct gut morphology adapted to diets of tree foliage, forbs, bark and twigs, but not graminoids (Clauss et al., 2010). Moose in winter provide a good model for studying nutrient balancing in wild ruminants since their relatively confined feeding niche, in combination with the reduced diversity of available forage plant species, makes reconstructing their botanical and nutritional composition from faeces relatively straightforward and economical. Moreover, moose are currently the only non-domestic ruminant in which macro-nutritional balancing has been experimentally tested in captivity (Felton et al., 2016), which provides a basis for hypothesis testing in free-ranging moose.

The nutritional composition of plant species can vary widely across taxa but can also be quite similar. If moose aim for a particular nutritional target as is suggested by the nutrient balancing hypothesis, the most direct way to reach this target would be to forage on a food item that already contains the nutrients in the desired proportions, that is a nutritionally balanced food. Depending on the characteristics of the habitat, such foods might be abundant, rare or altogether absent. In case of the latter, the alternative is then to combine different complementary foods to reach the nutritional target (Simpson & Raubenheimer, 2012). As a result, moose in different habitats could show the same nutritional diet composition but arrive there via very different botanical diets (in an extreme example via one nutritionally balanced plant species versus multiple nutritionally complementary species). Differences in botanical diet compositions therefore do not have to correspond to differences in the nutritional composition of diets (Hypothesis 1).

In an experimental study using six captive moose, Felton et al. (2016) found strong support for the nutrient balancing hypothesis in captive moose. Given free choice and unlimited access to high-protein and high-carbohydrate pellets, moose mixed their intake during each meal instead of maximizing either protein or carbohydrates. The accumulated trajectory of these non-random intakes converged on a nutritional target that closely resembled the nutritional balance of available protein (AP) to NPM (AP:NPM) of willow twigs (*Salix* spp., hereafter simply referred to as *Salix*). Similarly, in free-choice situations after periods of artificially imbalanced feeding, moose consistently returned to this point in nutrient space. These behaviours, in particular the complementary feeding, have not yet been confirmed in free-ranging moose. Based on the experimental findings of Felton et al. (2016), we expected the observed diets of free-ranging moose to resemble the AP:NPM ratio of *Salix* browse (Hypothesis 2) through either feeding on large amounts of *Salix* directly or mixing other food items that would be nutritionally complementary for reaching the *Salix* target.

Evidence for nutritional balancing in free-ranging moose was recently reported by Ma et al. (2019) for China and by Felton et al. (2021) for a large area in southern Sweden. In the latter case, the authors analysed rumen contents and found that moose tightly regulated the ratio of AP to total non-structural carbohydrates (TNC) plus lipids across different populations and diet types. Based on these findings, we hypothesized that the nutritional composition

TABLE 1 The hypotheses and predictions tested in this study. Not all predictions could be tested at all study sites. The superscript numbers behind each prediction indicate the study sites included in the analyses (1: Montane, 2: Interior Boreal, 3: Coastal-boreal Archipelago, 4: Coastal-boreal Mainland, 5: Boreo-nemoral; see also Figure 1). AP refers to available protein, NPM to non-protein macronutrients, TNC to total non-structural carbohydrates and NDF to neutral detergent fibre.

Hypothesis	Predictions tested	Support found	Section/figure
1. Botanical differences in diet composition do not directly correspond to nutritional differences	(1a) Pairwise dissimilarities between botanical diet compositions are not correlated with the corresponding pairwise dissimilarities in macro-nutritional composition of diets$^{1\text{-}5}$	None	3.1
	(1b) Botanical dissimilarity between diets is greater than macro-nutritional dissimilarity$^{1\text{-}5}$	Strong	3.1
2. The nutritional target with respect to AP:NPM resembles *Salix* spp. twigs	(2a) The nutritional rail for *Salix* spp. intersects the average observed diet$^{1\text{–}5}$	Medium	Figure 2
	(2b) Complementary forage items facilitate reaching the *Salix*-target$^{1\text{–}5}$	Strong	Figure 2
3. Macro-nutritional balancing occurs most strongly between AP and TNC	(3a) The correlation between AP and TNC is stronger in observed diets than in average diets based on food availability^{4,5}	Strong	3.3
	(3b) The AP:TNC ratio shows less variation across diets than the ratios between AP and other macronutrients$^{1\text{–}5}$	None	Figure 3
4. The starvation signal (use of body reserves strategy) is related to diet composition	A lower starvation signal corresponds to:		
	(4a) higher digestibility diets$^{1\text{–-}3}$	Weak	3.4
	(4b) higher AP:TNC ratios and AP:NDF ratios$^{1\text{–}3}$	None	3.4

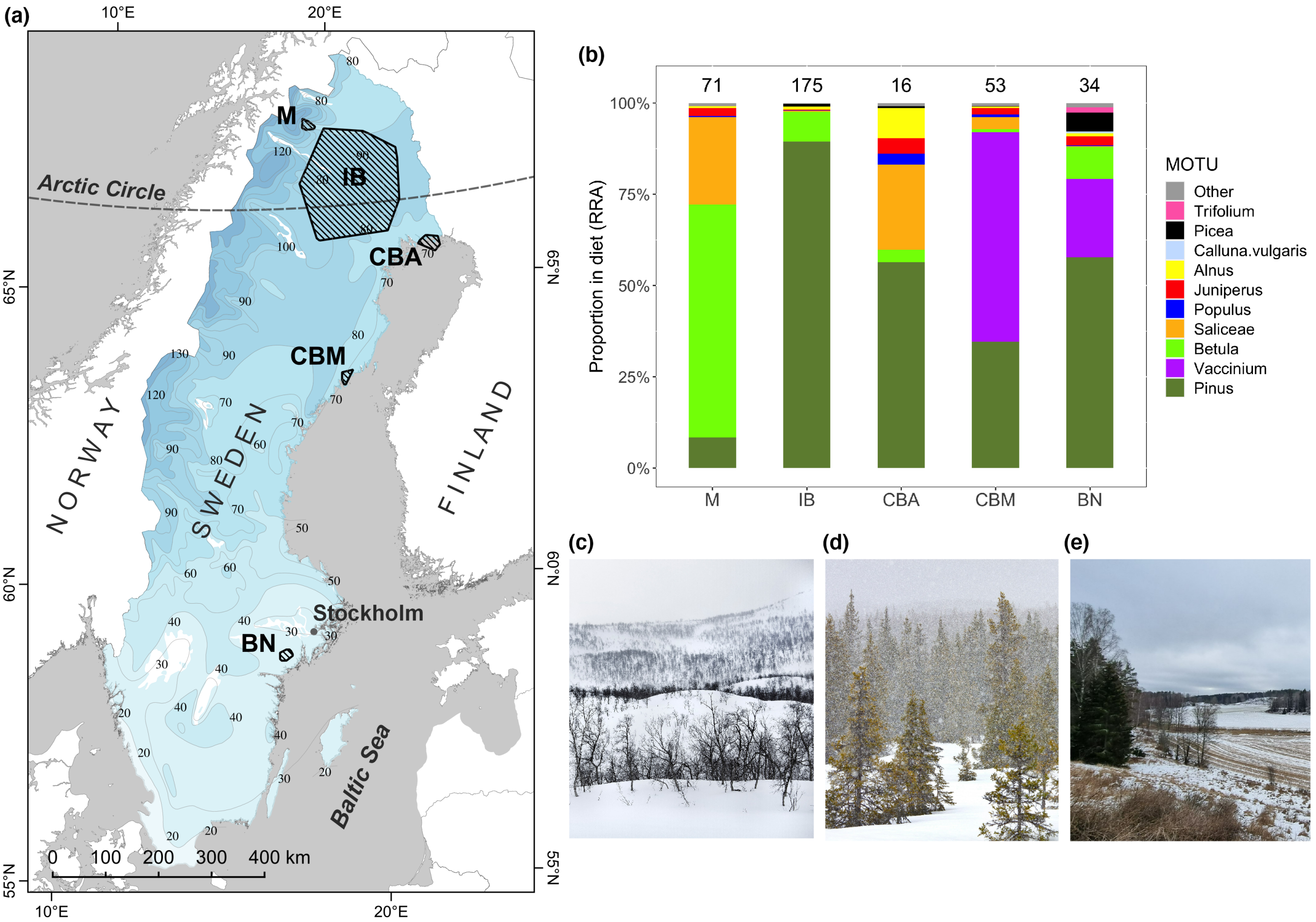

FIGURE 1 (a) Location of the five study sites, Montane (M), Interior Boreal (IB), Coastal-boreal Archipelago (CBA), Coastal-boreal Mainland (CBM) and Boreo-nemoral (BN). The blue gradient shows the average maximum snow depth in cm according to the Swedish Meteorological and Hydrological Institute (available at: www.smhi.se/data/utforskaren-oppna-data/storsta-snodjup-medel). (b) Average composition of moose winter diets based on relative read abundances (RRA) of 10 MOTUs (molecular operational taxonomic units) determined via DNA metabarcoding of faecal samples. Sample sizes are indicated above the bars. The three small pictures on the bottom right show characteristic landscapes for the different study sites: mountain birch forests at the Montane site (c), boreal forests of predominantly pine and spruce at the Interior Boreal, Coastal-boreal Archipelago and Coastal-boreal Mainland sites (d), and a mosaic of forests and fields at the Boreal-nemoral site (e).

of the moose diets we reconstructed from botanical diet compositions would show a similarly tight relationship between AP and TNC (Hypothesis 3).

Lastly, we were interested in assessing relationships between moose winter diet compositions and potential starvation responses. In highly seasonal environments with prolonged periods of food limitations, animals may adapt to such constraints by reducing metabolic expenditure in combination with foraging and starvation bouts (McCue, 2010). Well-established metabolic markers for prolonged starvation in animals are ketone bodies (Hall & Hall, 2021; Moyes & Schulte, 2008) such as Acetoacetate. Thus, we used the concentration of Acetoacetate in moose serum to investigate whether the metabolic state can be linked to diet composition (Hypothesis 4). We expected that a lower metabolic signal (less Acetoacetate) would be associated with higher digestibility diets (Berteaux et al., 1998; Tollefson et al., 2010). Furthermore, Ma et al. (2019) reported that during winter in China, moose populations with higher protein diets (i.e. higher AP:TNC ratios) showed higher population densities. This suggests that increasing ratios of protein to other macronutrients (until the nutritional target balance is reached) may be beneficial for moose and thus correspond to lower levels of starvation.

2 | MATERIALS AND METHODS

2.1 | Study sites and moose sample collection

Faecal samples from moose were collected during winter at five study sites, spanning a latitudinal gradient of approximately 1000 km (from 58.95°N, 17.12°E to 67.90°N, 18.87°E, Figure 1).

The Boreal-nemoral and Coastal-boreal Mainland study sites are characterized by a mosaic of forests, mires, and agricultural land. Common tree species include Scots pine *Pinus sylvestris*, Norway spruce *Picea abies*, birches *Betula* spp., willows *Salix* spp. and poplars

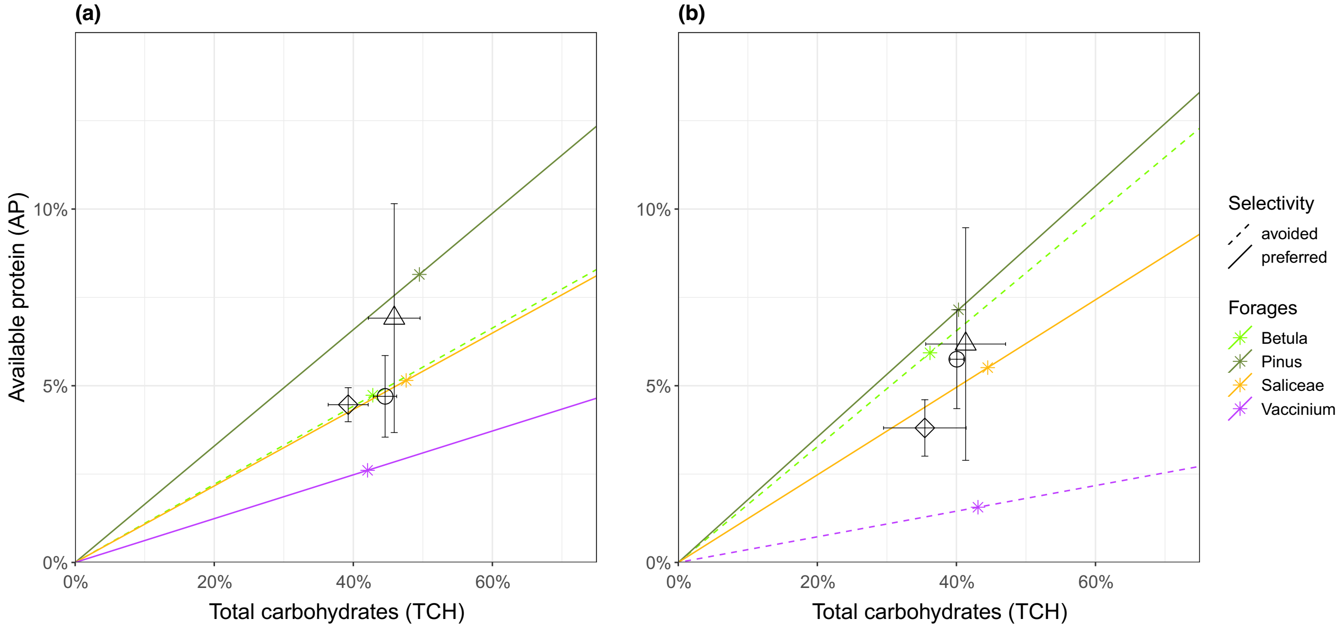

FIGURE 2 Macro-nutritional composition of moose winter diets in the dimensions of available protein (AP) and non-protein macronutrients (total carbohydrates, TCH) at the (a) Coastal-boreal Mainland and (b) Boreo-nemoral study sites. Circles correspond to the average observed diet composition, diamonds indicate the average diet composition if the available forage plants had been eaten in proportion to their occurrence, and the triangles denote the forage plants averages (i.e. if all forage species had been eaten in equal proportions). The error bars show the standard deviation. The colours correspond to the different forage plants with stars indicating the lab results for the macronutrient content and lines the resulting nutritional rails (solid lines: 'preferred' forages (Jacob's $D > 0$); dashed lines: 'avoided' forages (Jacob's $D < 0$)). For better visibility, only four nutritional rails are shown; for the full results see Figure S3.

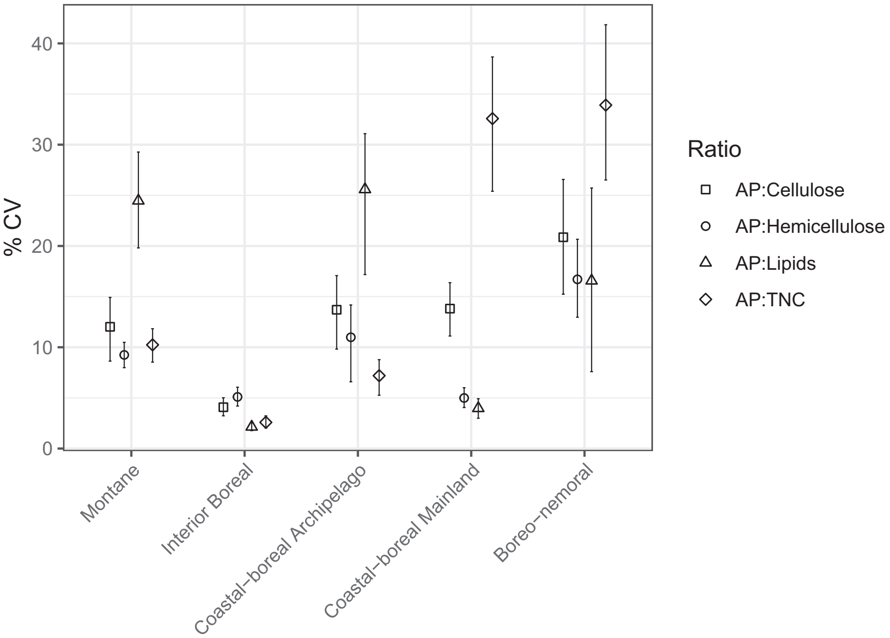

FIGURE 3 The coefficient of variation in percent (%CV) for four ratios (indicated by different point shapes) between available protein (AP) and other macronutrients at the five study sites. TNC stands for total non-structural carbohydrates. Shown are the bootstrap means of 1000 resampling iterations (points) with the error bars indicating the 95% bootstrap confidence interval.

Populus spp. The forest field layer is dominated by ericaceous shrubs (primarily from the genera *Vaccinium*, *Calluna* and *Empetrum*), mosses and lichens. Agriculture is more dominant at the southernmost Boreal-nemoral study site and comprises small- to medium-scale pastoral and arable farms. Main crops are pasture grasses and forbs, followed by cereals. In each of the two sites, we used sampling grids of 1x1km square transects (11 at the Coastal-boreal Mainland and 12 at the Boreal-nemoral site, spaced on average 3–6 km apart) to collect moose faecal pellets during the winter months from November 2016 to February 2017, and November 2017. We collected fresh faecal samples along the whole length of each transect (4 km), leaving at least 200m between samples to maximize the probability of sampling different individuals (i.e. during each round of collections, we assumed each faecal sample to represent the diet profile of a different individual). We considered faecal samples as fresh if they were not covered by snow and still had a shiny surface

(Hemami & Dolman, 2005). From each pellet group, we randomly selected one pellet and stored it at room temperature in a 20 mL scintillation tube with silica desiccant until further processing (Taberlet et al., 2018).

Based on Ecoregions 2017 (Dinerstein et al., 2017), we labelled the northernmost study sites as Montane, Interior Boreal and Coastal-boreal Archipelago. The Montane study site is located in the Swedish mountains at approximately 650 m a.s.l. In winter, the area is characterized by deep snow. The primary available food source for moose is mountain birch *Betula pubescens* with *Salix* shrubs, juniper *Juniperus communis*, poplar trees and a few scattered stands of pine also occurring. The Interior Boreal site encompasses large tracts of boreal forest, dominated by pine and spruce trees. The vegetation at the Interior Boreal and Coastal-boreal Archipelago sites is similar to the Coastal-boreal Mainland site, except that the deeper snow cover (~70 cm) prevents moose from accessing the forest field layer, and agriculture is largely missing. The Coastal-boreal Archipelago site encompasses several islands and is partly located within a national park (Haparanda Skärgård). Forestry occurs at the Boreo-nemoral, Coastal-boreal Mainland and Interior Boreal sites, but is absent at the Montane site and infrequent and small scale at the Coastal-boreal Archipelago site.

The sample collection for the three northern study sites is described in Fohringer et al. (2021). Briefly, faecal samples were collected from the rectum of free-ranging adult moose that were captured and released between the winters (February–March) of 2008 and 2017, which in northern Sweden correspond to winter conditions with snow melt rarely starting before mid-May. The faecal samples were placed into 50 mL screw cap tubes and stored at −20°C. Whole blood was drawn from the jugular vein of the immobilized animals into 9 mL S-Monovette® Z-Gel collection tubes and stored at −20°C. To evaluate the possible effects of sex, body size, and age on the diet, we recorded the sex of each individual and measured total length (TL) as the distance (in mm) from the tip of the muzzle to tip of the tail following the contour line. Age was estimated based on tooth wear (Ericsson & Wallin, 2001). The moose captures and handling were in accordance with ethical permits (A124-05, A116-09, A50-12 and A14-15) granted by the Swedish Animal Ethics Committee. All other fieldwork did not require permits.

2.2 | Food availability and selectivity

We measured food availability alongside the faecal sample collection on the transects at the Coastal-boreal Mainland and Boreo-nemoral sites. Using the step-point method (Coulloudon et al., 1999; Evans & Love, 1957), we recorded vegetation hits above the snow on a pole within the browsing height stratum of moose (0–3 m; Nichols et al. (2015)) approximately every 40 m (for 100 measurements per 4 km transect). The vegetation hits are analogous to moose bites and can easily be transformed into proportions to provide a quantitative measure of food availability, which can then be compared to the proportions of food items in moose diets. To determine selectivity, we used Jacobs (1974) index (D), which relates the use of a food item (i.e. its proportion in the diet, r) to its relative availability in the environment (p):

$$D = (r - p) / (r + p - 2rp).$$

The index ranges from −1 to 1, with negative values indicating utilization below relative availability ('avoidance') and positive values indicating utilization above relative availability ('preference'); a value of zero corresponds to the utilization of a food item in proportion to its relative availability. To account for the non-normality of the selectivity data, we used Wilcoxon tests to evaluate whether Jacob's *D* for forages differed from zero.

2.3 | Vegetation sample collection and nutritional analyses

We collected twigs from trees and shrubs from 11 plant species: grey alder *Alnus incana*, downy birch (also known as mountain birch), silver birch *Betula pendula*, heather *Calluna vulgaris*, juniper, Norway spruce, Scots pine, aspen *Populus tremula*, *Salix*, bilberry *Vaccinium myrtillus* and cowberry *Vaccinium vitis-idaea*. These species together with *Trifolium* spp. comprised >95% of moose diets across the five study sites. For further details, please see Appendix S1.

Vegetation samples were collected to imitate moose browsing, that is fresh, unbrowsed twigs were cut from the first 10 cm of tree side shoots and from the top of shrubs of several specimens until we reached ca. 150 g wet weight. The twig diameter was kept at ≤4 mm (Spaeth et al., 2002; Vivås et al., 1991). On subsequent collections, sampling locations were slightly offset (~10 m) to avoid sampling reactive effects to browsing by the plants. Vegetation samples were weighed and dried at 60°C for 48 h and then ground using a laboratory cutting mill using a 1 mm sieve (Krizsan & Huhtanen, 2013). We pooled collected plant material from each species across transects in each study area, thereby incorporating between-tree and between-transect variation in nutritional content (Felton et al., 2021). The nutritional properties used in this study are detailed in Table S1. The chemical analyses were performed by the DairyOne Forage Laboratory.

2.4 | DNA metabarcoding and diet data

The DNA metabarcoding process is described in Spitzer et al. (2021). In short, DNA extraction and purification was carried out on a QIASymphony SP platform using the DSP DNA minikit (Quiagen) according to the manufacturer's instructions. We used a universal primer for mammals (MamP007 in Giguet-Covex et al., 2014, corresponding to primers Mamm02 in Taberlet et al., 2018) to confirm that the field-collected faecal pellets originated from moose. To determine the botanical diet composition, we used the universal primer pair Sper01_F & Sper01_R (Taberlet et al. (2018), formerly g/h primers of Taberlet et al. (2007)), which amplify the P6-loop of the *trnL* intron of chloroplasts. After PCR, sequencing was performed on an Illumina HiSeq 2500 platform. Diet compositions were quantified based on relative read abundances of annotated sequences, that is, molecular operational taxonomic units. For more details, please see Appendix S2. We converted botanical diet compositions to nutritional compositions by multiplying the proportion of each plant species in a faecal sample by its nutritional content (Felton et al., 2016; Ma et al., 2019).

2.5 | Metabolic 'starvation' marker

Biomarkers that indicate starvation responses of individual moose included in this study were determined by Fohringer et al. (2021) by means of ^{1}H NMR-based metabolomics. Among those biomarkers, the authors highlighted elevated concentrations of ketone bodies as indicators of late starvation. Elevated concentrations of ketone bodies in serum indicate nutritional ketosis, a metabolic state that occurs when reduced carbohydrate resources are available (Hall & Hall, 2021; Moyes & Schulte, 2008). Out of the two ketone bodies that the ^{1}H NMR analysis by Fohringer et al. (2021) provided, we used Acetoacetate as the molecule indicating increased ketone body production in moose.

2.6 | Hypothesis testing

Of the constituents measured in the forage plants (Table S1), we focused on AP, lipids, TNC, cellulose and hemicellulose as the macronutritional components when testing our hypotheses as these correspond to the fractions that are metabolizable by moose.

Moose winter diets generally do not differ largely between sexes (Felton et al., 2016; Ma et al., 2019). We assessed possible differences between male and female moose for the three study sites where sex was known (Montane, Interior Boreal and Coastal-boreal Archipelago). Differences in body size (TL) were tested for each age group using t-tests with Bonferroni corrections of *p*-values. To evaluate differences between the sexes in the botanical and nutritional diet composition, we calculated pairwise Bray–Curtis dissimilarities between individual diets (i.e. faecal samples) and ordinated the results using non-metric multidimensional scaling (NMDS) (Kartzinel et al., 2015). We then tested for dietary differences using permutational multivariate analysis of variance (perMANOVA) in the R package VEGAN (Oksanen et al., 2017; Pansu et al., 2019). All statistical analyses were carried out in R version 3.6.1 (R Core Team, 2019) at a significance level of alpha = 0.05.

2.6.1 | Differences in botanical diet composition do not directly relate to nutritional differences (Hypothesis 1)

To test whether the differences in botanical diet compositions were uncorrelated to the nutritional differences (prediction 1a), we first constructed two pairwise Bray–Curtis dissimilarity matrices, one for the botanical and one for the macro-nutritional diet compositions, with each faecal sample representing one diet profile. We then used a Mantel test with 999 permutations to evaluate the correlation between the two matrices. Bray–Curtis dissimilarities range from 0 (identical composition) to 1 (no shared items), allowing for direct comparisons between dissimilarity matrices. We used a *t*-test to investigate whether pairwise dissimilarities were greater for botanical diet compositions than for macro-nutritional compositions (prediction 1b).

2.6.2 | *Salix* twigs resemble the AP:NPM Target of moose in winter (Hypothesis 2)

To test predictions 2a and b (that the nutritional rail for *Salix* intersects the average observed diets and that reaching the *Salix* target is facilitated by complementary food items), we followed a graphical approach using the GFN as in Felton et al. (2016), relating the proportion of AP in observed moose diets to the proportion of NPM.

The pellets and *Salix* twigs used in the original experimental feeding trial by Felton et al. (2016) showed fairly constant lipid values of ~2%, which is much less variation compared to what we found across the forage plant species in our study (Table S3). We suspect the high lipid levels of conifers to partially result from non-digestible cuticular waxes and resin oils. For our results to be more comparable to the feeding trial, we therefore excluded lipids from the NPM fraction, which thus corresponded to total carbohydrates (TCH = TNC + hemicellulose + cellulose).

2.6.3 | Macro-nutritional balancing occurs most strongly between AP and TNC (Hypothesis 3)

Using the data from the two study sites where food availability had been measured (Coastal-boreal Mainland and Boreo-nemoral, Figure S4), we evaluated whether AP and TNC were more strongly correlated in the observed diets than in hypothetical diets proportional to food availability (prediction 3a). To test the strength and significance of the correlations, we used Pearson's product moment correlation coefficient.

To test the second prediction (3b) that the AP:TNC ratio would be less variable than the ratios between AP and the other macronutrients (cellulose, hemicellulose and lipids), we calculated the coefficient of variation in percent (%CV = SD/mean*100) for those ratios from diet compositions at all five study sites. Since the %CV relates the standard deviation to the mean of a given dataset, it is a suitable metric for direct comparisons across datasets but corresponds to only a single value. To evaluate the variation associated with the %CV, we used a bootstrap approach and resampled the data for each study site with replacement prior to calculating the %CV. We repeated this procedure 1000 times for each site.

2.6.4 | The starvation signal (use of body reserves strategy) is related to diet composition (Hypothesis 4)

We used the relative concentration of Acetoacetate as the response variable in generalized linear models (gaussian family) set to test our predictions that a lower starvation signal corresponds to higher digestibility diets (prediction 4a; predictor: IVT-digestibility; Table S1), and higher ratios of AP:TNC and AP:NDF (predictions 4b). The predictions for Hypothesis 4 could only be tested for the study sites where blood samples were analysed for Acetoacetate, that is at the Montane, Interior Boreal and Coastal-boreal Archipelago sites. To account for a possible area effect, study site was included as a covariate in the models, and we estimated both its direct effect and that of the interaction with the main predictors on the response. To assure normality, we Tukey-transformed the response variable using the *transformTukey* function from R package RCOMPANION (Mangiafico, 2020).

3 | RESULTS

In total, 349 faecal samples from moose were successfully amplified to reveal the diet composition (Figure 1b, Table S2). We found large differences in botanical diet composition across the five study sites. At the Montane site, moose diets were dominated by mountain birch and *Salix*, whereas the Interior Boreal diets consisted almost completely of pine. At the Coastal-boreal Archipelago site, diets were diverse, consisting of approximately 50% pine, with the other half composed of birch, willow, poplar, juniper and relatively large amounts (~10%) of alder. At these three most northerly sites, dwarf shrubs such as *Vaccinium* spp. (corresponding mostly to bilberry and cowberry [see Appendix S2], hereafter referred to as *Vaccinium*) were largely absent due to the deep snow, whereas *Vaccinium* shrubs comprised ca. 50% at the Coastal-boreal Mainland and 20% at the Boreal-nemoral sites. Boreal-nemoral diets showed the highest amount of spruce in moose diets (~5%), a conifer that is usually avoided by moose (Cederlund et al., 1980). The corresponding macro-nutritional diet compositions were less distinct (Figure S1). The results of the nutritional analyses for the moose forage plants are presented in Table S3. Most of the macro-nutrients were not significantly correlated, except for cellulose and TNC (Pearson's $r = -0.70$, $p = 0.016$; Table S4).

TL between male and female moose differed only at ages four ($\bar{x}_{TLm} = 280\,\text{cm} \pm 7$ SD; $\bar{x}_{TLf} = 266\,\text{cm} \pm 11$ SD; $p < 0.001$) and five ($\bar{x}_{TLm} = 285\,\text{cm} \pm 7$ SD; $\bar{x}_{TLf} = 270\,\text{cm} \pm 9$ SD; $p < 0.001$). NMDS and perMANOVA revealed differences in diet composition between the sexes only at the Interior Boreal study site (botanical: stress = 0.09; perMANOVA, pseudo-$F_{1,153} = 4.92$, $p = 0.008$; nutritional: stress = 0.02; perMANOVA, pseudo-$F_{1,153} = 5.81$, $p = 0.012$) but not for the Montane and Coastal-boreal Archipelago sites. These differences resulted from males consuming slightly larger amounts of pine than females ($\bar{x}_m = 93\% \pm 9$ SD, $\bar{x}_f = 87\% \pm 15$ SD; Welch's *t*-test with Bonferroni correction, $p = 0.034$). The corresponding mean proportions for AP, cellulose, hemicellulose, TNC and lipids were nearly identical for the sexes, but the variance was higher in females for cellulose (Levene's test, $p = 0.033$) and lipids (Levene's test, $p = 0.045$).

3.1 | Differences in botanical diet composition do not directly relate to nutritional differences (Hypothesis 1)

We found no support for the prediction that dissimilarities in botanical diet composition would be unrelated to macro-nutritional composition (prediction 1a). The Mantel test showed a strong correlation between the Bray–Curtis pairwise dissimilarity matrices of botanical and macro-nutritional diet compositions (Mantel-$r = 0.89$, $p = 0.001$). Despite this correlation, the differences among botanical diet compositions (mean Bray–Curtis dissimilarity: 0.53 ± 0.35 SD) were significantly greater than among the corresponding macro-nutritional differences (mean Bray–Curtis dissimilarity: 0.13 ± 0.09 SD; Welch's *t*-test: $t = 269.15$, $p < 0.001$), which aligns with our prediction 1b.

3.2 | *Salix* twigs resemble the AP:NPM Target of moose in winter (Hypothesis 2)

We found support for prediction 2a at three of the five study sites. The nutritional rail for *Salix* (the presumed nutritional target) intersected the average observed diet at the Coastal-boreal Mainland and Montane sites and came close to intersecting the average observed diet at the Boreal-nemoral site (Figure 2 and Figure S5). This suggests that *Salix* represents a nutritionally balanced food for moose in winter and supports experimental findings by Felton et al. (2016). Pine and *Vaccinium* on their own appear to be nutritionally imbalanced with respect to AP and NPM as their nutritional rails do not intersect the *Salix* intake target and the observed diets. They are, however, nutritionally complementary, that is moose can reach their target by combining their intake from these two foods, which supports prediction 2b. Pine was preferred by moose at both study sites, whereas *Vaccinium* was preferred at the Coastal-boreal Mainland site but underutilized at the Boreo-nemoral site; *Salix* was preferred at the Coastal-boreal Mainland site and consumed slightly above availability at the Boreo-nemoral site (Figure S2).

The observed average diets did not intersect the *Salix* target at the Coastal-boreal Archipelago and Interior Boreal sites (Figure S5), with the observed diets at the latter site being the most distant from the *Salix* target (Figure S6).

3.3 | Macro-nutritional balancing occurs most strongly between AP and TNC (Hypothesis 3)

AP and TNC were significantly correlated in the observed diets (Coastal-boreal Mainland: $r = -0.75$, $p < 0.001$; Boreal-nemoral:

$r = -0.99$, $p < 0.001$) but not in the diets based on food availability (Coastal-boreal Mainland: $r = 0.11$, $p = 0.69$; Boreal-nemoral: $r = 0.23$, $p = 0.41$), which supports prediction 3a. We did not find support for the prediction (3b) that the variation of the AP:TNC ratio would be less than the ratios between AP and other macronutrients; the coefficient of variation was either similar or higher than that of other AP:Macronutrient ratios. No consistent pattern for any AP:Macronutrient ratio was found across the study sites (Figure 3).

3.4 | The starvation signal (use of body reserves strategy) is related to diet composition (Hypothesis 4)

We found weak evidence for prediction 4a. After controlling for the area effect, there was no significant negative relationship between digestibility and the levels of Acetoacetate in serum ($F[1227] = 3.05$, $p = 0.08$) but the low p-value suggests that such an effect might exist. We found no support for the predictions under 4b; after controlling for the area effect, there was no significant effect of the AP:TNC ratio ($F[1227] = 0.82$, $p = 0.37$) or the AP:NDF ratio ($F[1227] = 1.52$, $p = 0.22$) on the levels of Acetoacetate in serum. Acetoacetate levels were similarly low in moose with diets that contained more than 30% of either pine or birch and the highest in moose whose diets contained high proportions of *Salix* (Figure S7).

4 | DISCUSSION

Using faecal DNA metabarcoding and chemical analyses of forage plants, we assembled a thorough account of the botanical and nutritional composition of moose winter diets over a large area in Sweden. Our results were consistent with a number of predictions arising out of four general hypotheses related to nutrient balancing but were inconsistent with others.

4.1 | Differences in botanical diet composition do not directly relate to nutritional differences (Hypothesis 1)

Contrary to our prediction, the more diets differed in their botanical composition, the more they also differed in nutritional composition. However, on average, the dissimilarities between nutritional diet compositions were much lower than dissimilarities between botanical compositions. In winter, the diversity of available food items can be much reduced, which restricts the food space in which an animal can manoeuvre. For example, at the Montane study site, diets consisted almost completely of mountain birch, and of pine in the interior boreal diets. In both instances, this was probably due to the low availability of alternative food resources. Under such conditions, the difference in botanical diet composition would directly correlate with the nutritional differences between birch and pine. It would therefore be interesting to repeat our analyses using summer diets, when the full botanical and nutritional diversity of plants is available to foraging moose.

4.2 | *Salix* twigs resemble the AP:NPM target of moose in winter (Hypothesis 2)

Geometric analysis of the moose diets supported our prediction that moose would aim for a nutritional target that corresponds to the AP:TCH ratio of *Salix* as had been suggested by the results from feeding trials reported in Felton et al. (2016). At the Coastal-boreal mainland and Boreo-nemoral sites where *Vaccinium* was accessible due to low snow depth, moose mixed pine and *Vaccinium* in their diets. These two food items fall on opposite sides of the *Salix* target, which makes them nutritionally complementary. These results suggest that if *Salix* were more abundantly available, moose might directly increase *Salix* in their diets instead of mixing pine and *Vaccinium*. This could reduce the intake of pine, which would contribute to mitigating the moose-forestry conflict over pine damage in Sweden under the prerequisite that moose numbers are kept constant. Recent findings by Felton et al. (2022) lend support to this hypothesis.

Vaccinium was preferred by moose at the Coastal-boreal Mainland site but scored as avoided at the Boreo-nemoral site. This may be linked to feeding competition from smaller deer species such as fallow deer, which are more abundant at the Boreo-nemoral site (Spitzer et al., 2021). Competition over complementary foods may thus influence macronutrient balancing in moose.

At the sites where *Vaccinium* shrubs were not accessible due to deep snow, moose still combined high AP:TCH ratio pine with lower ratio browse but, on average, were not able to reach the *Salix* target. This does not necessarily carry negative costs as nutrient balancing can also occur over longer periods of time (Simpson & Raubenheimer, 2012). Longer-term studies throughout the year are needed to better understand nutrient balancing in moose, particularly regarding their rules of compromise. We also caution against valuing forage items solely based on their AP:TCH ratios. For example, moose could gain a ratio similar to the target by eating abundantly available spruce and birch as is illustrated by the hypothetical diet based on food availability, which came close to the observed diets at the Coastal-boreal Mainland and Boreo-nemoral sites. Both birch and spruce, however, were consumed in lower proportions than pine and *Vaccinium* at those sites and scored as 'avoided' on the selectivity index. Birch was the least digestible of all analysed food items. Perhaps combining more digestible pine and *Vaccinium* to reach the *Salix* target is a better strategy for moose than simply eating more birch. Moreover, it is possible that plants with a suitable AP:TCH ratio contain other components such as plant secondary metabolites which render them less palatable for moose. Spruce, which is typically avoided by moose (Cederlund et al., 1980), contains higher concentrations of phenolics than pine (Shipley et al., 1998). For example, Duncan et al. (1994) showed that the total concentration of monoterpenes in the needles of Sitka spruce *Picea sitchensis* had a significant negative influence on the amount of browsing by red deer. Clearly, the picture of nutrient balancing in moose is more complex than the AP:TCH ratio and requires further investigation. An implication for the management of moose food resources is that nutritionally complementary forage plants should be managed synchronously.

4.3 | Macro-nutritional balancing occurs most strongly between AP and TNC (Hypothesis 3)

Similar to Felton et al. (2021, using moose rumens), our data showed significant correlations between AP and TNC in observed diets but not in the diets reconstructed based on food availability. This provides further evidence for nutritional balancing in wild moose, especially since AP and TNC were not correlated across the different forage plants. Contrary to our expectations, however, the variation of the AP:TNC ratio in diets across the study sites was not consistently lower than the ratios of AP to other macronutrients. In some respects, our data are not directly comparable to Felton et al. (2021). In their study, the authors investigated moose diets in southern Sweden, where winter is less severe, the variety of available forages higher, and supplementary feeding more frequent and intensive. Moreover, the nutritional composition of rumen content differs from the composition of the plant material eaten due to transformations in the rumen (Van Soest, 1994). Retention time of forage particles in the digestive tract of ruminants depends on particle size and density (Clauss et al., 2011). In moose, rumen content largely represents 1 or 2 days (on average ca. 31 h; Schwartz et al., 1988), whereas faecal pellets may reflect longer-term diet compositions spanning about two (Schwartz et al., 1988) to a maximum of 10 days (Hjeljord et al., 1982). Despite these differences, our findings support the view that balancing carbohydrates may be more important for moose (and perhaps other ruminants) than for monogastric animals, possibly resulting from the need to maintain suitable rumen conditions (e.g. pH levels) for the rumen microflora. It would be desirable for future studies to focus on the consequences different communities of rumen microflora can have on the geometric patterns of nutritional regulation.

4.4 | The starvation signal (use of body reserves strategy) is related to diet composition (Hypothesis 4)

Analyses were limited to the three most northerly sites where blood samples had been studied (Montane, Boreal and Coastal-boreal Archipelago). Food choices in these areas were more limited than further south and the gradient in the digestibility of available forage might not have been strong enough to make a difference. This may explain why we found only weak statistical support for the relationship between food digestibility and the starvation index. Our data also did not quantify the overall amount of food intake, which may be more influential on the level of starvation than diet composition or quality. Foraging herbivores sometimes also behave counterintuitively. For example, it has been shown that large herbivores faced with poor quality food do not simply compensate by increasing intake, but instead draw on their body reserves until food of higher quality becomes available (Meyer et al., 2010). It is possible that under harsh winter conditions, reduced metabolic rate (Græsli et al., 2020) and limited food variety at the northernmost study sites, moose foraged less selectively than further south. The high Acetoacetate levels in *Salix*-dominated diets were surprising because most results point to *Salix* as an especially suitable winter forage for moose. This may have resulted from wintering at locations where *Salix* was the most accessible forage but available only at low biomass, causing moose as capital breeders to heavily rely on their body reserves for energy. Another but less likely explanation could be that the individuals that had primarily fed on *Salix* (as revealed by analysis of their faecal samples) may have been near critical levels of starvation (for reasons unknown) and may then specifically have sought out *Salix* as the highest quality food available in an effort to improve their condition.

5 | CONCLUSIONS

Our results provide further evidence for nutrient balancing in wild ruminants. Across a large spatial gradient moose showed very variable botanical diet compositions but much reduced differences in the nutritional composition of observed diets. Using the GFN, we found evidence that free-ranging moose in winter appear to mix two nutritionally complementary foods, pine and *Vaccinium*, to reach an intake target corresponding to the AP:TCH ratio of *Salix*.

The correlation between AP and TNC highlights the potential importance moose seem to assign to the balancing of non-structural carbohydrates. It would be interesting to investigate whether this pattern is linked to maintaining suitable conditions for the commensal rumen microbiome and whether it is consistent across the seasons and other ruminant species.

Food digestibility was only weakly negatively associated with Acetoacetate levels in serum (i.e. 'starvation'). It would be informative to test whether the relationship would be stronger when using actual moose rumen fluid.

We found no link between higher protein diets and level of starvation, which further suggests that protein may not be the prioritized macro-nutrient in moose diets. Nutrient balancing can occur in the context of different life-history objectives and depend on specific site conditions. It would be interesting to explore whether the patterns of macronutrient balancing that we observed during the winter also apply to other seasons and to moose outside of Sweden.

Lastly, we highlight the importance of identifying nutritionally complementary food plants for moose and other wild ruminants for forest and wildlife management and suggest that such foods should be managed synchronously.

AUTHOR CONTRIBUTIONS

Robert Spitzer, Annika M. Felton and Joris P.G.M. Cromsigt designed the study; David Raubenheimer substantially contributed to

conception and design, particularly to the visualization and interpretation of data; Robert Spitzer and Christian Fohringer collected the data; Joris P.G.M. Cromsigt, Marietjie Landman, Fredrik Widemo, Wiebke Neumann and Navinder J Singh contributed to acquisition of data; Pierre Taberlet, Robert Spitzer and Christian Fohringer conducted laboratory analyses; Eric Coissac performed bioinformatics; Robert Spitzer analysed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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CONFLICT OF INTEREST STATEMENT

None of the authors have a conflict to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available on Dryad at:

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Detailed methods: Vegetation sample collection.

Appendix S2. Detailed methods: DNA metabarcoding.

Table S1. Nutritional properties analysed for moose winter forage plants.

Table S2. MOTUs, corresponding plant species and proportions in moose winter diets.

Table S3. Nutritional properties of moose winter forage plant species.

Table S4. Pearson correlations between nutritional components.

Figure S1. Nutritional composition of moose winter diets.

Figure S2. Selectivity (Jacob's D) for moose forages.

Figure S3. Moose winter diets (AP & TCH) at the CBM and BN study sites.

Figure S4. Food availability.

Figure S5. Moose winter diets (AP & TCH) at the Montane, Interior Boreal and Coastal-boreal Archipelago study sites.

Figure S6. Moose winter diets (AP & TCH) in relation to the nutritional target (*Salix* spp.).

Figure S7. Relationship between birch and pine in moose diet and Acetoacetate in serum.

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