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Assessing Plant-Wax Markers as a Tool to Estimate intake and diet Composition in Beef Cattle

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ASSESSING PLANT-WAX MARKERS AS A TOOL TO ESTIMATE INTAKE AND DIET COMPOSITION IN BEEF CATTLE

By

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Ronald M. Lewis

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Assessing Plant-wax Markers as a Tool to Estimate Intake and Diet Composition in Beef Cattle

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University of Nebraska, 2017

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Estimating feed efficiency of ruminants in grazing environments is difficult due to challenges in measuring intake and diet composition of animals that are freely grazing. Plant-wax markers, especially *n*-alkanes (**ALK**), have been shown to be a potential tool to calculate intake and diet composition.

Two indoor experiments were conducted in successive years to assess ALK reliability to facilitate estimate DMI (**EDMI**) and diet composition. Heifers were fed a ration of 69.8% corn silage and 30% ground alfalfa with a daily supplement containing a ALK marker (C_{32}). Using a pooled fecal sample increased the correlation between observed DMI and EDM I (in 2015, $r = 0.79$; in 2016, $r = 0.65$) when compared to daily intakes methods. Furthermore, the EDM I was sensitive to diet composition estimates due to the forages having two distinctly different concentrations of C_{31} and C_{33} .

A series of grazing studies followed each experiment. The predominant plant species in all studies (smooth brome grass and Kentucky bluegrass) had ALK profiles that allowed 10% difference in diet compositions to be distinguished ($P < 0.02$). Differences in concentrations of marker C_{33} between plants resulted in unrealistic EDM I. When

EDMI based on the C31:C32 ratio were compared to observed intakes from the indoor studies, the results were highly variable ($0.01 < r^2 < 0.99$), which could be due to many factors including animal behavior and forage availability. Despite the lack of fine demarcations, sensible intakes were obtained in a grazing setting. The plant-wax methodology therefore shows promise for commercial use.

Key words: cattle, diet composition, estimation, grazing intake, *n*-alkanes, plant-wax markers

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Chapter I: Review of Literature

INTRODUCTION

Currently, the United States (US) is the largest beef producer in the world (USDA, 2015). As of January 1st, 2016, there was a total of 92.0 million cattle in the US (USDA, 2016a). The US beef industry is a multi-billion-dollar industry, with Americans eating 4.1 billion pounds of beef each year (USDA, 2016a).

Given its size, the beef cattle industry will be essential to feeding the US and global populations. The US exported over 2.5 billion pounds of beef in 2014 (USDA, 2016a), making it the fourth largest exporter of beef. The world's population is currently increasing rapidly and is expected to reach over 9 billion people by 2050 (UN, 2015). According to Delgado (2003), impoverished people in developing countries will increase their animal product consumption as their incomes increase above the poverty line. In addition to a growing desire for meat, the FOA (2012) predicts that cereal grain demand will increase as well over the next 15-30 years.

Population growth will impact land use and availability. Land needs for cereal grains will displace cattle. Furthermore, as population size increases land will be lost to agriculture due to expanding urban development. These factors will lead to less land available for further increase in meat production. Due to a finite amount of land, the increased demands could cause overgrazing, which will result in land degradation (FAO, 2003). Cattle producers will need to focus on two goals in order to remain competitive as

a source of protein: (i) have cattle that efficiently grow in rangeland environments; and, (ii) have production practices that are sustainable.

According to the USDA, as of 2007 the US had 400 million acres of cropland and 600 million acres of grassland, pasture and range (USDA, 2016b). Livestock in the US are typically raised on crop residues, or in locations where crops are not easily grown, such as rangeland in the western US. The Bureau of Land Management (**BLM**) manages 245 million acres of public lands, including 155 million acres grazed primarily by cattle and sheep (Gorey, 2016). Rangeland condition, including the presence of certain plants, is greatly reduced under heavy rates of grazing (Johnson et al., 1951). In order to protect native lands, the BLM administers permits and leases to ranchers. Land grazing is conditionally based on forage availability and season of use (Gorey, 2016). Unregulated grazing may cause damage to soil, plants, streams and springs. Currently, grazing management practices are designed to increase productivity, reduce soil erosion and sustain plant populations (Gorey, 2016). Grazing animals obtain and retain their energy through a complex system of biological and physiological characteristics. Bailey (2006) found some cattle have distinct and consistent grazing patterns. Large herbivores, such as cattle, can be presented with as many as 50 different plant-communities a day (Senft et al., 1987). Lands, managed by the BLM, will become an important resource for the cattle industry, but a better understanding on how to manage and raise sustainable animals will be needed to allow usage of these lands, whilst limiting the permanent impact of cattle grazing.

Cattle maintenance energy constitutes 70–75% of the cost of feeding (Cottle and Pitchford, 2014). According to Nkrumah et al. (2007), there is a substantial amount of genetic and phenotypic variation in beef cattle efficiency. Variation in energetic efficiencies can be utilized by breeding programs to produce more energy efficient cattle. Increasing feed efficiency would decrease the cost of production by decreasing the amount of input, such as forage or grain, needed.

A reliable tool is needed to identify energy efficient cattle in complex systems, such as rangeland. Plant waxes, vascular plant's extra cellular lipophilic barrier, could be a potential tool and has been shown to be useful to estimate intake, digestibility and diet composition (Dove and Mayes, 2005). In order to select genetically more efficient cattle, one must know intake, diet composition and digestibility of plants consumed (Cottle, 2013). Once identified selection can focus on cattle that thrive in specific environments with relatively few inputs. More efficient cattle will be needed to feed the Earth's growing population, while more systematic grazing schemes may possibly be less destructive to the environment.

PLANTS

Plant Waxes

The extracellular barrier in plants serves as the interface with the environment and is responsible for controlling the transfer of water, solutes and gasses (Molina, 2010). Cutin and suberin are the 2 different types of insoluble plant polyesters of fatty acids that, when combined with glycerol, make up the wax barrier. The cutin is responsible for

creating the structure of the cuticle, while the suberin varies by cell type and forms due to environmental stressors. Cutin and suberin are structurally similar, but have different chemical compositions. The cuticle covers the external portion of the epidermal cell wall of leaves, primary stems, flowers and fruits; additionally, there is an internal cuticle that occurs in seeds and in the lining of substomatal cavities. The hydrophobic portions of the cuticle are called waxes and consists of insoluble polymers, cutin and cutan and a mixture of epicuticular and intracuticular lipids. The C₁₆ and C₁₈ oxygenated fatty acids and glycerols create the cutin, while the cutan is believed to consist of hydrocarbon (Molina, 2010). Epicuticular lipids are projections that protrude from the cuticle, which is usually a complex mixture of aliphatic lipid compounds (Dawson et al., 2000). Aliphatic lipids are nonaromatic hydrocarbons that can be straight-chain, branched-chain or cyclic. The following are therefore entirely separate from previous structures in plant waxes: long-chain *n*-alkanes (**ALK**), ketones, fatty acids, long-chain alcohols (**LCOH**) and aldehydes (Molina, 2010).

Cuticle can differ between species and is greatly dependent on plant function. Warm-season plants (C₄) have thicker cuticles in order to survive droughts. Cool season grasses (C₃) are required to be more metabolic efficient due to decreases in sunlight for photosynthesis, so their cuticle facilitates this with parenchyma bundle sheaths (Wilson and Kennedy, 1996). Morphological differences lead to variation in the chemical composition of plant waxes. Changes in plant wax composition can also be seen in different parts of the plants, with leaves and flowers usually having the highest concentration of wax (Dawson et al., 2000). Several studies report variations in plant waxes among plant species and parts (Tulloch, 1976; Dove and Mayes, 1991; Dove and

Mayes, 1996). Plant waxes plus additional variations in nutrient value, such as lignin content and particle size, effect digestion as well as intake.

Ruminal Plant Digestion

Ruminants rely on a diverse microbial population within their rumens for the digestion of nutrients. Microbial populations include bacteria, protozoa fungi and virus. Microorganism degrade and ferment plant cells turning them into volatile fatty acids (VFA) and protein, that are utilized by the host animal. The metabolic rate is largely impacted by ruminal fermentation and the types of microorganisms within the rumen. The rumen has a relatively constant temperature with a pH that is slightly acidic due to saliva's buffering capacity (Masson and Phillipson, 1951). The rumen has a relatively constant supply of fluid and VFA that flow into the lower digestive tract and absorbed predominantly by the small intestine wall. The flow of liquid is based on the rate of digestion (k_d) and the rate of passage. True digestibility (**TD**) equals k_d divided by the total rate of fluid disappearance, which is due to digestion and passage (Mertens, 1987).

Differences in the morphology of plant epidermis and vascularization influence the rate of microbial digestion (Wilson and Kennedy, 1996). Depending on the plant part or species additional rumination might be necessary to break down plant matter. The cuticle, which contains plant waxes, is rarely digested by ruminal microbes but will crack due to stressors such as fungi, rumination and pH. Cracks in the cuticle and cell wall allow microbes access to the inner components of the cell, which are digested (Akin, 1979). The ALK in plant waxes pass through to the feces essentially unchanged (Wilson and Mertens, 1995; Chavez et al., 2011).

Plant Morphology

Digestion, as well as plant-wax composition, are affected by plant morphology. Changes due to age of the plant have been shown to change the nutrient content of the plant, which effects intake and animal production. For example, in the northern Great Plains plant morphology is controlled by air temperature, while the quantity of forage is a function of soil water and nutrients (Frank and Hofmann, 1989). The primary forage found and utilized by grazing animals in this region are cool season grasses (Frank et al., 1985).

There are several ways to measure morphological development, which include the Huan Growth Scale (**HGS**) and growing degree days (**GDD**). The HGS uses visual methods of measuring plant morphological developments, such as leaf sprouts (Huan, 1973). Although the HGS accurately depicts plant growth it can be quite laborious to obtain. The GDD, however, uses daily maximum and minimum temperature to predict growth (Frank and Hofmann, 1989). The GDD is measured as:

$$GDD = \sum \frac{(daily\ maximum\ temperature + daily\ minium\ temperature)}{2} \quad (Eq. 1.1)$$

Frank and Hofmann (1989) found a direct linear relationship between HGS and GDD for cool-season grasses ($r^2 = 0.62$ to 0.96), although warm-season grasses have a quadratic relationship ($r^2 = 0.95$ to 0.96).

The start date for accumulating GDD poses difficulty when wishing to predict plant development (Frank and Hofmann, 1989). Typically, the starting date for GDD is

the first day after March 15 when the average air temperature surpassed 0°C for 5 consecutive days (Frank et al., 1985). Start dates can depend on year and region of plant growth.

Although morphology is based on GDD, plant biomass is controlled in large part by water availability. Variability in plant water use is due to the evaporative potential of atmosphere, quantity of water available and plant characteristics (Power, 1983). Plant characteristics include leaf area and leaf area duration, a measure of green leaf retention over time, which highly affects water usage. Plant characteristics are also affected by harvest rates; however, Frank and Hofmann (1989) found that DM yields on moderately or heavy grazed pastures, across years with different precipitation, was largely due to species composition and prior grazing management.

Grazing causes defoliation of the plants within a pasture, which causes added stress on plants thereby affecting growth, nutrient quality of the forage and water efficiency. Harrison and Romo (1994) evaluated regrowth of smooth brome grass (*Bromus inermis*) after defoliation in relation to stage of the growth, moisture availability and GDD. After defoliation, smooth brome grass began to accumulate forage between 45-75 GDD when moisture was favorable; however, during dry years smooth brome grass regrowth was minimal with regrowth occurring between 110-140 GDD. They found that total annual production (35 to 139 g/m²) was unaffected by defoliation, but due to growth conditions, such as rainfall and temperature.

As maturity increases forage quality decreases (Nelson and Moser, 1994). As the plant matures, the ratio of leaf to stem changes, which is believed to be the greatest contributing factor to the decreased nutritive value (Ugherughe, 1986).

FEED INTAKE

Changes in plant nutrients has a large impact on the digestibility of feeds, which in turn affects intake. Animal intake and the digestibility of feeds are the result of a complex interaction of animal, diet and feeding environment (Conrad et al., 1964; Baumgardt, 1970). Factors that affect DMI include temperature, light intensity, water availability and latitude. Maturity also impacts DMI; it is influenced by harvest and storage methods, and forage composition, which particularly change lignin concentrations (Van Soest, 1994).

Cattle daily requirements of nutrients – protein, water, vitamins and minerals – also heavily influence intakes. The necessary concentrations of these nutrients is largely dependent on the stage of development of the animal. The concentration of nutrient varies between feeds and affects how much DMI an animal needs.

Cattle nutrient consumption is limited by the capacity of their digestive tract, which is especially in forage diets. Forage quality is highly correlated to intake. With diets that are nutrient dense gut fill is no longer the limiting factor of intake. With highly digestible feed, intake becomes the result of chemostatic and physiological mechanisms, which can differ among animals (Lalman, 2003).

Rumen microflora have been shown to be different across animals, which affects fermentation. Microorganisms produce volatile fatty acids (VFA), which are consumed by the host animal (Russel, 2002). Changes in microbial populations influence the composition of the VFA in rumen. These changes affect efficiency due to loss of energy during fermentation. Propionic acid is the most energy efficient VFA and is converted to ATP with no loss of carbon. Furthermore, when energy is converted to ATP proton slippage can occur, which decreases the efficiency of energy available. All these factors impact energy efficiency, which in turn influences intake.

Calculating Intake

Historically the most widely used method for calculating intake (I) is:

$$I = O / (1 - D) \quad (\text{Eq. 1.2})$$

where D is the proportion digestibility of the feed and O is the total fecal output (Dove and Mayes, 1996).

Digestibility is usually estimated by using *in vitro* methods that have been calibrated with *in vivo* measurements. However, there are potential sources of error to this method (Dove and Mayes, 1991). The first problem is that animals used for *in vivo* measurements are often mature animals fed near maintenance, but digestibility could be different in various stages of life. Secondly, digestibility is measured by an average of individuals, but there are variations between individuals. Lastly, animals on pasture may select different plants, which would alter the digestibility when compared to the *in vivo* animal.

In a confinement setting, feed intake can be recorded by measuring the amount of feed offered and refused. Laboratory settings are also necessary to collect fecal output and to determine whole-diet digestibility. Once animals leave a confined location, such as grazing on pasture, it becomes increasingly more difficult to calculate intake and feed efficiency.

The Beef NRC (2016) suggests that DMI (kg/d) for growing animals can be estimated by summing intakes requirements for net energy of maintenance ($NE_{m,DMI}$) and net energy of growth ($NE_{g,DMI}$). The $NE_{m,DMI}$ is calculated by taking the required daily net energy for maintenance ($NE_{m,intake}$; Mcal/d) and dividing it by the maintenance concentration of the diet ($NE_{m,diet}$; Mcal/kg). The $NE_{m,diet}$ of the feed is a function of the ME:

$$NE_{m,diet} \text{ (Mcal/kg)} = 1.37ME - 0.138ME^2 + 0.0105 ME^3 - 1.12$$

(Eq. 1.3)

The ME is estimated as 0.82 times the DE of the feed, while 4.4 Mcal of DE is equal to 1 kg of TDN. Therefore, ME is proportional to 3.62×10^{-3} TDN. Forage quality can be determined using TDN, which is calculated using proximate analysis based on CP, crude fiber (DCF), digestible nitrogen-free extract (NFE) and 2.25 times ether extract (DEE) (Rasby and Martin, 2016). The $NE_{m,diet}$ is used to calculate the $NE_{m,intake}$ for yearlings in the following way:

$$NE_{m,intake} = SBW^{0.75} * [(0.2435 * NE_{m,diet}) - (0.0466 * NE_{m,diet}^2) - 0.0869]$$

(Eq. 1.4)

where **SBW** is the shrunken body weight (NRC, 2016).

Energy utilization for growth is not as efficient as energy for maintenance.

$NE_{g,DMI}$, like $NE_{m,DMI}$, is calculated by taking the required daily net energy for gain ($NE_{g,intake}$; Mcal/d) and dividing it by the dietary concentration of net energy for gain ($NE_{g,diet}$; Mcal/kg). The $NE_{g,diet}$ concentration of the feed is a function of the ME:

$$NE_{g,diet} \text{ (Mcal/kg)} = 1.42ME - 0.174ME^2 + 0.0122 ME^3 - 1.65$$

(Eq. 1.5)

The $NE_{g,diet}$ is used to calculate the $NE_{g,intake}$ using empty BW (**EBW**) and empty BW gain (**EBG**) in the following way:

$$NE_{g,intake} = 0.0635 * EBW^{0.75} * EBG^{1.097}$$

(Eq. 1.6)

The EBW is calculated by multiplying SBW by 0.891, while EBG is calculated by multiplying shrunken BW gain by 0.956. With low-quality diets intakes have been over estimated, while with high-quality diets intakes have been underestimated, when using Eq. 1.4 to Eq. 1.6.

Lignin

Lignin is generally accepted as a primary entity responsible for limiting the digestion of forages (Van Soest, 1994). When plants mature, lignification begins forming a secondary cell wall. Lignin covalently bind to cell wall polysaccharides creating gross linkages (Ralph et al., 1995). The amount of lignin varies between plants. Mowat et al. (1969) conducted a study evaluating 56 forages and found lignin varied between 3.7 and 19.1%, and as plant matured lignin percent increased.

Temperature

In addition to lignin, cattle environment has also shown to affect performance. Intake declines as rectal temperature increases which occurs under hot environmental conditions. The DMI can be a function of core body temperature (Johnson et al., 1963; Hahn, 1995). Core body temperature can be effected by breed type, color of the animal, temperature, humidity and radiant energy. Hahn et al. (1992) found that an air temperature above 25°C decreased *Bos taurus* performance when cattle were not protected from solar radiation.

Due to the complexity of animal environment interaction, the Temperature Humidity Index (**THI**) has been used as a tool to better understand animal stress. The THI takes into account temperature and humidity to create an index that is correlated to body temperature. The THI can be calculated in many ways. The NRC (1971) method of measuring THI is:

$$THI = (1.8 \times T_{db} + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T_{db} - 26.8)]$$

(Eq. 1.7)

where T_{db} is dry-bulb temperature and RH is relative humidity. As a rule of thumb THI ranges are normal ($THI < 74$), alert ($74 < THI < 79$), danger ($79 < THI < 84$) and emergency ($THI > 84$) (LCI, 1970). Cattle are affected by the high THI, but are able to regulate their body heat through lower THI at night (Eirich et al., 2015). Therefore, the high and low THI must be reviewed in order to better understand animal stress and its relationship to intake. According to the Nebraska extension, overnight temperatures above 22.8 °C or three nights in row above 21.1 °C lead to a risk of heat stress (Eirich et al., 2015).

MARKERS FOR PREDICTING INTAKE

Traditionally, calculating intake and digestibility involves needing to know total amount of feed consumed along with total collection of fecal matter, which is not possible in unconfined settings. For this reason, fecal output is usually estimated by using indigestible markers. An “ideal” fecal marker has complete recovery in feces, accurate quantitative measurement and no effect on the animal or diet (Dove and Mayes, 2006).

Markers

Common markers to evaluate intake include chromium sesquioxide and even-chain ALK, which are administered by combining with oil in gel capsules or being placed on shredded paper. Synthetic forms of ALK have been safely used as markers in sheep,

goats, cattle and horses (Ferreira, 2010; Chavez et al., 2011). There are relatively few even-chain ALK found in nature with over 90% of ALK being odd-chained; therefore, feeding a known concentration of even-chained ALK can be used to dose animals to calculate intake (Chavez et al., 2011).

There are many sources of ALK, including plants, animals, sedimentation and petroleum products (Gromalt and Albaiges, 1987; Morrison and Boyd, 1992). Longer chain ALK have better fecal recovery rate (Dove and Mayes, 1991). Therefore, longer chain ALK are typically used to estimate intakes (Dillon, 1993; Olivan et al., 2007; Brosh et al., 2003). Fecal recovery rates of synthetic and natural ALK are directly proportional to the length of the carbon chain. A number of recovery rates have been reported, with rates for C₂₅ ranging from 0.430 to 0.724, while C₃₅ rates range from 0.879 to 0.999 (Brosh et al., 2003; Dillon, 1993; Dove and Mayes, 1991; Elwert et al., 2004; Elwert et al., 2008; Olivan et al., 2007). Chavez et al. (2011) found that either synthetic dotriacontane (C₃₂) or hexatriacontane (C₃₆) can be used as ALK markers.

Dosing

Variation in the concentration of the daily dose of any marker would lead individual samples to no longer represent mean fecal marker concentration, which leads to an incorrect estimation of intake. In order to minimize the source of error in dosing, controlled-release devices were created. Possible devices include slow-release gel capsules and controlled release capsule (CRC) (Dove and Mayes, 1991). The CRC is a metal cartridge that administers a constant dosage via a spring mechanism.

The CRC cartridge remains in the rumen for the remainder of the animal's life unless the animal is fistulated. Due to this, the CRC can be inconvenient if the animal was needed for other experiments or needed x-rays because the capsule cannot be removed unless surgery is performed. Considering the CRC cannot be monitored in non-fistulated animals, consistency of release by the device may be a concern. Chavez et al. (2011) used fistulated cattle to measure actual dosage of CRC and found that CRC would over and underestimate the intake due to inconsistencies in the release rate.

Initial concerns with once a day or pulse dosing of markers might have been unnecessary. Chavez et al. (2011) found that after 10 days of dosing with a daily supplement sprayed with C_{32} , intake was accurately estimated. Lippke (2002), who used a single large or pulse dose at the beginning of a trial, found such a strategy can be used to calculate intake. The pulse dose fecal output (O) is calculated by:

$$O = A_i * M^{-1} * EPR \quad (\text{Eq. 1.8})$$

where A_i is the dosage, M is the marker concentration at time of dosing and EPR is the exponential passage rate. Validation of alternative dosing methods, namely pulse or daily dosing, to the CRC has allowed for grazing research to be more easily completed.

Sample Collection

Easy and accurate techniques to collect and store samples without the loss of ALK is pertinent to using plant-wax markers as a feasible tool to estimate feed intake and diet selection. Techniques for collection and processing of samples should be tested to determine their accuracy. Chavez et al. (2011) found that after 10 days of a dosing with a

daily supplement containing ALK or with a CRC, the type of fecal collection (fecal grab or total fecal collection) did not impact the ALK concentrations in feces. Additionally, there was no difference in ALK concentrations in total and grab fecal samples. An oral daily dosage of ALK led to a continuous and uniform flow of marker ALK throughout the digestive system. The accuracy and precision of intake calculations are affected by sampling and measurement precision of both plant and fecal marker and can be further be affected by drying and collection protocol (Cottle and Romano, 2013). The fecal material must be dried and stored for ALK analysis, but the drying method can cause a loss in ALK (Dove and Mayes, 2006). Chavez et al. (2011) showed that freeze-drying, or oven-drying to a constant weight at 60 °C, did not impact fecal concentrations of ALK..

EXTRACTION OF PLANT WAXES

Dried and stored plant and fecal samples can have plant waxes extracted by using the protocols of Dove and Mayes (2006). Plant waxes extraction starts by precisely weighing the sample: 0.2 grams for plant matter and 0.1 g for feces. An internal standard for the desired wax also must be weighed and added to the sample. Next, the sample must have all the other (non-hydrocarbon) lipid removed. This is accomplished by heating the feces or plant matter in ethanolic KOH solution, which hydrolyzes triacylglycerols and other esters. Heptane and water are added to the solution, adequately mixed, and the non-aqueous portion aspirated and evaporated to create a crude extract. The crude extract is then dissolved in heptane and placed on a silica column. The addition of heptane elutes the ALK from the columns, which leaves LCOH, sterols, triterpenols and pigments in the column. The heptane ALK fraction is evaporated and then dissolved in dodecane before

being placed in gas chromatography (GC) vials.

The ethanolic KOH solution hydrolyzes esters containing the LCOH and volatile long-chain fatty acids (VLCFA). The silica column still contains LCOH and VLCFAs, which can be separated into a crude alcohol extract using a solvent with a higher polarity such as ethyl acetate/heptane (20:80). Alcohols in this fraction can be analyzed by GC, but peak shape is generally poor. By converting the alcohols to either acetate or trimethylsilyl (TMS) derivatives better results can be obtained. Acetate forms are more chemically stable than TMS derivatives, but TMS derivatives are more suitable for GC analysis because they are better-defined on the mass spectra. The crude alcohol extract can contain sterols, which may potentially interfere with the GC analysis. Aminopropyl solid phase extraction columns can effectively remove these sterols. Additionally, by increasing the polarity of solvents that run through the columns, secondary and primary alcohols and sterols can be collected.

GAS CHROMOTOGRAPHY

The extracted samples are then assessed using GC (Dove and Mayes, 2006). The GC sample, either feces or plant, is injected into the instrument and enters a gas stream that transports the sample into a separation tube known as the "column" (Thet and Woo, 2013). The gas that carries the sample is helium. The various components in the sample are separated inside the column. The detector measures the amount of the components that exit the column depending on weight. Peaks are typically measured using GC peak integration software. Peak locations are identified using an external sample, which contains known ALK of various carbon lengths. The peaks in the sample, now with

identified locations, are compared to internal standards concentrations, which allows concentrations of sample ALK to be calculated.

NEAR INFRARED SPECTROMETRY

In addition to GC, near infrared reflectance spectroscopy (**NIRS**) is a promising tool to calculate the concentration of natural ALK, diet composition, intake and digestibility (Keli et al., 2008; Decruyenaere et al., 2009). With NIRS, wavelengths in the near-infrared region of the electromagnetic spectrum (780–2500 nm) are used to generated vibrations that can be recorded (Bokobza, 1998). Different chemical bonds such as O–H, C–H and N–H vary in their strength and therefore the amount of energy required for the bond vibration is different. Using known chemical vibrations from pure samples the chemical bonds of a sample can be mapped. The NIRS has been routinely used for prediction of chemical composition and energy value of feeds (Keli et al., 2008). The NIRS gives rapid results and unlike GC is non-destructive to samples (Decruyenaere et al., 2009). However, NIRS relies heavily on calibration equations and, according to Keli et al. (2008), there is a need for improvements of these calibration equations to accurately differentiate between varieties of plants.

CALCULATING INTAKE

Once the concentration of ALK and LCOH in plants and feces are measured through GC or NIR, intake can be calculated. From Dove and Mayes (1991), intake was calculated by looking at the concentration of odd-chain ALK in herbage (**H_i**) and feces (**F_i**). Furthermore, the digestibility of the herbage (**D_i**) was defined as:

$$D_i = 1 - (H_i/F_i) \quad (\text{Eq. 1.9})$$

If the daily dose of an ALK is A_j and herbage intake is I , then fecal output (**O**) was obtained as:

$$O = (A_j + IH_j)/F_j \quad (\text{Eq. 1.10})$$

Using Eq. 1.2, 1.9 and 1.10, intake can be estimated as:

$$I = \frac{O}{1-D} = \left[\frac{A_j + IH_j}{F_j} \right] / \left[1 - \left(1 - \frac{H_i}{F_i} \right) \right]$$

$$(H_i * F_j) * I = (F_i * A_j + F_i IH_j)$$

$$I = \frac{A_j * F_i / F_j}{H_i - (F_i H_j) / F_j} \quad (\text{Eq. 1.11})$$

Intake, when using ALK, is calculated using Eq. 1.11 due to its simplicity. However, when multiple feeds are being consumed, Eq. 1.11 is transformed to take into account multiple herbages. The following equation is used for a two plant diet:

$$\frac{A_j * F_i}{P * [(F_j * H_{i1}) - (F_i * H_{j1})] + (1-P) * [(F_j * H_{i2}) - (F_i * H_{j2})]} \quad (\text{Eq. 1.12})$$

where H_{i1} is the odd-chain alkane for plant 1, H_{i2} is the odd-chain alkane for plant 2, H_{j1} is the even-chain alkane for plant 1, H_{j2} is the even-chain alkane for plant 2 and P is the proportion of plant 1 in the diet. The equation can be simplified to:

$$\frac{A_j * F_i}{[P * (F_j * H_{i1}) - P * (F_i * H_{j1})] + [(1-P) * (F_j * H_{i2}) - (1-P) * (F_i * H_{j2})]} \quad (\text{Eq. 1.13})$$

$$\begin{aligned}
& \frac{A_j * F_i}{[P * (F_j * H_{i1}) - P * (F_i * H_{j1})] + [(1-P) * (F_j * H_{i2}) - (1-P) * (F_i * H_{j2})]} * \frac{\frac{1}{F_i}}{\frac{1}{F_i}} \\
& \frac{A_j}{\left[\frac{1}{F_i} * P * (F_j * H_{i1}) - \frac{1}{F_i} * P * (F_i * H_{j1}) \right] + \left[\frac{1}{F_i} * (1-P) * (F_j * H_{i2}) - \frac{1}{F_i} * (1-P) * (F_i * H_{j2}) \right]} \\
& \frac{A_j}{\frac{1}{F_i} * P * (F_j * H_{i1}) - P * H_{j1} + \frac{1}{F_i} * (1-P) * (F_j * H_{i2}) - (1-P) * H_{j2}} \\
& \frac{A_j}{\frac{F_j}{F_i} [P * (H_{i1}) + (1-P) * (H_{i2})] - [P * H_{j1} + (1-P) * H_{j2}]} \quad (\text{Eq. 1.14})
\end{aligned}$$

As species of plants increases, Eq. 1.14 can be expanded to take into account all species.

An additional consideration when estimating intake is as carbon-chain length increases recovery rates also increase. However, sequential ALK tend to have similar recovery rates. Commonly the ratio of C₃₂:C₃₃ ALKs are used to estimate feed intake (Dove and Mayes, 1991). However, the C₃₁ marker can also be used to measure intake (Lewis et al., 2003). Robustness of prediction can be visualized by comparing predicted intakes using C₃₁:C₃₂ and C₃₂:C₃₃.

STATISTICS

Plant-wax Profiles

The ALK concentration of the herbage is necessary to predict intake but when the diet is not a single food, estimating these concentrations can be complex. Plant-waxes vary between species and these differences are a potential way to identify diet

composition of grazing cattle. Plant marker profiles, including ALK and LCOH, need testing to determine if they are sufficiently distinct before diet composition can be estimated. If plants are indistinguishable prior to consumption, they most certainly will not be recognizable in the feces.

A principal component analysis, along with a biplot, can be utilized to visualize which markers help explain variation among plant species. Additionally, statistical models can be developed to test for differences in plant species ALK concentrations. These models allow the variation between species and within separate studies or sampling periods, to be understood. Depending on the model structure, various programs can be used. For fixed effects models, the GLIMMIX procedure in SAS 9.3 (SAS Inst., Inc., Cary, NC), or the “lm” function in R (R Development Core Team), are typically used. More complex, mixed or random effect models can be fitted using the PROC MIXED procedure of SAS or the “lmer” function in R. The standard errors of the parameter estimates, along with 95% confidence intervals, can also be examined to better visualize the distinctiveness of the markers in each plant species on offer.

Diet Estimation

Diet estimates can be obtained using various statistical methods. These methods include Bayesian hierarchical linear unmixing model (**BHLU**; Vargas et al., 2017) and non-negative least squares (**NNLS**; Dove and Moore, 1995; Lewis et al., 2016). The BHLU method uses information about the percent contribution of plants to the diet, prior means, and a matrix of their covariances to inform the model as to the reliability of prior information. The smaller the covariance values, the more the diet composition estimates

depend on the values of the prior means. A thorough description of the BHLU model can be found in Vargas Jurado et al. (2017). The Lawson-Hanson NNLS implementation of NNLS, found in the “NNLS” package in R, also can be utilized to estimate diet composition. The NNLS, however, does not take into account prior information. Given its non-negative constraint, this least squares method ensures that estimated contributions of plants to a diet are either zero or positive.

A “blind” laboratory study can be used to determine the distinctiveness of the ALK profiles of plant species (Vargas Jurado et al., 2015). Samples of known proportions of plants can be constructed and the ALK concentrations of the mixtures determined. From these samples, NNLS and Bayesian methods can be used to estimate botanical compositions. Observed (or known) botanical compositions can then be regressed on those estimated. The goodness-of-fit of the regression line then help determine the robustness of the estimation.

Additional tools to understand the reliability of the diet composition and intake estimates are: (i) Tukey’s multiple-comparison test; (ii) orthogonal contrasts; (iii) the regression of natural log observed on natural log estimated values; (iv) Kulczynski similarity index (K_{SI}) ; and, (v) Lin’s concordance correlation coefficient (ρ_c). Tukey’s multiple-comparison test can be used to create simultaneous pairwise comparisons. In orthogonal contrasts, each set of contrasts equally subdivides model sums of squares into independent partitions and will identify what percentages of forages in a diet can be delineated. A log transformation will transform skewed distributions towards normality, which allows the variations in the data to be more interpretable. Lastly, both K_{SI} and ρ_c

coefficients estimate the similarity between 2 different measurements (Oosting, 1956; Ferreira et al., 2009).

Chavez et al. (2011) were able to estimate diet composition and intake when using 2 forages. However, it remains unclear if plant waxes can be reliably used in a pasture setting. Animals in pasture often have a wide variety of plants to select from and their selection will change depending on what is available, the season and their stage of life. In a study conducted by Reis et al (2015), cattle grazed a pasture containing tall fescue, bermudagrass, red clover and other plants. The goal of the study was to determine if observed DMI (**ODMI**) could be used to rank cows, based on efficiency, reliably under grazing conditions. The estimated DMI (**EDMI**) were calculated for lactating and non-lactating Angus cows using ALK markers. Chavez et al. (2011) found that cows shifted their consumption preferences over time. The ALK provided useful predictions of intake for lactating cows but did not accurately rank post-weaning cows when compared to the ODMI. Cows categorized as post-weaning were in their final stage of lactation when ODMI was measured but were dry when EDMIs were calculated. The discrepancies between ODMI and EDMIs therefore may be in response to different energy states rather than errors in the estimation process. A single EDMIs may not accurately depict an animal's performance throughout its whole life cycle.

Due to there being few different ALKs, only a few species of plants can be delineated at a time (Bugalho, 2002). According to Dove and Mayes (2005), LCOH and fatty acids show promise for discriminating a greater number of plants in the diet although their analysis required additional steps, which increases the likelihood for error.

Fraser et al. (2006) used different combinations of ALK and fatty alcohols to accurately estimate diet compositions from fecal profiles of animals grazing a complex sward.

Vargas Jurado et al. (2015) reliably estimated the amount of fescue in an exclusively fescue and red clover diet. However, including the LCOH added no improvement. Vargas Jurado et al. (2015) speculated that with other species of plants, or with a more complex mixture, LCOH may help with their discrimination in the diet.

CONCLUSION

Plant-wax markers could potentially change the way cattle are selected and managed. By allowing producers to better predict which animals have lower maintenance costs and by determining which plants cattle preferred for grazing, both animal selection and ecosystem management could be improved. Knowing which plants are being selected will help with land management and conservation allowing managers to pull animals from the pastures with undesirable diet selection.

HYPOTHESES AND OBJECTIVES

We hypothesize that plant wax markers, such as ALK, can be used to estimate feed intake and diet composition. In order to validate the use of plant wax makers we have 2 objectives: (i) to test the utility of using plant wax markers to estimate dietary choice and intake in cattle under controlled (indoor) conditions; and, (ii) using this validated methodology, to assess those variables in cattle under grazing conditions. Further, combining information garnered from the indoor and grazing studies, we intend to assess how individual animals perform throughout a growing season.

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Chapter II: Evaluation of *n*-Alkanes to Estimate Dry Matter Intake in Individually Fed Cattle

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ABSTRACT: Within grazing systems, determining differences in efficiencies is difficult due to the complexity of measuring forage intakes. Plant waxes, particularly *n*-alkanes (**ALK**), have shown potential for estimating intakes due to their indigestibility. Therefore, within forage-based systems, ALK markers may be used to estimate dietary choices and DMI. To test the reliability of this methodology, in 2 indoor experiments, heifers (in 2015, 26 heifers with BW 460 ± 79 kg; in 2016, 16 heifers with BW 354 ± 45 kg) were individually fed a total mixed ration (**TMR**) of 69.8% corn silage (**CS**), 30% ground alfalfa and 0.2% salt. In addition, they were dosed daily with an internal ALK marker (C_{32}), fed in a supplement. Fecal samples and individual intakes were collected for 5 d. The ALK concentrations of the fecal samples, and the TMR and its component plants, were measured. The relative concentrations of C_{31} and C_{33} to C_{32} , after fecal recovery adjustments, were used to calculate estimated DMI (**EDMI**). The objectives of this experiment were 3-fold: (i) to compare the effect of 3 fecal evaluation strategies on EDM; (ii) to determine the sensitivity of EDM to losses in dosed marker through wastage of the supplement; and, (iii) to ascertain the impact of estimates of diet composition on EDM. The reliability of EDM was tested by regressing observed DMI (**ODM**) on EDM and by their correlation. Regardless of fecal method, the slopes differed from one ($P < 0.04$) and the intercepts differed from zero ($P < 0.01$); however, there were still moderately high correlations between ODM and EDM ($r > 0.51$). Pooled fecal samples increased the reliability of the estimates (in 2015, $r = 0.79$; in 2016, $r = 0.65$). The EDM were sensitive to marker loss. However, if marker loss was consistent, EDM would be systematically either over or underestimated. If the diet was not considered a single food (TMR), the accuracy of the estimate of diet composition was

extremely important when calculating intakes. Due to the relative concentrations of C_{31} and C_{33} in CS and alfalfa, the non-negative least squares method for diet estimation overestimated the amount of CS present in the diet ($CS\% 0.79 \pm 0.02$), which increased EDMI. As the complexity of diets increase evaluating the efficacy of using ALK profiles to delineate the components of the diet becomes increasingly important.

Key words: cattle, diet composition, estimation, feed intake, *n*-alkanes, plant-wax

INTRODUCTION

Feeding cattle is expensive with maintenance energy constituting 70 to 75% of the cost (Cottle and Pitchford, 2014). Variation in feed efficiency has been observed in beef cattle, which, if exploited, would allow for genetic improvement (Nkrumah et al., 2014). Pragmatically, to achieve such change, a process is needed to reliably and easily identify efficient animals.

Plant-waxes contain *n*-alkanes (**ALK**) among their components, which essentially are indigestible when consumed by ruminant animals (Wilson and Mertens, 1995; Chavez et al., 2011). Several studies have reported significant variations of ALK among plant species, thereby providing unique profiles useful for distinguishing individual plants (Tulloch, 1976; Dove and Mayes, 1991; Bugalho et al., 2004; Ali et al., 2005). Additionally, there are relatively few even-chains ALK found in nature; by dosing animals with synthetic even-chained ALK, these markers can be used to estimate feed intakes and digestibility (Dove and Mayes, 2005; Ferreira, 2010; Chavez et al., 2011).

Our objective was to evaluate 3 factors that may impact the reliability of intake estimates based on plant-waxes. First, we compared 3 strategies for combining ALK concentrations obtained from fecal samples to estimate DMI (**EDMI**). One strategy entailed analyzing a single fecal sample per animal by pooling daily collections. The other strategies each entailed analyzing daily fecal samples. Second, we investigated the effect of losses in the daily dose of an internal ALK marker on EDM. Since some wastage was possible, ingestion of the marker was assumed to be complete or as percentage increments of that offered. The cattle were fed a total mixed ration (**TMR**)

consisting of approximately 70% corn silage (CS) and 30% ground alfalfa. Some sifting of the feedstuff was possible. Therefore, lastly, we considered the impact of differences in the composition of individual animals' diets, estimated using ALK, on EDM1.

MATERIALS AND METHODS

This study was conducted at the Roman L. Hruska U.S. Meat Animal Research Center (USMARC), Clay Center, NE. Animals were raised in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010), and their care was approved by the both the MARC and University of Nebraska-Lincoln Animal Care and Use Committees.

n-Alkane Dosing

The *n*-alkane C₃₂ (Dotriacontane, CAS# 544-85-4; Minakem, SAS, France) was used as an internal marker to estimate feed intake. Approximately 1.3 mg/kg BW per d of C₃₂ was added to 0.23 kg of feed supplement. In the 2015 experiment, the supplement was Producer's Pride All Stock Sweet Feed (12.0% CP DM; 18.0% crude fiber DM; Tractor Supply Company). The internal marker was melted onto 20 g ground soybean hull before mixing with the feed supplement. In the 2016 experiment, the supplement was Producer's Pride Calf Starter (16.2% CP DM; 12.5% crude fiber DM; Tractor Supply Company). The internal marker was mixed directly with the supplement.

The same daily dosage was used for all heifers within a study, and was based on the predicted BW of the heaviest heifer at the start of a dosing period. Those target weights (doses) ranged from 380 kg (495 mg/d) to 480 kg (625 mg/d).

Experiment

Two indoor experiments were conducted between the months of March and May in 2015 and 2016.

Exp. 1. In the first wk of April 2015, 40 spring 2014 born commercial MARC II heifers were moved to the USMARC Area 25 Building 45, which contained a Calan Broadbent Feeding System (American Calan, Northwood, NH). The heifers were split between 2 pens and began a 20 d adaptation period. The feed doors were secured open, and the heifers were offered ad libitum access to a TMR (**HF01**; 69.8% CS, 30% ground alfalfa hay and 0.2% salt, as DM) in the feed bunks. The chemical composition of the TMR, and its two component plants, are provided in Table 2.1. Heifers were carefully monitored to confirm that they were eating (visually observed at least twice daily), and their preferences for particular feed door positions noted.

At the end of the adaptation period (d 1), 26 well-adapted heifers were chosen and retained for the remainder of the study. These animals were equipped with a sensor key that would unlock a single feed door, and the doors were locked. Where possible, assignments to feed doors were based on preferences during the adaptation period. Each morning, starting at 8:00 a.m., feed bunks were filled with known weights of HF01. For the following 2 wk, refusals were weighed back weekly. Starting on d 8, prior to refilling the bunk, 0.23 kg of the feed supplement was offered. On d 15, and continuing for 10 d, the internal marker was added to the supplement.

Fecal sample collection began d 22, and continued for 4 d thereafter. Starting at 8:00 a.m., animals were moved into a nearby working facility, placed in a squeeze chute where their heads were secured. A rectal fecal sample was collected. During this same 5 d period, residual feed was removed and weighed daily. A sample of HF01, and orts from each animal's bunk, were also collected. Animals were weighed starting at 8 a.m. on d 1, 2, and 22 to 26. On d 22 to 26, BW were recorded coincident with collection of fecal samples. At the start of each weighing event, the accuracy of the weigh scale was validated.

Exp. 2. The design of the second indoor experiment was very similar to that of Exp. 1, and therefore only delineating elements will be presented. On March 22, 2015 born commercial MARC II heifers were placed in a pen with a Calan Broadbent Feeding System in MARC Area 25 Building 45. After 9 d of ad libitum access to HF01 in open gates, 16 heifers deemed adapted to the facilities were identified, fitted with keys, and the doors locked. These heifers were allowed an additional 6 d (15 d in total) to habituate to the feeding system before the start of the study on April 6 (d 1).

Similar to Exp. 1, each morning starting at 8:00 a.m. feed bunks were filled with known weights of HF01 in order for heifers to feed ad libitum. For approximately 2 wk, refusals were weighed back weekly. Starting on d 12, prior to refilling the bunk, 0.23 kg of the feed supplement was offered. On d 19, and continuing for 10 d, the internal marker was added to the supplement. Daily fecal samples and feed intakes were collected starting on d 26, which continued for 4 d, following the same procedures as in Exp. 1. Animals

were weighed on d 1, 2 and 26 to 30. On d 26 to 30, BW were recorded coincident with collection of fecal samples.

Laboratory analyses

Laboratory analyses were conducted by 2 technicians with each evaluating all samples (both fecal and foodstuffs) collected within a yr.

Sample preparation and extraction. The feed and fecal samples collected were place in a 50°C oven until dried and ground through a 1 mm mesh screen using a Wiley Mill (Thomas Scientific, Swedesboro, NJ). The ground samples were then stored in a dry cool location until analyzed.

Sample extractions were performed in duplicate (Dove and Mayes, 2006). A 0.2 g sample of a forage or TMR, or 0.1 g of fecal matter, was weighed, and 0.1 g of an internal standard solution containing 0.3mg/g of *n*-docosane (C₂₂) and *n*-tetratriacontane (C₃₄) was added to each sample tube as an internal standard. They were heated with 1 M ethanolic KOH for 16 h at 90°C. Heptane and distilled water were added to each sample and heated to 50°C; the top (non-aqueous) layer was collected and evaporated. Hydrocarbons were collected by solid phase extraction by heptane elution through a silica-gel column (3 mL 20 µm PE, Biotage LLC, Charlotte, NC). The ALK were re-dissolved in *n*-dodecane for gas chromatographic analysis.

Gas chromatography. Quantification of ALK was carried out with a gas chromatograph (GC) on an Agilent 7820A GC (Agilent Technologies, Wilmington, DE). Derivatized ALK fractions were injected (0.5µl) with a 7650A Automatic Liquid

Sampler onto a bonded-phase, non-polar column (Agilent J&W DB-1 column, 30 m, 0.53 mm internal diameter and 0.5 μ m film thickness). Helium served as the carrier gas at a constant flow of 4 mL/min. Temperature was 280°C for the injector and 340°C for the detector. The column was held at 140°C for 6 min, then increased at 50°C/min to 215°C with an iso-thermal hold of 1 min, and a second temperature ramp of 6°C/min to 320°C with a 4 min hold time.

Samples of ALK standard solution mixtures (C₂₁ to C₃₆; Sigma-Aldrich, St. Louis, MO) were included in the GC analyses to identify peaks and standard response factors. Chromatographic data were analyzed using Agilent ChemStation software (Rev. C.01.06 [61]). Peak areas were determined with auto-integration and manual review of chromatograms. The ALK concentrations were calculated relative to known amounts of the internal standards (C₂₂, and C₃₄), according to Dove and Mayes (2006).

Potential Factors Impacting Intake Estimates

Fecal evaluation. The EDMIs were estimated using 3 strategies for analyzing the ALK concentration of the fecal samples: (i) from a single fecal sample formed by pooling collections from 5 d, estimating intakes from the pool; (ii) from separate fecal samples collected each of 5 d, averaging intake estimates; and, (iii) from a mathematical average of the ALK concentrations of the 5 daily fecal samples, estimating intakes from the average. The pooling strategy required lab analyses of a single pooled fecal sample per animal, while the other strategies entailed analyzing multiple samples per animal. Analyzing a single sample would be more cost effective. Therefore, one aim of these

comparisons was to determine if the pooling strategy offered as reliable an approach for obtaining EDM I as the other strategies.

Loss of internal marker. The daily dose of internal marker was mixed with a supplement, which was fed. Although heifers were monitored to encourage their eating of their entire supplement, some wastage was possible. Therefore, the effect of losses in daily dose of the internal ALK marker was evaluated by assuming that 100%, 97.5%, 95%, and 90% of the supplement offered was consumed.

Diet composition. The TMR fed was a chopped feed. Some sifting by individual animals was therefore possible. We considered the possible impact of variation in the composition of the diet on EDM I in 4 ways: (i) all animals consuming the TMR, considered as a single homogenous food; (ii) all animals consuming the TMR but its composition determined as a mathematical composite (70% CS and 30% alfalfa) of the ALK profiles of its parts; and, the diet composition of individual animals estimated from the ALK profiles of the plants and their own fecal samples using either (iii) non-negative least squares (NNLS) or (iv) a Bayesian technique considering 3 heavily weighted prior assumptions regarding the CS composition of the diet (proportionally, 0.80, 0.70 and 0.60 CS). The latter two approaches allowed for sifting of the TMR by cattle.

Statistical Analyses

Statistical analyses were conducted in R (R Core Team, 2017) and SAS (SAS, 2000).

Estimating diet composition. Diet composition of individual heifers was estimated using both NNLS and a Bayesian approach. The "NNLS" package in R was used to obtain the solutions by NNLS. Using an algorithm developed by Jurado Vargas et al. (2017), a Gaussian distribution was used for the Bayesian analyses. Since only 2 plants were evaluated (CS and alfalfa), the prior mean was fully specified by the proportion of CS in the diet. Three prior means for the CS dietary proportion were considered: 0.80, 0.70 and 0.60. In all cases, the prior covariance matrix (order 2×2) was defined such that its diagonal elements were 1×10^5 and its off-diagonal elements were zero. That covariance structure placed heavy weight on prior information in the estimation process.

To better understand the extent to which the ALK profiles of CS and alfalfa could be used to delineate their relative contributions to the diet, the concentrations of C_{27} , C_{29} , C_{31} and C_{33} in pure plants were combined (weighted) to obtain theoretical mixtures containing, proportionally, from 1.0 to 0.0 of each plant, at 0.05 increments. Variation in the estimated diet compositions was evaluated by principal component analysis (**PCA**) using the "prcomp" function of R.

Using the NNLS and Bayesian approach the composition of the derived mixtures were estimated from the weighted concentrations of the 4 ALK. Those data were then used to explore our potential to discriminate incremental changes in the composition of such mixtures (e.g., proportionally 0.95 vs. 0.90 or 0.85 CS in a diet). An orthogonal contrast was created based on interesting proportional changes in CS (i.e., diet

compositions with CS percentages near 70%). The following model was fitted using the "lm" package in R:

$$y_{ij} = \mu + m_i + e_{ij} \quad (\text{Eq. 2.1})$$

where y_{ij} was the proportion of CS estimated from an extract ($j = 1, \dots, 4$) for the mathematically derived mixture m_i ($i = 1.0, \dots, 0.05$, in 0.05 increments of CS) with μ the overall mean proportion of CS in the mixture. The theoretical concentration of ALK was fitted as a fixed effect, with the residual e_{ij} the random effect. This model was fitted separately for diet composition derived from NNLS and a Bayesian analysis (prior mean of, proportionally, 0.5 CS).

Estimating intake. Fecal ALK concentrations were adjusted for incomplete recoveries based on their ALK carbon length. The adjustments were based on the fit of a beta regression by Vargas Jurado et al. (2017) using data on recovery rates published in the literature (Dillon 1993; Olivan, 2008; Brosh et al., 2003; Dove, 1996; Ferreira et al., 2007; Elwert et al., 2006; Elsert et al., 2008). The recovery rates used were 70.3% (C_{27}), 78.5% (C_{29}), 84.8% (C_{31}), 87.4% (C_{32}), and 89.6% (C_{33}).

An EDM was obtained for each combination of fecal evaluation method (3 levels), loss of internal marker (4 levels) and diet composition (5 of the 6 possible levels, which excluded the bayesian approach with 60% CS). Three prior means for the CS proportion in the diet were considered in the Bayesian analyses. However, one of those analyses (a prior mean of 0.60) generated diet compositions that suggested far lower

intakes of CS than was plausible; that scenario was therefore ignored when considering intakes.

For each combination of factors, the ALK concentrations of either C_{31} or C_{33} (odd-chained) and C_{32} (dosed internal marker) in feces, and the observed ALK in the feedstuff (TMR or the pair of pure plants), were used to obtain 2 estimates of each EDM I:

$$EDMI = \frac{A_j \times (F_i / F_j)}{H_i - ((F_i \times H_j) / F_j)} \quad (\text{Eq. 2.2})$$

where A_j was the daily dose of C_{32} , F_i and F_j were the adjusted concentrations of odd-chain (C_{31} or C_{33}) and dosed (C_{32}) ALK in the feces, respectively, and H_i and H_j were the observed concentrations of the odd-chain and dosed ALK in the feedstuff, respectively. Diet compositions had been estimated in several ways. Where appropriate, those estimates were used to adjust Eq. 2.2 to account for the proportional contribution of CS and alfalfa in the diet of the individual animal when estimating intake. Intake estimates based on the ratios $C_{31}:C_{32}$ and $C_{33}:C_{32}$ were considered separately or as their average.

Regression. As a measure of reliability, observed DMI (**ODMI**) were regressed on EDM I for each fecal evaluation and diet composition estimation method, as well as marker loss (Piñeiro et al., 2008). Using the "lm" package of R, the hypotheses tested were that the slope was not different from unity, and that the intercept was not different from zero.

ANOVA. Variation in intakes associated with the odd-chained ALK (C_{31} or C_{33}) used in the estimation process was evaluated in relation to fecal evaluation and diet

composition estimation method with ANOVA. For greater simplicity, the percentage of marker loss was not considered since it is a constant in the formula used to estimate intakes (A_j in Eq. 2.2); therefore, marker loss only has a proportional effect on those estimates. The evaluations were conducted with the “lme4” package in R fitting the linear mixed model:

$$y_{ijklm} = \mu + F_i + D_j + M_k + (FD)_{ij} + (DM)_{jk} + (FDM)_{ijk} + R_l + A_{(l)m} + e_{ijklm} \quad (\text{Eq. 2.3})$$

where y_{ijklm} was the EDM I for animal A_m [$m = 1, \dots, n$, where n was the number of animals within a year (in 2015, $n = 26$; in 2016, $n = 16$)] for the fecal evaluation method F_i ($i = 1, 2$ or 3 , for the 3 methods used to combine ALK profiles of fecal samples), diet composition method D_j ($j = 1, \dots, 5$, for the 5 statistical analyses used to obtain EDM I), and marker ratio m_k ($k = 1$ or 2 , for the 2 ALK marker ratios, $C_{31}:C_{32}$ and $C_{33}:C_{32}$ used to obtain the EDM I), in year R_l ($l = 1$ or 2 , for 2015 and 2016, respectively), with μ the overall mean EDM I. Fecal evaluation method, diet composition method, marker ratio, and their interactions [$(FD)_{ij}$; $(FM)_{ik}$; $(DM)_{jk}$; $(FDM)_{ijk}$] were fitted as fixed effects. Random effects were year (R_l), animal nested within year [$A_{(l)m}$], and the residual (e_{ijklm}).

Using the average of two marker ratios, variation in EDM I due to fecal evaluation method, loss of internal marker and diet composition method were tested using the “lme4” package from R (R Core Team, 2017). The linear mixed model fitted was:

$$y_{ijklm} = \mu + F_i + P_j + D_j + (FP)_{ij} + (FD)_{ik} + (PD)_{jk} + (FPD)_{ijk} + R_l + A_{(l)m} + e_{ijklm}$$

(Eq. 2.4)

where y_{ijklm} was the EDM I for animal A_m [$m = 1, \dots, n$, where n was the number of animals within a year (in 2015, $n = 26$; in 2016, $n = 16$)] for the fecal evaluation method F_i ($i = 1, 2$ or 3 , for the 3 methods used to combine ALK profiles of fecal samples), percentage loss of internal marker P_j ($j = 1, \dots, 4$, for the 4 presumed percentage intakes of the internal markers, namely 100%, 97.5%, 95% and 90%, respectively), and diet composition method D_k ($k = 1, \dots, 5$, for the 5 statistical analyses used to obtain EDM I) in year R_l ($l = 1$ or 2 , for 2015 and 2016, respectively), with μ the overall mean EDM I. Fecal evaluation method, marker loss, and diet composition method, and their interactions [$(FP)_{ij}$; $(FD)_{ik}$; $(PD)_{jk}$; $(FPD)_{ijk}$] were fitted as fixed effects. Random effects were year (R_l), animal nested within year [$A_{(l)m}$] and the residual (e_{ijklm}). Additionally, this same model was fitted where the response variable (y_{ijklm}) was the ODMI subtracted by EDM I for each animal.

In 2015, heifers had been randomly allocated to 2 adjacent pens in the building. In preliminary analyses, the effect of pen, and its interactions with the other main effects, on EDM I were tested fitting a similar model to Eq. 2.4. Rather than animal nested within year, animal was nested within pen. No significant pen effect was found and it therefore was omitted from the analyses. In addition, differences in ODMI between pens was evaluated. Again, no significant pen effect was detected.

Simple and Spearman's rank correlations were obtained among ODMI and the various estimates of intake.

RESULTS

The daily BW and ODMI for the heifers during the 5 d fecal collection period are summarized as boxplots (Figs. 2.1 and 2.2). Average BW and ODMI were higher in 2015 than in 2016 heifers ($P < 0.001$). In the 2015 experiment, animals on d 1 ate substantially less feed than the other 5 d. Limitedorts present on d 1 suggested limited amounts of feed were provided on d 1, which caused variability in ODMI among days in the 2015 experiment.

Diet Composition

The ALK concentrations of the TMR, its component plants, and the supplements, are provided in Table 2.2. The mean ALK concentrations for TMR, its component plants, and a mathematical composite formed assuming a 70% CS and 30% alfalfa diet, are graphed in Fig. 2.3. The C_{27} , C_{29} , C_{31} and C_{33} ALK concentrations of the CS and alfalfa sample from 2015 were used to derive theoretical mixtures containing, proportionally, from 1.0 to 0.0 of each plant, at 0.05 increments. These ALK profiles of these derived mixtures, and of the TMR fed in 2015, were evaluated by PCA (Fig. 2.4). Nearly all the variation was defined by the first principal component (99.9%). There was strong separation between the pure CS and alfalfa samples. However, the TMR, which contained 70% CS, appeared to more closely coincide with a derived mixture with 80% CS.

Diet composition estimates of the TMR were obtained from the ALK concentrations (Table 2.2) of the pure plants using Bayesian and NNLS approaches. Pure

plant samples were only available in 2015. Their ALK concentrations, however, were reanalyzed to evaluate the CS proportion in the TMR fed in 2016 for consistency; as noted earlier, a different technician analyzed the 2016 samples. The results are provided in Table 2.3. There appears to be a discrepancy between the estimated and actual CS composition of the TMR in both 2015 and 2016. There were also inconsistencies between the estimates derived from the Bayesian and NNLS methods. The NNLS overestimated the CS composition of TMR (76% in 2015 and 86% in 2016), while the Bayesian method had lower estimates (64% in 2015 and 74% in 2016).

The composition of TMR was also estimated based on the chemical composition of the ration and its components. When estimating the CS composition of TMR based on CP, its value was consistent with NNLS (78% CS in 2015; 89% CS in 2016). However, such was not true for estimates based on the other chemical measurements, which suggested extremely low CS contents in the TMR (ADF: 20%; NDF: 11%; lignin: 38%).

Orthogonal contrasts were constructed to compare means of the estimated diet compositions obtained from the theoretical mixtures of the 2 plants (Table 2.4). Our intent was to test the reliability of delineating incremental differences in those compositions. In diets containing 60% or more CS, differences of as little as 10% in the CS composition of the diet (e.g., 65 – 60% vs. 75 – 70%) could be distinguished ($P = 0.01$) from estimates obtained with the Bayesian approach. With NNLS, such discrimination was slightly less robust ($P = 0.06$). Neither approach could distinguish 5% increments in diet composition ($P > 0.31$).

Feed Intakes

Fecal evaluation. Feed intakes were estimated using 3 strategies for evaluating fecal samples. The first strategy was obtaining EDM I from the ALK profile of the pooled fecal samples. The second and third strategies were based on the ALK profiles of the daily fecal samples. In the second strategy, intake estimates were obtained from the daily samples, which were then averaged; in the third strategy, the ALK concentrations of the daily samples were averaged, and those averaged values were then used to estimate intakes. The correlation between EDM I and ODMI for each fecal evaluation strategy is provided in Table 2.5. When the estimates were based on pooled fecal samples, EDM I were more strongly correlated to ODMI. In both years, effectively the same EDM I were obtained ($r = 1.0$) from the strategies where evaluations were based on the individual daily fecal samples.

Results from the regression of ODMI on EDM I as well as a paired two-tailed t-test for each fecal sampling technique are provided in Table 2.6 considering TDM as a single food. Regardless of fecal evaluation strategy, the intercept differed from zero ($P < 0.001$) and the slope differed from one ($P < 0.001$). Despite slopes and intercepts being significantly different from their expected values, evaluations based on the pooled fecal samples provided the best fit (Fig. 2.5).

The correlation between ODMI and EDM I were lower in the 2016 as compared to 2015 experiment. There were differences between the two experiments, particularly the number and ODMI of heifers. The range in ODMI in 2015 was 7.7 to 11.2 kg/d; in 2016, that range was 5.3 to 7.4 kg/d. This corresponds with the smaller BW of the 2016 as

compared to 2015 heifers. The lower DMI would be more prone to errors due to scaling. Additionally, there was a smaller sample size in the 2016 experiment, with 10 fewer animals. With less animals evaluated, a single animal's intake may have greater impact on the fit of the regression of ODMI on EDMI. Still, despite the lower correlations, based on a paired two-tailed t-test, EDMI obtained from pooled fecal samples with TMR as the diet did not differ from ODMI in 2016 ($P > 0.26$). However, this was not the case in 2015 ($P < 0.01$).

Loss of internal marker. The sensitivity of the estimates to potential losses in the amount of internal markers consumed by heifers was evaluated. The scenarios considered were for 100%, 97.5%, 95% and 90% of the supplement fed ingested. Slopes and intercepts from the regression of ODMI on EDMI are provided in Table 2.7, with the fit of the regressions plotted in Fig. 2.6. Those results are for a single food (TMR) with a pooled fecal sample evaluated. In both yr, as the percentage loss of marker increased, the slopes became numerically closer to one, while the intercept essentially did not change. Still the fits were poor, with the intercepts and slopes far different than their expected values ($P < 0.01$). The median EDMI consistently decreased as the percentage of marker lost increased, although variation in EDMI was unaffected (Fig. 2.7).

Diet composition. Diet compositions were estimated using the Bayesian method with 3 prior means (0.80, 0.70 and 0.60) reflecting the proportion of CS in the diet, and NNLS. In order to visualize the variation in the estimated CS composition of the diets obtained using these procedures, a boxplot was created for the 4 estimated CS (Fig. 2.8). The Bayesian estimates appeared to be more sensitive to differences in fecal ALK

concentrations of individual animals (in 2015, SD 0.06 kg/d; in 2016, SD 0.03 to 0.05 kg/d) than NNLS (in 2015, SD 0.02 kg/d; in 2016 SD, 0.02 kg/d). Generally, the estimated proportion of CS in the diet was higher with NNLS.

The EDM I obtained with the various strategies for estimating diet composition were regressed separately on ODMI. The slopes and intercepts from the fits are shown in Table 2.8. In both yr, and for all strategies, the intercepts ($P < 0.01$) and slopes ($P < 0.03$) differed from their expected values. The EDM I also were displayed as boxplots (Fig. 2.9). There was considerable variation in the DMI across strategies with, in general, greater variation among animals in the estimated as compared to observed values particularly for the 2016 experiment.

A Spearman's rank correlation was calculated to compare the ODMI and EDM I (Table 2.9). The ranking of observed and estimated intakes were in greater agreement in 2015 when assuming a composite diet ($r = 0.71$); in 2016, that concordance was higher when considering TMR as a single food ($r = 0.78$). The Bayesian method and NNLS performed more poorly.

Interactions

Intakes were estimated based on the $C_{31}:C_{32}$ and $C_{33}:C_{32}$ ratios in the two plants and feces. Variation in EDM I due to the ALK ratio used, and fecal evaluation and diet composition estimation method, were evaluated with ANOVA. There was no 3-way interaction among these factors ($P = 0.99$), but there was a 2-way interaction between diet composition ($P = 0.001$) and fecal evaluation ($P = 0.001$) method with the ALK ratio.

The EDM I based on ALK ratio and diet composition is shown in Fig. 2.10; a similar pattern was seen for the interaction with fecal evaluation method. Although the difference between EDM I for ALK ratio changed depending on the level of the other factor, in all cases the average EDM I obtained from C₃₃:C₃₂ were higher than those from C₃₁:C₃₂.

Variation in EDM I, derived from the average of the ALK ratios, explained by the fecal evaluation and diet composition method, and the percentage loss of internal marker, also was explored with ANOVA. There was no 3-way, and with one exception, no 2-way interactions among these factors ($P > 0.94$). The exception was presence of a fecal evaluation by diet composition interaction ($P = 0.03$). Despite the presence of the interaction, regardless of diet composition, EDM I obtained from pooled fecal samples were lower than those based on the daily fecal samples (Fig. 2.11). The same patterns were seen when the estimated intakes were expressed as a difference from the observed intakes. The 3 main effects defined substantial variation in EDM I ($P = 0.001$), and in EDM I expressed as a difference from ODMI ($P = 0.001$). The values reported in Table 2.10 illustrate the size of those effects. As a general conclusion, when considering the foodstuff offered as TMR – a single food – evaluated using a pooled fecal sample, the estimates of intake were most reliable.

DISCUSSION

Under confined (housed) conditions, the results of several studies have shown that feed intakes can be reliably estimated using ALK (Mayes et al., 1986, Dove and Olivan, 1998; Dove et al., 2002; Lewis et al., 2003). Our findings were less convincing.

Fecal Evaluation

Fecal collection is an important part of calculating intake. Intake estimates were not adequate regardless of the fecal collection approach adopted. Despite relatively high correlations between ODMI and EDM I (in 2015, $0.79 > r > 0.67$; in 2016, $0.65 > r > 0.61$), the slope and intercept of the regression of ODMI on EDM I differed from their expected values of one and zero, respectively ($P < 0.03$). However, using a pooled fecal sample decreased the number of samples evaluated, increased the correlation between ODMI and EDM I, and improved the reliability of the estimates (slope closer to one; intercept closer to zero).

Loss of internal marker

Intakes were underestimated in both yr, which may reflect the level of dosing with the internal marker. A presumed loss in the amount of internal marker ingested consistently decreased EDM I further. Incorporating the internal marker into a supplementary feed risks loss in the dose due to residual product left in storage bags and wastage during feeding. The potential loss of marker may have contributed to those underestimates.

Accuracy in the dosing strategy is clearly important to avoid both under and overestimating intakes. In 2015, the supplement had a target concentration of 2,524 mg/kg of C₃₂ in the supplement. However, when the C₃₂ concentration of retained samples of the supplement were measured, its concentration was 2,494 mg/kg (SD 58 mg/kg). On the other hand, in 2016 the supplement had a target dosage of 2,176 mg/kg of

C₃₂. When measured in retained samples of the supplement, its value was 2,337 mg/kg (SD 128 mg/kg). The target dose of C₃₂ was used to calculate EDMI. Unintended extraneous dosing with the internal marker would contribute to underestimation of intakes since the fecal concentration of C₃₂ would be increased. Overdosing, therefore, could have contributed to the underestimation of intakes in 2016. If dosing level was inconsistent across supplements – both high and low – the average of estimates would not be affected. However, such variability would likely introduce greater variation (noise) in EDMI among animals. Supplements should be randomly tested to confirm the level and consistency of the dosage of the internal marker.

Diet Composition

The concentrations of C₂₇, C₂₉, C₃₁ and C₃₃ in the CS and alfalfa differed. In CS, the concentrations of all 4 ALK were uniformly low (2 to 16 mg/kg). In the alfalfa, their concentrations were higher (above 11 mg/kg), particularly C₃₁ (210 to 388 mg/kg). This disparity likely confounded estimates of both diet compositions and feed intakes, especially since the TMR was predominantly CS (70%).

The NNLS and Bayesian methods were used to estimate diet compositions of individual animals. With NNLS, individual animals were deemed to have 75 to 82% CS in their diets. With the Bayesian methods, the diet compositions estimated across animals were, on average, similar to that of the mean of the respective prior distribution assumed (80, 70 or 60% CS). The information content of the ALK profiles of the two plants was, apparently, insufficient to regress the diet composition estimates for the extreme scenarios (80 and 60%) towards the 70% CS in the actual TMR. The Bayesian estimates

also suggested considerable variation in the diet composition of individual animals. The TMR was a homogenous food. Although some sifting of the ration was possible, its extent was likely limited.

The opportunity to discriminate incremental changes in the CS composition of diets was also tested theoretically by forming a continuum of mixtures with alfalfa based on their ALK concentrations. At best, 10% increments could be discerned. Given the ALK profiles of these 2 plants, precisely estimating their proportional contributions to the diet was challenging.

Reliable estimation of feed intake not only depends on consistent dosing of the internal marker and on diet composition, but also on the concentrations of the C_{31} and C_{33} in the feedstuff, and thereby feces. Intakes are determined from the ratios of the natural markers to the dosed marker (C_{32}). If there is too little C_{31} or C_{33} in the feces, intakes are less accurately estimated. As noted earlier, the CS had low concentrations of both these ALK while alfalfa had higher concentrations of C_{31} particularly. That difference also contributed to the sensitivity of EDM I to the estimates of diet composition. There was an interaction between the ALK ratio ($C_{31}:C_{32}$ or $C_{33}:C_{32}$) used to estimate intake and diet composition ($P = 0.001$), which illustrates the susceptibility of these estimates to the ALK concentrations of plants in the diet.

When ODMI were regressed on EDM I the slopes differed from one ($P < 0.03$) and intercepts differed from zero ($P < 0.01$). Despite that result, Spearman's rank correlations between ODMI and EDM I were still moderately high (for TMR, $r > 0.58$), which indicates that the EDM I were still indicative of ODMI. Even though estimates

were inexact, they in general ranked animals correctly, and thereby remain useful for genetic selection.

CONCLUSION

Intake estimates, considering the TMR as a single food, were best when based on a pooled rather than daily fecal samples. That is advantageous. Extracting and measuring the ALK concentrations of samples is costly. Minimizing the number of fecal samples needed to reliably estimate intakes is key to the pragmatic application of the plant-wax methodology.

Consistent daily dosing of an internal ALK marker facilitates reliable intake estimates. In this study, C₃₂ was mixed with a supplement, which was fed daily. Underdosing because of wastage of the supplement by animals was deemed a possibility. It appears that if dosing levels were less than planned, underestimation of intakes occurs. Even if dosing level was higher than intended, if not accounted for in the estimation process, intakes also would be underestimated. Furthermore, variable dosing levels would contribute to variation in estimated intakes. Strategies to ensure administration of a consistent dose of the internal ALK marker are requisite for reliable estimates of intake.

The robustness of intake estimates depends on the reliable evaluation of diet composition, and thereby the concentrations and profiles of the ALK of plants contributing to the diet. Based on visual inspection, there appeared to be relatively little sifting of the TMR. However, there appeared to be large variation, especially in the Bayesian approaches, in the percent CS estimated in the diet. This discrepancy could

reflect the plants themselves. Although the plant-wax profiles of CS and alfalfa were distinct, the concentrations of ALK in CS were uniformly low. Since CS was predominant in the diet, the quality of the estimates of diet may have been affected. Additionally, the 2 most significant marker are C_{31} and C_{33} , due to their use when estimating intakes. If the concentrations of C_{31} and C_{33} differ appreciably among plants, small differences in diet composition may significantly impact EDMl.

Incorporation of additional plant-waxes markers, such as long-chain alcohols, may improve matters. If they lead to more accurate prediction of diet composition, intake estimates would also become more reliable. Still, studies intended to assess the use of plant-waxes to estimate diet composition and intakes need to consider the inherent characteristics of the plants themselves. As the number of plants species contributing to a diet increases, delineating their profiles becomes increasingly important.

Although EDMl did not perfectly reflect ODMI, they were sufficiently robust to rank animals on intake. Clearly, refinements of the tool remain. However, our results suggest that plant-wax markers provide a method to delineate feed efficiencies among animals within livestock breeding programs.

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Table 2.1 Chemical composition of the total mixed ration (TMR) and its component plants

Year	Foodstuff	ADF, % DM	NDF, % DM	CP, % DM	Lignin, % DM
2015	Alfalfa	34.6	47.0	22.8	9.32
	Corn silage	16.9	24.5	8.1	2.31
	TMR ¹	31.1	44.6	11.4	6.65
2016	TMR ¹	37.6	47.1	9.7	7.67

¹69.8% corn silage, 30% ground alfalfa hay and 0.2% salt, as DM.

Table 2.2 Mean (SD) alkane concentrations (mg/kg) for the total mixed ration (TMR), its component plants, and supplement

Year	Foodstuff	C ₂₇	C ₂₉	C ₃₁	C ₃₃	C ₃₂
Main Diet						
2015	Alfalfa	11.14	61.46	210.15	16.81	6.58
		(0.29)	(0.86)	(5.09)	(0.32)	(0.04)
	Corn silage	1.90	5.45	8.92	5.76	0.00
		(0.03)	(0.06)	(0.11)	(0.11)	(0.00)
2016	TMR ¹	6.38	28.02	80.22	10.86	0.00
		(0.21)	(1.23)	(1.49)	(0.38)	(0.00)
	Alfalfa ²	21.07	108.06	387.65	30.26	11.79
		(0.78)	(4.55)	(14.22)	(0.46)	(0.33)
	Corn silage ²	3.46	8.98	15.69	9.95	1.33
		(0.26)	(0.48)	(0.68)	(0.69)	(0.03)
	TMR ¹	7.54	44.97	107.58	17.45	1.85
		(0.44)	(2.21)	(4.61)	(1.94)	(1.76)
Supplement						
2015	Supplement ³	2.98	5.60	11.42	8.14	2494.22
		(0.10)	(0.16)	(0.24)	(0.07)	(58.34)
2016	Supplement ⁴	11.04	9.36	13.16	5.91	2303.64
		(1.51)	(0.28)	(0.29)	(0.46)	(98.95)

¹69.8% corn silage, 30% ground alfalfa hay and 0.2% salt, as DM.

²Samples collected in 2015, but extracted by a second technician and used for 2016 analysis.

³Supplement in 2015 contained 0.23 kg of Producer's Pride All Stock Sweet Feed (Tractor Supply Company), 20 g of soybean hulls and 625 mg of *n*-dotriacontane (C₃₂).

⁴Supplement in 2016 contained 0.23 kg of Producer's Pride Calf Starter (Tractor Supply Company) and 495 mg of *n*-dotriacontane (C₃₂).

Table 2.3 Prediction of percentage corn silage in the total mixed ration in each year based on the Bayesian method and non-negative least squares (NNLS)

Year	Bayesian ¹	SD	NNLS	SD
2015	64	0.002	76	0.01
2016	74	0.01	86	0.02

¹Assumed a Gaussian prior distribution with prior mean of, proportionally, 0.70 for corn silage, and a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

Table 2.4 Orthogonal contrasts of theoretical diet mixtures consisting of varying percentages of corn silage with diet composition estimated using non-negative least squares (NNLS) and a Bayesian method.

Method	Contrast 1	Contrast 2	Estimate	SE	<i>P</i> -value
NNLS	100-55	45-0	0.55	0.02	0.001
	100-80	75-55	0.25	0.03	<0.001
	100-95	90-85	0.10	0.05	0.06
	75-70	65-60	0.10	0.05	0.06
	100	95	0.05	0.07	0.50
	90	85	0.05	0.07	0.50
	75	70	0.05	0.07	0.49
	65	60	-0.05	0.07	0.49
	45-25	20-0	0.23	0.03	<0.001
	45-40	35-30	0.10	0.05	0.05
	45	40	0.05	0.07	0.49
	35	30	0.05	0.07	0.49
	20	15	0.05	0.07	0.48
	5	0	0.01	0.07	0.87
Bayesian ¹	100-55	45-0	0.55	0.02	<0.001
	100-80	75-55	0.25	0.02	<0.001
	100-95	90-85	0.09	0.04	0.01
	75-70	65-60	0.10	0.04	0.01
	100	95	0.04	0.05	0.48
	90	85	0.05	0.05	0.32
	75	70	0.05	0.05	0.32
	65	60	-0.05	0.05	0.33
	45-25	20-0	0.25	0.02	<0.001
	45-40	35-30	0.10	0.04	0.01
	45	40	0.05	0.05	0.33
	35	30	0.05	0.05	0.33
	20	15	0.05	0.05	0.32
	5	0	0.03	0.05	0.51

¹Assumed a Gaussian prior distribution with prior mean of, proportionally, 0.70 for corn silage, and a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

Table 2.5 Correlation between observed and estimated DMI across fecal evaluation strategies in the 2015 (above diagonal) and 2016 (below diagonal) experiment

	Observed	Estimated		
		Pool ¹	Daily avg. ²	Math ³
Observed		0.79	0.69	0.67
Pool	0.65		0.74	0.72
Daily avg.	0.51	0.85		1.00
Math	0.56	0.86	1.00	

¹ Intakes estimated from the *n*-alkane contents of a single fecal sample formed by combining collections from 5 consecutive d.

² Intakes estimated from the *n*-alkane contents of separate fecal samples collected 5 consecutive d, and then averaging the intake estimates.

³ Intakes estimated from averaging the *n*-alkane contents of fecal samples collected 5 consecutive d, then estimating intake from that average.

Table 2.6 Parameter estimates for the regression of observed on estimated DMI (kg DM/d) for intakes estimated using 3 fecal evaluation methods, with the diet defined as the total mixed ration

Year	Fecal method ¹	β_0 ² (SE)		β_1 ³ (SE)		r^2	<i>P</i> -value		
							β_0 ⁴ =0	β_1 ⁵ =1	T-test ⁶
2015	Pooled	4.18	(0.80)	0.60	(0.09)	0.62	<0.001	<0.001	<0.001
	Daily avg.	5.34	(0.85)	0.39	(0.08)	0.47	<0.001	<0.001	<0.001
	Math	5.39	(0.88)	0.39	(0.09)	0.45	<0.001	<0.001	<0.001
2016	Pooled	3.27	(0.90)	0.48	(0.15)	0.42	<0.001	0.04	0.26
	Daily avg.	4.16	(0.89)	0.35	(0.16)	0.26	<0.001	<0.001	0.01
	Math	3.94	(0.87)	0.39	(0.15)	0.32	<0.001	<0.001	0.01

¹ Fecal methods were: Pooled – intakes estimated from the *n*-alkane contents of a single fecal sample formed by combining collections from 5 consecutive d; Daily avg. – intakes estimated from averaging the *n*-alkane contents of fecal samples collected 5 consecutive d, then estimating intake from that average; Math – intakes estimated from the *n*-alkane contents of separate fecal samples collected 5 consecutive d, and then averaging the intake estimates.

² β_0 , intercept (kg/d).

³ β_1 , slope (kg/d per kg/d).

⁴ $\beta_0 = 0$, test of intercept equal to zero.

⁵ $\beta_1 = 1$, test of slope equal to one.

⁶ T-test, a paired t-test between observed and estimated DMI.

Table 2.7 Parameter estimates for the regression of estimated on observed DMI (kg DM/d) for intakes estimated assuming 4 percentage losses of internal marker, with the diet defined as the total mixed ration for pooled fecal samples

Year	Marker loss (%)	β_0^1 (SE)		β_1^2 (SE)		r^2	<i>P</i> -value	
							$\beta_0^3 = 0$	$\beta_1^4 = 1$
2015	100.0	4.17	(0.80)	0.58	(0.09)	0.63	<0.001	<0.001
	97.5	4.18	(0.80)	0.60	(0.09)	0.62	<0.001	<0.001
	95.0	4.18	(0.80)	0.61	(0.10)	0.62	<0.001	<0.001
	90.0	4.17	(0.80)	0.65	(0.10)	0.62	<0.001	<0.001
2016	100.0	3.27	(0.90)	0.48	(0.15)	0.42	<0.01	<0.01
	97.5	3.28	(0.90)	0.49	(0.15)	0.42	<0.01	0.01
	95.0	3.27	(0.90)	0.51	(0.16)	0.42	<0.01	0.01
	90.0	3.27	(0.90)	0.53	(0.17)	0.42	<0.01	0.01

¹ β_0 , intercept (kg/d).

² β_1 , slope (kg/d per kg/d).

³ $\beta_0 = 0$, test of intercept equal to zero.

⁴ $\beta_1 = 1$, test of slope equal to one.

Table 2.8 Parameter estimates for the regression of observed on estimated DMI (kg DM/d) for intakes estimated when diet composition was obtained using a Bayesian approach or non-negative least squares

Year	Method ¹	β_0 ² (SE)		β_1 ³ (SE)		r^2	<i>P</i> -value	
							β_0 ⁴ =0	β_1 ⁵ =1
2015	Bayes 1	7.14	(0.83)	0.16	(0.06)	0.21	<0.001	<0.001
	Bayes 2	6.31	(0.94)	0.27	(0.09)	0.29	<0.001	<0.001
	Bayes 3	5.82	(0.97)	0.38	(0.11)	0.34	<0.001	<0.001
	NNLS	4.79	(0.84)	0.34	(0.06)	0.54	<0.001	<0.001
	Composite	4.33	(0.78)	0.46	(0.07)	0.62	<0.001	<0.001
	TMR	4.17	(0.80)	0.58	(0.09)	0.63	<0.001	<0.001
2016	Bayes 1	3.67	(0.96)	0.33	(0.13)	0.32	<0.01	<0.001
	Bayes 2	3.62	(1.08)	0.42	(0.18)	0.28	<0.01	0.01
	Bayes 3	3.78	(1.15)	0.47	(0.23)	0.23	<0.01	0.03
	NNLS	2.30	(0.73)	0.44	(0.08)	0.67	<0.01	<0.001
	Composite	3.16	(0.87)	0.50	(0.15)	0.46	<0.01	<0.01
	TMR	3.27	(0.90)	0.48	(0.15)	0.42	<0.01	<0.01

¹Method of estimation of diet composition: Bayes – Bayesian method with 3 prior means (0.8, 0.7 and 0.6 for Bayes 1, 2 and 3, respectively) reflecting the proportion of CS in the diet; NNLS – Non-negative least squares; Composite – Mathematical composite assuming 70% corn silage and 30% alfalfa in the diet. Evaluations were for a pooled fecal sample with no loss in internal marker.

² β_0 , intercept (kg/d).

³ β_1 , slope (kg/d per kg/d).

⁴ $\beta_0 = 0$, test of intercept equal to zero.

⁵ $\beta_1 = 1$, test of slope equal to one.

Table 2.9 Spearman's rank correlation between observed and estimated DMI based on various methods¹ for determining diet composition in the 2015 (above diagonal) and 2016 (below diagonal) experiment

	Observed	Bayes 1	Bayes 2	NNLS	TMR	Composite
Observed		0.42	0.42	0.65	0.71	0.70
Bayes 1	0.55		0.97	0.78	0.58	0.58
Bayes 2	0.59	0.99		0.80	0.63	0.63
NNLS	0.78	0.88	0.89		0.94	0.94
TMR	0.58	0.84	0.85	0.85		1.00
Composite	0.60	0.86	0.86	0.87	1.00	

¹Diet composition was estimated as: Bayesian method with 3 prior means (0.8 and 0.7 for Bayes 1 and 2, respectively) reflecting the proportion of CS in the diet; NNLS – Non-negative least squares; TMR – total mixed ration as a single food; and, Composite – Mathematical composite assuming 70% corn silage and 30% alfalfa in the diet. Evaluations were for a pooled fecal sample with no loss in internal marker.

Table 2.10 Comparison of main effects for observed DMI subtracted by estimated DMI for each diet composition and fecal evaluation method, and loss of marker percentage

Factor (effect)	Level	Estimate	(SE)
Diet composition ¹	Bayes 1	-2.94	(1.36)
	Bayes 2	-0.80	(1.36) ^a
	Composite	-0.69	(1.36) ^a
	NNLS	-3.38	(1.36)
	TMR	0.57	(1.36)
Fecal evaluation ²	Pool	-0.78	(1.36)
	Daily avg.	-1.88	(1.36)
	Math	-1.69	(1.36)
Marker loss (%) ³	100.0	-1.89	(1.36)
	97.5	-1.64	(1.36)
	95.0	-1.39	(1.36)
	90.0	-0.88	(1.36)

¹Method of estimation of diet composition: Bayes – Bayesian method with 3 prior means (0.80, 0.70 and 0.60 for Bayes 1, 2 and 3, respectively) reflecting the proportion of CS in the diet; NNLS – Non-negative least squares; Composite – Mathematical composite assuming 70% corn silage and 30% alfalfa in the diet.

² Fecal methods: Pooled – intakes estimated from the *n*-alkane contents of a single fecal sample formed by combining collections from 5 consecutive d; Daily Avg. – intakes estimated from averaging the *n*-alkane contents of fecal samples collected 5 consecutive d, then estimating intake from that average; Math – intakes estimated from the *n*-alkane contents of separate fecal samples collected 5 consecutive d, and then averaging the intake estimates.

³ Assuming 4 percentage internal marker consumptions: 100%, 97.5%, 95% and 90%.

^a Estimates within a factor with different superscripts do not differ ($P < 0.05$).

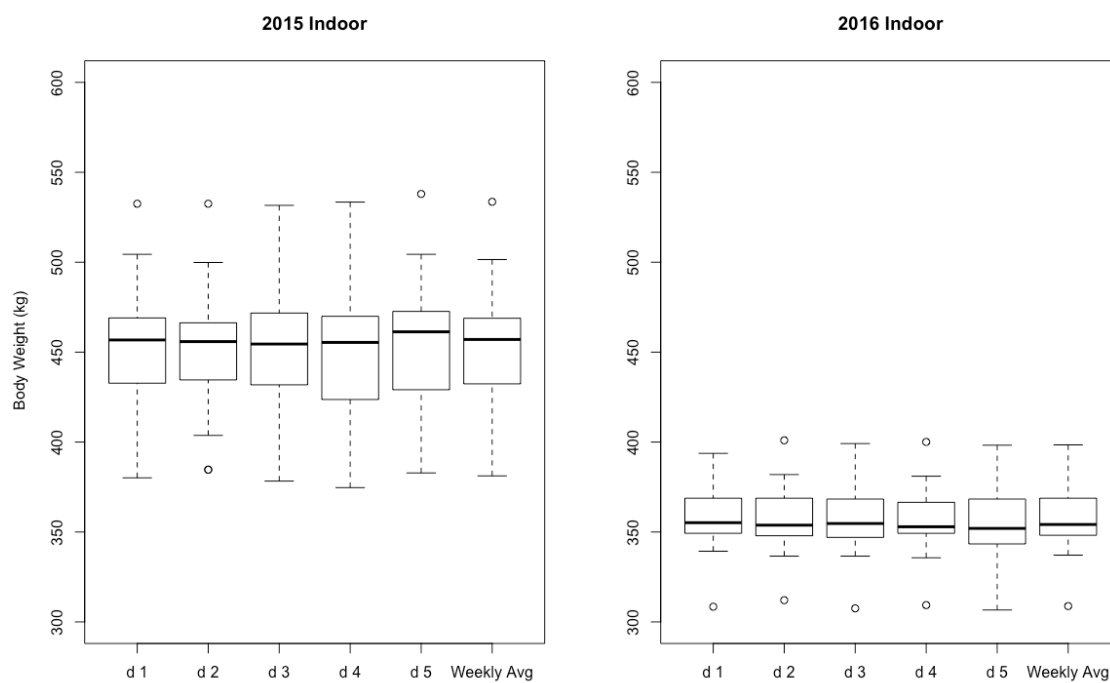


Fig. 2.1 Boxplot of the daily and corresponding average weekly BW for the 2015 and 2016 experiments.

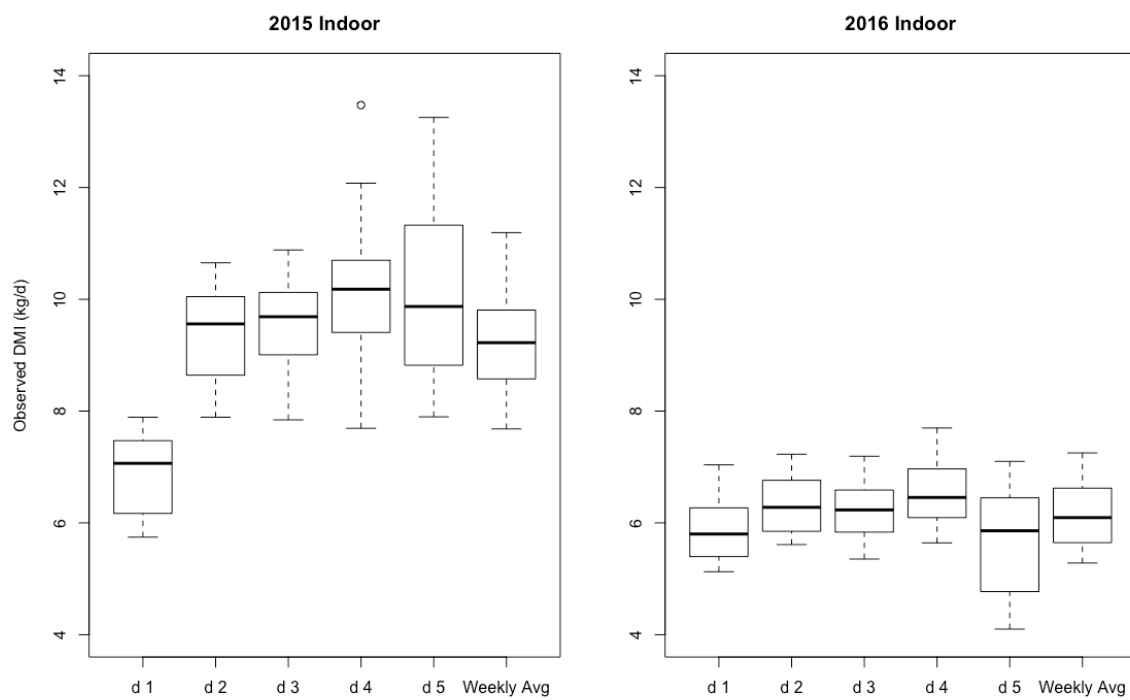


Fig. 2.2 Boxplot of the observed daily and corresponding weekly average DMI for the 2015 and 2016 experiment.

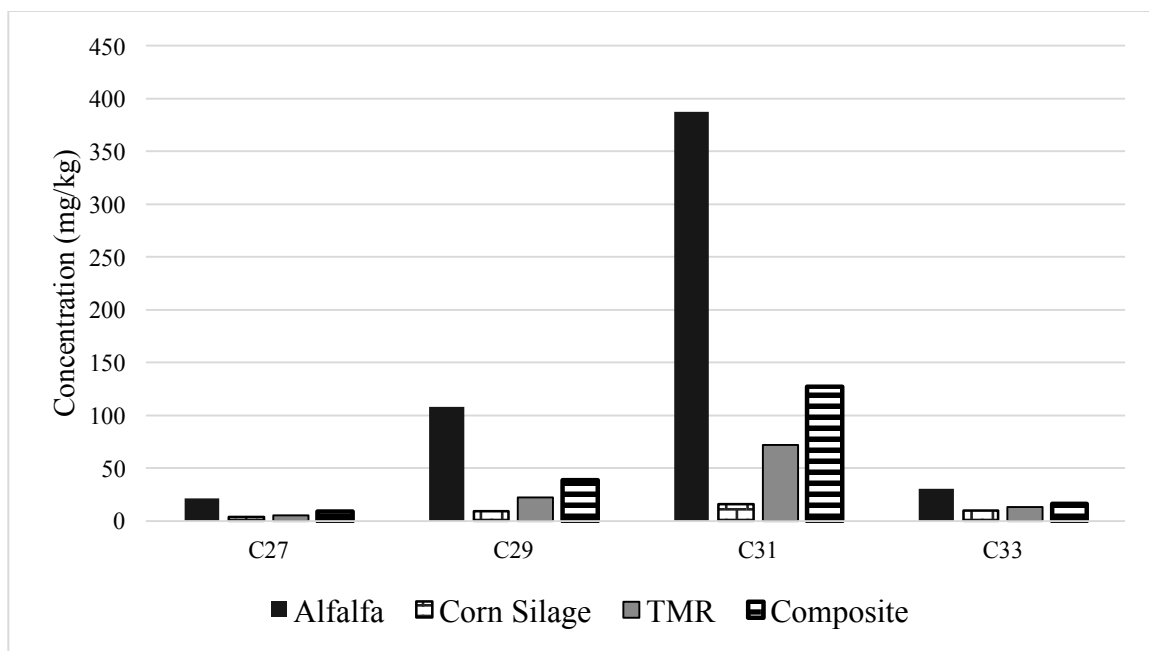


Fig. 2.3 Mean alkane concentrations (mg/kg) for the total mixed ration (TMR), its component plants and the composite (70% corn silage and 30% alfalfa).

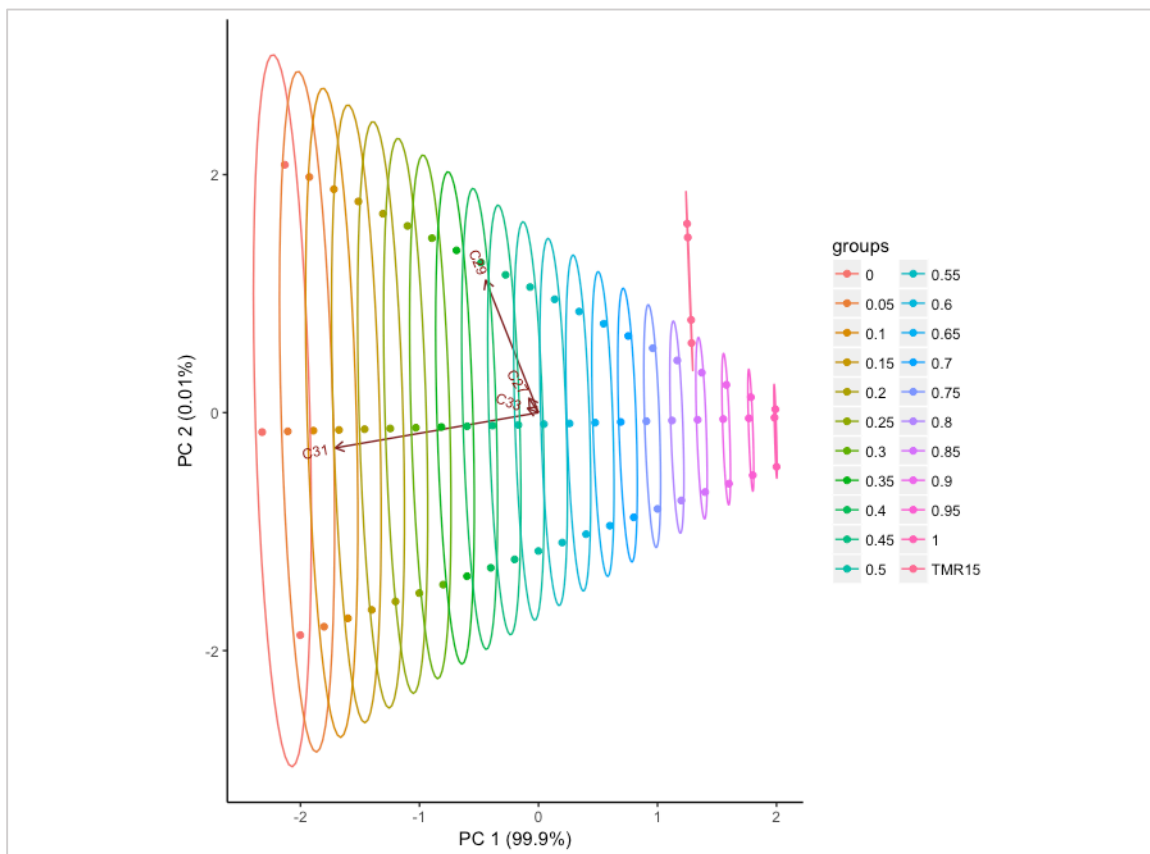


Fig. 2.4 Principal component analysis of theoretical mixtures of corn silage and alfalfa containing, proportionally, from 1.0 to 0.0 of each plant, at 0.05 increments, derived from the concentrations of C_{27} , C_{29} , C_{31} and C_{33} *n*-alkanes in the pure plants. For comparison, the measured *n*-alkane profile of the total mixed ration in 2015 also was included.

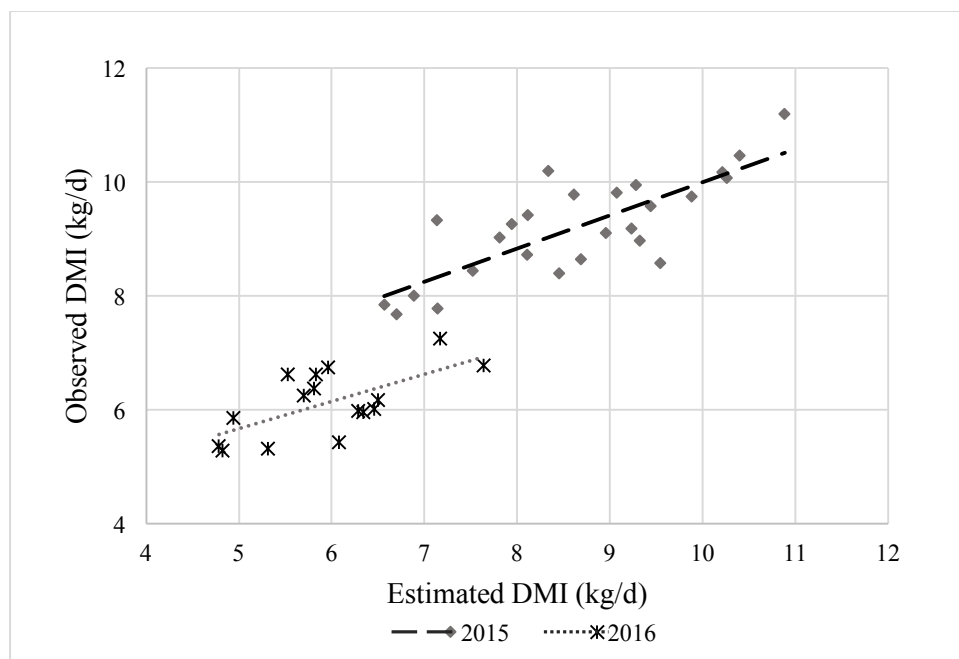


Fig. 2.5 Plot of the regression of estimated DMI, obtained from pooled fecal samples, on observed DMI when considering the total mixed ration as a single food.

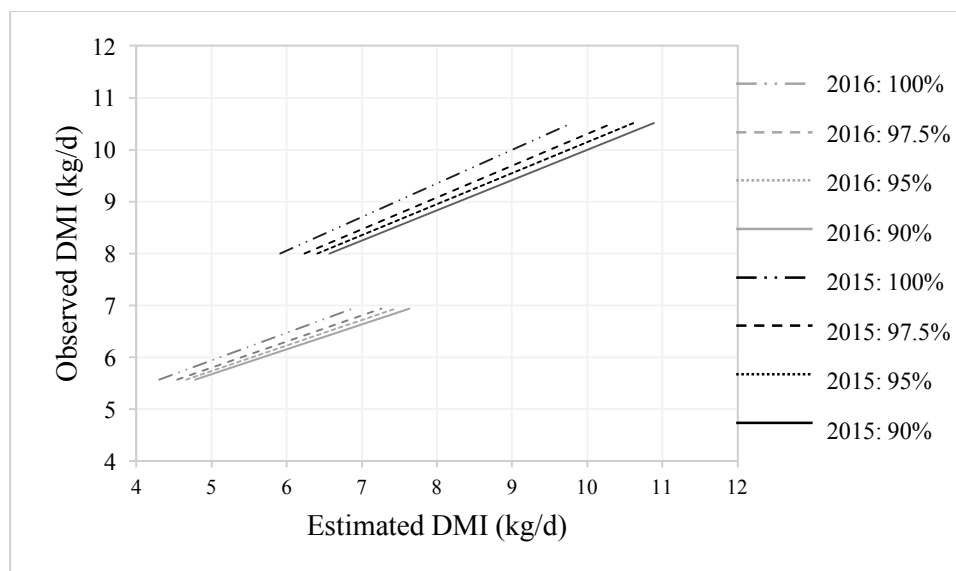


Fig. 2.6 Plot of the regression of observed on estimated DMI, obtained from various percentages of internal marker loss for the 2015 and 2016 experiment. The estimated values were based on a pooled fecal sample and considering the total mixed ration as a single food.

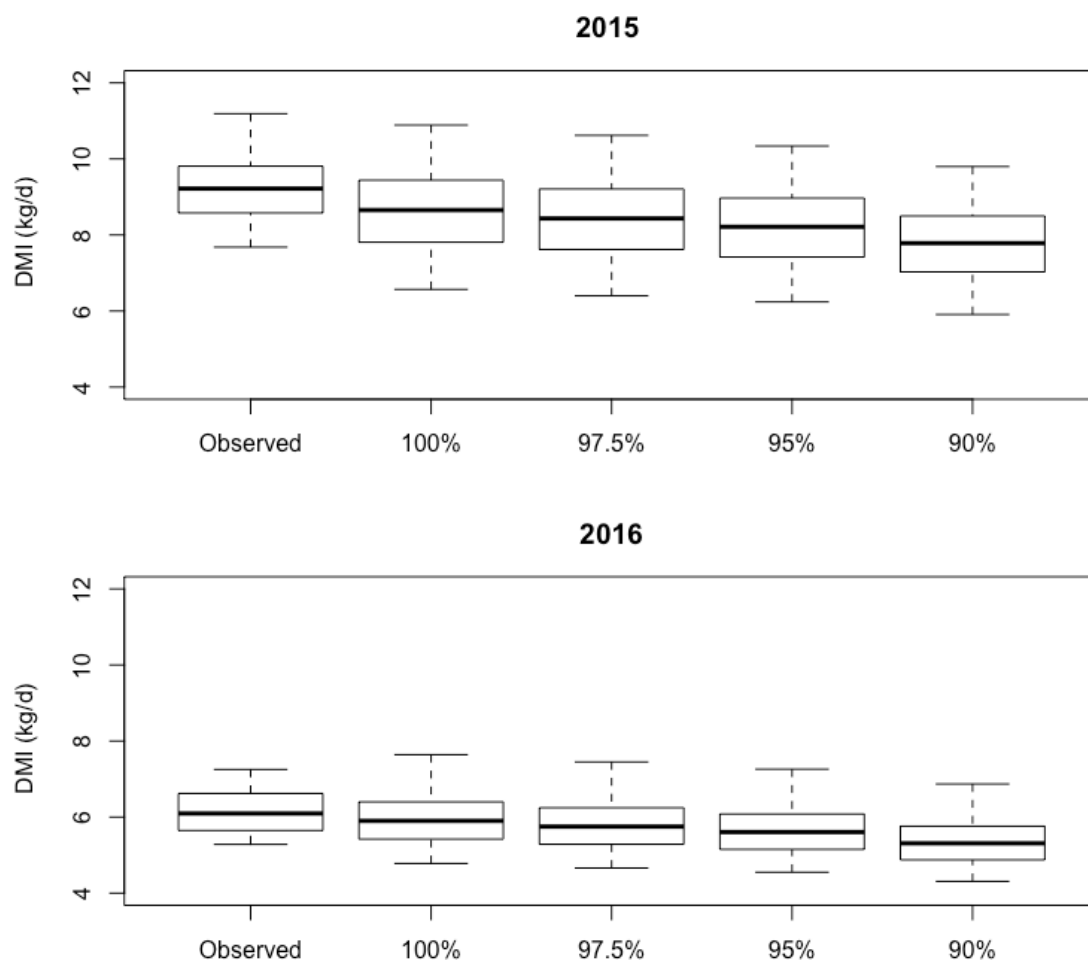


Fig. 2.7 Boxplots of the estimated DMI for various percentage losses of internal marker for the 2015 and 2016 experiment from pooled fecal samples and considering the total mixed ration as a single food.

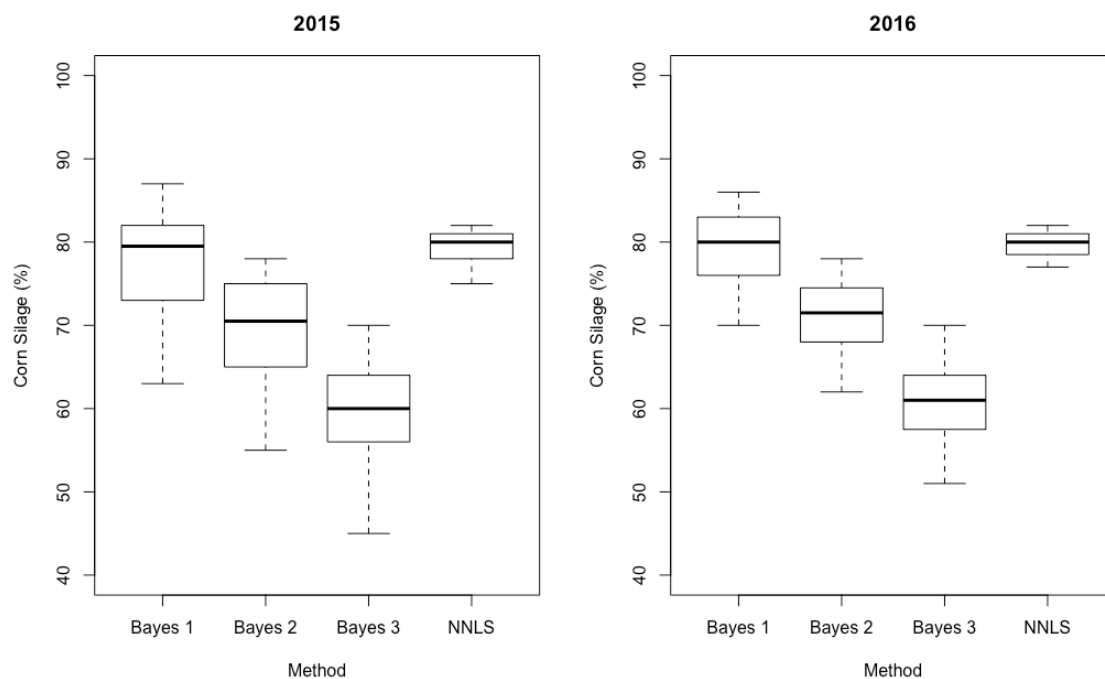


Fig. 2.8 Diet composition estimated using the Bayesian method, with 3 prior means (0.80, 0.70 and 0.60 for Bayes 1, 2 and 3, respectively) reflecting the proportion of CS in the diet, and non-negative least squares (NNLS) for the 2015 and 2016 experiment.

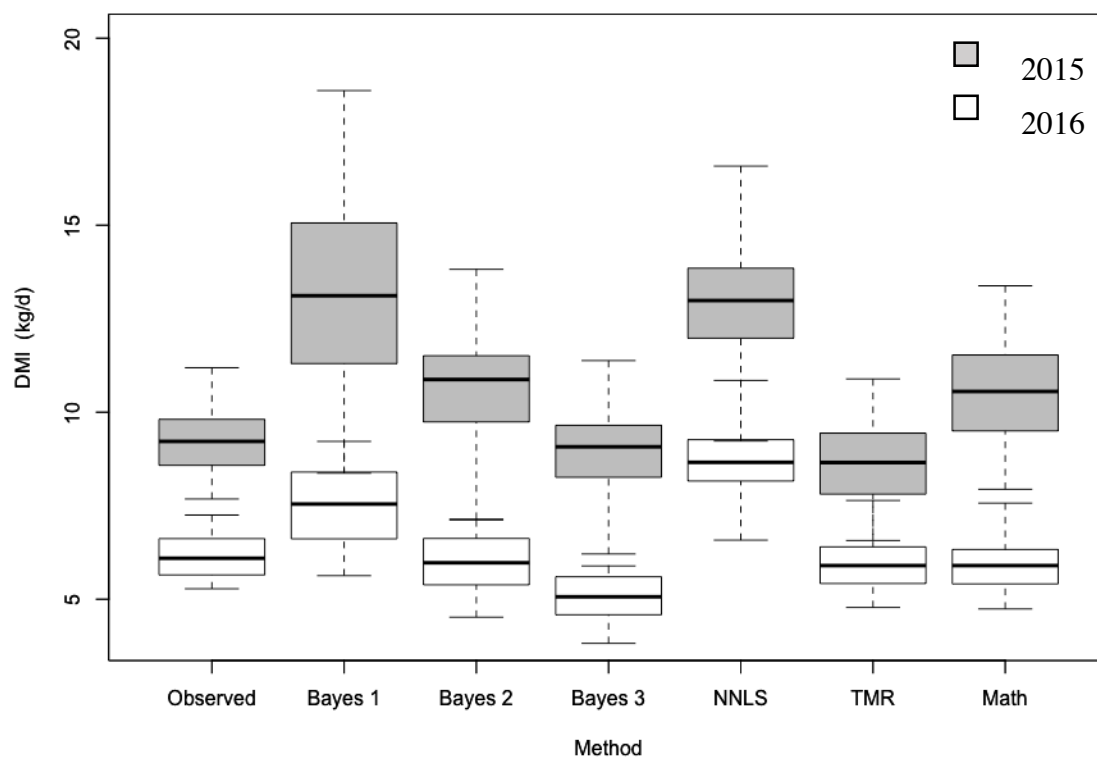


Fig. 2.9 Boxplots of observed and estimated DMI, where estimated values were derived using the various approaches for determining diet composition, for the 2015 and 2016 experiment. Diet composition was estimated as: Bayesian method with 3 prior means (0.80, 0.70 and 0.60 for Bayes 1, 2 and 3, respectively) reflecting the proportion of CS in the diet; NNLS – Non-negative least squares; TMR – total mixed ration as a single food; and, Composite – Mathematical composite assuming 70% corn silage and 30% alfalfa in the diet. Evaluations were for a pooled fecal sample with no loss in internal marker.

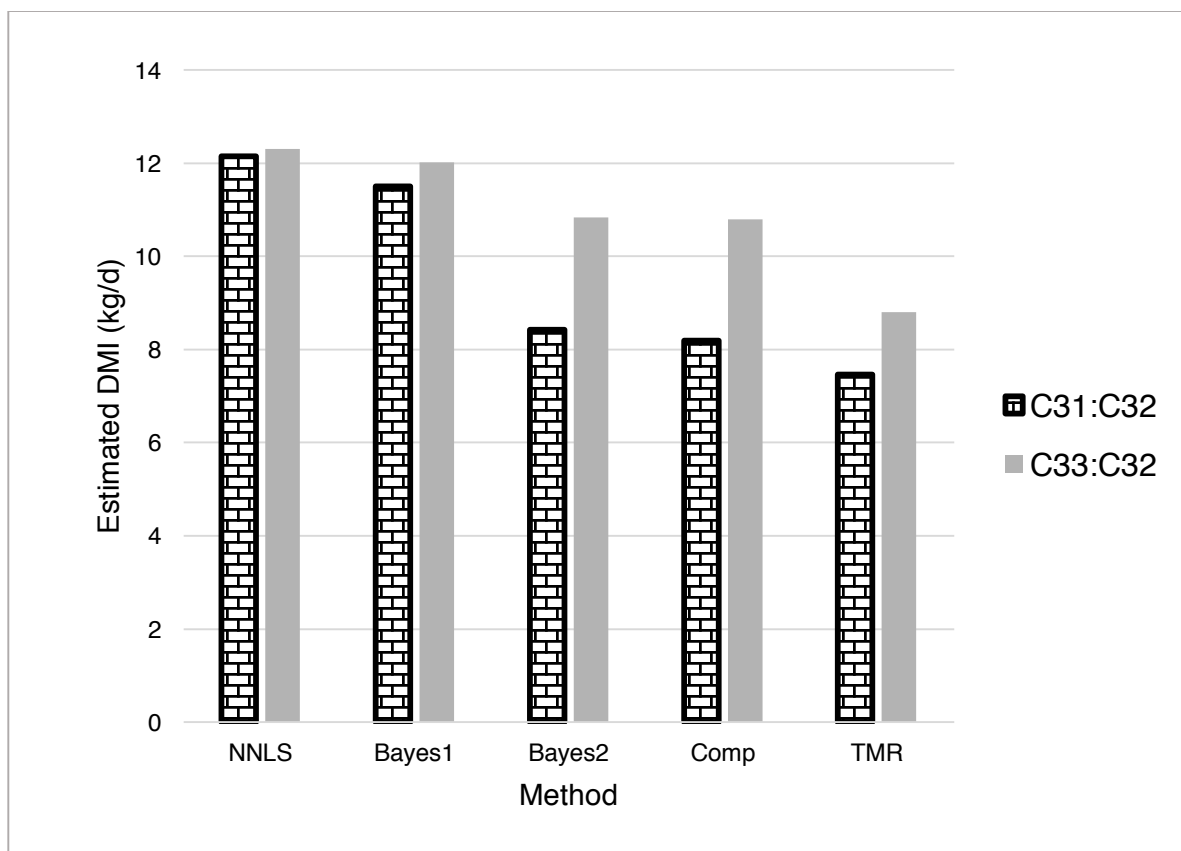


Fig. 2.10 A histogram of marker ratio ($C_{33}:C_{32}$ or $C_{31}:C_{32}$) and diet composition method on estimated DMI for the 2015 and 2016 experiment. There was an interaction between marker ratio and diet composition method ($P = 0.001$). Diet compositions were based on: a Bayesian method with 2 prior means reflecting the proportion of CS in the diet (0.80 and 0.70 for Bayes1 and Bayes2, respectively); non-negative least squares (NNLS); mathematical composite (Comp.) assuming 70% corn silage and 30% alfalfa in the diet; and, total mixed ration (TMR).

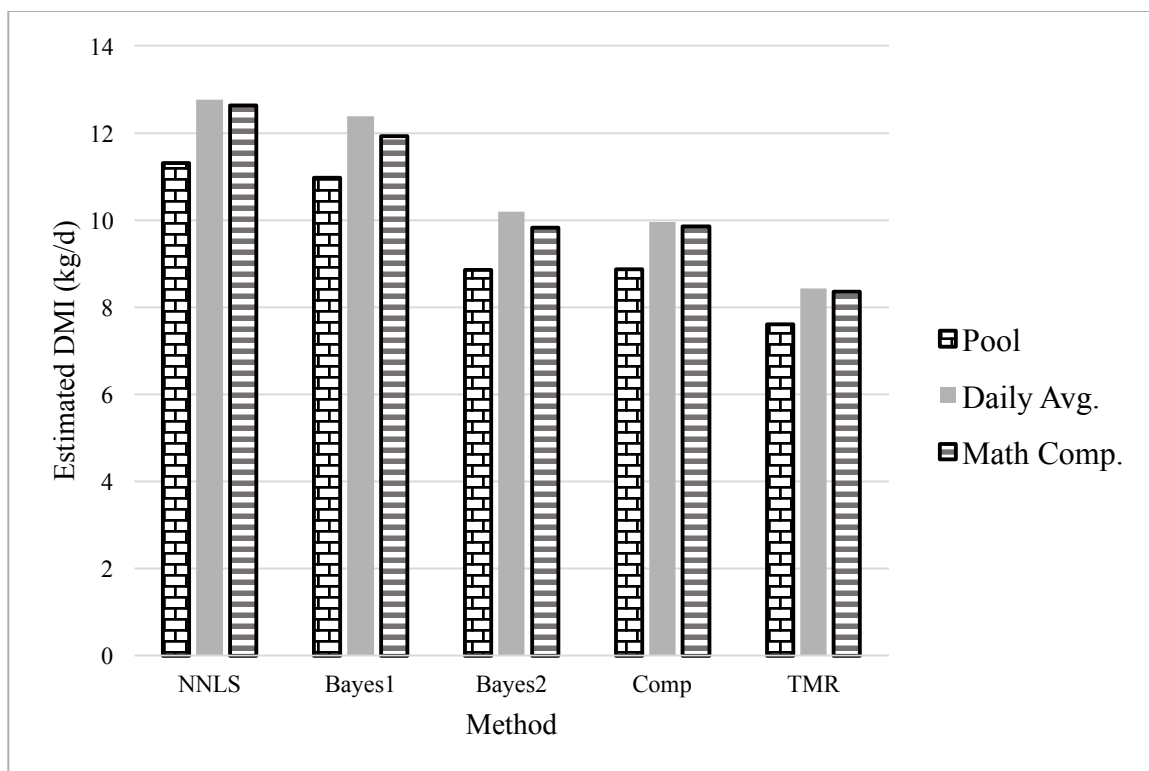


Fig. 2.11 A histogram of estimated DMI based on diet composition and fecal using the average intake estimated from markers $C_{31}:C_{32}$ and $C_{31}:C_{33}$ for the 2015 and 2016 experiment. There was an interaction between fecal and diet composition methods ($P = 0.03$). Diet compositions were based on: a Bayesian method with 2 prior means reflecting the proportion of CS in the diet (0.80 and 0.70 for Bayes1 and Bayes2, respectively); non-negative least squares (NNLS); mathematical composite (Comp.) assuming 70% corn silage and 30% alfalfa in the diet; and, total mixed ration (TMR). The fecal evaluation methods considered were: *n*-alkane contents of a single fecal sample formed by combining collections from 5 consecutive d (Pool); averaging the *n*-alkane contents of fecal samples collected 5 consecutive d, then estimating intake from that average (Daily avg.); and, the *n*-alkane contents of separate fecal samples collected 5 consecutive d, and then averaging the intake estimates (Math).

Chapter III: Evaluation of *n*-Alkanes to Estimate Dry Matter Intake in Grazing Cattle

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ABSTRACT: Determining intake of cattle in a grazing setting can be difficult due to the diversity of plants. A technique based on *n*-alkanes (**ALK**) has been shown to reliably estimate DMI (**EDMI**) and diet composition in controlled (indoor) settings. The question remains whether this method can be used in range and pasture systems due to the botanical complexity of grazing diets. Grazing experiments were conducted in 2015 (3 sequential studies) and 2016 (4 sequential studies). The 2015 experiment included 18 heifers with 6 different heifers grazing new yet adjoining pastures each study. The 2016 experiment included 12 heifers split between 2 adjacent pastures, which they grazed continuously throughout the 4 studies. Heifers were dosed for 12 d with an internal ALK marker (C_{32}) mixed in a supplement. Fecal samples were collected the final 5 d of dosing, with plant samples collected on d 1 and 5 of the fecal collection period. Plant and fecal ALK concentrations were determined. We had 4 objectives: (i) to determine if the plants found in predominantly smooth brome grass (**SB**) pasture could be delineated based on their ALK profiles; (ii) to estimate diet compositions from the ALK concentrations of plants and fecal samples; (iii) to estimate intakes using additional information garnered from an internal ALK marker; and, (iv) to compare intakes across sequential grazing studies. The predominant plant species were SB and Kentucky bluegrass (**KBG**). Based on their ALK profiles, 10% tradeoffs in the SB and KBG composition of diets could be delineated ($P < 0.02$). Intakes obtained based on $C_{33}:C_{32}$ ratios appeared to be substantially over and underestimated ($2.34 \text{ kg} < \text{EDMI} < 37.3 \text{ kg/d}$), which may be a consequence of the concentrations of C_{33} in SB, the predominant plant species grazed. When EDM I from the sequential studies were correlated with observed DMI obtained from a housed study, there was little consistency ($0.09 < r < 0.79$), perhaps reflecting

external factors on intake. External factors include weather, forage availability and nutrient requirements unique to a grazing environment. When comparing EDM I across outdoor studies, they were less variable; in 3 of the 4 studies in 2016, cattle intakes did not change ($P > 0.15$). Despite the lack of fine demarcations, sensible intakes were obtained in a grazing setting. The plant-wax methodology therefore shows promise for commercial use.

Key words: cattle, diet composition, estimation, grazing intake, *n*-alkanes, plant-wax markers

INTRODUCTION

Cattle performance is affected by a number of external forces including environmental stressors and nutrition, which impact intake and diet composition. Plant waxes, found in plants and feces, have been used to estimate both entities (Dove and Charmley, 2008; Oliveira et al., 2008). Such application of plant-wax markers could potentially improve cattle selection and management. By allowing producers to better predict which animals have lower maintenance costs, and by determining which plants cattle prefer to graze, both animal selection and ecosystem management could be improved.

Grazing animals have an opportunity to consume preferred plants, which increases the complexity of estimating their dietary composition. The number of plant species that can be delineated is determined by both the plant-wax markers available and variability in their profiles (Bugalho et al., 2004). With the wide variety of plants seen in most landscapes, it is challenging to accurately discern the composition of diets, which effects the estimation of intakes (Lewis et al., 2016).

This study was designed to assess the reliability of using *n*-alkanes (**ALK**), a key component of plant-waxes, to estimate diet selection and intake in cattle in a grazing setting. The ALK are saturated hydrocarbons found in the cuticular wax of most foliage. Four aspects of the estimation process were considered: (i) determining if the plants found in predominantly smooth brome grass pastures could be delineated based on their ALK profiles; (ii) estimating diet compositions from the ALK concentrations of the plants and individual animal fecal samples; (iii) estimating these animals intakes using

additional information garnered from an internal ALK marker; and, (iv) comparing intakes across sequential grazing studies. Environmental characteristics, including weather and plant biomass, were integrated into the definition of the grazing system.

MATERIALS AND METHODS

This study was conducted at the Roman L. Hruska U. S. Meat Animal Research Center (USMARC), Clay Center, NE. Animals were raised in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010), and their care was approved by the both the USMARC and University of Nebraska-Lincoln Animal Care and Use Committees.

n-Alkane Dosing

The *n*-alkane C₃₂ (Dotriacontane, CAS# 544-85-4; Minakem, SAS, France) was used as an internal marker. Dosing levels were based on the BW of the heaviest animal within a study, which ranged from 560 mg/d (430 kg) to 660 mg/d (510 kg). The internal markers were added directly to 0.23 kg of a feed supplement, which was a mix of equal weights of a calf starter feed (Producer's Pride Calf Starter, Tractor Supply Company) and either crimped oats or cracked corn. The grains were colored with a food dye by mixing 20 g of a dye with 1.13 kg of a grain. In the first year of outdoor studies the dyes used were FD&C Blue 1, FD&C Red 40 or FD&C Yellow 5; in the second, year FD&C Green#3, FD&C Red #3 or FD&C Yellow #5 were instead used. The combinations of a grain and food dye resulted in 6 varieties of the supplement within a year. Rutter et al. (2012) found that fecal pats of cattle fed similarly dyed supplements could be clearly

distinguished after 3 d. In addition, 60 g of liquid molasses was added to encourage consumption of the entirety of the supplement.

Experiment

Two sets of outdoor experiments were conducted between May and September in 2015 and 2016.

Exp. 1. Twenty-four heifers were placed in a smooth brome grass (**SB**; *Bromus inermis*) dominant pasture for 21 d. These heifers had recently completed a housed study in which their intakes of a forage-based diet (70% corn silage and 30% ground alfalfa hay) had been measured. Six heifers were then randomly assigned to 1 of 4 sequential grazing studies. Although 4 outdoors studies were originally planned, only 3 were conducted. This was due to the longer timeframe needed for the first group of animals to adapt to the outdoor feeding system used (described later). The sampling periods for these 3 studies occurred July 20 to 31 (study 1), August 17 to 28 (study 2), and September 14 to 25 (study 3), 2015. Weather information was obtained from a personal weather station (ID: KNEHARVA2), which was located at latitude N 40 ° 37 ' 4", longitude W 98 ° 5 ' 38 ", and elevation 552 m.

Prior to the start of a study, the 6 heifers were placed in a roughly 197 by 75 m pasture of predominately SB (Fig. 3.1) with a custom-built, portable Calan Broadbent Feeding System (**PCFS**; American Calan, Northwood, NH). The PCFS consisted of 3 doors installed on each side (6 doors in total) of a 183 cm (width) by 436 cm (length) hay wagon (Pequea Wagon Gears Model 806, Pequea Machine Inc., New Holland, PA). The

unit included a steel frame supporting an overhead tarp and a pair of solar panels (Solarland SLP100-12U, Wholesale Solar, Ontario, CA), which charged two 12-volt batteries powering the doors. The PCFS was vertically aligned with the water trough and approximately 610 cm away. The tongue (tow hitch) of the unit was positioned opposite to the water trough.

Throughout the adaptation period feed doors were secured open with a limited amount of the supplement provided daily at 8:00 a.m. Typically 7 d were allowed for the heifers to become familiar with the feeding system. The exception was the first study where a longer adaptation period was required (21 d). Animals were carefully monitored to confirm that they were accessing the PCFS (visually observed at least twice daily), and their preference for a door noted.

Following the adaptation period, on d 1 of a study period, the 6 heifers were weighed, fitted with keys, and moved to a new 2-acre SB dominant pasture along with the PCFS. The PCFS was positioned as before. The doors were locked open with a small amount of the calf starter feed provided daily.

Starting on d 15, and continuing for 10 d thereafter, the heifers were fed 0.23 kg of a dyed supplement – the mix of calf starter feed and a grain – and the internal marker starting at 8:00 a.m. in the PCFS. Starting on d 22, fecal samples were also collected daily for 5 d. Uniquely dyed, fresh fecal pats from the individual heifers were collected by simply walking through the paddock. This avoided gathering the cattle and thereby interrupting their grazing.

On d 8, 15 and 19 of the study (d 29, 36 and 40 of study 1), a survey of the paddock was performed to determine forage types and biomass. Samples were collected from within a quadrat, a hoop of 0.178 m². Upon entering the paddock, the quadrat was thrown randomly toward the middle of the paddock. The plants within the quadrat were identified and collected. Dead matter was discarded. Four additional quadrats were collected with random throws following a crisscross pattern. If the weight of a sample collected from the quadrats was insufficient for laboratory analyses, grab samples also were randomly collected across the paddock. When sufficient weights of grab samples were available, and depending on phenology, plants were separated into parts (i.e., leaf; stem; flower and seed head).

Exp. 2. In 2016 at the end of a housed study, 12 of the heifers were chosen for use throughout 4 outdoor studies. As with Exp. 1, their intakes of a forage-based diet had been measured in the preceding study. The sampling periods for the subsequent outdoor studies occurred May 23 to June 3 (study 1), June 20 to July 1 (study 2), July 25 to August 5 (study 3), and August 22 to Sept 2 (study 4), 2016. Weather information was again obtained from the personal weather station.

At the start of the first outdoor study, the heifers were randomly divided into 2 groups (6 heifers each). To encourage quicker adaptation to the PCFS once moved to pasture, the 2 groups of heifers were confined in separate pens for 4 d with ad libitum access to the forage-based diet provided via PCFS. The doors were left open.

The 2 groups of heifers, and their PCFS, were then moved to two separate 88 by 393 m (8.5 acre) predominantly SB pastures (Fig. 3.2), where they grazed the remainder

of the season. The PCFS was positioned as in Exp. 1. The doors were locked with a limited amount of calf starter feed provided daily at 8:00 a.m. A temporary cross-fence was erected for the first month constraining the heifers to a 2-acre area within their pasture that included the PCFS. This was to encourage their access to the unit. At the end of the first study, the cross-fence was removed.

Similar to Exp. 1, each study began (d 1) with about a 2 wk period in which the heifers were offered a limited amount of calf starter feed at pasture in the PCFS. However, the doors were locked throughout this period since, with the exception of study 1, the heifers were already habituated to their pasture and PCFS.

Within a study, starting d 17 and continuing for 10 d, the heifers were fed the dyed supplement – the mix of calf starter feed and a grain – and the internal markers. Starting on d 24, fecal samples were also collected daily for 5 d by walking through the paddock. On d 17, 24 and 28 forage samples were collected following the same protocols as in Exp. 1. At the end of each study (d 28), the heifers were weighed and then returned to their same pasture.

During study 1, one heifer refused to eat from the PCFS. Starting on the second outdoor study, this heifer was hand fed daily with a supplement (calf-starter feed and crimped oats) mixed with the internal markers but a different colored dye (FD&C Blue 5). A further heifer was added to this group for studies 2 through 4, which did use the PCFS.

Laboratory analyses

Sample preparation and extraction. The forage and fecal samples collected over all studies were placed in an oven at 50°C until dried, ground through a 1 mm mesh screen using a Wiley Mill (Thomas Scientific, Swedesboro, NJ), and then stored in a dry cool location until analysis.

Equal DM weights of the daily fecal collections on an animal within a study were combined to form a pooled sample for analysis. The ALK profiles of the whole plants collected on d 1 and 5 of fecal collection periods in each pasture and study were analyzed separately. When available, grab samples from the entire pasture were used; if unavailable, samples from the quadrat farthest from the water source were used.

Sample extractions were performed in duplicate (Dove and Mayes, 2006). A 0.2 g of a forage sample or 0.1 g of fecal matter were weighed, and 0.1 g of an internal standard solution containing 0.3 mg/g of *n*-docosane (C₂₂) and *n*-tetratriacontane (C₃₄) was added to each sample tube as internal standards. These samples were heated with 1 M ethanolic KOH for 16 h at 90°C. Heptane and distilled water were added to each sample and were heated to 50°C; the non-aqueous layer was collected and evaporated.

Hydrocarbons were collected by solid phase extraction by heptane elution through a silica-gel column (3 mL 20 µm PE, Biotage LLC, Charlotte NC).. The ALK were re-dissolved in *n*-dodecane for gas chromatographic analysis.

Gas chromatography. Quantification of ALK was carried out with a gas chromatograph (GC) on an Agilent 7820A GC (Agilent Technologies, Wilmington, DE).

Derivatized ALK fractions were injected (0.5 μ l) with 7650A Automatic Liquid Sampler onto a bonded-phase, non-polar column (Agilent J&W DB-1 column, 30 m, 0.53 mm internal diameter and 0.5 μ m film thickness). Helium served as the carrier gas at a constant flow of 4 mL/min. Temperature was 280°C for the injector and 340°C for the detector. The column was held at 140°C for 6 min, then increased at 50°C/min to 215°C with an iso-thermal hold of 1 min, and a second temperature ramp of 6°C/min to 320°C with a 4 min hold time.

Samples of ALK standard solution mixtures (C_{21} to C_{36} ; Sigma-Aldrich, St. Louis, MO, USA) were included in the GC analyses to identify peaks and standard response factors. Chromatographic data were analyzed using Agilent ChemStation software (Rev. C.01.06 [61]). Peak areas were determined with auto-integration and manual review of chromatograms. The ALK concentrations were calculated relative to known amounts of the internal standards (C_{22} and C_{34}), according to Dove and Mayes (2006).

Statistical Analyses

Weather. Precipitation patterns were studied to understand biomass availability of the pastures. Daily maximum and minimum temperatures were used to calculate growth degree days (**GDD**) to evaluate morphological differences between the 2 yr. Dry bulb temperature along with relative humidity was used to calculate Temperature Humidity Index (**THI**; NRC, 1971). Days exceeding a THI index of 84 were inspected to determine if evening temperatures allowed animals to cool (Eirich et al., 2015).

Estimating diet composition. In order to determine the observed botanical composition of each pasture on each d forages were collected, the weight of biomass from the 5 quadrats were summed by species, and then species percentages calculated for the pasture in its entirety. The percentage values from d 1 and 5 the of fecal sampling period were then averaged to represent the botanical composition of a pasture and study.

Two predominate plant species, SB and Kentucky bluegrass (**KBG**; *Poa pratensis*), were present in the pastures during both experiments. The concentrations of the 4 ALK in these plants, and in the fecal sample, were used to estimate diet compositions. Estimates were obtained for individual animals for each study using the average ALK concentrations from the plants collected on d 1 and 5 of the fecal collection period.

Two statistical methods were used to obtain the diet composition estimates: non-negative least squares (**NNLS**) and a Bayesian approach. The "NNLS" package in R (R Core Team, 2017) was used to obtain the solutions by NNLS. Using an algorithm developed by Jurado Vargas et al. (2017), a Gaussian distribution was assumed for the Bayesian analyses. Since only 2 plants were evaluated (SB and KBG), the prior mean was fully specified by the proportion of SB in the diet. The pastures in 2015 were typically 90% SB; in 2016, the pastures were typically 80% SB. In order to capture that difference, distinct sets of prior means were tested in each year: 0.95, 0.90 and 0.85 in 2015, and 0.90, 0.80, and 0.70 in 2016. In all cases, the prior covariance matrix (order 2 x 2) was defined such that diagonal elements were 1×10^{15} and off-diagonal elements were zero. That covariance structure placed high weight on prior information in the estimation

process. Analyses using an alternative covariance structure with off-diagonal elements of 1×10^{15} , which would place little weight on prior information, failed to converge.

Boxplots were created using the "graphics" package in R to view the spread in the estimates of diet composition derived from NNLS and the Bayesian analyses.

In order to characterize how precisely SB and KBG could be delineated in a mixed diet based on their ALK profiles, the C_{27} , C_{29} , C_{31} and C_{33} concentrations of pure plants from a single collection (d 1 in pasture 2 of study 2 in Exp. 2) were used to derive theoretical mixtures containing, proportionally, from 100 to 0% CS, at 5% increments. Variation among the derived ALK profiles of the mixtures were evaluated with principal component analysis (**PCA**) using the “prcomp” function of R. The results were graphed with the “ggbiplot” package.

Using NNLS and the Bayesian approach, the composition of the derived mixtures were estimated from the weighted concentrations of the 4 ALK. In the Bayesian analysis, the prior mean for the proportion of SB in the diet was set at 0.5, which contained a covariance structure that placed a low weight on prior information (off-diagonal elements 1×10^{15}). Those data were then used to explore our potential to discriminate incremental changes in the SB composition of such mixtures (e.g., proportionally 0.95 vs. 0.90 or 0.85 SB in a diet) using orthogonal contrasts. The following model was fitted using the "lm" package in R:

$$y_{ij} = \mu + m_i + e_{ij} \quad (\text{Eq. 3.1})$$

where y_{ij} was the percentage of SB estimated for an extract ($j = 1, \dots, 4$) for the mathematically derived mixture m_i ($i = 1.0, \dots, 0.05$, in 0.05 increments of SB) with μ the overall mean proportion of SB in the diet. The theoretical concentration of ALK was fitted as a fixed effect, with the residual e_{ij} the random effect. This model was fitted separately for diet compositions estimated with the two statistical methods.

To account for potential lab-based measurement errors, similar to Vargas Jurado et al. (2015) and Lewis et al. (2016), a blind study was designed to understand the distinctiveness of the ALK profiles of the 2 plant species. Plant samples from the same collection d as used to derive the theoretical mixtures (d 1 in pasture 2 of study 2 in Exp. 2) were used to generate plant mixtures with different SB percentages. The mixtures reflected pasture compositions consisting, proportionally, of 1.00 to 0.60 SB in 0.05 increments. The concentrations of C_{27} , C_{29} , C_{31} and C_{33} in the mixtures were measured. Diet compositions were then estimated by NNLS and the Bayesian method with the same procedures used with the theoretically derived mixtures.

Estimating feed intake. Feed intakes were estimated assuming SB and KBG alone contributed to the diet. Since diet composition contributes to intake estimates, the 2 diet composition estimates were used: NNLS and a Bayesian approach. Values for prior means for the SB and KBG contributions to diets were based on their observed contributions to the botanical composition of a study within pasture. For the 2015 experiment, study 1 had a prior mean of 0.90 for SB; for studies 2 and 3, that prior mean was 0.95. For the 2016 experiment, for studies 1 and 3 in both pastures, study 2 in pasture 1, and study 4 in pasture 2, the prior mean for SB was 0.80. Whereas, for study 2 in

pasture 2, and study 4 in pasture 1, the Bayesian analyses were based on a prior mean of 0.90 for SB.

Fecal C_{27} , C_{29} , C_{31} and C_{33} concentrations were adjusted for incomplete recoveries based on the carbon length of the ALK. The adjustments were based on the fit of a beta regression by Vargas Jurado et al. (2017) using data on recovery rates published in the literature (Brosh et al., 2003; Dillon, 1993; Dove, 1996; Elwert et al., 2004; Elwert et al., 2008; Olivan et al., 2007). The recovery rates use were 70.3% (C_{27}), 78.5% (C_{29}), 84.8% (C_{31}), 87.4% (C_{32}) and 89.6% (C_{33}).

The adjusted fecal ALK for markers C_{31} and C_{33} (odd-chain marker) and C_{32} (dosed internal marker) were used to estimate DMI (**EDMI**):

$$EDMI = \frac{A_j * F_i / F_j}{H_i - (F_i H_j) / F_j} \quad (\text{Eq. 3.2})$$

where A_j was the daily dose of C_{32} , F_i and F_j were the adjusted concentrations of odd-chain (C_{31} or C_{33}) and dosed (C_{32}) ALK in the feces, respectively, and H_i and H_j were the observed concentrations of the odd-chain and dosed ALK in a plant, respectively. Diet compositions had been estimated in several ways. Those estimates were used to adjust Eq. 3.2 to account for the proportional contribution of SB and KBG in the diet of the individual animal when estimating intake.

Regression. Observed DMI (**ODMI**) measured in the heifers in the housed study were regressed on the EDMi obtained using the diet compositions derived from the NNLS and Bayesian analyses. Despite animals being housed in different systems, there

was still interest in the relationship between intake in controlled and grazing environments. If EDMl perfectly reflected ODMI the regression line would have a slope of one and an intercept of zero. Using the "lm" package of R, the hypotheses tested were that the slope was not differed from unity, and that the intercept was not differed from zero.

Observed intakes from the indoor studies, and EDMl from the outdoor studies, were also compared using pairwise t-tests. Since the animals in Exp. 2 had EDMl for each of the 4 outdoor studies, the consistencies of those intakes were also compared by regressing EDMl from one outdoor study on another, and with pairwise t-tests.

ANOVA. Variation in concentrations of individual ALK in SB and KBG in the 2015 experiment were tested using the MIXED procedure in SAS 9.3 (SAS Inst., Inc., Cary, NC). The mixed model fitted was:

$$y_{ijkl} = \mu + S_i + F_j + (SF)_{ij} + D_{(ij)k} + e_{ijkl} \quad (\text{Eq. 3.3})$$

where y_{ijkl} was the concentration of an ALK (C_{27} , C_{29} , C_{31} or C_{33}) obtained for an extract ($l = 1$ or 2) collected in study S_i ($i = 1, \dots, 3$, for the 3 studies) of plant F_j ($j = 1$ or 2 , for SB or KBG, respectively) on day D_k ($k = 1$ or 2 , for the Monday and Friday plant sample, respectively, harvested during the same wk as fecal collections), with μ the overall mean concentration of the ALK. Study and plant, and their interaction $[(SF)_{ij}]$, were fitted as fixed effects. Random effects were day nested within the study by plant interaction $[D_{(ij)k}]$, and the residual error (e_{ijkl}). When forming test statistics, the error term for plant, study and their interaction was $D_{(ij)k}$.

In the 2016 experiment, variation in concentrations of individual ALK among plants were tested using the MIXED procedure in SAS 9.3 (SAS Inst., Inc., Cary, NC):

$$y_{ijklm} = \mu + P_i + S_j + F_k + (PS)_{ij} + (PF)_{ik} + (SF)_{jk} + (PSF)_{ijk} + D_{(ijk)l} + e_{ijklm}$$

(Eq. 3.4)

where y_{ijklm} was the concentration of an ALK (C₂₇, C₂₉, C₃₁ or C₃₃) obtained for an extract ($m = 1$ or 2) collected from pasture P_i ($i = 1$ or 2 , for the 2 pastures) in study S_j ($j = 1, \dots, 4$, for the 4 studies) of plant F_k ($k = 1$ or 2 , for SB or KBG, respectively) on day D_k ($k = 1$ or 2 , for the Monday and Friday plant sample, respectively, harvested during the same wk as fecal collections), with μ the overall mean concentration of the ALK. Study and plant, and their interaction $[(SF)_{ij}]$, were fitted as fixed effects. Random effects were pasture and its interaction with study $[(PS)_{ij}]$, plant $[(PF)_{ik}]$ and study by plant $[(PSF)_{ijk}]$, day nested within the pasture by study by plant interaction $[D_{(ijk)l}]$, and the residual error (e_{ijklm}). When forming test statistics, the error term for study was $(PS)_{ij}$, for plant was $(PF)_{ik}$, and for study by plant interaction was $(PSF)_{ijk}$.

RESULTS

Weather

Daily high and low temperature, and GDD, are plotted against Julian date in Fig. 3.3 for 2015 and 2016. Thirty yr average high and low temperatures are also shown. High and low daily temperatures, and GDD, appear similar in the 2 yr. The cumulative

precipitation between March and September in 2015 and 2016 were 58.57 and 41.78 cm, respectively (Fig. 3.4). Precipitation differed across yr; this was especially true in June 2016, with 18.54 cm less rainfall than in June 2015. Compared to a 30 yr average, 2016 was a drier year.

The daily THI index was calculated. On days where THI was in excess of 84, a threshold value for heat stress (Eirich et al., 2015), evening temperatures were considered to assess the extent animals cooled overnight. There was no d in which the threshold for THI was exceeded with evening temperatures remaining above 23.9°C, suggesting no overt heat stress.

Plants

The chemical composition – ADF, NDF, CP and lignin – of SB was measured for each study and pasture in both yr, and for KBG in 2016. Although present, an insufficient quantity of KBG was collected in 2015 to analyze by study. The average values of the chemical compositions for each study and pasture are provided in Table 3.1.

The biomass availability (kg/m^2) was determined for each yr (for 2015, Fig. 3.5; for 2016, Fig. 3.6). In 2015, the percentage SB and KBG in pasture were, on average, 95.0% (SD 0.05%) and 4.8% (SD 0.06%), respectively. In 2016, there was slightly more biodiversity in the pastures grazed. In one pasture, the percentage SB and KBG were 82.0% (SD 0.06%) and 13.0% (SD 0.05%), respectively; in the second pasture, those values were 85.0% (SD 0.07%) and 11.0% (SD 0.04%), respectively. Between 4.0 and

5.0% of the plants in 2016 were alternative species (i.e., rush; fescue sedge; needle leaf sedge; Virginia ground cherry)

Concentrations of ALK for SB and KBG are given in Table 3.4. In 2015, C_{27} concentrations did not differ between SB and KBG ($P = 0.22$) across studies ($P = 0.59$). However, for C_{29} concentrations there was a weak and haphazard species by study interaction ($P = 0.054$). The C_{31} and C_{33} concentrations were consistently lower in SB than KBG ($P < 0.03$) regardless of the study.

In 2016, there was no interaction between species and study across pastures in the concentrations of any of the 4 ALK ($P > 0.16$). The C_{27} and C_{29} concentrations in SB and KBG did not differ ($P > 0.12$) but consistently decreased over the grazing season (for C_{27} , $P = 0.009$; for C_{29} , $P = 0.063$). The C_{31} concentrations did not differ by species ($P = 0.31$) or by study ($P = 0.25$). Only C_{33} concentration was consistently lower in SB than KBG ($P = 0.053$) regardless of study ($P = 0.165$), as also found in 2015.

Diet composition

The concentrations C_{27} , C_{29} , C_{31} and C_{33} for SB and KBG from d 1 of study 2 in pasture 2 from Exp. 2 are shown in Fig. 3.7. These values were used to form a theoretical continuum of their mixtures by allowing the contribution of each plant to be exchanged at 5% increments. The diet compositions of the mixtures were then estimated by NNLS and a Bayesian method (prior mean for SB of 0.5). The relationships between the 4 ALK and the theoretical diet mixtures were assessed with PCA (Fig. 3.8). The first principal component explained a majority of the variation (80.7%), while the second principal

component explained most of the rest (the explained 19.0%). The C_{31} and C_{33} concentrations of the 2 plants appeared to be most discriminating, followed by C_{29} .

Using the estimates from both statistical approaches, orthogonal contrasts were constructed to compare means of the estimated diet compositions obtained from the theoretical mixtures of the 2 plants (Table 3.2). With both approaches, differences of 10% or higher in the SB composition of the diet (e.g., 65-60% vs. 75-70%) could be distinguished ($P < 0.01$). However, smaller demarcations (5%) could not be detected ($P > 0.23$).

Fabricated diets were also prepared by combining pure SB and KBG in proportions of 0.95 to 0.05 through 0.60 to 0.40, at 0.05 increments. Plant samples collected on d 1 of study 2 in 2016 were used. The relationships between the ALK concentrations and SB percentage of the mixtures, and the pure plants were evaluated with PCA (Fig. 3.9). Similar to the evaluation of the theoretical mixtures, the first principal component (78.9%) explained most of the variation, while the second principal component explained most of the rest (20.7%). Again, the main discriminated ALK were C_{31} and C_{33} , followed by C_{29} . Orthogonal contrasts were constructed using these measured values to determine the extent to which the mixtures could be delineated (Table 3.3). Diet compositions based on the NNLS and Bayesian approaches could be distinguished at 10% increments ($P < 0.02$) but not at 5% increments ($P > 0.09$).

The concentrations of the 4 ALK in SB and KBG by pasture and study are provided in Table 3.4. These values were used to estimate diet composition using 3 Bayesian methods, specific to a year (for 2015, prior means for SB of 0.95, 0.90 and

0.85; for 2016, prior means for SB of 0.90, 0.80 and 0.70), and NNLS. The distributions of these estimates are shown in Fig. 3.10 for the 2015 experiment, and in Fig. 3.11 and Fig. 3.12 for pasture 1 and 2, respectively, for the 2016 experiment. These plots include as observed values the proportion of SB found in a pasture for a study during the 5 d fecal collection period. Depending on study, there appeared to be substantial differences between the observed values and diet composition depending on the approach used in its estimation.

Feed intakes

The concentration of internal ALK marker (C_{32}), along with the natural occurring ALK (C_{27} , C_{29} , C_{31} and C_{33}), in the supplement was tested to confirm the level of dosing. Those concentrations are provided in Table 3.5. In both yr, the measured C_{32} in the supplement was in general higher than that planned.

Intakes were calculated with NNLS and the Bayesian approach that best depicted the observed proportion of SB in a study and pasture. These EDMIs are provided for individual animals in Tables 3.6 and 3.7 for 2015 and 2016, respectively. When EDMIs were obtained from the ratio $C_{33}:C_{32}$, there appeared to be extremely low (e.g., 2.34 kg/d) and high (e.g., 37.3 kg/d) values. Based on paired t-tests, EDMI derived from $C_{31}:C_{32}$ and $C_{33}:C_{32}$ differed depending on the study (Table 3.8). For these reasons, EDMIs using $C_{33}:C_{32}$ were deemed unreliable and not used when comparing ODMI to EDMI nor EDMI across yr.

The ODMI measured in the indoor study were regressed on the EDM I obtained from the $C_{31}:C_{32}$ ratio with diet composition derived either from the Bayesian or NNLS techniques (Table 3.9). The results varied by yr and study. For example, in study 2 in pasture 1 of the 2016 experiment, the intercept did not differ from zero ($P > 0.88$) and the slope did not differ from one ($P > 0.85$) regardless of the method used to estimate diet composition. However, in other studies, slopes and intercepts significantly differed from their expected values. Differences in goodness-of-fit of the regressions was reflected by considerable variability in r^2 values across studies, ranging from 0.01 to 0.63. The pairwise t-tests echoed that variability ($0.001 < P < 0.45$).

In 2016, heifers had been randomly allocated to 2 adjacent pastures. As defined in Eq. 3.3 and 3.4, pasture was considered a random effect. The variance in BW and in EDM I in the heifers within a pasture were compared by study using a homoscedastic t-test. The extent of variability in heifer BW ($P > 0.21$) and in EDM I ($P > 0.32$), regardless of diet estimation technique, was similar between pastures. Performance levels of heifers within the 2 pastures therefore appear to have been consistent.

Regardless of diet estimation technique, the ranking of animals in terms of estimated amounts of food consumed changed. For diet compositions estimated using both statistical methods, the EDM I from one study were regressed on the EDM I for the other 3 studies (Table 3.10). In some cases – for instance the regression of EDM I of study 1 on the EDM I of either study 2 or 4 – the prediction were relatively poor. However, in many other cases, the predictions were more reliable. For example, based on the Bayesian approach, the intercept and slope from the regression of EDM I in study 2 on 3 did not

differ from one (0.85; $P = 0.77$) or zero (0.32; $P = 0.95$); the estimates were equally reliable when based on NNLS (slope 0.85, $P = 0.74$; intercept -0.08, $P = 0.99$). However, this was not necessarily always the case. Furthermore, the r^2 values from the fit of the regressions differed appreciably, ranging from 0.01 to 0.48. Consistent with those results, based on the paired t-tests, EDM I also varied between studies.

Lastly, the EDM I obtained when diet compositions were estimated with NNLS or the Bayesian technique were compared across studies in the 2016 experiment (results not shown). Intakes estimated in studies 1 and 2 did not differ ($P > 0.08$) based on the statistical method used to obtain diet compositions. However, for studies 3 and 4, intakes based on the Bayesian approach and NNLS differed appreciably ($P < 0.04$).

DISCUSSION

Plants and Weather

The estimation of feed intakes using ALK in confined conditions has been shown to be reliable in a number of studies (e.g., Mayes et al., 1986; Dove and Olivan, 1998; Dove et al., 2002; Lewis et al., 2003). Those intake estimates are affected by diet composition, which can be highly variable in grazing situations. Plant species differ in their ALK profiles (Bungelho, 2004; Ali, 2005; Lewis et al., 2016). Furthermore, changes in phenology have been shown to correspond with changes in the ALK concentrations of plants and their parts. Therefore, plants must be sampled throughout the growing season to capture variations associated with maturity.

The maturity level of plants has been shown to be directly correlated to GDD, while plant biomass is largely controlled by precipitation patterns (Power, 1983). By comparing GDD across yr, a concordance in plant maturity levels can be uncovered. In this experiment the GDD in the 2 yr were quite similar suggesting that the plants matured at the same rate. Therefore, despite differences in the botanical compositions of the pastures, their results are comparable.

Diet Composition

Based on the ALK profiles of SB and KBG, 10% but not 5% differences in their respective contributions to a diet could be delineated. There were large differences in the C_{33} concentrations of KBG [108.2 (SD 20.4) mg/kg] and SB [12.4 (SD 3.5) mg/kg] yet smaller differences in their C_{31} concentrations [KBG 244.1 (SD 55.7) mg/kg; SB 180.1 (SD 44.4) mg/kg]. As a consequence, EDM I based on the $C_{33}:C_{32}$ ratio appeared to be much more sensitive to fluctuations (or error) in diet composition estimates than those based on the $C_{31}:C_{32}$ ratio. Some feed intake estimates calculated from the $C_{33}:C_{32}$ ratio were highly implausible. Although final intake estimates have been derived by averaging the intakes from these sets of ratios (Lewis et al., 2003) doing so appears inappropriate in this circumstance.

Within each year, as the season progressed, the proportion of SB in the pasture changed. This may lead to changes in individual animal choices as to the composition of their diet. Still, pragmatically, that selectivity is bounded by biomass of plants on offer. As the season progressed, the reliability of the diet compositions estimated using NNLS became more suspect, as they differed appreciably from observed botanical compositions

of the pastures. With the Bayesian approach, where the prior mean could be set to more closely reflect the characteristics of pasture, the observed and estimated diet compositions aligned more closely. A clear illustration of this finding was seen in pasture 2 of study 4 in 2016 (Fig. 3.11). Based on plant biomass, the pastures were 88% SB. However, from NNLS the estimated composition of the animal's diet was, on average, 33% (SD 6%) SB. As the season progressed lignin contents in the SB increased, while it stayed relatively consistent in the KBG. An increase of lignin is less desirable due to nutrient utilization by the animal and would likely affect cattle's decision to select more KBG (Van Soest, 1994). However, such a diet seems extremely unlikely even if cattle were quite selective; it would entail KBG contributing more than half of the forage consumed when its availability in the pastures was quite limited at that time (Fig. 3.6).

However, compelling estimates of diet composition to closely align with the observed botanical composition from an expansive grazing area also may be misguided. In 2016, between the 2 pastures, the proportion of the biomass that was SB differed, particularly in study 4. Variation among quadrats within pastures was also noticed. Therefore, depending on the location in which an animal prefers to graze, the plant mixture may differ. Due to variations in the botanical composition of pastures, a clearly preferable method to estimate diet composition across the grazing season was not transparent.

Feed Intake

Feed intake of cattle is affected by many factors including temperature, forage quality, stage of animal growth, gut fill, and animal behavior. As a consequence, intakes

can be quite variable. It therefore is not unreasonable that intakes in controlled (indoor) conditions may differ from those in a grazing situation, particularly when animals differ in age or stage of maturity and are offered different foods. An animal's behavior in a confined setting also may not reflect its behavior while grazing. With pasture intakes estimated over short durations – in this and other studies, a 5 d interval – the reliability of those estimates may be lessened. The heifers in this experiment were growing [in 2015, from 447 (SD 34) to 468 (SD 31) kg; in 2016, from 394 (SD 27) kg to 457 (SD 22)]; their nutrient requirements therefore also may have changed across studies. Differences between intakes in the indoor and outdoor experiment perhaps should be anticipated.

In the series of outdoor studies, the physical environment remained the same despite some changes in weather conditions and forage quality across the season. It therefore may be expected that individual animal intakes across the sequential outdoor studies would be more similar than with the indoor intakes. In 3 of the 4 outdoor studies in 2016, intakes did not differ ($P > 0.07$). The exception was study 2, where intakes were estimated to be higher ($P < 0.04$). Those higher intakes may be in response to the cattle being allowed access to the full pasture; in study 1, they were confined to only one-third of the grazing area limiting the forage biomass on offer. Furthermore, in early June 2016 there was very little rain (Fig. 3.4). However, during the 2 wk period coinciding with study 2, which occurred in late June, it rained 1.48 cm. An increase in biomass was seen in pasture 1 (2.32 kg/m²) but less so in pasture 2 (1.42 kg/m²; Fig. 3.6). That variation may in part be due to the forage sampling strategy or actual differences in botanical composition between pastures. Nevertheless, this increased biomass may help explain the increased intakes.

CONCLUSION

It has been demonstrated that historically feed intakes can be reliably estimated with plant-wax markers in controlled indoor studies. However, the estimation process becomes more challenging in a grazing environment where, for instance, the botanical composition of the sward may be diverse and changeable across the season. The diet composition of individual animals will impact the quality of the intake estimates. Therefore, the ability to clearly discriminate plants based on their ALK profiles, and thereby accurately determine the dietary choices of individual animals, affects EDMI. If plants cannot be distinguished, and differ appreciably in their C_{31} or C_{33} concentrations, then EDMI will be less accurate. It is therefore important to examine the ALK profiles of the plants in a pasture when defining a strategy for estimating diet composition and intakes.

Considering intakes measured in a controlled setting as useful predictors of intakes in a grazing environment appears spurious. A number of factors, including weather conditions, plant phenology and grazing behaviors, likely contribute to that discrepancy up and beyond estimation errors. Still, despite the lack of fine demarcations, sensible intakes could be obtained in a grazing setting. The plant-wax methodology therefore shows promise for commercial use.

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Table 3.1 Chemical composition of forages by year, study and pasture

Species	Year	Study	Pasture	Crude Protein (CV ³)		ADF (CV ³)		NDF (CV ³)		TDN ⁵ (CV ³)		Lignin (CV ³)	
Smooth ¹ brome grass	2015	1	n/a	7.35	(0.18)	39.05	(0.03)	63.50	(0.01)	58.00	(0.02)	6.10	(0.06)
		2	n/a	6.65	(0.32)	41.70	(0.13)	66.20	(0.05)	55.00	(0.11)	6.93	(0.15)
		3	n/a	6.20	(0.13)	47.35	(0.03)	71.35	(0.01)	48.55	(0.03)	8.00	(0.12)
	2016	1	1	8.00	(0.18)	34.45	(0.11)	58.60	(0.03)	63.30	(0.07)	3.96	(0.15)
			2	9.60	(0.13)	40.40	(0.04)	67.05	(0.02)	56.50	(0.04)	4.97	(0.07)
		2	1	6.00	(0.40)	43.00	(0.14)	65.80	(0.03)	53.55	(0.13)	6.20	(0.22)
			2	5.75	(0.09)	42.30	(0.13)	65.20	(0.07)	54.35	(0.11)	6.33	(0.32)
		3	1	4.85	(0.52)	47.10	(0.06)	68.05	(0.04)	48.85	(0.06)	7.12	(0.09)
			2	6.80	(0.03)	44.15	(0.02)	66.95	(0.02)	52.20	(0.02)	7.02	(0.19)
		4	1	7.90	(0.56)	45.70	(0.11)	67.10	(0.04)	50.45	(0.12)	7.45	(0.28)
			2	6.60	(0.82)	47.65	(0.12)	71.40	(0.06)	48.20	(0.14)	7.47	(0.12)
Kentucky ² bluegrass	2016	1	1	9.05	(0.08)	43.90	(0.08)	65.90	(0.03)	52.55	(0.08)	4.54	(0.16)
			2	8.50	(0.09)	43.35	(0.19)	67.50	(0.12)	53.10	(0.17)	5.86	(0.53)
		2	1	7.50	(0.11)	41.10	(0.09)	65.15	(0.04)	55.65	(0.08)	4.90	(0.38)
			2	6.60	(0.18)	43.60	(0.09)	67.30	(0.02)	52.85	(0.09)	5.38	(0.16)
		3	1	9.40	(n/a) ⁴	42.30	(n/a) ⁴	67.70	(n/a) ⁴	54.40	(n/a) ⁴	4.79	(n/a) ⁴
			2	8.60	(n/a) ⁴	44.80	(n/a) ⁴	70.50	(n/a) ⁴	51.50	(n/a) ⁴	4.61	(n/a) ⁴
		4	1	12.70	(n/a) ⁴	39.50	(n/a) ⁴	67.60	(n/a) ⁴	57.60	(n/a) ⁴	4.92	(n/a) ⁴

¹Chemical composition was calculated for both yr.

²Chemical composition was only calculated for Kentucky bluegrass in 2016. In 2015, insufficient forage sample was available to analyze for each study.

³ Coefficient of variation (CV).

⁴ Chemical composition was only calculated for 1 day due to lack of biomass collected.

⁵ TDN values were calculated using ADF.

Table 3.2 Orthogonal contrasts of derived (theoretical) forage mixtures consisting of various percentage of smooth brome grass (SB).

Method	Percent SB in mixture		Estimate (SE)		P-value
	Contrast 1	Contrast 2			
NNLS ¹	100-55	45-0	0.55	(0.01)	<0.001
	100-80	75-55	0.25	(0.02)	<0.001
	100-95	90-85	0.10	(0.03)	<0.001
	75-70	65-60	0.10	(0.03)	<0.001
	100	95	0.04	(0.04)	0.33
	90	85	0.05	(0.04)	0.26
	75	70	0.05	(0.04)	0.26
	65	60	-0.05	(0.04)	0.26
	45-25	20-0	0.25	(0.02)	<0.001
	45-40	35-30	0.10	(0.03)	<0.001
	45	40	0.05	(0.04)	0.26
	35	30	0.05	(0.04)	0.26
	20	15	0.05	(0.04)	0.26
	5	0	0.04	(0.04)	0.33
	100-55	45-0	0.55	(0.01)	<0.001
	100-80	75-55	0.25	(0.02)	<0.001
Bayesian ²	100-95	90-85	0.09	(0.03)	<0.001
	75-70	65-60	0.10	(0.03)	<0.001
	100	95	0.04	(0.04)	0.38
	90	85	0.05	(0.04)	0.23
	75	70	0.05	(0.04)	0.23
	65	60	-0.05	(0.04)	0.24
	45-25	20-0	0.25	(0.02)	<0.001
	45-40	35-30	0.10	(0.03)	<0.001
	45	40	0.05	(0.04)	0.23
	35	30	0.05	(0.04)	0.23
	20	15	0.05	(0.04)	0.24
	5	0	0.04	(0.04)	0.39

¹ Non-negative least squares method for estimating diet composition.

² Bayesian method for estimated diet composition assuming a Gaussian prior distribution with prior mean of, proportionally, 0.5 smooth brome grass, and a covariance matrix with diagonal elements 1×10^{15} and off-diagonal elements of zero.

Table 3.3 Orthogonal contrasts of extracted forage mixtures consisting of various percentage of smooth brome grass (SB)

Method	Percent of SB in mixture		Estimate (SE)		<i>P</i> -value
	Contrast 1	Contrast 2			
NNLS ¹	100-65	0	0.84	(0.04)	<0.001
	100-85	80-65	0.18	(0.02)	<0.001
	100-95	90-85	0.10	(0.03)	0.01
	80-75	70-65	0.09	(0.03)	0.01
	100	95	0.03	(0.05)	0.54
	90	85	0.05	(0.05)	0.35
	80	75	0.08	(0.05)	0.09
	70	65	0.04	(0.05)	0.38
Bayesian ²	100-65	0	0.83	(0.04)	<0.001
	100-85	80-65	0.18	(0.02)	<0.001
	100-95	90-85	0.09	(0.03)	0.02
	80-75	70-65	0.09	(0.03)	0.01
	100	95	0.03	(0.05)	0.60
	90	85	0.05	(0.05)	0.30
	80	75	0.08	(0.05)	0.10
	70	65	0.04	(0.05)	0.37

¹ Non-negative least squares method for estimating diet composition.

² Bayesian method for estimated diet composition assuming a Gaussian prior distribution with prior mean of, proportionally, 0.5 smooth brome grass, and a covariance matrix with diagonal elements 1×10^{15} and off-diagonal elements of zero.

Table 3.4 Mean (SE) *n*-alkane concentrations of forages by year, study and pasture

Year	Study	Species	Pasture	C27	C29	C31	C33	C32
2015	o1	Kentucky bluegrass	1	4.70 (0.04)	35.97 (2.61)	159.37 (2.59)	90.50 (4.22)	8.30 (0.26)
		Smooth brome grass		5.54 (0.31)	35.18 (4.34)	110.96 (8.29)	7.36 (0.37)	4.49 (0.38)
	o2	Kentucky bluegrass	2	5.04 (0.24)	50.23 (3.45)	232.06 (23.20)	120.57 (3.89)	8.48 (0.43)
		Smooth brome grass		3.37 (0.59)	26.27 (3.12)	118.66 (16.01)	7.58 (0.84)	2.60 (0.63)
	o3	Kentucky bluegrass	3	5.52 (0.25)	34.77 (3.85)	183.57 (3.44)	119.32 (9.24)	8.50 (0.56)
		Smooth brome grass		5.43 (0.67)	43.21 (2.12)	174.73 (21.59)	14.96 (1.75)	5.99 (1.29)
2016	O1	Kentucky Bluegrass	1	25.68 (3.43)	207.85 (19.52)	366.00 (7.79)	79.46 (7.42)	6.41 (0.34)
			2	32.95 (3.52)	123.74 (4.25)	237.49 (23.11)	96.49 (15.36)	8.16 (0.32)
		Smooth brome grass	1	21.66 (6.74)	74.54 (10.70)	230.02 (30.96)	12.53 (0.27)	3.79 (0.16)
			2	9.99 (1.54)	68.73 (1.91)	225.61 (6.55)	13.75 (1.30)	4.56 (0.17)
		Kentucky bluegrass	1	19.83 (1.50)	102.16 (10.34)	241.96 (12.38)	97.29 (2.10)	5.18 (0.58)
			2	10.67 (1.25)	104.37 (11.50)	259.03 (3.54)	106.10 (2.97)	5.82 (0.16)
	O2	Smooth brome grass	1	10.93 (2.68)	58.00 (11.59)	210.26 (17.89)	15.95 (2.60)	3.98 (0.61)
			2	9.92 (2.54)	54.86 (10.58)	245.01 (34.38)	17.72 (1.60)	4.60 (0.84)
		Kentucky bluegrass	1	4.89 (0.47)	70.07 (11.78)	283.67 (13.34)	140.61 (1.12)	18.91 (5.30)
			2	6.25 (0.59)	66.21 (3.51)	281.78 (12.22)	130.95 (12.18)	8.19 (0.55)
		Smooth brome grass	1	5.06 (1.01)	45.45 (0.93)	175.07 (2.17)	14.33 (1.14)	4.57 (0.75)
			2	4.66 (0.47)	50.86 (2.36)	183.27 (7.10)	11.15 (0.87)	2.95 (0.37)
	O3	Kentucky bluegrass	1	6.20 (0.28)	64.89 (1.14)	200.37 (25.89)	83.37 (17.37)	9.49 (0.80)
			2	4.13 (0.12)	54.11 (10.13)	239.36 (36.68)	125.79 (11.41)	12.04 (6.98)
		Smooth brome grass	1	4.72 (0.18)	43.03 (1.81)	154.76 (5.39)	12.06 (0.49)	5.72 (0.46)
			2	8.86 (1.35)	42.80 (3.55)	152.82 (23.78)	8.49 (1.32)	14.15 (5.66)

Table 3.5 Mean (SD) *n*-alkane concentrations of supplements by year and study

Year	Study	Dye	Grain	Measured (mg/kg)					Planned (mg/kg)	Error (%) ¹
				C27	C29	C31	C33	C32	C32	C32
2015	2	Blue	Oat	9.46	7.23	12.07	6.24	2676.09	2667.14	0
				(1.47)	(0.90)	(2.18)	(0.46)	(294.35)		
		Yellow	Corn	8.14	7.62	10.45	8.44	3376.27		-27
				(0.91)	(2.82)	(0.90)	(1.47)	(167.66)		
2016	1	Green	Oat	8.72	7.31	9.48	6.05	2400.26	2263.94	-6
				(3.20)	(1.14)	(1.26)	(0.79)	(116.78)		
		Yellow	Corn	6.13	6.77	7.52	6.66	2587.94		-14
				(0.63)	(0.79)	(0.51)	(0.36)	(87.02)		
	2	Green	Corn	4.38	6.23	8.86	6.08	2620.04	2263.94	-16
				(0.66)	(0.67)	(0.88)	(0.33)	(106.56)		
		Red	Oat	5.34	6.72	8.69	6.16	2196.86		3
				(0.62)	(1.00)	(1.33)	(1.00)	(107.05)		
	3	Red	Oat	5.52	7.58	9.70	6.19	2368.26	2263.94	-5
				(0.77)	(1.61)	(1.76)	(0.53)	(109.15)		
		Yellow	Corn	5.20	6.94	9.69	5.87	2460.58		-9
				(0.63)	(0.69)	(0.37)	(0.37)	(291.00)		
	4	Blue	Oat	9.33	8.20	13.85	5.83	2604.00	2263.94	-15
				(0.37)	(0.68)	(1.31)	(0.14)	(108.06)		
		Green	Oat	11.90	8.69	13.41	8.00	2496.79		-10
				(6.15)	(5.14)	(5.53)	(4.88)	(366.36)		

¹ Error percent was calculated by subtracting the measured dose by the planned dose and dividing that difference by the planned dose.

Table 3.6 Estimated intake (kg/d) in 2015 experiment obtained from either the C₃₁:C₃₂ or C₃₃:C₃₂ *n*-alkanes ratio where diet composition was estimated using either a Bayesian approach or non-negative least squares (NNLS)

Study	Animal ID	C ₃₁ :C ₃₂		C ₃₃ :C ₃₂	
		Bayesian	NNLS	Bayesian	NNLS
O1 ¹	6857	7.34	7.79	3.80	5.35
	6141	9.41	9.93	5.77	7.86
	7038	6.42	6.73	3.40	4.43
	6403	4.86	5.11	2.86	3.75
	7021	7.63	8.01	4.53	6.06
	6319	6.94	6.95	2.60	3.96
O2 ²	6980	8.17	8.17	2.93	4.19
	6487	9.39	9.39	3.60	5.39
	7157	8.24	8.24	2.99	3.59
	6233	5.26	5.27	2.34	2.76
	6037	8.28	8.28	3.06	8.25
	6278	6.47	6.47	6.14	4.39
O3 ²	6990	8.55	8.55	6.88	5.83
	6308	10.43	10.42	7.32	8.67
	7153	9.40	9.40	7.86	10.52
	6958	8.67	8.46	10.14	9.39
	6810	9.84	9.84	9.96	5.36
	6485	9.37	9.43	7.07	10.05

¹ Bayesian method assuming a Gaussian prior distribution with a prior mean of, proportionally, 0.90 for smooth brome grass, with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

²Bayesian method assuming a Gaussian prior distribution with a prior mean of, proportionally, 0.95 for smooth brome grass, with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

Table 3.7 Estimated intake (kg/d) in 2016 experiment obtained from either the $C_{31}:C_{32}$ or $C_{33}:C_{32}$ *n*-alkanes ratio where diet composition was estimated using either a Bayesian approach or non-negative least squares (NNLS)

Study	Pasture	Animal ID	$C_{31}:C_{32}$		$C_{33}:C_{32}$	
			Bayesian	NNLS	Bayesian	NNLS
O1	1 ¹	4557	7.34	7.79	3.80	5.35
		4581	9.41	9.93	5.77	7.86
		4597	6.42	6.73	3.40	4.43
		4627	4.86	5.11	2.86	3.75
		4676	7.63	8.01	4.53	6.06
	2 ¹	4551	6.94	6.95	2.60	3.96
		4555	8.17	8.17	2.93	4.19
		4568	9.39	9.39	3.60	5.39
		4592	8.24	8.24	2.99	3.59
		4674	5.26	5.27	2.34	2.76
O2	1 ¹	4710	8.28	8.28	3.06	8.25
		4553	6.47	6.47	6.14	4.39
		4581	8.55	8.55	6.88	5.83
		4597	10.43	10.42	7.32	8.67
		4676	9.40	9.40	7.86	10.52
	2 ²	4551	8.67	8.46	10.14	9.39
		4555	9.84	9.84	9.96	5.36
		4592	9.37	9.43	7.07	10.05
		4674	8.70	8.73	7.82	8.23
		4710	11.37	11.41	9.38	8.50
O3	1 ¹	4557	8.95	8.72	10.96	10.25
		4581	8.16	7.84	11.00	9.19
		4597	8.55	8.27	11.04	8.72
		4676	8.03	7.64	12.07	8.04
		4679	7.52	7.05	12.33	7.47
	2 ¹	4551	9.80	9.18	17.17	10.00
		4555	8.77	8.57	11.43	9.78
		4568	6.95	6.40	16.40	8.72
		4674	8.36	7.68	22.49	10.62
		4710	9.97	9.58	12.42	9.89
O4	1 ²	4553	6.82	6.02	17.37	5.98
		4557	7.24	6.60	12.69	6.59
		4581	7.88	6.73	37.34	6.68
		4597	8.92	7.91	19.02	7.90
		4676	7.18	6.58	13.13	6.52
		4679	8.87	7.99	19.66	7.89
	2 ¹	4551	8.63	6.87	13.12	4.71
		4555	6.62	6.11	10.50	6.39
		4674	7.57	6.82	17.98	8.41

¹ Bayesian method assuming a Gaussian prior distribution with a prior mean of, proportionally, 0.80 for smooth brome grass, with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

² Bayesian method assuming a Gaussian prior distribution with a prior mean of, proportionally, 0.90 for smooth brome grass, with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

Table 3.8 Comparison of intakes calculated using $C_{31}:C_{32}$ vs. $C_{33}:C_{32}$ ratios.

Outdoor	Year	Study	Pasture ³	r	T-test ⁵
Bayesian	2015	o1 ²	1	0.97	<0.001
		o2 ¹	2	0.45	0.04
		o3 ¹	3	0.87	<0.001
	2016	o1	1 ²	0.93	<0.001
			2 ²	0.91	<0.001
		o2	1 ²	0.71	0.06
			2 ⁴	0.05	0.36
		o3	1 ²	0.70	<0.001
			2 ²	0.09	0.03
		o4	1 ⁴	0.07	0.02
			2 ²	0.10	0.10
NNLS	2015	o1	1	1.00	<0.001
		o2	2	0.98	0.03
		o3	3	1.00	<0.001
	2016	o1	1	0.95	<0.001
			2	0.31	0.01
		o2	1	0.66	0.21
			2	0.05	0.29
		o3	1	0.83	0.03
			2	0.27	0.03
		o4	1	1.00	0.01
			2	<0.001	0.94

¹Bayesian method assuming a Gaussian prior distribution with a prior mean of, proportionally, 0.95 for smooth brome grass, with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

²Bayesian method assuming a Gaussian prior distribution with a prior mean of, proportionally, 0.90 smooth brome grass, with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

³Pastures were different across years.

⁴Bayesian method assuming a Gaussian prior distribution with a prior mean of, proportionally, 0.80 for smooth brome grass, with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

⁵T-test, a paired t-test between estimated DMI using $C_{31}:C_{32}$ and $C_{33}:C_{32}$ ratios.

Table 3.9 Parameter estimates for the regression of observed indoor intakes (kg DMI/d) on estimated intakes (kg DMI/d) obtained using C₃₁:C₃₂ ratios

Method	Year	Study	Pasture	β_0 ⁴ (SE)		β_1 ⁵ (SE)		r^2	r	<i>P</i> -value		
										β_0 ⁶ =0	β_1 ⁷ =1	T-test ⁸
Bayesian	2015	o1 ¹	n/a	9.70	(2.18)	-0.05	(0.28)	0.01	0.18	0.01	0.02	0.05
		o2 ²	n/a	4.34	(2.02)	0.48	(0.20)	0.59	0.47	0.10	0.06	0.07
		o3 ²	n/a	6.75	(2.21)	0.31	(0.31)	0.20	0.42	0.04	0.09	0.01
	2016	o1	1 ¹	7.49	(1.58)	-0.16	(0.22)	0.16	0.47	0.02	0.01	0.43
			2 ¹	5.59	(2.78)	0.10	(0.31)	0.05	0.52	0.18	0.10	0.07
		o2	1 ¹	-1.58	(9.15)	0.95	(1.09)	0.28	0.17	0.88	0.97	0.08
			2 ³	5.04	(3.47)	0.19	(0.45)	0.06	0.21	0.24	0.17	<0.001
		o3	1 ¹	4.82	(1.26)	0.17	(0.16)	0.22	0.50	0.02	0.01	0.01
			2 ¹	2.99	(1.55)	0.32	(0.16)	0.56	0.29	0.15	0.02	0.02
		o4	1 ³	8.30	(2.08)	-0.24	(0.23)	0.26	0.69	0.03	0.01	0.05
			2 ¹	8.29	(2.29)	-0.32	(0.30)	0.54	0.19	0.17	0.14	0.15
NNLS	2015	o1	n/a	9.65	(2.22)	-0.05	(0.29)	0.01	0.09	0.01	0.02	0.03
		o2	n/a	3.81	(2.24)	0.56	(0.23)	0.59	0.79	0.16	0.13	0.27
		o3	n/a	6.75	(2.20)	0.31	(0.31)	0.20	0.44	0.04	0.09	0.01
	2016	o1	1	7.49	(1.56)	-0.15	(0.20)	0.16	0.49	0.02	0.01	0.28
			2	5.60	(2.79)	0.10	(0.32)	0.05	0.49	0.18	0.10	0.07
		o2	1	-0.02	(7.75)	0.79	(0.95)	0.26	0.17	1.00	0.85	0.08
			2	3.97	(3.48)	0.37	(0.51)	0.15	0.88	0.34	0.31	<0.001
		o3	1	4.82	(1.27)	0.17	(0.16)	0.22	0.51	0.02	0.01	0.01
			2	2.96	(1.36)	0.32	(0.14)	0.63	0.54	0.12	0.02	0.04
		o4	1	7.95	(1.94)	-0.22	(0.23)	0.22	0.39	0.03	0.01	0.45
			2	8.03	(6.46)	-0.34	(0.98)	0.11	0.85	0.43	0.40	0.20

¹Bayesian method assuming a Gaussian prior distribution with a prior mean of, proportionally, 0.90 for smooth brome grass, with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

²Bayesian method assuming a Gaussian prior distribution with a prior mean of, proportionally, 0.95 for smooth brome grass, with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

³Bayesian method assuming a Gaussian prior distribution with a prior mean of, proportionally, 0.80 for smooth brome grass, with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

⁴ β_0 , intercept (kg/d).

⁵ β_1 , slope (kg/d per kg/d).

⁶ $\beta_0 = 0$, test of intercept equal to zero.

⁷ $\beta_1 = 1$, test of slope equal to one.

⁶T-test, a paired t-test between observed and estimated DMI.

Table 3.10 Parameter estimates for the regression of estimated intakes, based on $C_{31}:C_{32}$ ratio, for the 2016 outdoor studies.

Method	X variable	Y variable	β_0 ⁶ (SE)		β_1 ⁷ (SE)		r^2	<i>P</i> -value		
								β_0 ⁸ =0	β_1 ⁹ =1	T-test ¹⁰
Bayesian ¹	O1 ³	O2 ⁴	9.71	(1.95)	-0.06	(0.26)	0.01	0.01	0.02	0.04
		O3 ³	5.55	(2.13)	0.36	(0.29)	0.28	0.06	0.09	0.15
		O4 ⁵	9.13	(1.98)	-0.14	(0.27)	0.06	0.01	0.01	0.32
	O2 ⁴	O1 ³	9.31	(8.73)	-0.22	(0.94)	0.01	0.35	0.27	0.04
		O3 ³	0.32	(4.45)	0.85	(0.48)	0.44	0.95	0.77	0.01
		O4 ⁵	4.43	(4.49)	0.40	(0.48)	0.15	0.38	0.28	0.02
	O3 ³	O1 ³	0.89	(5.15)	0.79	(0.63)	0.28	0.87	0.75	0.15
		O2 ⁴	5.04	(2.41)	0.52	(0.29)	0.44	0.10	0.18	0.01
		O4 ⁵	6.37	(3.24)	0.21	(0.39)	0.07	0.12	0.12	0.92
	O4 ⁵	O1 ³	10.98	(7.15)	-0.45	(0.88)	0.06	0.20	0.17	0.32
		O2 ⁴	6.30	(3.61)	0.36	(0.44)	0.15	0.16	0.22	0.02
		O3 ⁵	5.57	(4.82)	0.32	(0.59)	0.07	0.31	0.31	0.92
NNLS ²	O1	O2	9.55	(1.90)	-0.04	(0.25)	0.01	0.01	0.01	0.07
		O3	5.26	(1.96)	0.33	(0.26)	0.29	0.06	0.06	0.71
		O4	6.59	(1.56)	0.07	(0.20)	0.03	0.01	0.01	0.57
	O2	O1	9.05	(9.23)	-0.17	(1.00)	0.01	0.38	0.31	0.07
		O3	-0.08	(4.07)	0.85	(0.44)	0.48	0.99	0.74	<0.001
		O4	3.87	(3.47)	0.35	(0.37)	0.18	0.33	0.16	<0.001
	O3	O1	0.66	(5.38)	0.89	(0.69)	0.29	0.91	0.88	0.71
		O2	4.84	(2.30)	0.57	(0.30)	0.48	0.10	0.22	<0.001
		O4	4.43	(2.27)	0.35	(0.29)	0.26	0.12	0.09	0.13
	O4	O1	4.62	(8.50)	0.41	(1.19)	0.03	0.62	0.64	0.57
		O2	5.58	(3.90)	0.51	(0.55)	0.18	0.23	0.42	<0.001
		O3	2.37	(4.51)	0.75	(0.63)	0.26	0.63	0.72	0.13

¹Bayesian method assumed a Gaussian prior distribution with a prior mean based on observed pasture composition with a covariance matrix with diagonal elements of 1×10^5 and off-diagonal elements of zero. Animals A, B and C were in pasture 1, while animals D, E and F were in pasture 2.

²Non-negative least squares approach for estimating diet.

³A prior mean of, proportionally, 0.90 for smooth brome grass.

⁴Pasture 1 assumed a prior mean of, proportionally, 0.90 for smooth brome grass, while pasture 2 assumed a prior mean of, proportionally, 0.80 smooth brome grass.

⁵Pastures 1 assumed a prior mean of, proportionally, 0.80 for smooth brome grass, while pasture 2 assumed a prior mean of, proportionally, 0.90 for smooth brome grass

⁶ β_0 , intercept (kg/d).

⁷ β_1 , slope (kg/d per kg/d).

⁸ $\beta_0 = 0$, test of intercept equal to zero.

⁹ $\beta_1 = 1$, test of slope equal to one.

¹⁰T-test, a paired t-test between estimated DMI from the two studies.



Fig. 3.1 Pasture set up for the 2015 grazing study.



Fig. 3.2 Pasture set up for the 2016 grazing study.

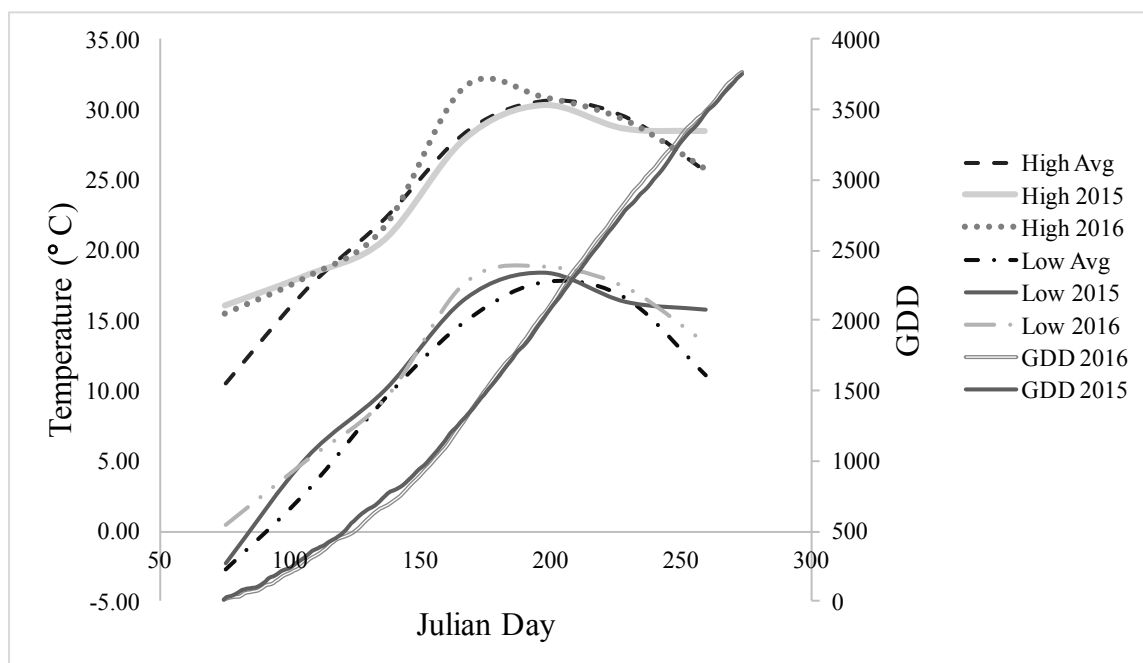


Fig. 3.3 Observed high and low temperatures, and growing degree days (GDD), relative to Julian date in 2015 and 2016. Thirty yr. high and low temperatures also are shown.

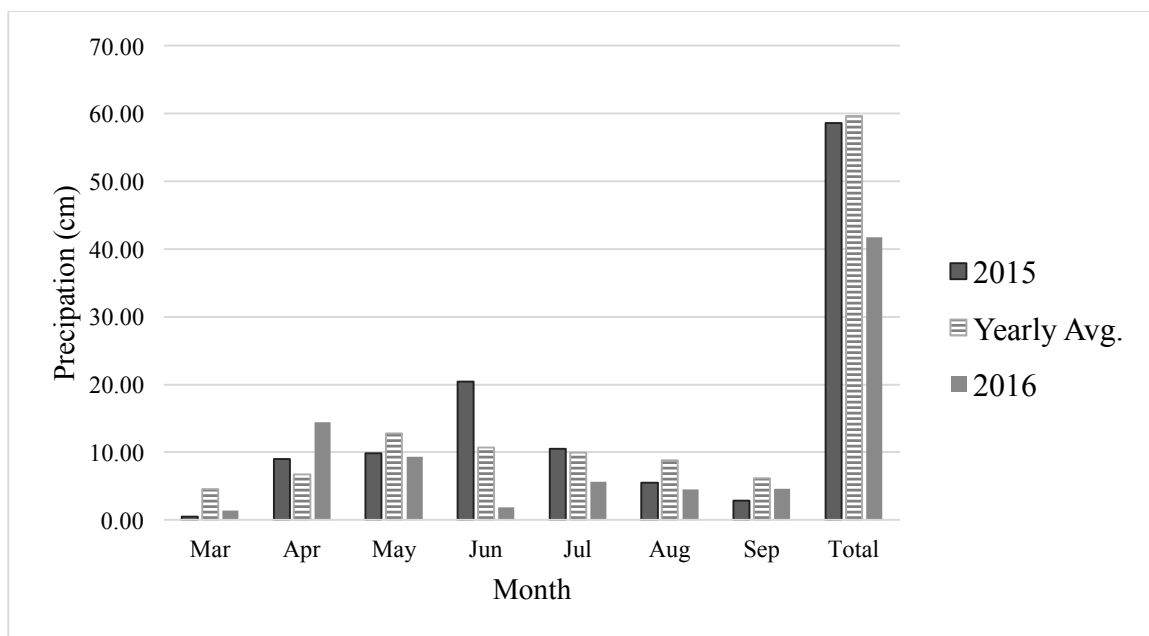


Fig. 3.4 Precipitation for 2015 and 2016 measured from the Harvard, NE weather station (40.618, -98.094; elevation 552 meters). Thirty yr average precipitations also are shown.

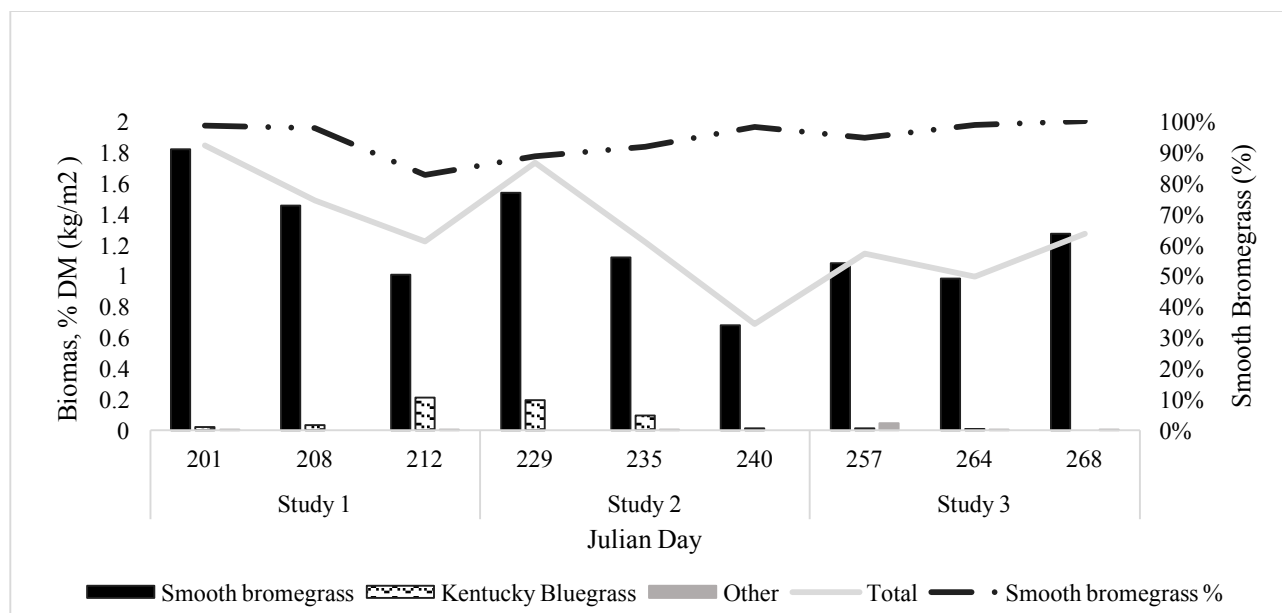


Fig. 3.5 Observed pasture composition for 2015. Biomass available (kg/m^2) by plant species on the left axis, while percent smooth brome grass on the right axis.

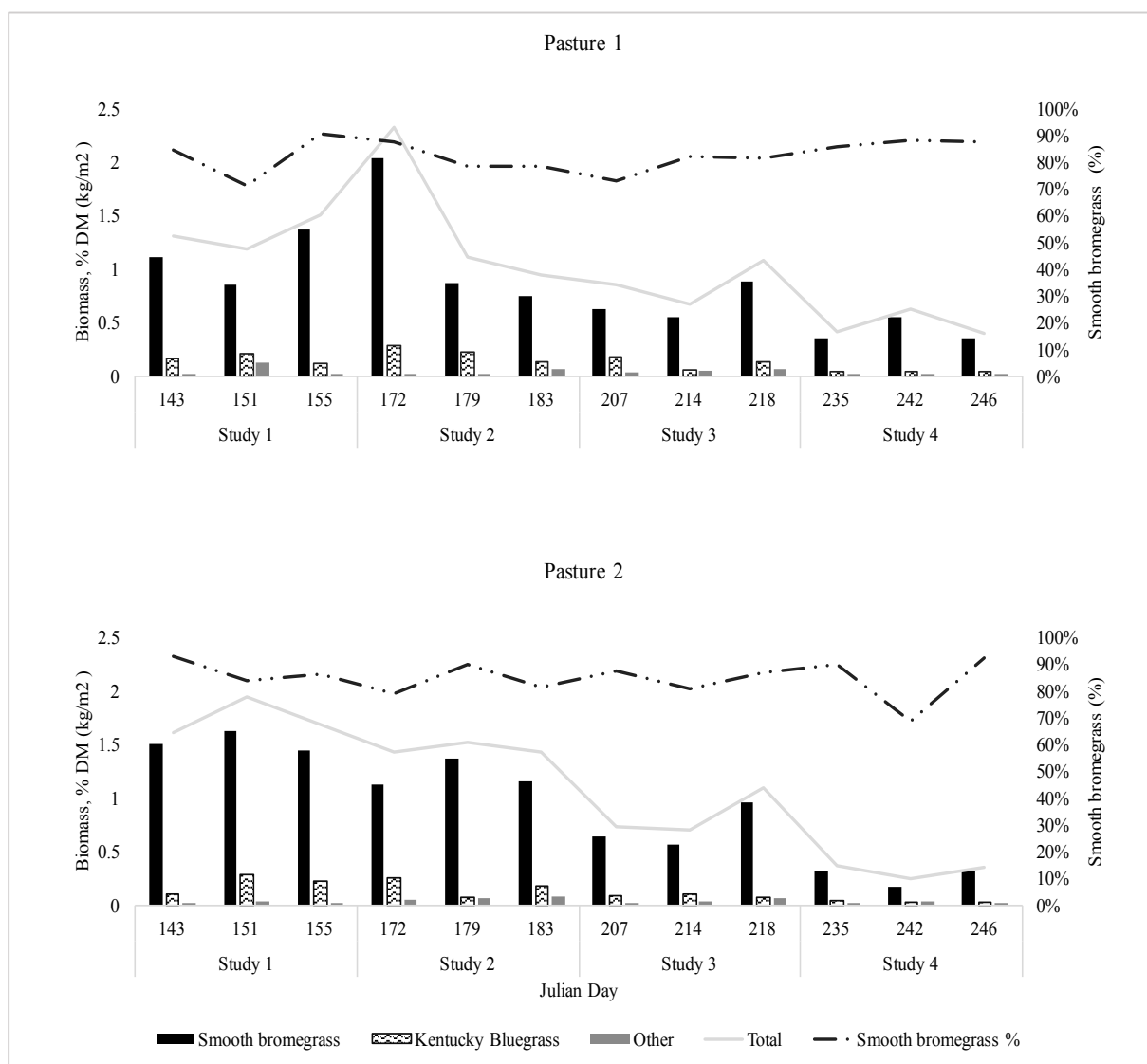


Fig. 3.6 Observed pasture composition for 2016 by pasture. Biomass available (kg/m^2) by plant species on the left axis, with smooth brome percent on the right axis.

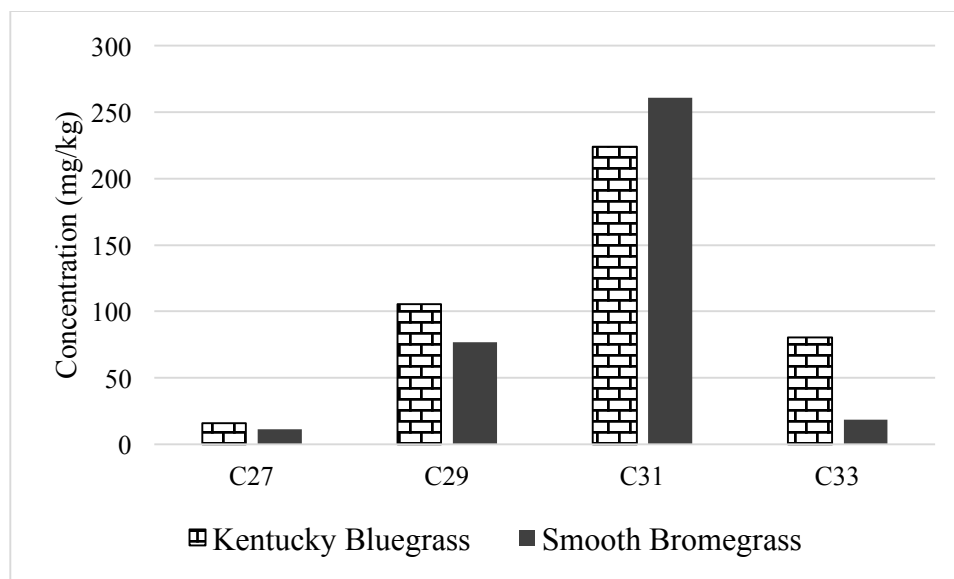


Fig. 3.7 *N*-alkane concentrations for Kentucky bluegrass and smooth brome grass collected on June 20, 2016 in pasture 2. These samples correspond with collections on d 1 of study 2 of that year.

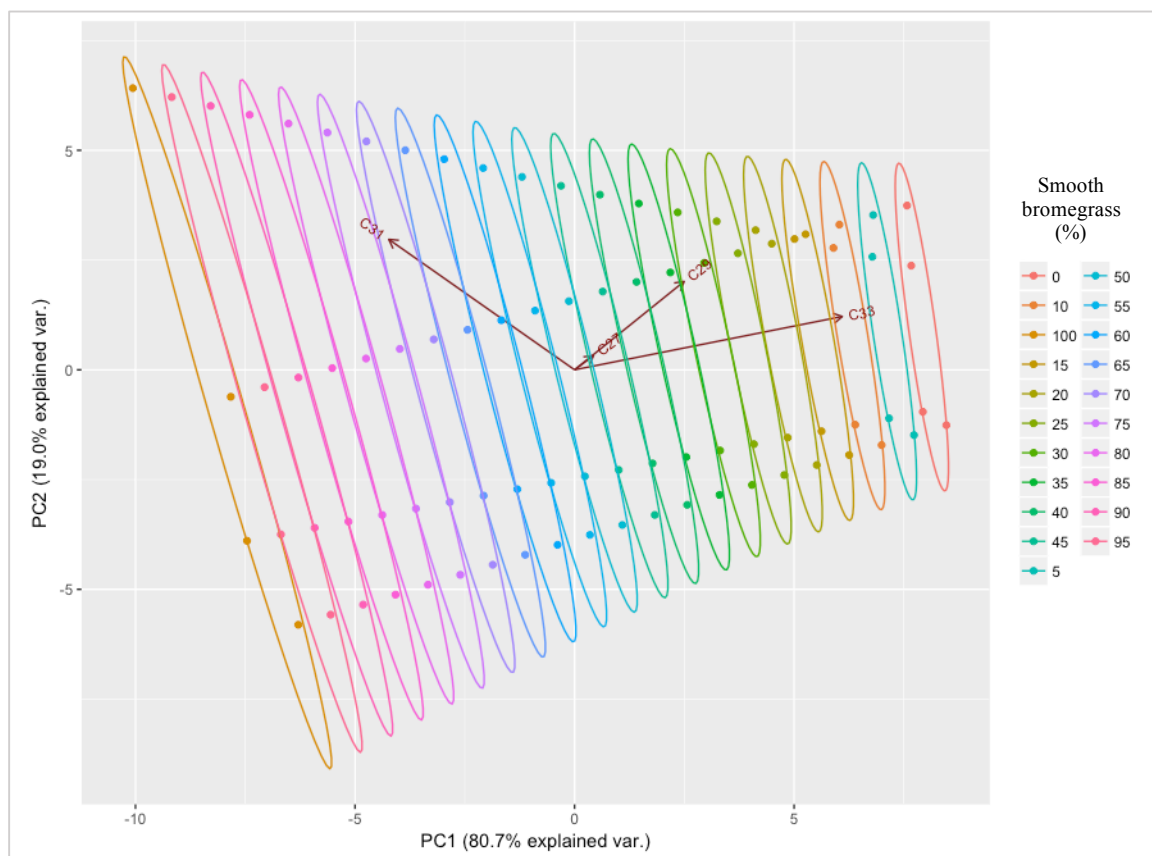


Fig. 3.8 Principal component analysis of forage mixture of different percentages of smooth brome grass and Kentucky bluegrass using theoretical values generated from the concentrations of C_{27} , C_{29} , C_{31} and C_{33} *n*-alkanes in the pure plants collected on d 1 of study 2 in pasture 2 of the 2016.

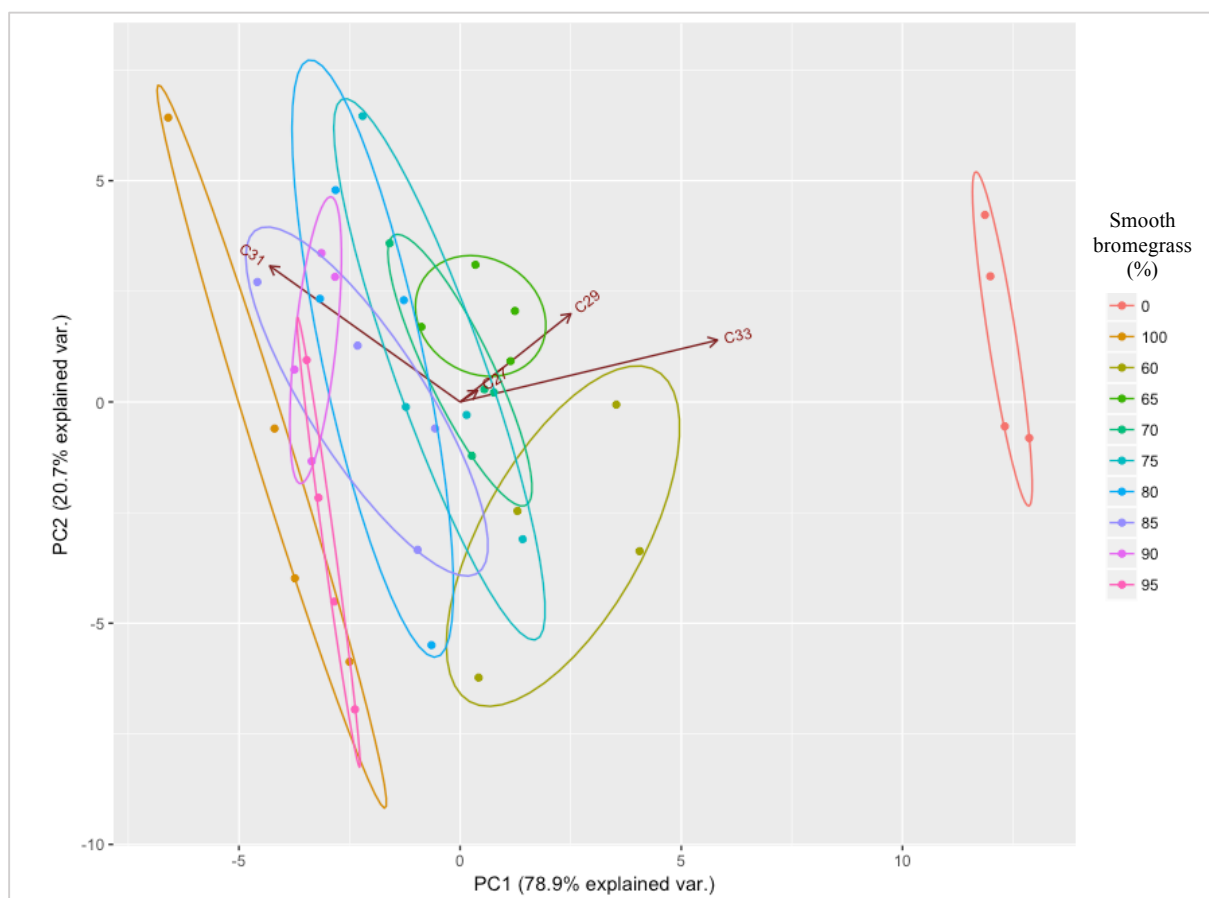


Fig. 3.9 Principal component analysis of forage mixtures formed by combining specified weights of smooth brome grass and Kentucky bluegrass collected on d 1 of study 2 in pasture 2 of the 2016.

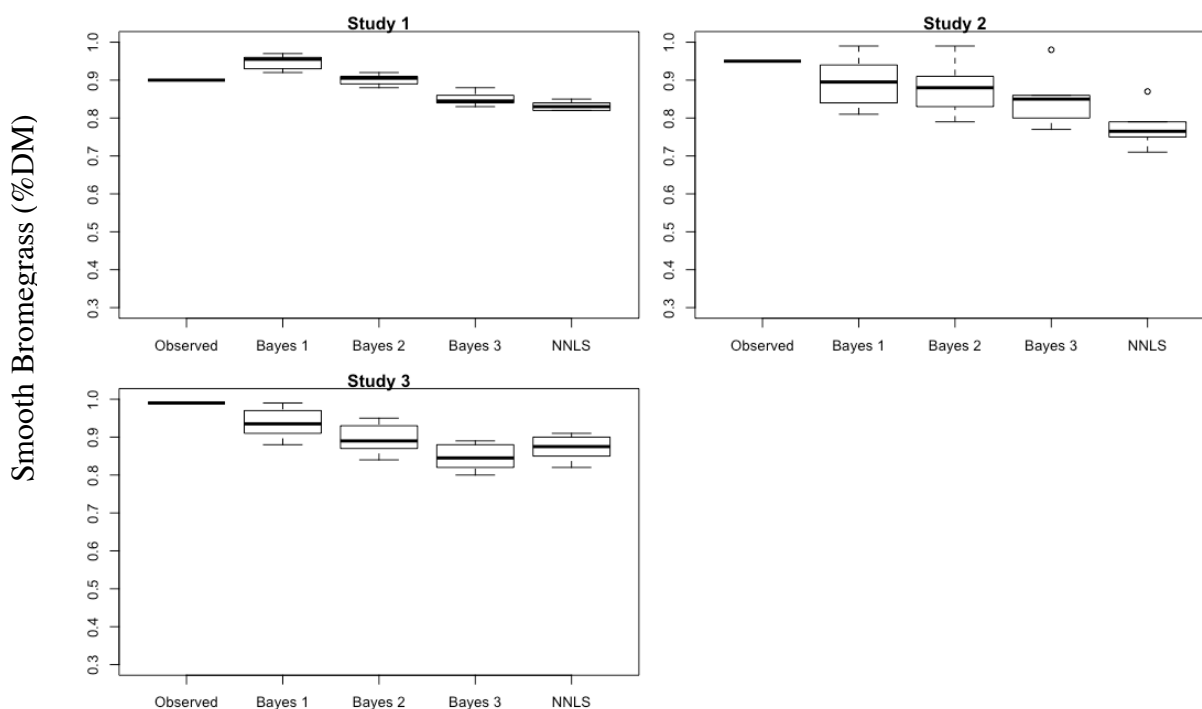


Fig. 3.10 Boxplot of diet composition estimates for the 2015 experiment. The Bayesian methods assumed a Gaussian prior distribution with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero. For Bayes 1, 2 and 3, the prior means were, proportionally, 0.95, 0.90 and 0.85, respectively, for smooth bromegrass. The NNLS is the non-negative least squares method for estimating diet composition.

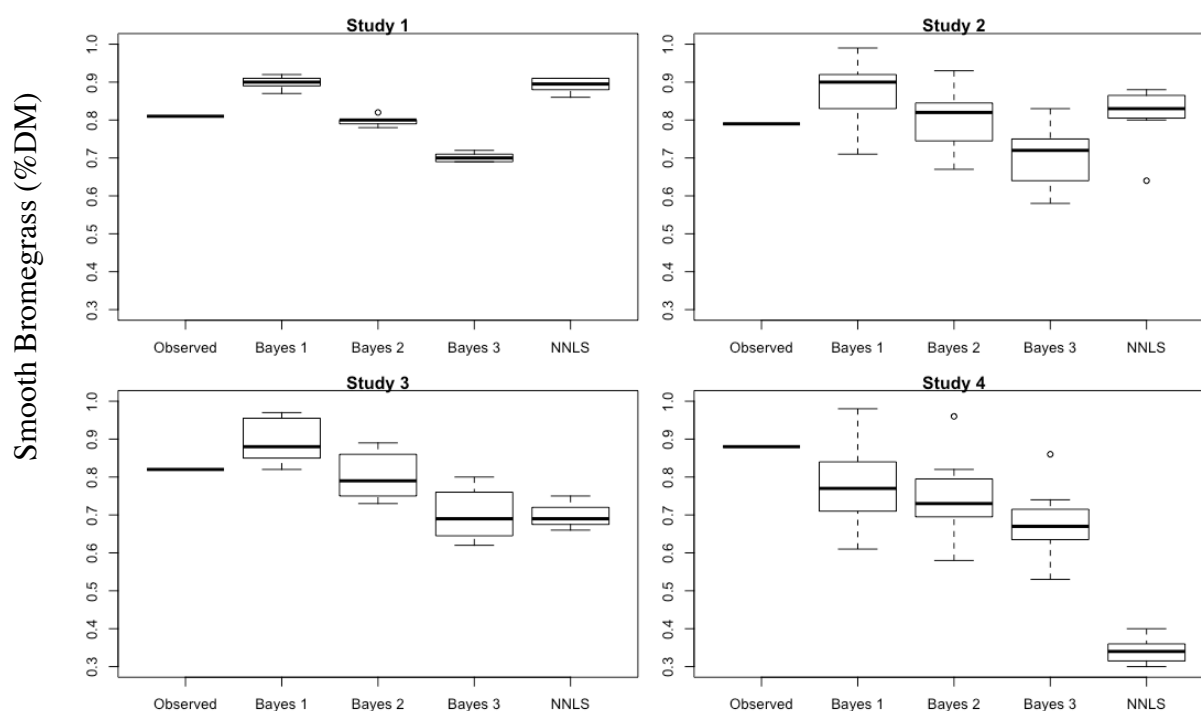


Fig. 3.11 Boxplot of diet composition estimates for pasture 1 in the 2016 experiment. The Bayesian methods assumed a Gaussian prior distribution with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero. For Bayes 1, 2 and 3, the prior means were, proportionally, 0.90, 0.80 and 0.70, respectively, for smooth bromegrass. The NNLS is the non-negative least squares method for estimating diet composition.

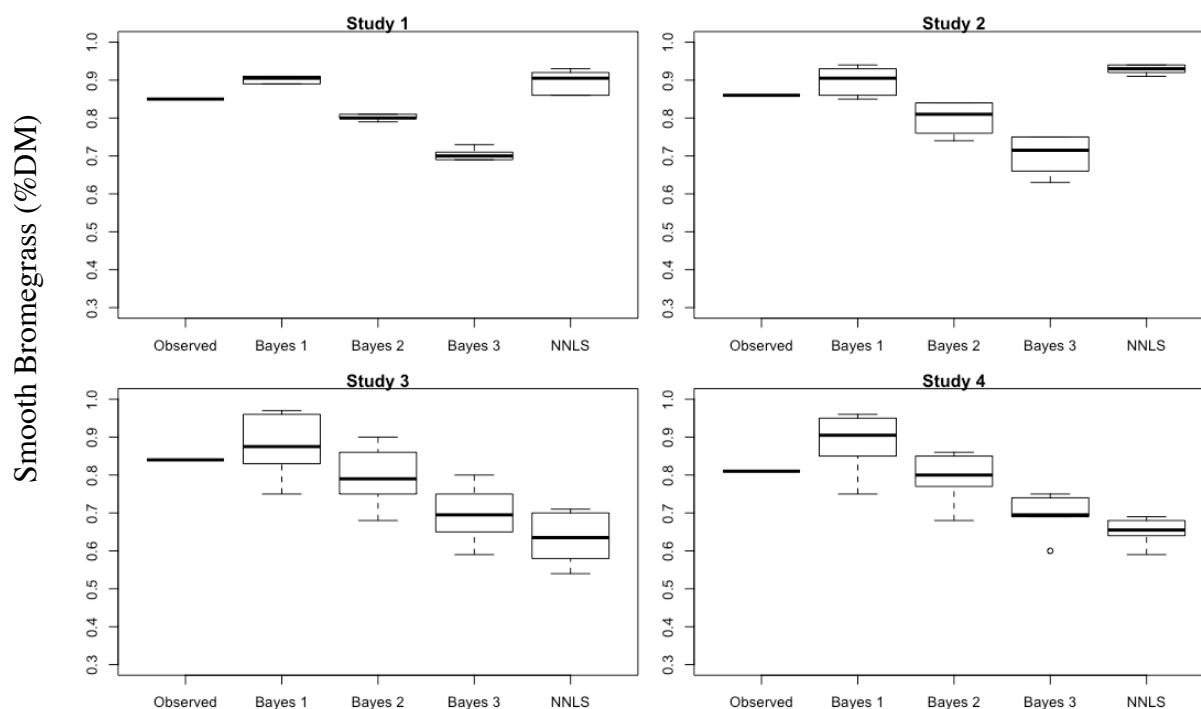


Fig. 3.12 Boxplot of diet composition estimates for pasture 2 in the 2016 experiment. The Bayesian methods assumed a Gaussian prior distribution with a covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero. For Bayes 1, 2 and 3, the prior means were, proportionally, 0.90, 0.80 and 0.70, respectively, for smooth bromegrass. The NNLS is the non-negative least squares method for estimating diet composition.

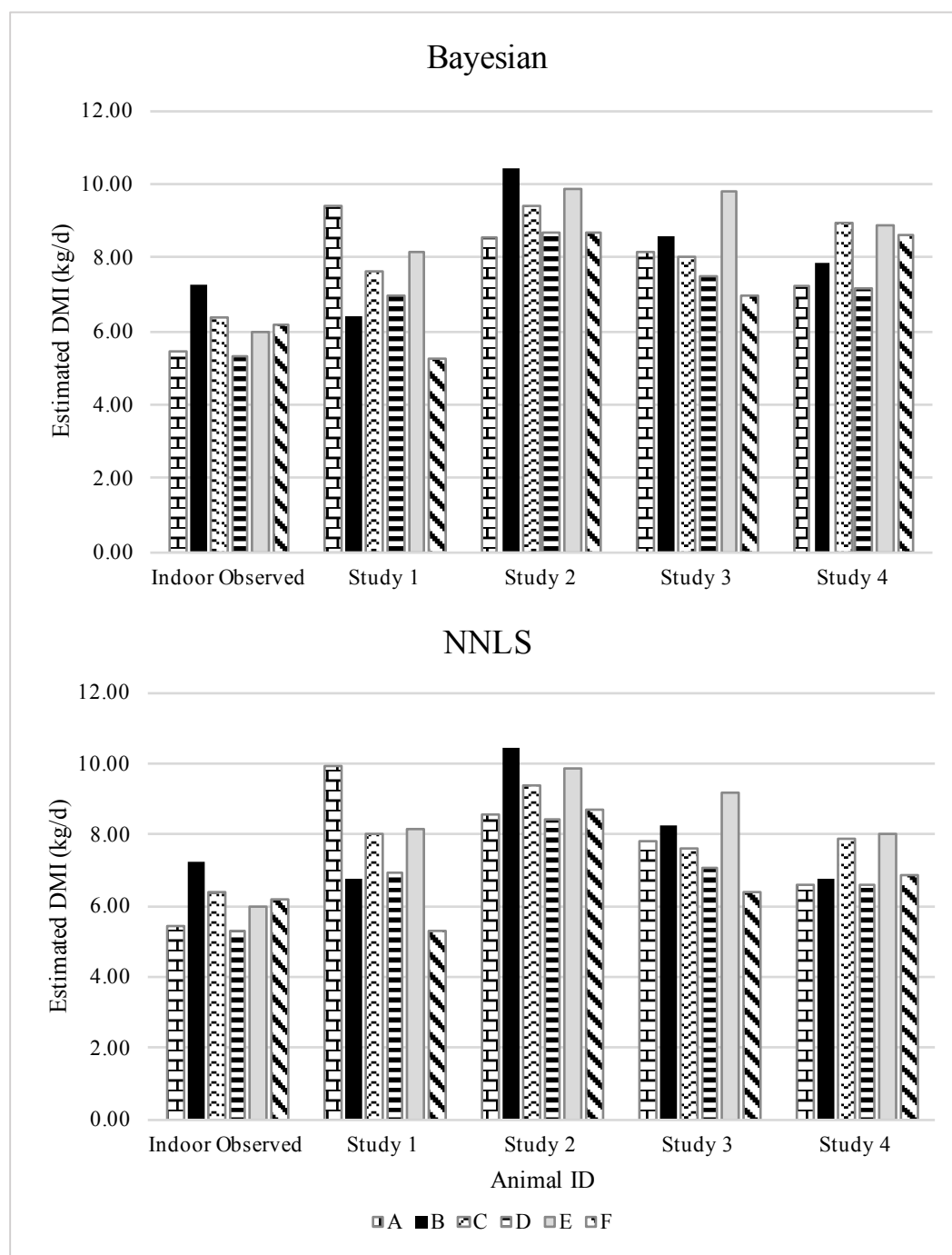


Fig. 3.13 Estimated DMI for a selection of individual animals in the 2016 experiment by study and diet composition estimation method. Animals A, B and C were in pasture 1, while animals D, E and F were in pasture 2. Diet compositions were estimated by non-negative least squares (NNLS) or a Bayesian method. For studies 1 and

3 in both pastures, study 2 in pasture 1, and study 4 in pasture 2 the Bayesian estimates were based on a prior mean of, proportionally, 0.80 for smooth brome grass. Whereas, in study 2 in pasture 2, and study 4 in pasture 1, the Bayesian estimates were based on a prior mean of, proportionally, 0.90 for smooth brome grass. All Bayesian techniques were estimated with a Gaussian prior distribution and covariance matrix with diagonal elements 1×10^5 and off-diagonal elements of zero.

Chapter IV: Synthesis

INTRODUCTION

Most of American beef cattle are raised on pasture. As the world's population grows there will be less area to raise cattle, but a larger demand for their products. By selecting cattle to become more energy efficient under less than desirable conditions, cattle may be able to fit in a particular niche and help feed the plant.

To select animals based on efficiency, one must know intake, diet composition and digestibility of plants consumed. Studies have shown that there is a substantial amount of variation in metabolic and grazing efficiencies, which indicates the possibility that these traits can be improved upon. When the availability of food is controlled, diet composition is known while feed intake and digestibility can be easily measured. However, traditional methods of measuring efficiency are nearly impossible for range settings. Plants contain waxes, which help protect the plant against environmental stressors. Plant-waxes, such as *n*-alkanes (ALK), are essentially indigestible by ruminant animals and have shown to be a promising tool to measure digestibility, intake and diet composition. The work presented in chapter II and III, consisted of 2 separate indoor studies, occurring in 2015 and 2016, which were then followed by a series of outdoor studies that consisted of the same animals as the indoor study.

INDOOR STUDIES

Our objective for the indoor studies was to evaluate 3 factors that impact the reliability of intake estimates based on plant-waxes. Firstly, we compared 3 strategies for combining ALK concentrations obtained on fecal samples to estimate DM intake (**EDMI**) daily. One strategy entailed analyzing a single fecal sample per animal by pooling daily collections. The other strategies analyzed daily fecal samples. Secondly, due to the supplement being fed, marker loss was possible; the effect of dosing loss on EDM I was, therefore, investigated. Since some wastage was possible, ingestion of the marker was assumed to be complete or as percentage increments of that offered. The cattle were fed a total mixed ration (**TMR**) consisting of approximately 70% corn silage (**CS**) and 30% ground alfalfa. Some sifting of the feedstuff by the cattle was possible. Therefore, as our last study, we considered the impact of differences in the composition of individual animals' diets, estimated using ALK, on EDM I.

OUTDOOR STUDIES

The outdoor study was designed to assess the reliability of using ALK to estimate diet selection and intake in cattle in a grazing setting. Four aspects were reviewed to better understand the ALK's utility: (i) validation that plants found in a predominantly smooth brome grass (**SB**) pasture can be delineated prior to consumption; (ii) estimation of diet composition using ALK markers compared to known species; (iii) estimation of DMI using ALK markers; and (iv) intake performance across the studies relative to previous performance and plant biomass available.

RELIABILITY

As a measure of reliability for the indoor study observed DMI (**ODMI**) were regressed on EDMl. (Piñeiro et al., 2008). We know the estimation is reliable if the slopes do not differ from unity and the intercept is not significantly differed from zero. The indoor experiments revealed a substantial increase in variation in EDMl as compared to ODMI, which made the regressions coefficient of ODMI on EDMl inconsistent with the regression of EDMl on ODMI. No method, when ODMI was regressed on EDMl, with any combination of fecal evaluation, diet composition or percentage loss of internal marker had slopes or intercepts that were not significantly different from the presumed values. However, when EDMl was regressed on ODMI the opposite was true, with all methods having slopes not significantly different from one nor intercepts different from zero.

The increased variation is likely due to a combination of errors, which include lab analyses, incorrect dosing (marker loss or supplement error) and the profiles of the feeds themselves. If feed intakes cannot be reliably measured in a confined setting, then there is likely no way to reliably obtain EDMl when diet composition and actual intake will be unknown. Although there is increased variation, the question remains if the EDMl represent the animals' intakes well enough so that animals can be ranked and selected based on EDMl calculated using ALK. Genetic selection can loosely be thought of as selecting animals based on ranks. If EDMl based on ALK reliability rank animals, then even though the EDMl are not exact, could be used for genetic selection. Spearman's rank correlations (Table 4.1) show that ALK profiles when using a single feed, can

reliability rank animals (in 2015, r 0.71; in 2016, r 0.61). However, in order to understand the inherent problems seen in the increased variation when using ALK to EDM I needs to be addressed.

From this work, several concerns arose that were believed to have increased variation in the EDM I. These issues include: (i) lab errors; (ii) administration of dosing; (iii) variation of intake due to animal behavior; (iv) the number of samples needing extractions; and (v) given their ALK profiles, the ability to distinguish plants and EDM I. The combinations of these issues resulted in increased variation in the EDM I.

Laboratory Errors

Variations in ALK concentrations between samples can be the results of many sources of error. Plants, first and foremost, must be properly identified and labeled before extraction. In this study, a few samples were not properly labeled or identified, but their profiles were unique enough to show this error. If the profiles were not unique, then this error could be presumed to be due to technician error or morphological changes present in the plant, which would reduce reliability.

Technicians. Technicians were internally consistent in their evaluation of ALK concentrations of fecal and forage samples; however, there was variation between technicians. This error can best be visualized by Fig. 4.1, which contains ALK concentrations of pure alfalfa and CS measured by 2 separate technicians. In order to calculate EDM I, these disparities make it necessary for one technician to extract all fecal and plant samples from a single study. These constraints significantly hinder the

flexibility of the ALK method for estimating intakes. The extraction technique is a test and well accepted protocol; therefore, the variability between technicians may be due to differences in their interpretation of the protocols. The technician's lab methods should be examined further to find potential sources of error that could lead to inconsistencies in ALK concentrations.

Gas chromatography. The gas chromatography (GC) machine was a significant source of error and a hindrance to progress. Even with preventative maintenance, continuous visual monitoring was required to ensure the GC was running correctly. The carrier liquid, *n*-dodecane, is considerably "sticky" which caused injection needles to clog. To counter this issue, the washing solution was changed from heptane to acetone, which helped; still, needle clogging still occurred. Injection errors, which causes variation in the estimate of ALK concentrations from the same extraction vial, also occurred. Injection errors were believed to be the result of the column or needle becoming contaminated. Injection errors caused a need for reruns, which cost time and money. Although large injection errors could be seen by visually monitoring the peaks, smaller, yet still significant, injection errors could go unnoticed until the run was finished and concentrations were calculated. Additionally, samples with dosed ALK and extracted using the Dove and Mayes (2005) protocol, caused clogging in the GC column due to high concentrations of ALK. Samples with known high concentrations of ALK should be diluted prior to running on the GC. Adaptations to the methodology the still needed to make extracting and running samples occur smoothly before this technique could be used in a production setting.

Dosing

During the course of these experiments incorrect dosing possibly occurred, which would add variation seen in EDM calculations. The indoor experiments' sensitivity analysis showed potential problems if animals were not consuming the full dosing due to wasting. Wasting of supplement could help explain under estimates of intake observed in the indoor experiments. Furthermore, dosing problems continued to occur in the outdoor experiments, because animals would not consume their supplement. In 2015, outdoor animals adapted so poorly to the supplement that study 1 had to be abandoned. While in 2016 only 6 animals out of the original 12 were observed consuming their dose supplement continuously. The difficulty animals had adapting to the supplement may have been due to palatability. Although animals would consume the supplement in confinement because they had no choice, the supplement could be less appealing to animals that are free choice grazing. A new more palatable supplement should be used in future experiments. Lessening the amount of dye, while still keeping the distinctiveness of the fecal pats, may help increase palatability or sweeteners may need to be added. A daily bolus, although more laborious, could be used to remove risk of animals failing to consume their full dose.

Collection Days

Animal daily intakes vary due to a number of interacting factors such as nutrition, digestibility, the rate of digestion and the rate of passage. This variation can be seen in the daily ODMI as well as in the daily fecal ALK concentrations. The results showed that pooling daily fecal samples better depicted an average intake when compared to the daily

fecal methods, as well as reduced analytical time. However, a 5 d collection period may not be long enough due to the rate of passage. The ODMI collected on the same day as the collected fecal samples does not account for intake prior to the collection period. Both indoor experiments had significant drops in intake the week fecal samples were collected, which was likely due to the added stress caused by being handled daily. Fecal pats at the beginning of the experiment may have higher ALK concentrations due to higher intakes the week prior due to rate of digestion. For these reasons, it is perhaps pertinent to increase the number of sampling days so that pooled fecals can better reflect the animals average intake. If increasing sampling days is not possible, observing individual intake the days before fecal pooling could also help minimize error. Furthermore, a recent study by Olivera et al. (2015) showed that EDM I can change throughout the day. These changes are likely due to gutfill and movement of digestive fluid. Due to this pooling, more samples may decrease the variability seen in EDM I.

The outdoor study had no known intakes, so EDM I had to be compared to previous ODMI. Comparing previous ODMI to EDM I from a different study affected by many environmental and animal variation is problematic. Animal intakes fluctuate and their behavior can change in various environments. Due to this, the inability for ALK profiles to replicate intakes from previous indoor ODMI is understandable. Animals' individual EDM I across sequential grazing studies show variation as well, which again can be due to environmental factors or metabolic needs. From the current research, it is still unclear if EDM I can accurately depict actual intakes.

Decreased sampling

Pooling fecal samples decreases the number of samples needing extracting, which decreases the amount of time and money spent on EDM. However, forages must also be extracted for each study. Forage ALK profiles have been shown to be dependent on morphological changes of the plant; therefore, current forage samples must be extracted each time EDM are calculated. These studies further confirmed that ALK concentrations can change throughout the season. A better understanding of morphological changes effects on ALK profiles could decrease the number of plant samples needing extraction. Morphological changes have been linked in SB to growing degree days (GDD). If ALK profiles for different plant species could be predicted based on weather and morphological changes then the amount of sampling and extractions could be decreased. Although decreasing the laborious attributes of this technique would make it more appealing as a commercial product, the variability seen across the studies, plant parts and the individual quadrants may make modeling ALK concentrations precisely, which is needed for EDM, incredibly difficult and costly.

Distinguishing diets

The results from the present work further confirmed that plant-wax makers such as ALK can create reasonably close EDM if diet were a considered a single food. However, animals grazing do not typically consume a homogeneous diet. Estimating diet composition is highly dependent on the ALK profiles of the plants. Both the indoor and outdoor study contained forage mixtures that were not able to delineate 5% changes in

the plant profiles. Small changes can effect EDM I especially when the diet components have highly variable ALK profiles.

For both indoor experiments, the non-negative least squares (NNLS) statistical method overestimated the amount of CS present in the TMR. Combined with the low concentrations of ALK in the CS, which was the majority of the diet, the overestimation of the CS composition of the diet contributed to EDM I based on the NNLS to overestimate intake. Despite this problem, in 2016, the NNLS method was able to best rank animals in accordance with their ODMI when using a Spearman's rank correlation ($r = 0.78$), but the correlation was lower in 2015 ($r = 0.48$). The inability for these diet composition estimation techniques to properly rank animals would prevent animals grazing an unknown diet to be reliably selected for differences in efficiency.

The ALK profiles caused further issues with the Bayesian approaches evaluated in the indoor experiment (Bayes 1, with prior mean of 80% for CS; Bayes 2, with prior mean of 70% for CS). In order for diet composition estimation to converge, the prior knowledge had to be heavily weighted. When prior knowledge was allowed to have minimal influence the diet composition estimation failed to converge, which likely was due to lack of distinction between the ALK profiles of alfalfa and CS. Although heavily weighting the prior knowledge may work in the indoor experiment, the animals on pasture were exposed to a far more variable diet, which means large emphasis on prior knowledge may not be appropriate. Like in the indoor experiments, the Bayesian methods for the outdoor experiments had to be heavily weighted in order for the diet composition estimations to converge.

There were further issues with diet composition prediction and EDM I during the course of the outdoor studies. The two dominant plants, SB and KBG, had significantly different concentrations of marker C_{33} . SB and KBG, much like the alfalfa and CS, could only be delineated at 10% increments. Markers C_{31} and C_{33} ratio with C_{32} are typically averaged to calculate EDM I. The vastly different concentrations of marker C_{33} in the diet components, SB and KBG, made EDM I based on marker C_{33} more sensitive to errors in diet estimation, which likely contributed to the extreme EDM I (e.g., as high as 37.3 kg/d). Such unrealistic intakes show the importance of examining plant profiles prior to diet composition estimates or EDM I. Unlike C_{33} , EDM I using marker C_{31} were reasonable, which was likely due to lack of substantial differences in C_{31} between SB and KBG.

Additional markers could be used to help distinguish plant profiles and increase the reliability of diet composition estimation. In addition to ALK, plant-waxes also include ketones, fatty acids, long-chain alcohols (**LCOH**) and aldehydes. Issues arise with the reliability of these additional compounds because they need additional steps beyond the ALK extraction to be separated. These additional steps allow the concentrations to be more affected by laboratory issues. The LCOH were extracted for the 2015 experiment samples but variation between samples caused these values to be unreliable; therefore, they were not used in these analyses.

Increasing the ability to distinguish plants will increase the strength in estimated diet composition and in turn increase the reliability of EDM I. However, if plants were similar in ALK profiles and nutrient content, it may not be necessary to distinguish

between plants. Common plants classification systems (C3 and C4) has not been a reliable way to group plant profiles. However, maybe additional species classification could be used to separate plants by ALK profiles and nutrient content. If the nutritive qualities are the same, then the chemostatic and physiological mechanism, which affect intake, would not be different between plants, making the need to tell plants apart unnecessary.

ADDITIONAL TOOLS

A single tool will not allow scientist to truly understand grazing efficiency in large open ranges. Instead, a variety of tools will need to be utilized to genetically select cattle that perform better. Tools, when added to plant-waxes, could increase the accuracy of genetic selection. These tools could include species grouping, DNA analysis and GPS tracking.

As pasture size increases, more sampling would need to occur to accurately depict the diversity of the pasture. The DNA analysis of the manure could decrease the number of plants assumed to be in the diet. Small segments of chloroplast DNA have been successfully amplified from manure, which suggests that plant DNA have the ability to survive the digestive system. Cattle have distinct and consistent grazing patterns. Large herbivores, such as cattle, can be presented as many as 50 different plant-community a day. The difference in grazing location changes what plants an animal is presented with, which in turn changes diet composition and potentially intake. By knowing where cattle are located, a number of plants animals are presented with may decrease. With all these

methods, decreasing a number of plants needing to be discriminated against will greatly help plant-wax markers ability to estimate diet composition and intake.

CONCLUSION

Grazing animals live in vastly different environments, often travel large distances, are presented with a large variety of plants and have different energy efficiencies, which makes it hard to depict what, and how much, they are eating. Due to the complexity of energy utilization and grazing systems, a reliable set of tools is needed to identify efficient cattle on pasture. The ALK profiles may provide a useful addition, but there is still need for a considerable amount of work to understand the sources of variation seen in the EDM calculations. Furthermore, if the variation is found to consistent EDM based on ALK profiles may still to be accurate rank animals based on their intake. Ultimately, by combining nutrition and genetics, cattle's future of feeding the earth's growing population may be ensured.

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Table 4.1 Spearman's rank correlation between observed and estimated DMI based on various methods¹ for determining diet composition in the 2015 (above diagonal) and 2016 (below diagonal) Exp.

	Observed	Bayes 1	Bayes 2	NNLS	TMR	Composite
Observed		0.42	0.42	0.48	0.65	0.71
Bayes 1	0.55		0.97	0.91	0.78	0.58
Bayes 2	0.59	0.99		0.97	0.80	0.63
NNLS	0.48	0.93	0.94		0.88	0.74
TMR	0.78	0.88	0.89	0.77		0.94
Composite	0.58	0.84	0.85	0.78	0.85	

¹Diet composition was estimated as: Bayesian method with 2 prior means (0.8 and 0.7 for Bayes 1 and 2, respectively) reflecting the proportion of corn silage in the diet; NNLS – Non-negative least squares; TMR – total mixed ration as a single food; and, Composite – Mathematical composite assuming 70% corn silage and 30% alfalfa.

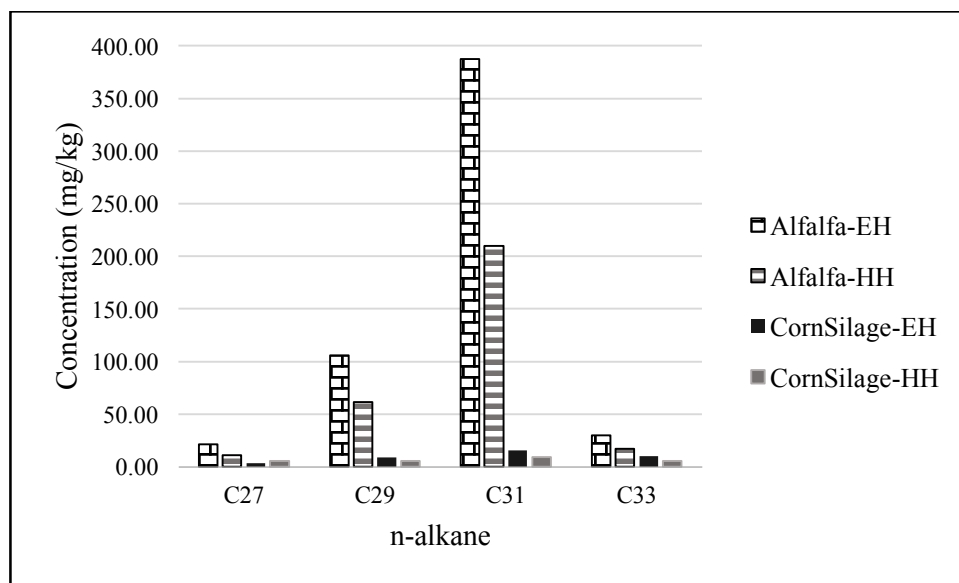


Fig. 4.1 N-alkane concentrations measured by 2 separate technicians (HH and EH) for the same alfalfa and corn silage samples.

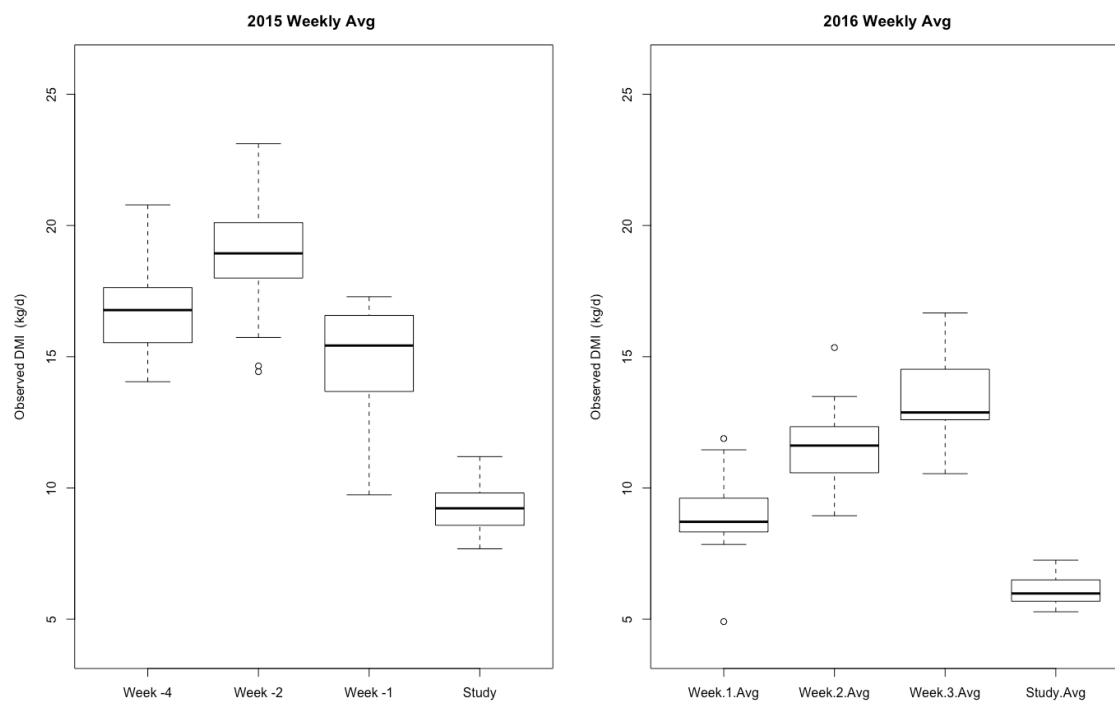


Fig. 4.2 Weekly averages of observed DMI leading up to and during the 2015 and 2016 indoor experiment.