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Management Factors Influence On The Fatty Acid Content And Composition Of Forages

Caleb Goossen
University of Vermont

MANAGEMENT FACTORS INFLUENCE ON THE FATTY ACID CONTENT AND COMPOSITION OF FORAGES

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Caleb P. Goossen

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Dissertation Examination Committee:

Sidney C. Bosworth, Ph.D., Advisor
Jana Kraft, Ph.D., Chairperson
Heather M. Darby, Ph.D.
Richard M. Single, Ph.D.
Cynthia J. Forehand, Ph.D., Dean of the Graduate College

ABSTRACT

Demand for ruminant-derived products high in beneficial fatty acids (FA) has led to a desire to maximize the alpha-linolenic acid (ALA) and total FA (ΣFA) content of forage crops. Several management factors affect the ALA and ΣFA content of forages, including phenology, species, season, and nitrogen fertility. Yet, the influence of harvest management decisions that affect wilting time of conserved forages is under-studied. Similarly, the majority of published research regarding ALA and ΣFA content is of cool season (C_3 photosynthetic) temperate perennial forage species, and not warm season (C_4 photosynthetic) annual species. Sample preservation methodologies used in published research are often too expensive and time consuming for desired practicality, or unreliable. This dissertation aids in addressing these deficiencies.

In the first study, forced hot air drying of forage samples was shown to be unreliable for accurate FA analysis, and an alternative methodology was established utilizing brief microwave pretreatment of small fresh weight samples prior to forced hot air drying, yielding results similar in accuracy to lyophilized samples. Oxidative losses of ground dried forage samples were also examined, again suggesting that microwave pretreatment prior to forced hot air drying is a fast, inexpensive and otherwise desirable choice for forage sample preservation in anticipation of later FA analysis. A second study investigated two warm season annual forage species (sorghum-sudangrass and pearl millet), showing that maturity-associated declines in whole plant ALA and ΣFA content are largely a product of an increasing ratio of ALA- and ΣFA-scarce pseudostem fractions, and only secondarily resultant of maturity associated declines within individual plant fractions. Lamina mass ratio was identified as a correlate with ALA and ΣFA content, at least as useful as two common correlates - crude protein and neutral detergent fiber content. A third study also showed the critical influence of crop maturity upon ALA and ΣFA content in two warm season annual forages (pearl millet and sudangrass), in addition to differences between species and those resultant from differing nitrogen fertility. Conserved forage harvest decisions were evaluated in the fourth study. No difference was found between wide and narrow swath treatments (70% and 40% of mower width, respectively) of AM and PM mown reed canarygrass, but there was evidence to suggest that AM mowing may allow for a higher content of ALA and ΣFA content relative to PM mowing. Ensiling was also found to decrease ALA content or proportion.

In conclusion, management choices promoting grazing and/or harvesting of a higher laminae proportion, optimizing nitrogen fertility, and suitable choice of species for meeting these goals may be the best way to maximize the ALA and ΣFA content of forages grown for livestock. AM mowing may reduce ALA and ΣFA content losses otherwise caused by overnight wilting of forages mown for conservation, and microwave pretreatment prior to forced hot air drying is an advisable sample preservation methodology for researchers furthering the study of forage ALA and ΣFA content, when lyophilization is impractical or too expensive.

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LIST OF ABBREVIATIONS

ALA	**Alpha-linolenic acid**
aNDF	**Neutral detergent fiber**
BRF	**Borderview research farm**
CLA	**Conjugated linoleic acid**
CLnA	**Conjugated linolenic acid**
CONS	**Conserved maturity**
CP	**Crude protein**
DM	**Dry matter**
FA	**Fatty Acid**
FAME	**Fatty acid methyl ester**
FD	**Freeze-dried**
FHA	**Forced hot air**
HREC	**Horticulture Research and Education Center**
LA	**Linoleic acid**
LMR	**Lamina mass ratio**
MUFA	**Monounsaturated fatty acid**
MW	**Microwaved**
N	**Nitrogen**
NDF	**Neutral detergent fiber**
NIRS	**Near-infrared reflectance spectroscopy**
PAST	**Pasture maturity**
PUFA	**Polyunsaturated fatty acid**
RA	**Rumenic acid**
SFA	**Saturated fatty acid**
UVMHREC	**University of Vermont Horticultural Research and Education Center**
VA	**Vaccenic acid**

CHAPTER 1: LITERATURE REVIEW

1.1. Research significance

Consumer interest in the fatty acid (**FA**) profile and content of ruminant-derived products is driving interest in the FA content of forage crops which make up a majority of ruminant animal diets. Generally, a decrease of saturated fatty acids (**SFA**, particularly 12:0, 14:0 & 16:0) is desired in ruminant milk and dairy products, in conjunction with an increase of unsaturated fatty acids, particularly oleic acid (18:1 9*c*), n–3 polyunsaturated FA (**PUFA**), conjugated linoleic acids (**CLA**) – principally rumenic acid (**RA**, 18:2 9*c*,11*t*), and its precursor vaccenic acid (**VA**, 18:1 11*t*) (Dewhurst *et al*., 2006). RA is produced in the rumen via microbial biohydrogenation of the PUFA linoleic acid (**LA**, 18:2 9*c*,12*c*) and alpha-linolenic acid (**ALA**, 18:3 9*c*,12*c*,15*c*; Jenkins *et al*., 2008). Up to 75% of the variability in milk RA content can be explained by feed content of LA and ALA (Mohammed *et al*., 2009). Very little of the PUFA that are desirable in milk can be synthesized *de novo* by ruminants, thus, long-chain FA must be ingested in dairy feed in order to be secreted into milk (Elgersma *et al*., 2006).

RA has been shown to reduce arthritic severity, and to be protective against colitis, in mouse models (Ferlay *et al*., 2017; Oh *et al*., 2017). And there is epidemiological evidence that RA may reduce breast and colorectal cancers in humans (Rodríguez-Alcalá et al., 2017), and *in vitro* evidence of anti-cancer activity (Oh et al., 2014), however, further evidence of *in vivo* activity in humans is not yet fully demonstrated (Ferlay et al., 2017). Because VA and conjugated linolenic acids (**CLnA**) can be metabolized to RA in humans, they are also sought after FA components of

ruminant-derived products. ALA is a precursor of RA, VA and CLnA in the rumen, and as such, increases of ALA in the diets of cattle can increase their ruminal outflow for incorporation into milk and meat. Increased dietary ALA also increases ALA concentration in milk (Hebeisen *et al*., 1993). ALA is an essential n-3 FA. Beyond minimum essential quantities of ALA, n-3 FA are considered desirable in higher quantities in the human diet to reduce the n-6:n-3 FA ratio of the diet. The higher dietary n-6:n-3 FA ratios common in modern Western diets are associated with inflammation related chronic diseases including coronary heart disease, diabetes, and arthritis (Simopoulos, 2008; Strandvik, 2011).

Ruminant-derived products, including meat and/or dairy, are the only significant source of RA and VA in the human diet (Dhiman *et al*., 1999; Bessa *et al*., 2000). RA levels can be increased by increasing the PUFA content of ruminant diets. Fresh green forages both contain high PUFA proportions, and when ingested, create a rumen environment with a pH that is favorable to the rumen microbes that produce VA and RA via biohydrogenation of ALA and LA. Thus, VA and RA are increased in high forage ruminant-derived products both by supplying more of their constituent precursors, and a microbial environment favorable to their creation (Bessa *et al*., 2000; Jenkins *et al*., 2008; Daley *et al*., 2010;). n-3 FA in cattle diets, such as ALA (the primary FA found in forage crops), are associated with improved fertility and reproductive success in dairy cattle (Cerri *et al*., 2009; Moallem *et al*., 2013; Soydan *et al*., 2017) and may even influence cattle offspring sex ratio (Marei *et al*., 2018). Additionally, increasing the

PUFA content of butter, by increasing the PUFA in dairy cattle diets, can create softer, more easily spreadable butter (Thomson and van der Poel, 2000).

Consumer demand for organic whole milk has less elasticity in demand response to price than organic skim milk (Liu *et al*., 2013), and more than half of respondents in an Italian study showed willingness to pay a price premium for n-3 FA enriched mozzarella cheese (Vecchio *et al*., 2016). This is likely due to perceived health benefits from beneficial FA in milkfat (Mitani *et al*., 2016; Kilcawley *et al*., 2018). There is evidence that interest of North American consumers in high CLA milk is unrelated to previous purchases of n-3 products (Peng *et al*., 2006), suggesting that "grass milk", *i.e.,* milk produced without grain feeding known for higher RA content (Benbrook *et al*., 2013, 2018), may independently appeal to consumers for both RA and n-3 PUFA content, in addition to consumers that simply desire to financially support grass-based agriculture.

At least one American supplier of organic "grass milk" closely monitors the FA content of their producers raw product to ensure minimum n-3 FA and CLA content, as well as a maximal n-6:n-3 FA ratio. In return for not feeding grain, grazing more than twice the minimum dry matter (**DM**) intake required by USDA organic standards, and meeting FA content expectations, these "grass milk" farmers receive a price premium of ~15% relative to the organic milk price (Benbrook *et al*., 2018).

Common forage crops typically contain 1.0% - 4.5% FA by DM (Halmemies-Beauchet-Filleau, 2013). As such, forage FA are a large dietary component of all ruminant diets, regardless of their inclusion of grain feeding. Changes in content of relatively energy dense FA in forages can therefore impact the bottom line of producers.

1.2. Fatty acids in forage crops

This work is focused upon herbage FA, and therefore seed FA, and seed-rich feedstuffs (*i.e.*, maize silage, *Zea mays* L.) are not considered here.

There are three main FA in the most common forage crops; ALA, LA, and palmitic acid (16:0), accounting for up to 93 g $100g^{-1}$ of ΣFA (Clapham et al., 2005). Of these, ALA is the predominant FA, frequently making up 50-75 g $100g^{-1}$ of ΣFA in

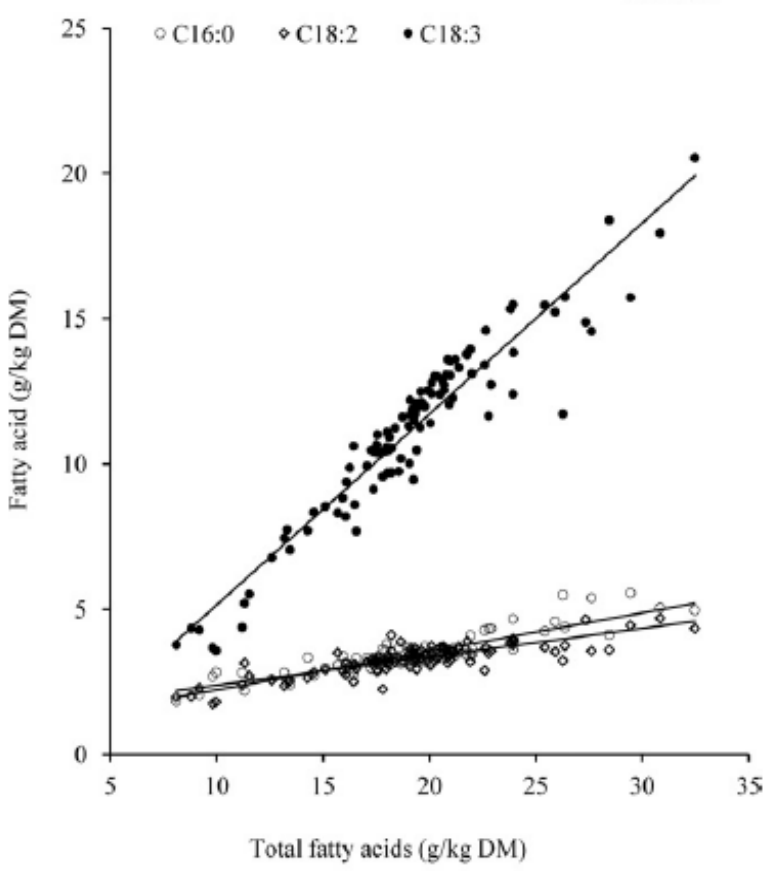

Figure 1.1. Changes in major individual fatty acids (FA, g kg^{-1} DM) in relation to changes in ΣFA content (g kg^{-1} DM) of grass silages (*n*=101). Adapted from Khan *et al.* (2012).

grasses (Dewhurst and Moloney, 2013; Garton, 1960; Glasser et al., 2013) and 40-50 g $100g^{-1}$ of ΣFA in legumes (Glasser et al., 2013). ALA is notably also the most variable

FA in grass silages, with changes in ALA content having the greatest impact upon ΣFA (**Figure 1.1.**).

Most of the FA in forage crops are located in thylakoid membranes within chloroplasts, with lipids constituting up to 40% of chloroplast DM. Galactolipids are the glycolipids that principally make up thylakoid membranes and consist almost entirely of ALA in most higher plants. Other common FA in forage crops such as LA and palmitic acid, and to a lesser extent stearic acid (18:0) and oleic acid, are more often found in the phospholipids and other glycolipids that make up other membranes within plant cells (Buccioni *et al*., 2012; **Figure 1.2.**). This distinction between ALA-rich thylakoid membrane and other cellular membranes has profound influence upon overall forage crop FA content and composition.

Differences between species or even cultivars are often listed as a large factor in forage FA content and composition, and have been demonstrated in individual studies (Boufaïed *et al*., 2003; Elgersma and Smit, 2005; Palladino *et al*., 2009), however, apart from borage (*Borago officinalis* L.), Clapham *et al*. (2005) found that many common grasses and forbs have similar ALA, LA, and palmitic acid ratios (**Figure 1.3.**). That the photosynthetic role of triunsaturated FA, like ALA, is highly conserved, and therefore FA composition of forage crop components is somewhat inflexible, is supported by the findings of a meta-analysis of forage FA studies by Glasser *et al*. (2013) that no major differences could be distinguished between species, or even botanical families.

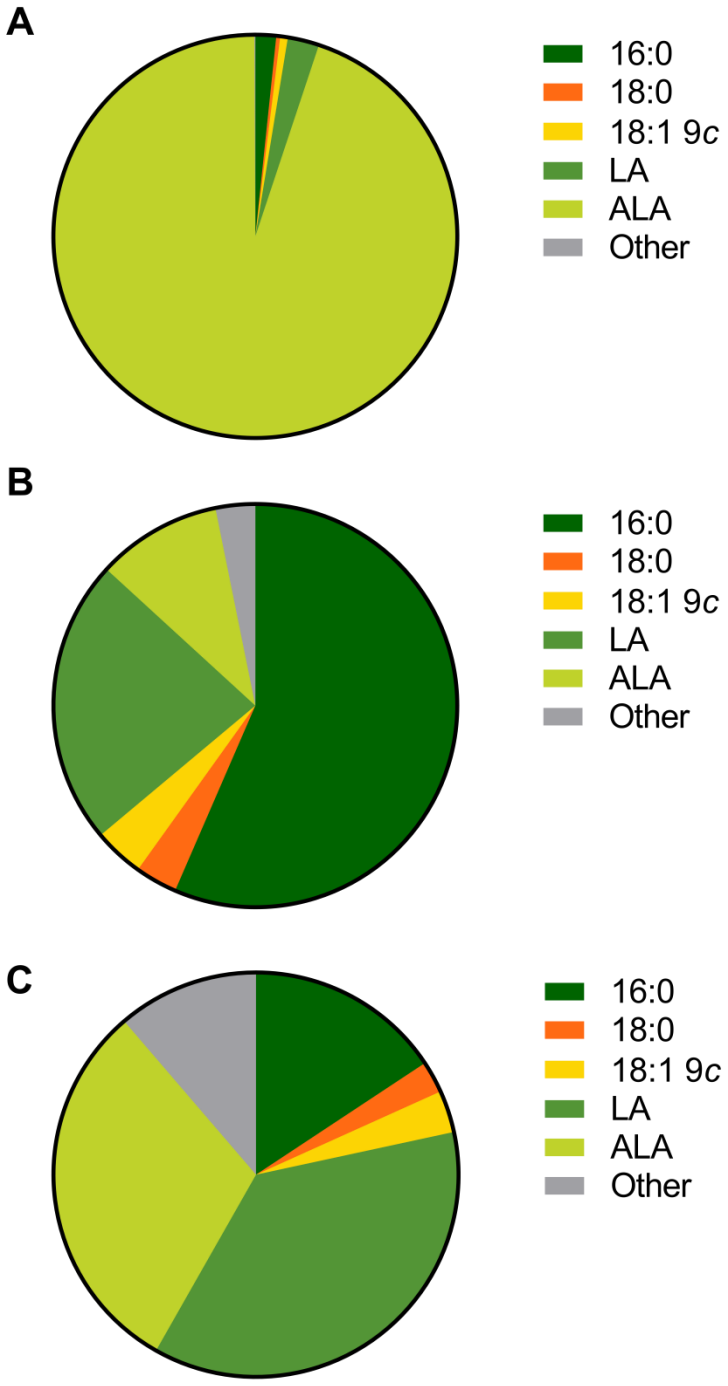

Figure 1.2. Fatty acid composition (g 100 g^{-1} ΣFA) of lipid fractions of red clover leaves: galactosyl glycerides, phospholipids, and triglycerides in panels A, B, and C, respectively. Adapted from Weenink, as cited in Buccioni *et al*. (2012).

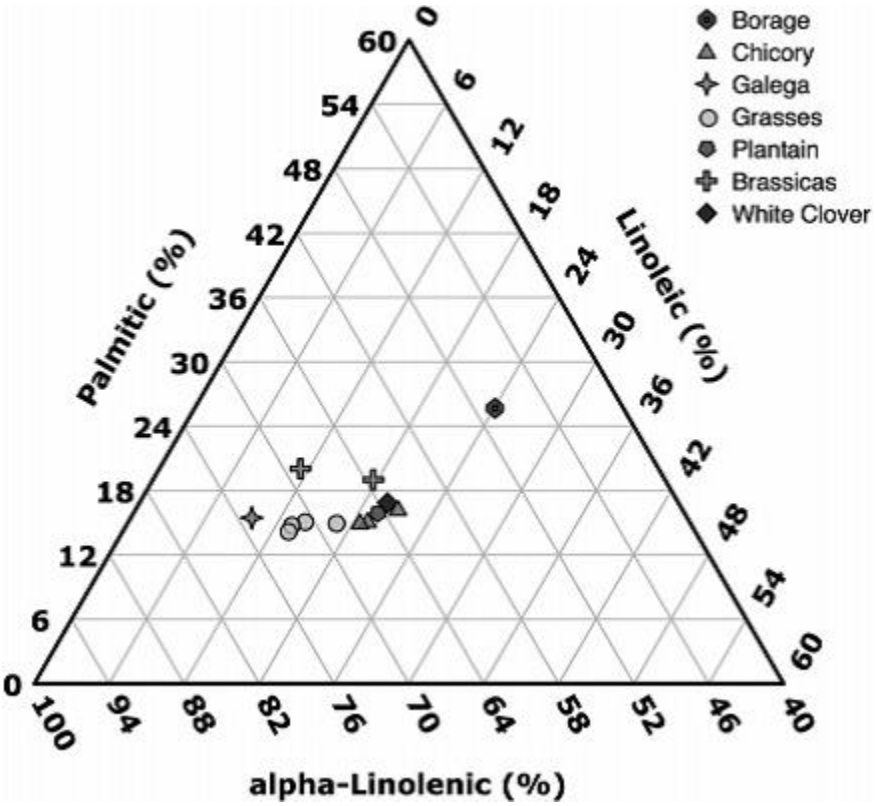

Figure 1.3. Relative proportions of alpha-linolenic acid (ALA), linoleic acid (LA), and palmitic acid in 13 forage crop selections.
Adapted from Clapham *et al*. (2005).

The lack of consistent differences between forage species should not be construed as suggesting a lack of genetic control on forage FA, however, as ryegrass studies have shown distinct gene pools to have different ALA and ΣFA content when receiving the same management (Dewhurst *et al*., 2001), a "stay green" trait can influence ALA and ΣFA content by retaining thylakoid membranes later in senescence than wild type (Dewhurst *et al*., 2002; Harwood *et al*., 1982), and the evidence of some possible ploidy level effects in perennial ryegrass (*Lolium perenne* L., Gilliland *et al*., 2002). It is likely that interactions with environmental and management influences (described in section

1.3.) simply confound and dwarf the effects of genetic control within and between species. Differences that have been presented between species may in fact be largely differences in gross plant architecture, and not finer genetic control of lipid membrane composition.

The vast majority of research describing forage FA is in temperate cool season (C_3 photosynthetic) perennial grass species. Very little research has been directed toward tropical (warm season, *i.e.*, C_4 photosynthetic) perennial grass species, and even less to annual warm season grass species, with the exception perhaps of maize silages. Warm season grasses typically have a lower proportion of lamina (leaf blade) tissue than cool season grasses (Atkinson *et al*., 2016), and as a result, may be expected to contain less FA overall and a lower proportion of ALA. Several investigations of perennial warm season grasses found greater palmitic acid proportions than ALA, and a very low ΣFA content (O'Kelly and Reich, 1976; Martins *et al*., 2016; Mojica-Rodríguez *et al*., 2017) though other studies of warm season grasses found ALA proportions that were much closer to results reported for cool season grasses in perennial species (Khan *et al*., 2015; Dias *et al*., 2017) and in annual species (Vargas *et al*., 2013; Bainbridge *et al*., 2017).

Unlike grasses and legumes which can be grouped within what are called "18:3 plants", some higher plants are referred to as "16:3 plants" because they utilize the prokaryotic lipid metabolism pathway to produce the n-3 FA hexadecatrienoic acid (16:3 7*c*,10*c*,13*c*), in addition to the eukaryotic lipid metabolism pathway that produces ALA, and use both of these triunsaturated FA in their membranes (Harwood, 1996). All of the forage crops investigated in this dissertation are 18:3 plants, however, some 16:3 plants

that may be used as livestock feed include *Plantago* spp. which may have very small quantities of 16:3 n-3, and the *Brassicaceae* family which may contain up to 30% of ΣFA as 16:3 n-3 (Mongrand *et al*., 1998). Hexadecatrienoic acid is largely absent from discussions of forage FA, and consequently may have been over-looked, such as in the investigation of Clapham *et al*. (2005) that included *Brassica* spp. and a *Plantago*.

1.3. Factors that influence FA levels in forage crops

1.3.1. Temperature

Temperature is a possible component of seasonal effects (**Section 1.3.6.**), and has also been suggested as a rationale for stark contrasts of ALA content reported in studies of warm season grasses (Dias *et al*., 2017; Mojica-Rodríguez *et al*., 2017). The nature of an unsaturated FA is to bend, or kink, at each double bonding site along the carbon chain (**Figure 1.4.**). In this fashion, a greater degree of unsaturation will increase the kinked nature of individual FA and subsequently decrease the density at which FA can be aligned in a lipid membrane, increasing the fluidity of that membrane. Throughout normal turnover of the chloroplast lipid membranes, saturation level is adjusted to maintain a rigidity and fluidity balance in response to temperature. A shift to lower temperatures activates desaturase enzymes, thereby increasing proportions of triunsaturated FA such as ALA (Xu and Siegenthaler, 1997), and both ALA and hexadecatrienoic acid in 16:3 plants (Falcone *et al*., 2004). Even rapid changes in rates of desaturation activity are slowly reflected in overall FA composition of lipids, suggesting that rapid temperature shifts have minimal influence and that changes in overall saturation are a mid- to long-term acclimation (Williams *et al*., 2000). In response to

higher temperatures, plants require an increased rigidity for optimal membrane performance, and thus, increase content of the fully saturated stearic acid (Harwood, 1996). Changes in thylakoid membrane FA saturation level may be as simple as replacing ALA in thylakoid membranes with diunsaturated LA throughout normal membrane lipid turnover, over the course of 60 to 100 hours, and as such, are not in response transient temperature fluctuations (Falcone *et al*., 2004; Larkindale and Huang, 2004; Narayanen *et al*., 2016).

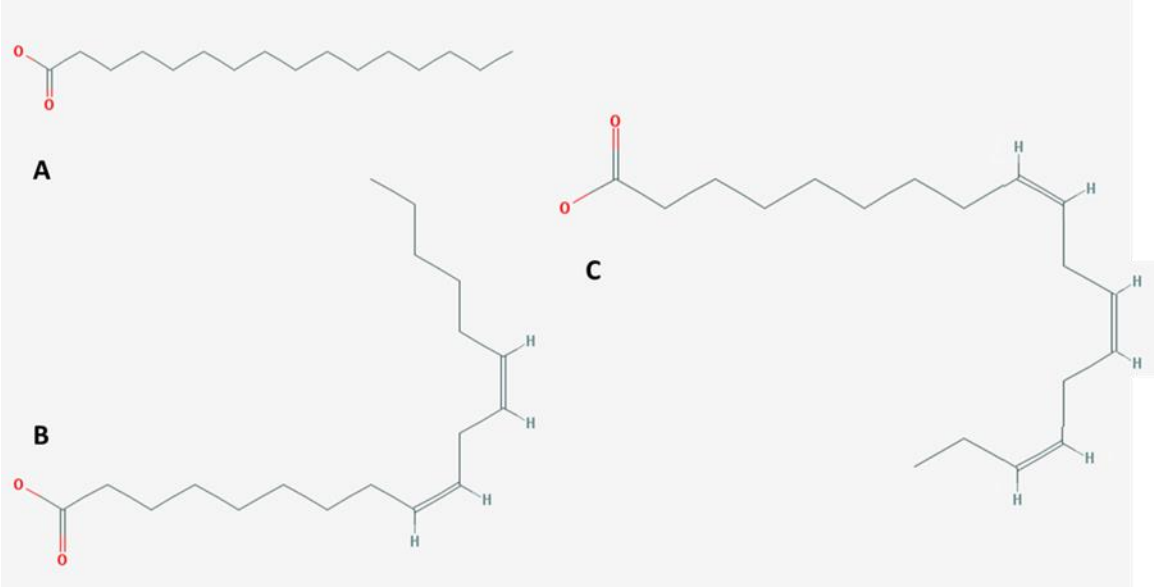

Figure 1.4. Structural models of A) the saturated fatty acid (FA) palmitic acid (16:0), B) the diunsaturated FA linoleic acid (18:2), and C) the triunsaturated FA alpha-linolenic acid (18:3), demonstrating kinks at double bonding sites.
Adapted from Kim *et al.* (2016).

In a less direct fashion, increased temperatures can also influence forage FA content and composition by increasing the rate of forage maturation and lignification (increasing neutral detergent fiber, **NDF**, within plant fractions), and by altering the relative proportions of lamina and stem/pseudostem fractions (Buxton and Fales, 1994).

Both of these impacts would serve to dilute the amount of ALA-rich thylakoid membranes on a whole plant DM basis.

1.3.2. Diurnal variation

Diurnal fluctuations in grass ALA and ΣFA content have been reported, and largely attributed to increases of nonstructural carbohydrates, and subsequently DM, throughout the photosynthetic day diluting a constant FA presence (Avondo *et al*., 2008; Doreau *et al*., 2007; Vibart *et al*., 2017). Conversely, Gregorini *et al*. (2008) reported no diurnal FA changes, and Scollan *et al*. (2003) and Vasta *et al*. (2012) reported an opposite diurnal effect, however, differences reported by Scollan *et al*. are likely to be resultant from genetic differences more than diurnal effects. This opposing diurnal trend is perhaps best explained by the works of Browse *et al*. (1981) and Ekman *et al*. (2007), which displayed light-dependent oleic acid synthesis diluting the proportion of ALA as the photosynthetic day progressed, and light-independent desaturation activity converting oleic acid into ALA overnight. However, their examples may only be practically applicable to emerging leaves where FA synthesis is greatest (Hawke *et al*., 1974).

As plants acclimate to shading, they may increase their chloroplast concentration, thereby increasing their content of ALA-rich thylakoid membranes, and increasing the degree of unsaturation of their overall FA (Marchin *et al*., 2017). Dewhurst and King (1998), however, found that complete shading (with black plastic) for 24 hours reduced ALA proportion and ΣFA content.

There is also some evidence that FA may be used in grass leaves for short term energy storage of photosynthetic gains throughout the day in the form of small amounts

of triacylglycerols (Perlikowski *et al*., 2016). The creation of these triacylglycerols has alternatively been proposed as short-term storage of ALA and LA during membrane remodeling (Narayanen *et al*., 2016) or in response to oxidative stresses related to photodamage, though more research is needed to verify that claim (Marchin *et al*., 2017).

1.3.3. Water relations

Maintaining lipid membrane stability and integrity is also critical in the success of plants in response to water stress, however, this response is not universal and varies even between very closely related plants. Osmotic water stress caused a decline in membrane lipids in a water stress sensitive *Arabidopsis thaliana* (L.) Heynh., while increasing the membrane lipids in a more tolerant close relative, *Thellungiella salsuginea* (Pall.) O.E. Schulz. In the water-stress tolerant species, amount of FA and level of FA unsaturation was increased (*i.e.*, more ALA and 16:3 n-3), suggesting that maintaining an adequate amount and greater ratio of unsaturated membrane FA is critical for plants to survive drought stress (Upchurch, 2008; Yu and Li, 2014). Similarly, withholding water for fourteen days to susceptible and tolerant wheat cultivars was shown to more greatly affect the membrane lipids of the susceptible cultivar (Quartacci *et al*., 1995). Perlikowski *et al*. (2016) has also attributed improved drought tolerance to earlier membrane lipid response and membrane regeneration when comparing two introgression genotypes of *Lolium multiflorum* x *Festuca arundinacea* L..

Water deficit can inhibit lipid biosynthesis, and stimulate lipolytic and peroxidative activities, which all can decrease membrane FA content (Upchurch, 2008). No publications examining conditions of excessive water were found.

1.3.4. Soil fertility

Supplying adequate nitrogen fertility has been identified as one of the few management practices through which a producer can increase the ALA proportion and ΣFA content of their forage crops, in a paradigm that is otherwise dominated by attempting to limit ALA and ΣFA losses (Glasser *et al*., 2013). Nitrogen fertility status (and subsequently crude protein content) has been linked with FA content since at least the works of Barta (1975) and Mayland *et al*. (1976), and Barta (1975) also identified that increasing potassium fertility status does not have a direct connection with FA content. Crude protein content is frequently reported in forage FA studies, and is considered the variable that best predicts ΣFA content (Glasser *et al*., 2013). Optimal nitrogen fertility allows for the greatest abundance of chloroplasts in grasses, and therefore, a greater content of ALA-rich thylakoid membranes (Dewhurst and Moloney, 2013). Boufaïed *et al*., (2003) suggested that nitrogen fertility associated FA increases in timothy (*Phleum pretense* L.) were purely an increase of chloroplast proportion within leaf fractions, as the nitrogen fertility also decreased the ratio of leaf to stem fractions, which would otherwise have likely decreased ALA and ΣFA content. Increasing phosphorus fertility status has been found to have little impact upon FA content and composition of grasses (Boufaïed *et al*., 2003; Lee *et al*., 2006).

1.3.5. Maturity

Forage crop maturity has repeatedly been proposed as a major factor in determining forage crop FA content and composition. The designs used to examine the

effect of plant maturity have varied however, with treatment designations that are of limited comparability between studies. A meta-analysis of published studies of forage FA content and composition by Glasser *et al*. (2013) determined that vegetation stage was the most influential impactor of forage FA, confirming the findings of Khan *et al*. (2012). As such, NDF, a measure of structural components in plant cells that increase with advancing maturity and is typically lowest in leaf tissue, is an important negative correlate of forage FA. Whole plant measures of NDF increase both from changes within aging plant cells, and from phenological shifts in plant fractions, such as flowering/culm production at reproductive maturity, or other decreases in leaf:stem ratio through stem elongation or leaf senescence. The works of Cabiddu *et al*. (2017) and Dias *et al*. (2017) have recently highlighted the greater importance of changes in relative plant fractions than of cell maturation changes within plant fractions, for FA decreases associated with advancing maturity in berseem clover (*Trifolium alexandrinum* L.) and elephant grass (*Pennisetum purpureum* Schumach.), respectively. The scale at which ratios of relative plant fractions change with advancing maturity, and subsequently the ratio of thylakoid membranes relative to overall plant DM, is likely a primary distinction in the FA content and composition of individual species.

1.3.6. Season

The meta-analysis by Glasser *et al*. (2013) also shows a clear seasonal variation in the average proportion of ALA reported in published literature (**Table 1.1.**), and concomitantly ALA and ΣFA content. Forage FA content and ALA proportion of ΣFA content typically decreases from spring until June and July, increasing again into the

autumn months. This seasonal variation is likely not a direct effect of its own, but resultant both from temperature differences and early season primary growth maturing to

Table 1.1. Effect of the time in the year (Northern Hemisphere) on FA[a] composition (g 100 g^{-1} ΣFA) and total FA content (g kg^{-1} DM[a]) of pure grasses and pasture. Adapted from Glasser *et al.* (2013).

Month[b]	16:0	18:0	18:1	18:2	18:3	Total FA
April	13.86	1.96	2.31	12.66	64.33	23.3
May	16.42	2.62	3.72	15.86	55.58	16.8
June	18.62	2.87	4.07	15.89	50.33	14.2
July	18.59	3.22	4.01	15.48	53.28	16.7
August	18.00	2.60	3.18	14.18	55.11	16.7
September	17.50	2.31	2.84	14.36	57.74	19.1
October	16.76	2.64	2.83	13.62	59.00	20.2
November	19.50	2.79	3.92	13.67	54.77	22.9
RMSE	2.05	0.51	1.17	1.90	5.21	4.5

[a] FA, fatty acids; DM, dry matter.
[b] The values are LS means by month: the mean value for each month thus corresponds to mid-month.

a reproductive stage in the early summer with subsequent regrowth cycles staying vegetative in the cool season perennial species that make up the majority of forage FA research. There are individual studies where a substantial FA decrease was not always found in the summer, or a progressive increase across the season was found (Dewhurst *et al*., 2002; Boufaïed *et al*., 2003; Elgersma *et al*., 2003b), which may be resultant from a later onset of sampling in the spring when grasses have reached a reproductive stage of maturity (missing vegetative early spring growth with its high FA content) and, at least in the case of Dewhurst *et al*. (2002), frequent cutting to avoid onset of flowering. Reports for legumes are mixed, possibly resultant from management decisions and/or maturity and phenological differences between studies, with white clover (*Trifolium repens* L.) decreasing in ALA and ΣFA from spring to summer, red clover (*Trifolium pretense* L.) increasing, and alfalfa (*Medicago sativa* L.) not differing greatly (Lee *et al*., 2006; Van Ranst *et al*., 2009).

1.3.7. Impacts of forage conservation

The meta-analysis of forage FA content and composition studies by Glasser *et al*. (2013) summarized that forage conservation practices such as haymaking and ensiling can sometimes influence ΣFA content and ALA proportion of grass and legume crops. Briefly, haymaking led to decreases in ΣFA content (-2.4 g kg^{-1} DM) and ALA proportion of ΣFA (-7.13 g 100 g^{-1} ΣFA) on average, though ALA proportion decreases were nearly twice as large in studies with poor drying conditions (-13.2 g 100 g^{-1} ΣFA). Ensiling, on the other hand, increased ΣFA content and did not alter ALA proportion in unwilted silages, and lowered ALA proportion by 5% in wilted silages.

FA losses during forage conservation may partially be a result of leaf shatter during harvest operations (Dewhurst *et al*., 2006), however, most forage conservation losses of FA are likely the result of lipolytic activity of endogenous plant enzymes, and subsequent oxidation of PUFA, particularly ALA. Some lipolytic enzyme activity is always occurring within plant cells, as thylakoid membranes are subject to constant turnover and replacement (Falcone *et al*., 2004). Wounding plants, such as mowing for harvest, stimulates a rapid stress response in which lipase enzymes liberate ALA and LA from lipid membranes. Lipoxygenase enzymes catalyze the deoxygenation of these PUFA, generating hydroperoxy PUFA which are the substrates for at least seven different enzyme families. Hydroperoxy PUFA are used to produce direct and indirect defenses to perceived herbivory, such as jasmonates and green leaf volatiles - the source of the "fresh cut grass" smell after mowing (Dar *et al*., 2015; Dewhurst *et al*., 2003; Turner *et al*., 2002; Venkatesan, 2015). The "stay-green" trait that slows or reduces senescence of

chloroplasts has been suggested as a potential breeding target as a mechanism to limit the accessibility of thylakoid membranes to lipoxygenase activity (Dewhurst *et al*., 2003).

Because endogenous plant enzymes are highly active, forage samples collected for research must be analyzed immediately upon collection or preserved for later analysis in a manner that minimizes lipolysis and subsequent oxidation (Christie and Han, 2010; Elgersma, 2015). Inconsistencies in sample preservation methodology may reduce the comparability of forage FA research, and/or be a confounding factor in perceived treatment effects.

1.3.8. Impact of wilting

Investigations into wilting losses of ALA and ΣFA in perennial ryegrass showed reductions after extended wilting periods (Dewhurst and King, 1998; Dewhurst *et al*., 2002; Elgersma *et al*., 2003; Van Ranst *et al*., 2009a; Warren *et al*., 2002), and Khan *et al*. (2011) found the proportion of ALA decreased primarily in an initial wilting phase (up to ~ 45 g DM 100 g^{-1} fresh weight) and that ΣFA content did not continue to decrease in long-term controlled lab wilting beyond that point; however, field cured samples dried more quickly and were of a much greater DM content (67 g DM 100 g^{-1} fresh weight) when they reached a similar ΣFA content. This suggests that the field-cured samples reached a DM content at which lipolytic enzyme activity was greatly reduced while there were still labile FA available to be lost when overnight re-wetting increased lipolytic activity, whereas lab-cured samples took longer to reach a critical DM content for reduced lipolytic activity and readily available pools of FA had already degraded. The potential significance of this DM point are further corroborated by the findings of Van

Ranst *et al*. (2009a), that lipolytic enzyme activity is greatly reduced in clovers (*Trifolium* spp. L.) as they wilted to 40 - 50 g DM 100 g^{-1} fresh weight.

Similar studies of timothy are less congruous than those of perennial ryegrass, as Boufaïed *et al.* (2003) and Lee *et al*. (2006) found a drop in ALA and ΣFA content in an initial wilt, but little further reduction in extended drying to hay, while Shingfield *et al*. (2005) found little change within a 6 hour wilt, but reductions after extended curing to hay, and Arvidsson *et al*. (2009a) found no effect on ALA or ΣFA content when wilting to 33.6 or 35.0 g DM 100 g^{-1} fresh weight.

1.3.9. Impact of ensiling

The impact of ensiling upon FA of conserved forage crops can be difficult to compare with published studies, as results have been mixed and many of the studies are confounded by the presence of un-sampled wilting prior to ensiling. Of the studies that sampled both after wilting and again after ensiling Arvidsson *et al*. (2009a) and Dewhurst and King (1998), observed no effect of ensiling on ΣFA content or ALA proportion, though Boufaïed *et al*. (2003) found increases in both ΣFA and ALA content. Of studies that compared unwilted forage before and after ensiling, Alves *et al*. (2011) and Boufaïed *et al*. (2003) both reported increases in ΣFA content, though only Boufaïed *et al*. (2003) found an increase in ALA content, and Liu *et al*. (2018) reported a decrease in ALA proportion, though no change in ΣFA content. In studies comparing fresh forage with silages made from wilted material, Vanhatalo *et al*. (2007) reported mixed results for ΣPUFA proportion - decreasing in grass and mature clover silages but increasing in young clover silages – otherwise, significant decreases in ALA proportion were

universal: Chow *et al*. (2004) and Van Ranst *et al*. (2009a) reported increases in ΣFA content, Whiting *et al*. (2004) reported decreases in ALA and ΣFA content, and Ding *et al*. (2013) and Elgersma *et al*. (2003) found decreases in both ALA proportion and ΣFA content, though Ding *et al*. reported varying degrees of ensiling decreases in both ALA proportion and ΣFA content, pursuant to applied pre-ensiling treatments.

Increases in ΣFA content of ensiled forages are typically suggested to be the result of DM losses associated with ensiling, such as effluent loss or respiratory/fermentative losses, essentially concentrating the remaining DM components, including FA (Lee *et al*., 2006; Baumont, as cited in Glasser *et al*., 2013). In at least one example (Liu *et al*., 2018) DM content decreased 15.6 g DM 100 g^{-1} fresh weight, possibly off-setting the reported ALA decrease, as ΣFA content of the resulting silage was not significantly different than the fresh forage it was made from.

It was posited by Elgersma *et al*. (2003) that ensiling changes in FA composition may be resultant from endogenous plant lipolytic enzyme activity in addition to microbial lipolytic enzyme activity. The examination of alfalfa (*Medicago sativa* L.) silage by Ding *et al*. (2013) confirms that both endogenous plant enzymes and microbial enzyme activity can reduce ALA content and proportion, and ΣFA content. If the two effectors can be assumed additive, endogenous plant enzymes were responsible for approximately 28 g 100 g^{-1} of the overall 40 g 100 g^{-1} ensiling reduction in ΣFA content found by Ding *et al*.

CHAPTER 2: MICROWAVE PRETREATMENT ALLOWS ACCURATE FATTY ACID ANALYSIS OF SMALL FRESH WEIGHT (100 g) DRIED ALFALFA, RYEGRASS, AND WINTER RYE SAMPLES

C.P. Goossen[a], S.C. Bosworth[ab], H.M. Darby[b], and J. Kraft[c*]

[a]Department of Plant and Soil Science, The University of Vermont, Burlington, VT, 05405, USA

[b]Extension System, The University of Vermont, Burlington, VT, 05405, USA

[c]Department of Animal and Veterinary Sciences, The University of Vermont, Burlington, VT, 05405, USA

[*]Corresponding author

Email: Jana.Kraft@uvm.edu

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2.1. Abstract

Accurate analysis of forage fatty acid (FA) profile and content, particularly polyunsaturated FA (PUFA), such as alpha-linolenic acid (ALA) and linoleic acid (LA), is vital for ongoing research optimizing the desired FA content of ruminant-derived foods. Lyophilization (*i.e.,* freeze-drying) is regarded as the gold standard for preserving labile constituents, such as PUFA, in fresh forage samples. This method, however, is expensive, time consuming, and generally impractical for the large number and size of forage samples in agronomic studies.

The objective of the first experiment was to evaluate the efficacy of a brief (1 min) microwave pretreatment prior to forced hot air drying (FHA) for preserving alfalfa (*Medicago sativa* L.) and Italian ryegrass (*Lolium multiflorum* L.) forage samples, relative to both a freeze-drying control and FHA drying alone. In a second experiment, similar drying methods were examined in winter rye (*Secale cereale* L.), as well as the decline of ALA proportion of total FA in ground samples stored 72 weeks.

In the first experiment, small fresh weight samples (100 g) receiving 1 min microwave pretreatment before FHA drying were found equivalent to freeze-dried samples in FA content and profile, and both were greater in ΣPUFA than samples that received FHA alone. Large fresh weight samples (400 – 500 g) receiving FHA alone, a common practice in agronomic studies, contained 1.5 – 2.5 fold lower total ALA content and 1.3 – 1.6 fold lower LA content than the freeze-dried control, while large fresh weight samples (400 – 500 g) receiving 1 or 2 min microwave pretreatment contained 1.2 – 2.2 fold lower total ALA content and 1.1 – 1.5 fold lower LA content

than the freeze-dried control. The second experiment revealed a reduction in ALA proportion of total FA of stored samples over a long period of time regardless of preparation method; however, FHA samples had a greater rate of reduction.

This study confirms that FHA drying alone is not adequate for forage sample preservation for FA analysis, and may lead to a significant underestimation of ALA, the principal FA in plants. This distorts measurements of other FA reported on a proportional basis (g $100g^{-1}$ total FA). In conclusion, a simple and inexpensive 1 minute microwave pretreatment prior to FHA drying results in FA analysis comparable to freeze-dried samples, provided that samples are of a small fresh weight (100 g).

2.2. Introduction

Growing consumer interest in the fatty acid (FA) content and composition of ruminant-derived dairy and meat products has led to the increased study of the FA content and profile of forage crops – particularly the content of total polyunsaturated FA (ΣPUFA), alpha-linolenic acid (ALA, 18:3 9*c*,12*c*,15*c*), and linoleic acid (LA, 18:2 9*c*,12*c*). Animals cannot synthesize ALA or LA *de novo*, thus, ALA and LA in ruminant-derived products result exclusively from the animals' diets. Therefore, accurate analysis of the FA profile of forages is vital for ongoing research optimizing the desired FA content of ruminant-derived foods.

The lipids of forage plants are primarily found in thylakoid membranes and are subject to constant turnover and replacement (Falcone *et al*., 2004). Moreover, the primary plant PUFA ALA is oxygenated to produce metabolites such as jasmonates and green leaf volatiles in response to plant stresses, *e.g.*, wounding (Turner *et al*., 2002; Vu

et al., 2012; Dar *et al*., 2015; Venkatesan, 2015; Sofo *et al*., 2016). As endogenous plant enzymes are highly active, plant tissues must either be analyzed immediately upon collection or preserved for later analysis in a manner that minimizes lipolysis and subsequent oxidation (Christie and Han, 2010; Elgersma, 2015; Sofo *et al*., 2016).

Samples of fresh forages in agronomic field studies are often large (≥400 g fresh weight) to be as representative of an entire plot as possible and to minimize the relative influence of any sampling errors (Foster and Wright, 1968). Sampling is commonly based on one sample (or occasionally a composite of subsamples) for both dry matter (DM) estimation and later analyses of forage quality. DM yield samples have been recommended to be as large as, or larger than, 900 g fresh weight (Hanson and Carnahan, 1956). Samples are typically recommended dried in a forced hot air drying oven, with various recommended temperatures and durations of time depending upon the constituents being investigated (Faithfull, 2002). In practice, however, the large quantity of samples that may be produced by multiple concurrent studies and the need to examine several constituents from each sample leads to a compromise drying temperature and duration being utilized for all samples.

Lyophilization (*i.e.*, freeze-drying) of frozen forage samples is generally considered the best drying method for preserving labile constituents of fresh forage samples (Heberer *et al*., 1985; Faithfull, 2002; Arvidsson *et al*., 2009; Pelletier *et al*., 2010; Elgersma, 2015). Freeze-drying, however, necessitates expensive equipment, is time intensive, and is generally impractical for a large number and size of fresh forage samples typically produced in agronomic field studies. Freeze-dried samples may not

dry as thoroughly as oven-dried samples, and special care must be taken to prevent rehydration before grinding as they can be stickier than oven-dried samples and may present difficulties in milling (Faithfull, 2002).

Microwave drying of plant tissues has been suggested as a means of complete sample drying (Hofman, 1965; Higgins and Spooner, 1986; Karn, 1986, 1991; Popp *et al*., 1996). Pelletier *et al*. (2010) found that a brief 1 min microwave pretreatment prior to forced hot air drying of fresh forage samples yielded non-structural carbohydrate estimates similar to those of freeze-dried samples, and much higher than forced hot air (FHA) dried samples that were not subjected to microwave pretreatment. Pelletier *et al*. (2010) hypothesized that the rapid heating of the microwave pretreatment denatures and therefore deactivates plant enzymes responsible for post-harvest respiration. This proposed mode of action is corroborated by similar sample preparation methods of highly labile constituents (*e.g.*, polyphenols and glucosinolates) in which microwaving was found to limit enzymatic degradation (Gulati *et al.*, 2003; Verkerk and Dekker, 2004; Niu *et al.*, 2015). In addition, heating samples at 75 °C for 15 min was utilized to deactivate endogenous plant lipases before sample freezing by Narayanan *et al.* (2016). Domestic microwave ovens are a suitable choice for sample preparation as they have been designed for even heating, utilizing lower frequency wavelengths than might otherwise be chosen for optimal energy to heat transfer, allowing deep penetration and avoiding excessive surface heating (Smith and Xiong, 2006).

In this paper we hypothesize that FHA drying alone may cause significant losses in ΣPUFA content during forage sample preservation and in storage post-grinding.

Additionally, we propose that 1 min microwave pretreatment of small fresh weight (100 g) forage samples prior to FHA drying prevents those ΣPUFA losses resultant from sample preservation. The objectives of this study were to I) evaluate FHA drying alone for the preservation of FA in forage samples relative to a freeze-dried control, II) evaluate brief (1 or 2 min) microwave pretreatment combined with FHA drying for the preservation of FA in forage samples relative to a freeze-dried control, III) quantify the ALA proportion decreases in dried ground forage samples during storage and, IV) examine how sample preservation method influences storage decreases of ALA proportion. To the best of our knowledge, no prior research has investigated the use of microwave pretreatment before FHA drying to preserve FA contents in forage plants. Additionally, no research we are aware of has considered storage losses of ALA in ground dried forage samples.

2.3. Materials and methods

2.3.1. Experiment 1 sampling

Samples from first and second growth (53 days regrowth) of alfalfa (*Medicago sativa* L.) were collected in 2015 on May 14 and August 28, respectively, at stage 3 (early bud) and stage 5 (early flower) as described by Kalu and Fick (1981). These were collected from two established stands at the University of Vermont Paul R. Miller Research and Educational Center (South Burlington, VT, USA) using handheld electric clippers (Gardena Accu Grass Shears ComfortCut, Husqvarna Professional Products Inc., NC, USA) cut at a height of 12 cm. Samples from first and fourth growth (43 days regrowth) of Italian ryegrass (*Lolium multiflorum* L. cv. ‘Green Spirit’) were collected

on June 24 and September 18, respectively, in 2015 from fields in Weybridge, VT and Jericho, VT, USA, cut at a height of 8 cm using the same handheld electric clippers. In both harvests, the grass stand was in a vegetative stage of growth with no evidence of reproductive culms. Crude protein and aNDF were determined by near infrared reflectance spectroscopy (FOSS NIRS DS2500; MN, USA) using 2015 NIRS Consortium calibrations (NIRSC, WI, USA). For both species and both sampling dates, the material was mixed thoroughly by hand on a large table to homogenize the sample. Simultaneously, any weeds and/or dead plant matter found were removed. Replicate piles were divided and randomly assigned to treatment groups as shown in **Table 2.1.** and described below.

2.3.2. Experiment 1 treatments

Drying treatments were applied as follows:

a) Freeze-dried (FD) - 100 g fresh weight samples were placed in 1 quart double zipper plastic freezer bags in a cooler with ice, and stored in a -80 °C freezer within 30 minutes after sorting. Three days later, the samples were freeze-dried for 48 hours (Labconco FreeZone, MO, USA). A large FD treatment was not pursued as the small sample was already at the maximum size limitation of the freeze-dryer used.

b) Small-sample forced hot air (FHA) - 100 g fresh weight samples were placed in 15.5 x 10 x 31.5 cm brown paper bags (ULINE, WI, USA) left opened and upright on the top shelf of a custom-built forced hot air drying room at the University of Vermont Horticulture Research and Education Center (UVMHREC) set at 44 °C.

c) Large-sample forced hot air (FHA) - 400 and 500 g fresh weight samples were placed in 47 x 35 cm cotton sample bags (Legend Inc., NV, USA) but otherwise prepared in the same manner as small FHA samples. This sample size is representative of the amount collected for DM and forage quality determination from small plot field trials (Foster and Wright, 1968).

d) Small-sample microwave pretreated (MW) - 100 g fresh weight samples were placed in paper bags identical to those used for small FHA samples and heated in a microwave oven (Emerson model: MW8778W, NJ, USA) at maximum power (800 W) for one minute. Subsequently, the paper bags were unfolded and placed opened and upright, intermixed on the same shelf of the UVMHREC drying room with all other FHA and MW samples. The small MW treatment was added to experiment 1 after first growth samples were analyzed, and is therefore only present in results of aftermath growth samples.

e) Large-sample microwave pretreated (MW) - 400 and 500 g fresh weight samples were placed in cloth bags identical to those used for large FHA samples and heated in the same microwave oven at maximum power for 1 min for first growth samples and 2 min for aftermath growth samples. With the inclusion of small MW samples in the comparison of aftermath growth samples, we chose to increase the duration of microwave pretreatment to 2 min for large MW samples as 1 min had proven insufficient in the results obtained from first growth samples. After microwave heating, samples were intermixed on the same shelf of the UVMHREC drying room with all other FHA and MW samples.

All FHA and MW samples were dried for five days. Upon retrieval from drying; FHA, MW, and FD samples were ground in a Wiley mill (Thomas Scientific, PA, USA) to pass through a 2 mm screen, then ground to pass through a 1 mm screen in a cyclone mill (UDY Corporation, CO, USA) and stored in 10 cm x 15.25 cm x 2 mm plastic sample bags (G.T. Bag Company, CA, USA) at approximately 20 °C in the absence of light.

2.3.3. Experiment 2

Winter rye (*Secale cereale* L.) was collected on December 8, 2014 from the UVMHREC at a height of 4 cm with the same electric clippers used in Experiment 1. The rye was in a vegetative stage of development having approximately three tillers per plant. The harvested material was handled in the same manner as in Experiment 1, and divided into eight replicate piles of homogenized material. Each replicate pile was divided again into three 100 g fresh weight samples that received one of the three study treatments: FD, FHA, or MW as described for the small treatments in Experiment 1.

Upon completion of freeze-drying, all dried samples (FD, FHA, and MW) were ground to pass through a 1 mm screen in a cyclone mill and stored in plastic bags as described for Experiment 1. FAME were prepared on the same day as grinding (week 0), and the sample bags were re-sealed after squeezing out excess air. On weeks 2, 4, 6, 8, 12, 24, 36, 48, and 72 the bags were opened, briefly mixed with a laboratory spatula, sampled to prepare FAME for analysis, and again re-sealed after squeezing out excess air. Bagged samples were stored out of the light in a cardboard box under normal laboratory conditions: 20 - 22 °C and 35% - 45% relative humidity. There was not

enough material to perform DM corrections at all samplings; data are therefore presented on a proportional basis (g individual FA $100g^{-1}$ ΣFA, **Table 2.4., Fig. 2.1.**), and on an assumed DM basis (g kg^{-1} forage DM, **Table 2.4.**) for week 0 samples only, as they were processed immediately following drying and grinding.

2.3.4. Forage fatty acid analyses

FAME were prepared from the dried and ground forage samples of Experiments 1 and 2 using a modified one step transesterification method of Sukhija and Palmquist (1988). Briefly; 1 mL of internal standard (1 mg 13:0 triacylglycerol mL^{-1} acetone), 2 mL of toluene, and 2 mL of 5% methanolic sulfuric acid were added to 500 mg of ground forage sample. The solution was incubated at 50 °C overnight. Five mL of 5% sodium chloride solution and 2 mL of hexane were added. The samples were mixed and centrifuged for 2 minutes, and the resulting hexane layer was collected. The extraction procedure was repeated twice with 1 mL of hexane. Four mL of 6% potassium bicarbonate solution were added, the samples were mixed and centrifuged for 2 minutes, and the resulting hexane layer was collected. Samples were then dried over anhydrous sodium sulfate and filtered through charcoal and silica gel. A 1% FAME solution was used for gas-liquid chromatographic analysis performed on a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a split injector (1 µL injection volume, 1:100 split ratio), flame ionization detector, and a SP-2560 fused-silica capillary column (100 m × 0.25 mm i.d. × 0.2 µm film thickness; Supelco, PA, USA). Hydrogen was used as carrier gas at a flow rate of 1 mL/min and fueling the detector at 40 mL/min. Other gases were purified air at 400 mL/min and nitrogen makeup gas at

30 mL/min. The injector and detector were both maintained at 250 °C. The oven was programmed as follows: initial temperature of 45 °C held constant for 4 min, then programmed to increase 13 °C/min to 175 °C and held for 27 min, then programmed to increase 4 °C/min to 215 °C, and held for 35 min. Flame ionization detector response peaks were integrated and quantified with GCsolution software (version 2.30.00). Identification of FAME was accomplished by comparison of relative retention times with commercial FAME standards (Nu-Check Prep #463 and #674; MN, USA). Fatty acid content was determined using the internal standard. A leveled 15 mL scoop subsample of the ground forage samples taken at time of FAME preparation was dried at 100 °C for 24 hours for DM mass correction of ground samples. The FA results are presented on a forage DM basis and as proportions (weight weight^{-1}) of total FA (ΣFA) detected with a chain length between 12 and 24 carbon atoms. The lowest level of detection was <0.001 g 100 g^{-1} ΣFA and FA less than 1 g 100 g^{-1} ΣFA are not reported.

2.3.5. Experiment 1 statistical analysis

Statistical analysis was performed separately for first growth samples and aftermath growth samples as treatments and replications varied between the two sampling groups. The GLIMMIX procedure in SAS version 9.4 (SAS Institute, Cary, NC, USA) was used for all FA measures with the following model:

$$Y_{ijk} = \mu + T_i + S_j + TS_{ij} + e_{ijk}$$

where Y_{ijk} = observation, μ = grand mean, T_i = effect of treatment (i = FD, small FHA, small MW, large FHA, large MW), S_j = effect of species (j = alfalfa, ryegrass), TS_{ij} =

interaction between treatment and species, and e_{ijk} = residual error (k = replications 1 – 6 or 1- 5).

Within the LSMEANS statement of the GLIMMIX procedure, a stepdown Dunnett's test was used to compare treatment means with means of the FD control for each species. Differences were considered significant with an adjusted $P<0.05$.

2.3.6. Experiment 2 statistical analysis

The GLM procedure in SAS version 9.4 (SAS Institute, Cary, NC, USA) was used for all FA measures of Week 0 data, with the following model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where Y_{ij} = observation, μ = grand mean, T_i = effect of treatment (i = FD, MW, FHA), and e_{ij} = residual error (j = replications 1 – 8).

Multiple comparisons were made upon least squares means with Tukey HSD P-value adjustments and the PDGLM800 macro for SAS (Saxton, 1998). Differences were considered significant with an adjusted $P<0.05$.

The MIXED procedure in SAS version 9.4 (SAS Institute, Cary, NC, USA) was used to compare the rate of decrease in ALA measures of ground forage samples over time with the following model:

$$Y_{ijk} = \mu + \tau_j + T_i + e_{ijk}$$

where Y_{ijk} = observation, μ = grand mean, τ_j = covariate week of storage (j = 0, 2, 4, 6, 8, 12, 24, 36, 48, 72 for long term analysis and j = 0, 2, 4, 6, 8, 12 for short term analysis), T_i = effect of treatment (i = FD, FHA, MW), and e_{ijk} = residual error (k = replications 1 – 8). Week of storage was designated as a repeated effect and a first order

autoregressive covariance structure assumed. Differences of slope were considered significant with a $P<0.05$.

2.4. Results

2.4.1. Experiment 1

In both the first growth and aftermath growth samples of Experiment 1, large FHA samples contained ΣFA contents that were approximately 1.8- and 1.4- to 1.5-fold lower, respectively, than FD samples for alfalfa and Italian ryegrass. Notably, reductions in ALA contents of large FHA samples were approximately 2.3- to 2.5- and 1.5- to 1.6-fold lower, respectively, than FD samples of alfalfa and ryegrass. LA contents were less affected, with 1.5- to 1.6- and 1.3- to 1.4-fold decreases in the large FHA samples relative to the FD samples, for alfalfa and ryegrass, respectively (**Tables 2.2., 2.3.**).

Overall, there were effects of treatment, species, and treatment by species interactions (**Table 2.2.**). For both the alfalfa and ryegrass, the FD treatment contained higher measures of many FA, including the predominant ALA and ΣPUFA, than the large 1 min MW or FHA treatments. There were no small (100 g) fresh weight MW samples in this first growth comparison. Exceptions included the following. On a DM basis (g FA kg^{-1} forage DM), the small FHA samples were similar to FD control in some FA measurements. Small FHA ryegrass samples were closest to FD control as they did not differ in total saturated FA (ΣSFA) or total monounsaturated FA (ΣMUFA) content. Small FHA alfalfa samples were only similar to FD control in LA and 24:0

content. All treatment groups of both species were lower in ALA and ΣPUFA content and therefore ΣFA content than the FD samples.

On a proportional basis (g $100g^{-1}$ ΣFA), LA content of small FHA ryegrass samples was not different from FD ryegrass samples, and again all treatment groups of both species were lower than FD samples in ΣPUFA content and subsequently the proportion of ΣSFA and ΣMUFA was greater than the FD control in all treatment groups (**Table 2.2.**).

Regardless of species, small 1 min MW samples did not differ from FD samples in the content or proportion of any measured FA; whereas, the large 2 min MW samples and the FHA samples (small and large) were lower for the most prevalent (ALA, ΣPUFA) and many other FA components, both on a DM and proportional basis (**Table 2.3.**).

Large 2 min MW samples of both alfalfa and ryegrass did not differ from FD samples in ΣSFA content. None of the alfalfa treatments differed from FD samples in ΣMUFA content on a DM basis, though both ryegrass FHA samples did (**Table 2.3.**).

With the exception of small 1 min MW samples, all alfalfa FA measured on a proportional basis differed from the FD control. In ryegrass samples, ΣMUFA content did not differ between any treatment and FD on a proportional basis. Large 2 min MW ryegrass samples were the only aftermath growth treatment, other than the small 1 min MW treatments, to not differ from FD in the ratio of n-6:n-3 FA (**Table 2.3.**).

2.4.2. Experiment 2

At week zero (FAME prepared immediately following grinding) the MW treatment yielded FA results that were equivalent to the FD treatment on a DM basis (**Table 2.4.**). However, as a proportion of ΣFA, MW samples had slightly more ΣSFA, less ΣPUFA and a higher ratio of n-6:n-3 FA compared to FD. ΣFA and 22:0 content did not differ between any treatments. FHA samples were lower than FD samples in LA and ALA. FHA samples did not differ from FD samples in LA content on a proportional basis or in the n-6:n-3 ratio from FD or MW samples. FHA samples were higher in content of most individual SFA and 18:1 9*c*, and in content and proportions of ΣSFA and ΣMUFA than MW samples, but did not differ in DM content of LA, ALA, and ΣPUFA.

Because there was not enough material to perform DM corrections at all samplings, the following results are presented on a proportional basis only.

The ground samples of all three preparation methods showed declines in ALA throughout 72 weeks of storage ($P<0.0001$, **Fig. 2.1.**). All three rates of decline were different from each other, though MW and FHA were most different (FD vs MW and FD vs FHA: $P=0.02$, MW vs FHA: $P<0.0001$). After 72 weeks, ALA content had decreased by 1.22 percentage points in MW samples, 1.77 percentage points in FD samples, and 2.34 percentage points in FHA samples ($P<0.0001$).

Only FHA samples exhibited a non-zero rate of decline in ALA proportion over a shorter time period (12 weeks), which decreased by 0.72 percentage points ($P<0.0001$, **Fig. 2.1.**). FD and MW rates of decline did not differ from zero ($P=0.13$ and 0.41,

respectively) or each other (P=0.62), but both differed from FHA (P=0.03 and 0.01, respectively).

Across 72 weeks of storage, ground samples of all three preparation methods showed a slight increase in LA as a proportion of ΣFA (P<0.001, **Fig. A.1.**). There was no statistical difference in rate of increase between the three methods. At the shorter 12 week timescale, only MW samples exhibited a non-zero rate of decline in LA proportion (P=0.03), despite a lack of statistical difference between the rates of decline of all three preparation methods.

2.5. Discussion

2.5.1. Microwave pretreatment

A brief 1 minute microwave pretreatment before forced hot air drying was found to be essentially as accurate as freeze-drying for preparing forage samples for FA analysis, when sample fresh weight was 100 g (**Tables 2.3., 2.4.**). Although large MW samples (400, 500 g) contained a greater numerical ALA and ΣFA content than FHA samples, particularly when microwave duration was increased to 2 minutes (**Table 2.3.**), all of the larger fresh weight samples contained lower amounts of ALA, and subsequently ΣFA content, than the FD control (**Tables 2.2., 2.3.**). Because we did not measure lipolytic enzyme activity, we can only speculate that either; I) larger fresh weight MW samples did not reach a sufficient temperature to inactivate all enzyme activity, and/or II) the increased quantity of confined material had a lesser ability to dry relative to the smaller quantity of material in the paper bags utilized for small samples,

and/or III) cotton sample bags utilized for large samples impeded drying relative to the open paper bags used for small samples.

In Experiment 1 aftermath growth samples, the small 1 min MW treatment did not differ from the FD treatment in any individual FA or group of FA for both alfalfa and Italian ryegrass species. With greater statistical power in Experiment 2, small differences were seen between MW and FD treatments in ΣSFA and ΣPUFA proportions, as well as the n-6:n-3 ratio. Yet, these differences are minimal enough that they are unlikely to be of biological significance.

The FD and small 1 min MW alfalfa samples from Experiment 1 contained similar ΣFA, ALA, and LA contents to those reported by Dierking *et al.* (2010), within which 24 day old regrowth samples were immediately frozen with liquid nitrogen and subsequently freeze-dried, whereas alfalfa samples that were dried *via* forced hot air alone were generally lower (**Fig. 2.2.**). The ΣFA, ALA, and LA contents of FD and small 1 min MW Italian ryegrass samples presented here were greater than those by Dewhurst *et al.* (2001) and Lee *et al*. (2009), within which aftermath samples ranging from 28 to 42 days regrowth were frozen and freeze-dried (**Fig. 2.3.**).

Our results not only suggest the efficacy of brief microwave heating to denature endogenous plant enzymes prior to forced hot air drying, as proposed by Pelletier *et al.* (2010) for total non-structural carbohydrate analysis, but also its potential for adaptation as a sample preservation method for FA analysis, provided small sample size.

2.5.2. Forced hot air drying

Forced hot air drying alone has proven to be an inferior method for preserving forage sample FA from degradation, particularly when sample fresh weights were 400 – 500 g (**Tables 2.2., 2.3.**). Similarly to MW samples, the impact of sample fresh weight in FHA samples may have been a result of less thorough heating, a slower drying rate of the larger samples, and/or the cotton bags used for them. Though differences in preservation method may be small in some instances (**Table 2.4.**), results from FHA drying are inconsistent at best.

In Experiment 2, though all winter rye samples were in optimal drying conditions (*e.g.*, small sample size, a near-empty drying room), FHA samples still contained lower ALA and ΣPUFA levels and higher ΣSFA than the FD samples, on both a DM content and proportional basis (**Table 2.4.**).

Our results confirm that forced hot air drying of large forage samples, often used in agronomic studies, is not a reliable method for FA analysis and also suggest that drying rate varies between forage species, and therefore, the amount of time needed to halt enzymatic degradation of labile FA. The impact of species shown in Experiment 1 is perhaps attributable to the more succulent nature of alfalfa and the greater surface area to volume ratio of ryegrass, however, differential susceptibility to lipolysis has been demonstrated among cultivars of perennial ryegrass (Chow *et al.*, 2004) and a reduced lipolytic activity in 'Green Spirit' ryegrass relative to alfalfa could be partly responsible for the difference between the species in ΣPUFA losses. This study did not monitor drying time directly, however, temperature data loggers used in the study

suggest that: I) large 1 min MW alfalfa samples dried more quickly than large FHA alfalfa samples (**Fig. A.2.**), II) small ryegrass samples dried more quickly than large ryegrass samples (**Fig. A.3.**), and III) ryegrass samples dried more quickly than alfalfa samples (**Fig. A.2., A.3.**).

The findings of the current study are in contrast to those of Arvidsson *et al.* (2009), wherein forced hot air drying of 500 g fresh weight samples at 60 °C and 35 °C for 1 and 6 days, respectively, was found to yield results comparable to frozen and freeze-dried samples. Arvidsson *et al.* (2009) concluded that freeze-drying samples is satisfactory but that forced hot air drying of samples is "just as good, or even better in some cases". The difference in results may indicate sub-optimal drying conditions in the drying room utilized for the current study (*e.g.*, a large quantity of samples and subsequently greater relative humidity, the sample bags used, *etc.*), or differences in drying rate and/or lipolytic activity of the species investigated, as Arvidsson *et al*. (2009) investigated timothy (*Phleum pratense* L.). The experimental design of Arvidsson *et al.* (2009) was perhaps of insufficient power to detect differences with small treatment sample sizes (n=2) consisting of potentially more heterogeneous material than the current study. Additionally, the authors only reported FA measures on a proportional basis (g individual FA 100 g^{-1} ΣFA) which can produce different findings than presentation on a DM content basis (g individual FA kg^{-1} DM), particularly when the individual FA being reported on is highly correlated with the ΣFA content of the sample (Mocking *et al*., 2012), as is typically the case with ALA in plants.

Although the lowest ΣFA, ALA, and LA contents in alfalfa in Experiment 1 were found in the large FHA treatments, these were greater than those reported by Whiting *et al.* (2004), in which samples of a relatively low reported NDF content (33% of DM) were dried in an oven at 60 °C for 48h and Boufaïed *et al.* (2003) wherein 500 g early flowering stage (10% bloom) samples were dried in a forced air oven for 55 °C for 48h (**Fig. 2.2.**). The ΣFA, ALA and LA content of large FHA samples of Italian ryegrass in Experiment 1 were comparable to the results found by Boufaïed *et al.* (2003) with 63 day old vegetative samples dried as above, and slightly higher than those found in Garcia *et al*. (2015), wherein vegetative first growth and 1 month aftermath growth samples were also preserved with forced hot air drying at 60 °C for 24h (**Fig. 2.3.**).

2.5.3. Storage loss of ALA in ground samples

At both short- and long-term timescales (12 and 72 weeks of storage, respectively) the rate of ALA loss in MW samples was the most different from the rate of ALA loss in FHA samples (**Fig. 2.1.**). This may have been due in part to a difference in the density of ground samples (FD: 0.183 g cm^{-3}, FHA: 0.210 g cm^{-3}, MW: 0.398 g cm^{-3}) as more tightly packing MW samples had less space between particles, and therefore both lower air volume and smaller inter-particulate sites for oxidation to occur. This does not explain why the ALA content in FHA samples decreased at a higher rate than in FD samples, however, as the FD samples had the lowest density of all methods. The effect of sample density is more likely on a smaller scale, as evidenced by the slight difference in rate of ALA decline between the most and least

dense samples (MW and FD, respectively) detectable after 72 weeks of storage, but not at 12 weeks.

It might be expected that every subsampling time point would accelerate oxidation of ALA in the sample as the bag was opened and the material was being re-mixed. Yet, during the first 12 weeks of storage, when sampling was most frequent, no significant rate of decline was observed in the FD and MW samples (**Fig. 2.1.**).

Because of the greater enzymatic degradation potential of ALA, and its primacy among thylakoid membrane FA, losses during preservation and storage can significantly affect ΣFA and ΣPUFA content reported. This can have a large impact upon reported contents of other individual FA and FA groups when presented on a proportional basis, as it may superficially increase their content (*e.g.*, in Experiment 1, LA contents were 1.4- to 1.6-fold lower in large FHA samples relative to FD samples, yet LA content was greater on a proportional basis, **Table 2.2.**, and in Experiment 2, the proportion of LA increased over the 72 weeks of storage, **Fig. A.1.**). Similarly, the n-6:n-3 ratio can be affected by the greater loss of the primary forage n-3 FA ALA than the primary forage n-6 FA LA (**Table 2.2., 2.3.**), although this is not always the case (**Table 2.4.**).

Our findings of ALA proportion decrease in a stored ground forage may initially appear to be in contrast to the preliminary findings of Elgersma and Wever (2008) wherein ALA proportion within rapidly dried grass did not change after five weeks or six months of storage, however, the immediate ALA content losses (25 – 45%) from

the extreme drying temperature utilized in that study (900 °C) may have precluded any further ALA decline in storage.

2.5.4. Recommended best practices

The effects of preservation method on FA in Italian ryegrass are likely less pronounced than on FA in alfalfa as a result of the greater surface area to volume ratio of Italian ryegrass, and therefore, shorter drying times required to inactivate enzymatic activity (**Tables 2.2., 2.3.; Fig. 2.2., 2.3.**). In addition to sample preservation method, some of the heterogeneity of the FA content in studies may be caused by experimental conditions (*e.g.*, season, edaphic conditions, plant phenology), differences in FAME preparation procedure and/or gas chromatographic analysis, or possibly a lack of DM correction at time of FAME preparation, which would further underrepresent the amount of FA present in a given sample (**Fig. 2.2., 2.3.**). DM corrections are frequently not stated in the methods of forage FA studies, and thus, it becomes difficult to distinguish whether they were I) performed, II) calculated from the DM ratio at harvest, or III) simply not reported. A lack of DM correction may likely underrepresent the amount of FA present in a given sample, however, will not affect results presented on a proportional basis. Presenting results on a proportional basis must be done with care however, with consideration to the innate fact that a change in the proportion of one FA will automatically increase or decrease the relative proportion of all other FA in the sample (Mocking *et al*., 2012). As ALA is the principal FA in plants, results presented as a relative proportion of ΣFA are relatively recalcitrant to changes in ALA content in

comparison with presentation on a DM basis, as a reduction or increase in ALA content will concomitantly produce a substantial reduction or increase in ΣFA content.

Utilizing small sample or subsample size and microwave pretreatment was demonstrated here to be a viable methodology to preserve the FA content of alfalfa, ryegrass, and winter rye forage samples. The successful MW treatments in the present study utilized 100 g fresh weight. While this is small for typical agronomic samples, which are frequently 400 g fresh weight or larger to ensure an adequately representative sample, FA analysis typically involves a small subsample (≤5 g DM). However, care must be taken at time of collection to ensure that samples or subsamples are adequately representative, as less material is available for mixing and homogenization via grinding post-drying.

Although small studies may allow for immediate preparation of FAME from harvested samples, most researchers will need to store samples for later FAME preparation and analysis. When facilities and time allow, freezing at -80 °C and subsequent freeze drying remains the gold standard for preservation of labile forage components. When freeze-drying is impractical or too expensive, our results suggest that utilizing a brief (1 min) microwave pretreatment of small samples or sub-samples (100 g fresh weight) prior to forced hot air drying can effectively mitigate enzymatic degradation of PUFA, though further studies are needed to confirm this hypothesized mode of action. The data presented in **Fig. 2.1.** suggest that FAME preparation should be undertaken as close to the time of sample drying and grinding as possible, ideally within 12 weeks if samples are stored thereafter at laboratory room temperature.

The direct comparison of FD and small 1 min MW sample preservation was limited to first growth winter rye and aftermath growth alfalfa and ryegrass samples. However, we propose that this is a sufficient proof of concept to recommend this methodology of sample preservation for FA analysis. Differences in sample fresh weight, and traits of individual species (succulence, surface area to volume ratio, lipolytic enzyme activity, *etc*.) may require further elaboration and explication of our findings to be generalizable to all forage species and sampling protocols. We suggest that 1 min of microwave pretreating a 100 g fresh weight sample, is a logical benchmark from which to start further investigations. Increased duration of microwave pretreatment may not have the same effect upon larger samples (**Table 2.3.**). Further research may be warranted to indicate whether larger fresh weight samples are experiencing FA losses because of increased post-microwaving drying time, or if the greater mass would simply require an increased microwaving duration to adequately inhibit FA losses.

2.6. Conclusion

The data presented in this paper confirm that forced hot air drying alone cannot be considered a reliable forage sample preservation method for later FAME analysis. Sample size, species and succulence all affect drying rates. However, a 1 minute microwave pretreatment was shown to be an inexpensive, time efficient and simple means to insure adequate preservation of forced hot air dried alfalfa, ryegrass, and winter rye forage samples when there is a risk that drying conditions may be sub-optimal, provided that sample fresh weights are 100 g. This work also demonstrated

that ALA proportion of dried ground forage samples decreases over time, and that the method of sample drying can influence the rate at which the ALA proportion decreases.

2.7. Acknowledgements

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2.9. Figures

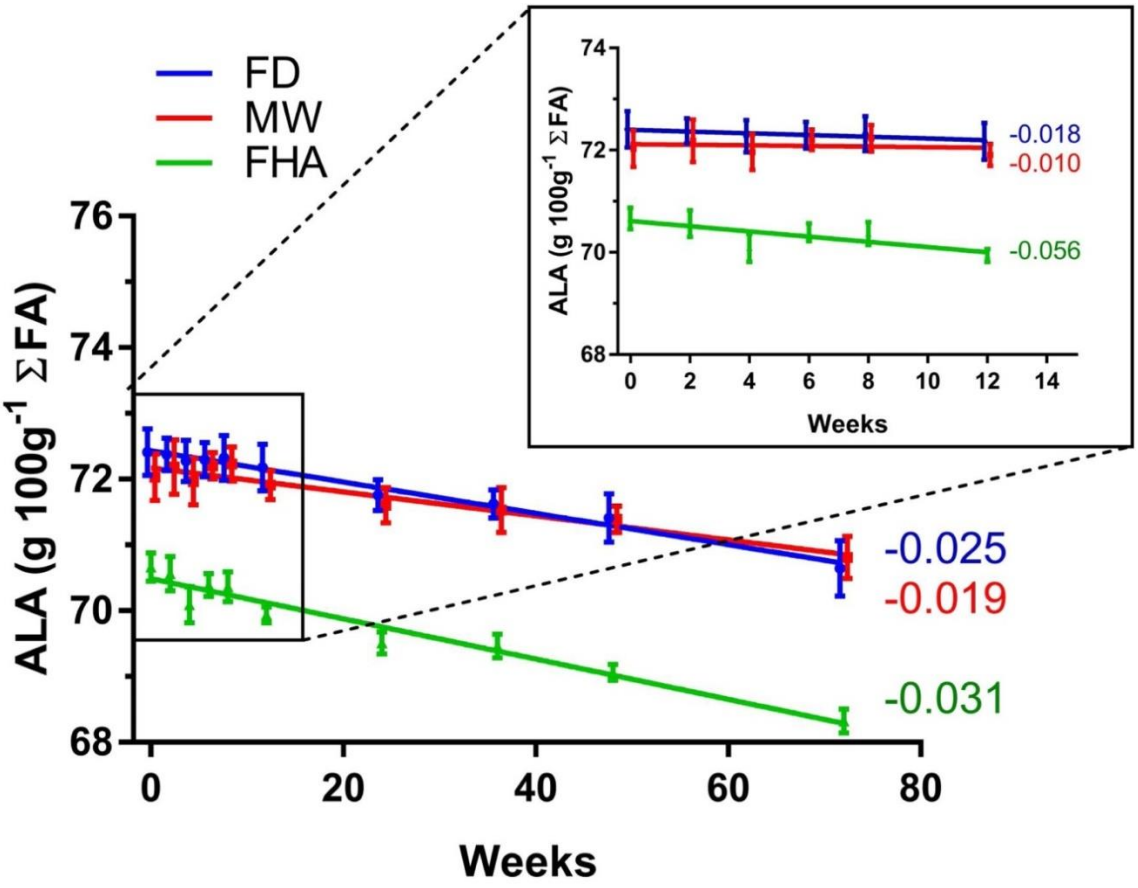

Figure 2.1. Alpha-linolenic acid (ALA) proportion of total fatty acids (ΣFA) decreases over time (*n* = 8). Slope of linear regressions reported in corresponding color to right of regression line. FD (blue) = Freeze-dried, MW (red) = microwave pretreatment prior to forced hot air drying, FHA (green) = forced hot air drying alone.

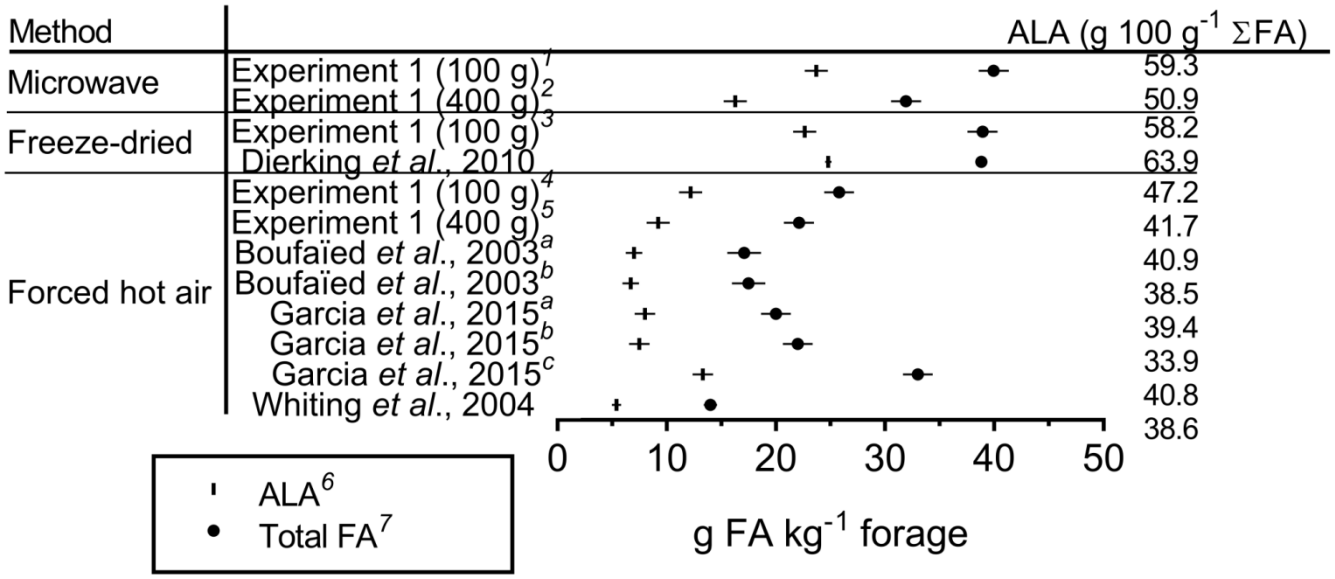

Figure 2.2. Comparison of Experiment 1 second growth alfalfa (*M. sativa* L.) results by methodology; least squares means and 95% confidence interval.

[1]100 g fresh weight, received 1 min microwave pretreatment prior to forced hot air drying. [2]400 g fresh weight, received 2 min microwave pretreatment prior to forced hot air drying. [3]100 g fresh weight, frozen and freeze-dried. [4]100 g fresh weight, forced hot air drying alone. [5]400 g fresh weight, forced hot air drying alone. [6]ALA = alpha-linolenic acid. [7]FA = fatty acid. [a]Spring samples; [b]summer samples; [c]autumn samples.

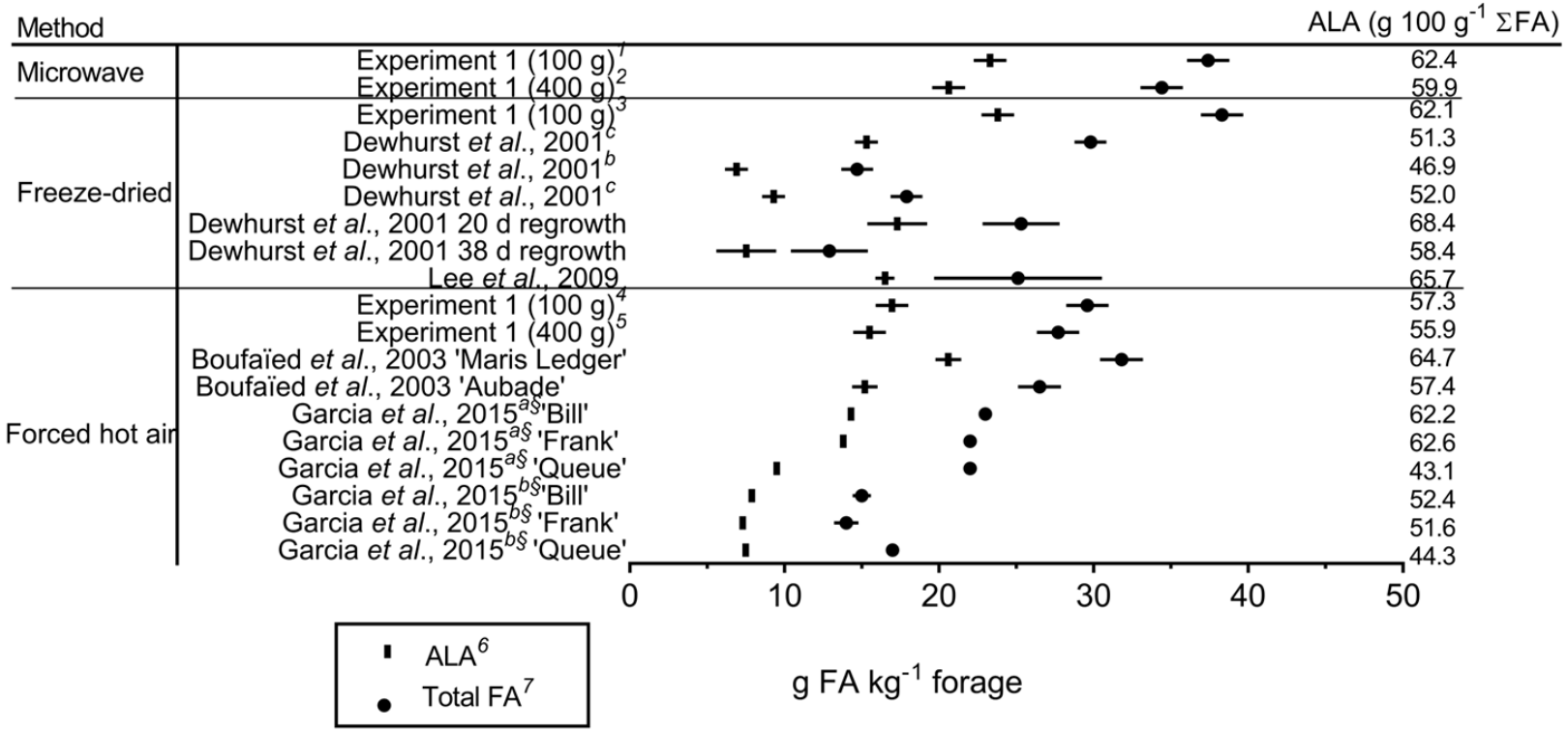

Figure 2.3. Comparison of Experiment 1 fourth growth Italian ryegrass (*L. multiflorum* L.) results by methodology; least squares means and 95% confidence interval (when calculable).

[1]100 g fresh weight, received 1 min microwave pretreatment prior to forced hot air drying. [2]400 g fresh weight, received 2 min microwave pretreatment prior to forced hot air drying. [3]100 g fresh weight, frozen and freeze-dried. [4]100 g fresh weight, forced hot air drying alone. [5]400 g fresh weight, forced hot air drying alone. [6]ALA = alpha-linolenic acid. [7]FA = fatty acid. [a]Spring samples; [b]summer samples; [c]autumn samples. §Confidence interval for ALA could not be calculated from published text.

Table 2.1. Treatment groups of first growth and aftermath growth samples.

	Experiment 1				Experiment 2
Species	Alfalfa	Ryegrass	Alfalfa	Ryegrass	Winter rye
Growth (cutting)	First	First	Second	Fourth	First
Growth stage	Early bud	Vegetative	Early flower	Vegetative	Vegetative
Collection date	May 14, 2015	Jun. 24, 2015	Aug. 28, 2015	Sep. 18, 2015	Dec. 8, 2014
aNDF[a] g kg^{-1} DM (SEM)	249 (2)	509 (2)	295 (5)	490 (3)	370 (3)
CP[b] g kg^{-1} DM (SEM)	271 (3)	165 (1)	247 (3)	214 (2)	251 (1)
Replicate subsamples	*n* = 6	*n* = 6	*n* = 5	*n* = 5	*n* = 8
Treatments[c]					
Freeze-dried (FD)	100 g	100 g	100 g	100 g	100 g
Forced hot air (FHA)	100 g	100 g	100 g	100 g	100 g
	500 g	400 g	400 g	400 g	
Microwaved (MW)[d]					
One minute MW	500 g	400 g	100 g	100 g	100 g
Two minutes MW			400 g	400 g	

[a]aNDF = neutral detergent fiber. Least squares means (standard error of the mean).
[b]CP = crude protein. Least squares means (standard error of the mean).
[c]Treatment sample fresh weights are presented in the table.
[d]MW microwave pre-treatment prior to forced hot air drying.

Table 2.2. Least squares means[a] of fatty acid content of first growth alfalfa and ryegrass samples prepared in four treatment groups[b]

	Alfalfa				Ryegrass					*P* value[c]		
Sample fresh weight (g)	100	500	100	400	100	400	100	400	**SEM**	**Trt**	**Sp**	**Trt x Sp**
Treatment	**FD**	**MW**	**FHA**	**FHA**	**FD**	**MW**	**FHA**	**FHA**				
g kg^{-1} DM												
16:0	6.08	5.40*	6.53*	5.18*	4.78	4.28*	4.68	3.92*	0.05	<.0001	<.0001	<.0001
18:0	0.66	0.79*	0.77*	0.81*	0.41	0.41	0.41	0.39	0.01	<.0001	<.0001	<.0001
18:1 9*c*	0.64	0.55*	0.83*	0.42*	0.49	0.43*	0.49	0.35*	0.01	<.0001	<.0001	<.0001
18:2 (LA)	7.82	5.10*	7.74	4.75*	4.54	3.66*	4.14*	3.29*	0.06	<.0001	<.0001	<.0001
18:3 (ALA)	22.31	10.19*	15.94*	9.61*	23.41	17.05*	20.40*	14.48*	0.34	<.0001	<.0001	<.0001
20:0	0.20	0.24*	0.22*	0.24*	0.10	0.12*	0.11	0.13*	<0.01	<.0001	<.0001	*ns*
22:0	0.30	0.31	0.32*	0.30	0.33	0.33	0.34*	0.34	<0.01	0.0015	<.0001	*ns*
24:0	0.30	0.33*	0.30	0.33*	0.23	0.23	0.23	0.24	0.01	0.0262	<.0001	*ns*
ΣFA	38.83	23.45*	33.19*	22.19*	34.82	27.08*	31.37*	23.71*	0.41	<.0001	*ns*	<.0001
ΣSFA	7.91	7.50*	8.54*	7.30*	6.12	5.68*	6.08	5.36*	0.06	<.0001	<.0001	<.0001
ΣMUFA	0.78	0.64*	0.94*	0.50*	0.74	0.67*	0.74	0.57*	0.01	<.0001	0.0003	<.0001
ΣPUFA	30.15	15.31*	23.71*	14.38*	27.96	20.72*	24.55*	17.78*	0.37	<.0001	<.0001	<.0001
n-6:n-3	0.35	0.50*	0.49*	0.50*	0.19	0.22*	0.20	0.23*	0.01	<.0001	<.0001	<.0001
g 100g^{-1} ΣFA												
18:2 (LA)	20.13	21.73*	23.33*	21.4*	13.04	13.52*	13.19	13.89*	0.14	<.0001	<.0001	<.0001
18:3 (ALA)	57.43	43.41*	48.01*	43.31*	67.19	62.97*	65.02*	60.99*	0.39	<.0001	<.0001	<.0001
ΣSFA	20.36	32.04*	25.73*	32.93*	17.60	21.00*	19.38*	22.65*	0.27	<.0001	<.0001	<.0001
ΣMUFA	2.00	2.73*	2.85*	2.26*	2.13	2.47*	2.36*	2.41*	0.05	<.0001	0.0025	<.0001
ΣPUFA	77.64	65.23*	71.43*	64.81*	80.27	76.53*	78.26*	74.94*	0.31	<.0001	<.0001	<.0001

[a] $n = 6$

[b] FD = Freeze-dried, MW = microwave pre-treatment prior to forced hot air drying, FHA = forced hot air (forced hot air drying alone); MW samples received 1 minute microwave pretreatment

[c] Model effects: Trt = effect of treatment, Sp = effect of species, Trt x Sp= effect of treatment x species interaction, *ns*= not significant

* denotes a value that is significantly different from the freeze-dried control of the same species (stepdown Dunnett adjusted $P < 0.05$

Table 2.3. Least squares means[a] of fatty acid content of aftermath growth of alfalfa and ryegrass samples prepared in five treatment groups[b]

	Alfalfa					Ryegrass						*P* value[c]		
Sample fresh weight (g)	100	100	400	100	400	100	100	400	100	400				
Treatment	**FD**	**MW**	**MW**	**FHA**	**FHA**	**FD**	**MW**	**MW**	**FHA**	**FHA**	**SE**	**Trt**	**Sp**	**Trt x Sp**
g kg^{-1} DM														
16:0	6.22	6.32	6.43	5.46*	5.13*	5.71	5.53	5.61	5.05*	4.90*	0.08	<.0001	<.0001	0.0033
18:0	0.96	0.96	1.01*	1.00	1.01*	0.50	0.48	0.51	0.51	0.51	0.01	0.0024	<.0001	*ns*
18:1 9*c*	0.71	0.70	0.80	0.64	0.75	0.82	0.79	0.81	0.59*	0.56*	0.05	0.0005	*ns*	0.0200
18:2 (LA)	6.82	6.74	5.84*	4.94*	4.41*	6.02	5.90	5.40*	4.99*	4.74*	0.09	<.0001	<.0001	<.0001
18:3 (ALA)	22.6	23.6	16.27	12.19	9.22*	23.8	23.3	20.62	16.96	15.50*	0.54	<.0001	<.0001	<.0001
20:0	0.21	0.20	0.22	0.25*	0.26*	0.12	0.13	0.13	0.14	0.16*	0.01	<.0001	<.0001	*ns*
22:0	0.34	0.33	0.35	0.34	0.35	0.35	0.33	0.35	0.36	0.37	0.01	0.0004	0.0094	*ns*
24:0	0.32	0.31	0.34	0.35*	0.37*	0.27	0.26	0.29	0.28	0.28	0.01	<.0001	<.0001	0.0358
ΣFA	38.9	39.9	31.92	25.79	22.11	38.3	37.4	34.40	29.59	27.7*	0.70	<.0001	0.0003	<.0001
ΣSFA	8.52	8.59	8.84	7.87*	7.62*	7.36	7.13	7.31	6.78*	6.69*	0.09	<.0001	<.0001	0.0114
ΣMUFA	0.88	0.87	0.94	0.75	0.85	1.05	1.01	1.05	0.80*	0.75*	0.05	<.0001	0.0227	*ns*
ΣPUFA	29.5	30.4	22.14	17.17	13.64	29.9	29.2	26.05	22.00	20.26*	0.61	<.0001	<.0001	<.0001
n-6:n-3	0.30	0.29	0.36*	0.41*	0.48*	0.25	0.25	0.26	0.30*	0.31*	0.01	<.0001	<.0001	<.0001
g 100g^{-1} ΣFA														
18:2 (LA)	17.5	16.9	18.32	19.20	19.95	15.7	15.7	15.72	16.88	17.12*	0.19	<.0001	<.0001	0.0003
18:3 (ALA)	58.1	59.2	50.93	47.20	41.68	62.1	62.3	59.87	57.32	55.93*	0.56	<.0001	<.0001	<.0001
ΣSFA	21.9	21.5	27.74	30.57	34.47	19.2	19.0	21.31	22.93	24.17*	0.31	<.0001	<.0001	<.0001
ΣMUFA	2.25	2.18	2.97*	2.91*	3.83*	2.74	2.69	3.03	2.72	2.70	0.17	<.0001	*ns*	<.0001
ΣPUFA	75.8	76.3	69.30	66.52	61.71	78.0	78.2	75.67	74.35	73.13*	0.39	<.0001	<.0001	<.0001

[a] $n = 5$

[b] FD = Freeze-dried, MW = microwave pre-treatment prior to forced hot air drying, FHA = forced hot air (forced hot air drying alone); 100 g MW samples received 1 minute microwave pretreatment, 400 g MW samples received 2 minute microwave pretreatment

[c] Model effects: Trt = effect of treatment, Sp = effect of species, Trt x Sp = effect of treatment x species interaction, *ns*= not significant

* denotes a value that is significantly different from the freeze-dried control of the same species (stepdown Dunnett adjusted $P < 0.05$)

Table 2.4. Least squares means[a] of fatty acid content of early vegetative winter rye prepared in three treatment groups

Treatment[b]	FD	MW	FHA	SEM	*P* value
g kg^{-1} DM[c]					
16:0	6.30 b	6.23 b	6.67 a	0.07	0.0002
18:0	0.45 b	0.44 b	0.48 a	<0.01	<0.0001
18:1 9c	0.41 b	0.41 b	0.52 a	0.01	<0.0001
18:2 (LA)	4.75 a	4.69 ab	4.59 b	0.04	0.0360
18:3 (ALA)	35.22 a	34.01 ab	32.29 b	0.44	0.0182
22:0	0.36	0.36	0.37	<0.01	*ns*
24:0	0.22 b	0.22 b	0.23 a	<0.01	0.0020
ΣFA	48.63	47.21	47.11	0.56	*ns*
ΣSFA	7.96 b	7.86 b	8.45 a	0.07	<0.0001
ΣMUFA	0.46 b	0.46 b	0.58 a	0.01	<0.0001
ΣPUFA	40.20 a	38.89 ab	38.08 b	0.48	0.0183
n-6:n-3	0.136 b	0.139 a	0.139 ab	<0.01	0.0334
g 100g^{-1} ΣFA					
18:2 (LA)	9.77 ab	9.93 a	9.74 b	0.05	0.0162
18:3 (ALA)	72.41 a	72.03 a	70.66 b	0.11	<0.0001
ΣSFA	16.37 c	16.66 b	17.94 a	0.07	<0.0001
ΣMUFA	0.95 b	0.97 b	1.23 a	0.01	<0.0001
ΣPUFA	82.65 a	82.36 b	80.82 c	0.08	<0.0001

[a]Least squares means without a common letter differ significantly; $P < 0.05$ (Tukey's HSD). $n = 8$
[b]FD = Freeze-dried, MW = 1 minute microwave pre-treatment prior to forced hot air drying, FHA = forced hot air (forced hot air drying alone)
[c]Samples were processed immediately following drying and grinding and therefore are presented on an assumed dry matter (DM) basis as there was insufficient material to perform DM correction.

CHAPTER 3: FATTY ACIDS DECREASE IN SUMMER ANNUAL GRASSES FROM RELATIVE INCREASES OF PSEUDOSTEM

C.P. Goossen[a], J. Kraft[b], and S.C. Bosworth[ac*]

[a]Department of Plant and Soil Science, The University of Vermont, Burlington, VT, 05405, USA

[b]Department of Animal and Veterinary Sciences, The University of Vermont, Burlington, VT, 05405, USA

[c]Extension System, The University of Vermont, Burlington, VT, 05405, USA

[*]Corresponding author

Email: sid.bosworth@uvm.edu

Core ideas

- Fatty acid content and composition of lamina and pseudostem are markedly different
- Whole plant fatty acid analysis is a non-ideal presentation for warm season annuals
- Lamina mass ratio is an important correlate of fatty acids in warm season annuals
- Managing for greater lamina intake may greatly influence animal fatty acid supply

3.1. Abstract

The decrease in alpha-linolenic acid (ALA) and total fatty acid (ΣFA) content in maturing grasses is exacerbated in tall summer annuals. ALA and ΣFA content were compared within plant fractions to determine if decreases are attributable to advancing maturity within fractions, or simply the change in proportion of the fractions. Vegetative and early head emergence sorghum-sudangrass were collected at one sampling date, and pearl millet were collected six times, from early vegetative to late boot stage. Lamina and pseudostem fractions were separated and whole plant FA composition was calculated from the weighted average of the fractions. As sorghum-sudangrass and pearl millet matured, ALA and ΣFA content decreased by 43% - 60% within pseudostems and on a whole-plant basis, though only by 10% - 13% in laminae. The ALA proportion of ΣFA decreased by 14% - 16% on a whole plant basis, despite not changing within constituent fractions.

3.2. Background

The content of alpha-linolenic acid (ALA) and total fatty acids (ΣFA) decreases with advancing maturity in C3 photosynthetic (*i.e.*, cool season) forage crops (Dewhurst *et al*., 2001; Boufaïed *et al*., 2003; Elgersma *et al*., 2003; Clapham *et al*., 2005; Glasser *et al*., 2013). There is a growing interest in C4 photosynthetic (*i.e.*, warm season) annual forage species, however, little research into FA has been performed (O'Kelly and Reich, 1976; Vargas M *et al*., 2013; Bainbridge *et al*., 2017; Dias *et al*., 2017).

Maturity-associated decreases in ALA and ΣFA content as well as lower proportions of ALA within ΣFA may be due, at least in part, to growth of pseudostem

fractions which contain less ALA and ΣFA than laminae fractions (Dewhurst *et al*., 2001; Elgersma *et al*., 2003, 2005; Dias *et al*., 2017).

Because many warm season annual grass species elongate and mature with large pseudostem fractions, we hypothesize that they will exhibit significant decreases in ALA and ΣFA content with advancing maturity, primarily due to the increasing dry matter (DM) proportion of the pseudostem fraction. The objective of this study was to determine the extent to which the content and proportion of ALA as well as the content of ΣFA change within plant fractions as they mature.

3.3. Materials and methods

3.3.1. Experiment 1

Sorghum-sudangrass (*Sorghum bicolor* nothosubsp. *drumondii* (Steud.) de Wet ex Davidse cv. 'Blackhawk BMR 12') samples were collected from a production field at The University of Vermont Horticultural Research and Education Center, South Burlington, Vermont, USA (44°25'N, 73°12'W) on August 24, 2015, cut at a height of 15 cm using handheld electric clippers (Gardena Accu Grass Shears ComfortCut, Husqvarna Professional Products Inc.). The field was mowed once previously and primarily consisted of vegetative aftermath growth. Early head emergence stage plants that escaped mowing were also collected from sections interspersed throughout the field. This allowed for the comparison of two forage maturity stages (minimally elongated vegetative stage versus elongated early head emergence stage) grown under largely the same environmental and edaphic conditions. Harvested tillers were mixed thoroughly by hand and any weeds and/or dead plant matter found were removed. Three replicate

samples of each treatment were separated between lamina (separated immediately above the ligule) and pseudostem fractions for both vegetative and elongated samples, respectively. Lamina and pseudostem samples (<150 g fresh weight) were placed in separate paper bags, microwaved at maximum power (800 W) for one minute, and dried as described in Goossen *et al*. (2018).

3.3.2. Experiment 2

Five replicate pearl millet (*Pennisetum glaucum* L. cv. 'Wonderleaf') samples were collected from a commercial farm field in Highgate Center, Vermont, USA (44°58'N, 73°01'W) six times over 19 days (July 18 – August 5, 2016) in the same manner as in Experiment 1. Pearl millet samples ranged from early vegetative stage on day one to late boot stage on day 19. All samples were divided into lamina and pseudostem fractions, microwaved onsite (as described above) before being transported to the same drying room as in Experiment 1.

3.3.3. Forage analysis

Nutritive quality and FA analyses were performed as described according to Goossen *et al*. (2018). Whole plant measures were calculated for each replicate as weighted averages by dry weight of the constituent plant fractions, *i.e.*, whole plant FA g kg^{-1} DM = (lamina FA g kg^{-1} DM x lamina proportion) + (pseudostem FA g kg^{-1} DM x pseudostem proportion). Variance of whole plant means estimates were weighted by the mean DM proportion of each plant fraction at each time point, *i.e.*, $var(p_iX_{lamina} + (1-p_i)X_{pseudostem}) = p_i^2var_{lamina} + (1-p_i)^2var_{pseudostem} + 2*cov(p_iX_{lamina}, (1-p_i)X_{pseudostem})$ where p = lamina DM proportion and $_i$ = time point. The small amount of pseudostem material

present at the first sampling date necessitated compositing dried samples for FA analysis, and as such, variance estimates could not exist for that fraction, and could not be calculated for the whole plant measure at that time point.

3.3.4. Statistical analysis

The GLM procedure in SAS version 9.4 (SAS institute, Cary, NC, USA) was used to perform a two-way ANOVA for Experiment 1 FA measures, testing the effects and interaction of maturity stage (vegetative or reproductive) and plant fraction (lamina or pseudostem). Differences of means were considered significant with a Tukey's HSD test adjusted $P<0.05$. The GLM procedure was also used to perform a one-way ANCOVA for Experiment 2 on plant fractions, using height as covariate. The CORR procedure in SAS was used to generate partial correlation coefficients.

3.4. Results

In Experiment 1, sorghum-sudangrass height was not recorded in this preliminary investigation, however, aftermath tillers were in a vegetative stage, with a lamina mass ratio (LMR; lamina DM / lamina + pseudostem DM) that was more than twice the LMR of the early heading stage first growth tillers (**Table 3.1., Figure B.1.**). Over the course of 19 days in Experiment 2, pearl millet grew from an average height of 54 cm to an average height of 139 cm, while the LMR decreased from 0.96 to 0.43 (**Table 3.1., Figure B.2.**).

In both Experiments, there was a substantial difference of ALA and ΣFA content and ALA proportion of ΣFA, between lamina and pseudostem fractions (**Table 3.2.; Figure 3.1.**). Though ALA and ΣFA content were affected by maturity stage and height

in Experiments 1 and 2, respectively, ALA proportion did not vary between maturity stages of the same plant fractions in Experiment 1 (**Table 3.2.**), nor with increasing height in Experiment 2 (P=0.7). In Experiment 2, despite a significant effect of height upon ALA content (P=0.03), neither lamina nor pseudostem fractions had a rate of change of ALA content or proportion different than zero. However, the rate of ΣFA content reduction in the pseudostem fraction was more than twice that of the lamina fraction (slopes = -0.11, -0.04, respectively; **Figure 3.1.**).

In Experiment 2, LMR, CP, and aNDF were all highly correlated with the three FA measures on a whole plant basis (**Table 3.3.**). Within plant fractions, lamina CP content was a stronger correlate of lamina ALA and ΣFA content than either LMR or lamina aNDF. However, lamina CP content was not associated with ALA proportion, with which LMR showed a slight negative correlation. In pseudostems, LMR was the strongest correlate with ALA and ΣFA content, though not with ALA proportion, which was more strongly correlated with pseudostem CP content.

3.5. Discussion

In agreement with our hypothesis, ALA and ΣFA content and ALA proportion declined rapidly with advancing maturity on a whole plant basis (**Table 3.2., Figure 3.1.**). ALA and ΣFA content declines were markedly less in lamina fractions, and ALA proportion was unchanged in lamina and pseudostem fractions.

In both experiments, ALA and ΣFA content declines were minor within lamina fractions, possibly a result of cell wall thickening as the laminae aged. ALA is vital for chloroplast function, which may explain why the ALA proportion of ΣFA did not

decrease within the lamina fraction. The decline of ALA and ΣFA in the pseudostem fraction is likely due to I) the greater lignification of living cells within this structurally important plant component, and II) a greater proportion of non- or minimally metabolically active tissue (*e.g.*, pith, xylem) with consequently less lipid-rich membranes, and very little ALA-rich chloroplast membranes.

This study provides evidence that declines in ALA and ΣFA content, associated with advancing maturity, are largely a function of a greater DM proportion of pseudostem fractions, which inherently contain less ALA and ΣFA. This has profound management implications for ruminant milk and meat producers concerned with the FA profile of their products, whom utilize these warm season annual forage species. ALA and ΣFA content decrease minimally within laminae fractions, providing an opportunity for management practices, such as reduced stocking pressure in grazing systems, and/or high mowing/chopping height, to capture a greater proportion of laminae material in older stands.

3.6. Acknowledgements

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3.9. Figure

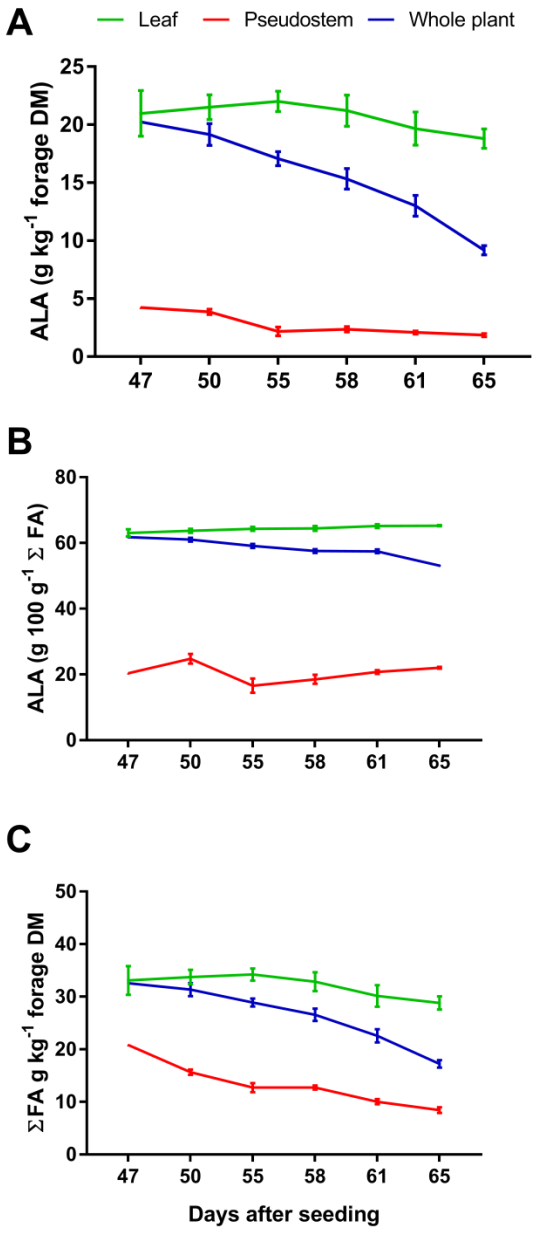

Figure 3.1. Alpha-linolenic acid (ALA) content, proportion, and total fatty acid (ΣFA) content (panels A, B, and C, respectively) of pearl millet over 19 days, by plant fraction (least squares means and standard error of means, *n*=5 replicate samples - except composited pseudostem samples at the first collection. Whole plant measure is a weighted mean of plant fractions.

Table 3.1. Sampling information for Experiments 1 and 2.

	Experiment 1		Experiment 2					
Species	Sorghum-sudangrass		Pearl millet					
Location	S. Burlington, VT, USA		Highgate Center, VT, USA					
Growth Stage[a]	Late veg.	Early head	Early veg.	Veg.	Elong.	Elong.	Flag leaf	Boot stage
Sampling Date	24 Aug.	24 Aug.	18 Jul.	21 Jul.	26 Jul.	29 Jul.	1 Aug.	5 Aug.
Days after seeding	-	-	47	50	55	58	61	65
Height (cm)	-	-	54	74	107	117	133	139
LMR[b]	0.69	0.32	0.96	0.87	0.75	0.69	0.62	0.43

[a]veg. = vegetative, elong. = elongating

[b]LMR = lamina mass ratio (lamina DM / lamina + pseudostem DM)

Table 3.2. Means comparison[a] and analysis of variance, effects of plant fraction and maturity stage on alpha-linolenic acid (ALA) content, proportion, and total fatty acid (ΣFA) content of sorghum-sudangrass (*n*=3 replicate samples).

Plant fraction and maturity		ALA (g kg^{-1} DM)		ALA (g 100 g^{-1} ΣFA)		ΣFA (g kg^{-1} DM)	
Lamina	Vegetative	26.8	a	66.1	a	40.5	a
	Reproductive	23.8	b	67.7	a	35.1	b
Pseudostem	Vegetative	5.1	c	24.1	b	21.1	c
	Reproductive	2.8	c	23.4	b	12.0	d
	SEM[c]	0.5		0.4		0.9	
Whole plant (calculated)[d]	Vegetative	20.1		58.2		34.5	
	Reproductive	9.5		49.0		19.4	
Effect		F-value	*P*-value	F-value	*P*-value	F-value	*P*-value
Plant fraction		1520	<.0001	14070	<.0001	592	<.0001
Maturity stage		23	0.0013	2	*ns*[b]	69	<.0001
Stage*fraction		0	*ns*	10	0.0141	5	*ns*

[a]Least squares means without a common letter differ significantly; $P < 0.05$ (Tukey's HSD)

[b]*ns* = non-significant

[c]SEM = standard error of the means

[d]Whole plant measures were not included in the analysis of variance

Table 3.3. Pearson correlation coefficients (*n* = 30) of lamina mass ratio (LMR), crude protein (CP) and neutral detergent fiber (aNDF) content with alpha-linolenic acid (ALA) content, proportion, and total fatty acid (ΣFA) content of a weighted mean of constituent fractions (calculated whole plant basis) as well as in lamina and pseudostem fractions of pearl millet.

		LMR[a]		CP[b]		aNDF[c]	
		r-value	*P*-value	r-value	*P*-value	r-value	*P*-value
Whole plant	ALA (g kg^{-1} DM)	0.87	<.0001	0.89	<.0001	-0.86	<.0001
	ALA (g 100 g^{-1} ΣFA)	0.86	<.0001	0.79	<.0001	-0.79	<.0001
	ΣFA (g kg^{-1} DM)	0.87	<.0001	0.92	<.0001	-0.87	<.0001
Lamina	ALA (g kg^{-1} DM)	0.33	0.08	0.70	<.0001	-0.41	0.03
	ALA (g 100 g^{-1} ΣFA)	-0.46	0.01	0.00	0.98	0.36	0.05
	ΣFA (g kg^{-1} DM)	0.43	0.02	0.76	<.0001	-0.5	0.005
Pseudostem	ALA (g kg^{-1} DM)	0.78	<.0001	0.04	0.83	-0.74	<.0001
	ALA (g 100 g^{-1} ΣFA)	0.02	0.93	-0.57	0.001	-0.15	0.42
	ΣFA (g kg^{-1} DM)	0.89	<.0001	0.3	0.1	-0.76	<.0001

[a]LMR = lamina mass ratio (lamina DM / lamina + pseudostem DM)
[b]CP = crude protein content of each plant fraction, and weighted mean on a whole plant basis
[c]aNDF= neutral detergent fiber content of each plant fraction, and weighted mean on a whole plant basis

CHAPTER 4: SUMMER ANNUAL FORAGES DECREASE IN FATTY ACID CONTENT WITH MATURITY, BUT INCREASE WITH ADDED NITROGEN FERTILITY

C.P. Goossen[a], J. Kraft[b], H.M. Darby[c], and S.C. Bosworth[ac*]

[a]Department of Plant and Soil Science, The University of Vermont, Burlington, VT, 05405, USA

[b]Department of Animal and Veterinary Sciences, The University of Vermont, Burlington, VT, 05405, USA

[c]Extension System, The University of Vermont, Burlington, VT, 05405, USA

[*]Corresponding author

Email: sid.bosworth@uvm.edu

KEYWORDS

C4 photosynthesis, summer annual forage, alpha-linolenic acid, pearl millet, sudangrass, n-3 fatty acids

4.1. Abstract

The extent to which forage management factors influence the fatty acid (FA) content and profile of traditional cool season (C_3 photosynthesis) forage species is well known. There are only limited reports of warm season (C_4 photosynthesis) annual forage species' FA content and composition, and no investigations to the effect of key management factors such as plant maturity at harvest and nitrogen fertility. In this study, main plot effects of plant maturity at harvest (60 cm vs. 90 cm height) and sub-plot effects of nitrogen fertility (39, 79, 118, and 157 kg N ha^{-1}) were investigated with pearl millet (*Pennisetum glaucum* L.) and sudangrass (*Sorghum* X *drummondii* (Nees ex Steud.) Millsp. & Chase).

Plant maturity had the greatest impact upon ALA and total FA (ΣFA) content in this study, with later maturity samples containing on average 3.2 and 4.7 g less ALA and ΣFA, respectively, per kg of forage dry matter than earlier maturity samples. There were interactions between plant maturity, cutting, site-year, and nitrogen fertility, however. Regrowth cuttings were lower in ALA and ΣFA, except for early maturity sudangrass samples in the first year, which was impacted by an unusually rainy spring.

N fertility had very limited effects upon ALA and ΣFA in the first year of sampling, where the drastic rainfall likely reduced the efficacy of N treatments, limiting FA. In the second year of sampling, differences between high and low N treatments were sizable in early maturity samples, but less so in later maturity samples.

Optimizing soil nitrogen fertility and managing for high proportion of laminae t may be of greater importance in maximizing ALA and ΣFA content in these tall growing summer annual species than in traditional cool season perennial pasture species.

4.2. Introduction

Interest in the health effects of fatty acids (FA) in ruminant derived-products, such as dairy and meat, has led to an increased consumer demand for milk and meat with a beneficial FA profile, which is strongly associated with pasture feeding. This association has been attributed to the higher pH rumen environment from forage feeding, and the relatively large supply of the n-3 polyunsaturated FA alpha-linolenic acid (ALA; C18:3 9*c*, 12*c*, 15*c*) in fresh pasture species (Elgersma, 2015). ALA is the chief FA in vegetative forages and an important source of the desired FA content and profile of ruminant milk and meat products.

Grazing availability from traditional perennial pasture can noticeably decrease during the hot dry months of the "summer slump", forcing many producers to supplement with conserved forages. Conserved forages, however, often have lower contents of ALA relative to fresh forages (Glasser *et al*., 2013) because of enzymatic degradation during wilting (Dewhurst *et al*., 2003), and because they are typically harvested at an advanced maturity and inclusive of more pseudostem components relative to grazed forages (Elgersma, 2015). For this reason, there is a growing interest in utilizing warm season annual forages in summer months to provide fresh grazing, which is associated with desired FA profile. Warm season annuals exceed the productivity of cool season forages in hot dry weather, and can also be critical as an "emergency planting". However,

compared to cool season grasses, warm season grasses typically have a lower proportion of lamina tissue, where the majority of forage FA are found (Atkinson *et al*., 2016), and there is limited research on the FA composition and content of warm season annual forage species. For example, individual and total FA (ΣFA) content was reported for several perennial warm season grasses (Khan *et al*., 2015; Mojica-Rodríguez *et al*., 2017; O'Kelly and Reich, 1976), for perennial elephant grass (*Pennisetum purpureum* Schum.) at two grazing heights and grazing intensities (Dias *et al*., 2017). FA composition was also presented at one growth stage and management condition for both pearl millet (*Pennisetum glaucum* L.) (Bainbridge *et al*., 2017) and Kikuyugrass (*Pennisetum clandestinum* Hochst. ex Chiov.) (Vargas *et al*., 2013).

In cool season forage species, plant maturity and applied nitrogen (N) fertility are among the most important management factors identified to affect the overall FA content and ALA proportion (Glasser *et al*., 2013), with ALA and ΣFA content typically decreasing in response to advancing maturity, and increasing in response to greater N fertility. Therefore, this study was designed to compare four levels of N fertility at two maturity stages in pearl millet (*Pennisetum glaucum* L. cv. 'Wonderleaf') and sudangrass (*Sorghum* X *drummondii* (Nees ex Steud.) Millsp. & Chase cv. 'Hayking'). N fertility levels were chosen to span from a low N application to an excessive N application (below and above typical agronomic recommendations, *i.e.*, annual application rates between 90 and 170 kg N ha^{-1}), and the two maturity stages were chosen to represent an early vegetative stage at which grazing within a stand would typically be started, and a later boot stage in which a conserved forage harvest would typically be performed and/or

grazing would typically be concluded. We hypothesized that the greater nitrogen fertility levels and earlier maturity stages would result in the greatest content of ALA, and therefore ΣFA content and ALA proportion, in both species.

4.3. Materials and methods

4.3.1. Field management and site description

The experiment was conducted over the course of 2013 and 2014 at The University of Vermont Horticulture Research and Education Center (HREC) in South Burlington, Vermont (44°25'N; 73°12'W) and at the Borderview Research Farm (BRF) in Alburgh, Vermont (45°0'N; 73°18'W). The HREC location consisted of excessively drained deep Windsor loamy sand soils (Mixed, mesic Typic Udipsamments; Soil Survey Staff, 2018) with a <5% slope and the BRF location consisted of somewhat excessively drained Benson rocky silt loam over shaly limestone (Loamy-skeletal, mixed, active, mesic Lithic Eutrudepts; Soil Survey Staff, 2018) with a slope between 3% and 8%. In the 2014 repetition of the study, the HREC location utilized the same field with a re-randomization of plots, and the BRF location utilized an adjacent field of the same soil type.

In 2013, the BRF and HREC locations were seeded on June 5th and June 10th, respectively, and in 2014, the BRF and HREC locations were seeded on June 16th and June 9th, respectively (**Table 4.1.**). Both sites were seeded at 15.25 cm row spacing with a 5-row research plot seeder (Carter MFG Co., Brookston, IN, USA). Pearl millet (*Pennisetum glaucum* L. cv. '*Wonderleaf*') was seeded at a rate of 22.4 kg ha^{-1} and sudangrass (*Sorghum* X *drummondii* (Nees ex Steud.) Millsp. & Chase cv. 'Hayking')

was seeded at a rate of 33.6 kg ha^{-1}. Seed was procured from King's Agriseeds (Ronks, PA, USA) both years. All sub-plots were seeded to be 2.3 m x 7.6 m though plot ends were trimmed off prior to each sampling and excluded.

4.3.2. Treatments

The study utilized a split-plot design, with whole-plot differences of maturity at harvest. The whole plot treatments were: "pasture maturity" (PAST) harvested at 60 cm height and "conserved maturity" (CONS) harvested after reaching 90 cm height, approximating a minimally elongated vegetative stage appropriate for grazing and an elongated boot to early head emergence stage appropriate for mechanical harvest, respectively (**Table 4.1.**). Split-plot treatments consisted of randomized combinations of the two species, pearl millet and sudangrass, and four rates of N fertility applied before each cutting cycle (**Table 4.1.**): 39, 79, 118, and 157 kg N ha^{-1}. All plots received the base rate of fertilizer (39 kg ha^{-1}) in the form of composted poultry litter to provide adequate general nutrition of P and K (5-3-4 NPK 'Pro Gro' supplying 10 kg P ha^{-1} and 26 kg K ha^{-1} in 2013, 4-3-3 NPK 'Cheep Cheep' supplying 13 kg P ha^{-1} and 24 kg K ha^{-1} in 2014; both produced by North Country Organics, Bradford, VT, USA). Plots receiving additional nitrogen treatments did so in the form of Chilean nitrate ($NaNO_3$; 16-0-0 NPK Allganic, SQM North America, Atlanta, GA, USA). In 2013 Chilean nitrate was applied immediately preceding seeding, and in 2014, approximately three weeks after germination to maximize uptake and utilization, and to minimize leaching losses of the highly soluble Chilean nitrate. All fertility treatments were re-applied approximately seven days following first growth cutting, with the exception of the PAST plots at the

BRF location in 2014 (**Table 4.1.**). The study was replicated five times at each location each year with the exception of the HREC location in 2013, which was limited to four replications. Samples were not collected in the first growth cutting of 2013 at the HREC location because of an unprecedented amount of rain that spring (2.7 fold higher than 20 year normal for the area, **Figure 4.1.**) and subsequent poor growth of all treatments, ostensibly due to loss of nitrogen fertility in the sandy soil. All plots at the HREC location in 2013 were mowed on July 17 and all fertility treatments were re-applied on July 21.

4.3.3. Sampling

Height (cm) was recorded at each sampling as a mean of three measurements per plot (Table 1). Chlorophyll content was estimated (SPAD units) with a Konica Minolta SPAD-502 (Chiyoda, Tokyo, Japan) at each sampling as a mean of ten measurements per plot: ten tillers representative of the plot were measured in the middle of the leaf length of the uppermost fully extended leaf, halfway between the midrib and the leaf margin. Forage yield was measured with a small plot research harvester (Carter MFG Co., Brookston, IN, USA) cutting a 0.9 m wide swath out of the middle of each plot at a stubble height of 15 cm. Immediately following yield harvests, four subsamples per plot were cut at the same height at random points along the unharvested plot area using handheld electric clippers (Gardena Accu Grass Shears ComfortCut, Husqvarna Professional Products Inc., NC, USA) and composited for forage quality analysis. Weeds were separated from each quality sample and dried separately. Five representative tillers from each quality sample were divided just above the ligule and dried separately as

lamina and pseudostem (stem, culm, and petiole, *i.e.*, leaf sheath) fractions to provide a measure of "lamina mass ratio" (LMR). LMR was calculated as the proportion of the dry matter (DM) weight of laminae relative to the total DM weight of laminae and pseudostem portions. Forage quality samples were dried for 7 days in a custom-built forced hot air walk-in drying room at the HREC location, set to 42°C in 2013 and 44°C in 2014. Dried samples were ground with a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA, USA) to pass through a 2 mm screen, and a cyclone forage mill (UDY Corporation, Fort Collins, CO, USA) to pass through a 1mm screen.

4.3.4. Fatty acid analysis

Fatty acid methyl esters (FAME) were extracted from the dried and ground forage quality samples using a modified one step transesterification method of Sukhija and Palmquist (1988) as described in Goossen *et al*., 2018a, with the exception of microwave pretreatment and DM correction at time of FAME preparation.

4.3.5. Statistical analysis

The MIXED procedure in SAS version 9.4 (SAS Institute, Cary, NC, USA) was used for all analyses of FA measures with a single repeated measures split plot model, with cutting within site-year as a repeat measure, sample as subject, and assuming an unstructured covariance matrix. Because of unbalanced data between locations, locations and years were analyzed as "site-years". Denominator degrees of freedom were computed using the Kenward-Roger approximation. Multiple comparisons were made upon least squares means with Tukey HSD *P*-value adjustments and the PDMIX800 macro for SAS (Saxton, 1998). Least squares means of interactions were limited to three terms.

Differences were considered significant with an adjusted $P<0.05$. Regrowth samples from the HREC location in 2013 were analyzed separately with the MIXED procedure, utilizing a simpler model that left out the effect of site-year, cutting cycle, and repeated measures, as the first growth cutting from that location was not sampled.

4.4. Results

Ranges of FA content were small for the majority of individual FA measured, but large for ALA (**Figure 4.2.**). ALA is the principal and most variable FA in forages. Because of this, only ΣFA content (g kg^{-1} forage DM), ALA content (g kg^{-1} forage DM), and ALA proportion (g 100 g^{-1} ΣFA) results are presented. Plant maturity, species, nitrogen fertility, and cutting were all found to have significant impacts upon ΣFA and ALA content and ALA proportions, however, differences between site-years were also substantial, and produced interactions with all of the above simple effects (**Tables 4.2., 4.3.**).

4.4.1. Plant maturity

Plant maturity was the most significant impactor of ΣFA content and ALA content and proportion in this study (**Table 4.2.**). Overall CONS plant maturity reduced ΣFA and ALA content by 4.7 and 3.2 g kg^{-1} forage DM, respectively and ALA proportion by 5.1 g 100 g^{-1} ΣFA (**Table 4.4.**) relative to PAST maturity. However, these effects of later plant maturity were not consistent across all site-years (**Figure 4.3.**), with no statistical difference in ALA content or proportion between PAST and CONS samples at the BRF location in 2014. Additionally, numerical decreases in ALA content and

proportion in CONS samples at the BRF location in 2013 and HREC location in 2014 were not statistically significant.

4.4.2. Nitrogen

Nitrogen fertility had the second greatest impact upon ΣFA content and ALA content and proportion in this study with the two highest treatment levels containing more of all three FA measures than the lowest two treatment levels, which were also different from each other (**Table 4.6.**). However, these overall averages are driven primarily by PAST sample results from the 2014 site-years, as well as CONS samples from the BRF location in 2014 differing between the lowest and highest N treatment level (**Figure 4.4.**).

4.4.3. Cutting

Regrowth cuttings were lower in ΣFA and ALA content by 1.4 and 0.9 g kg^{-1} forage DM, respectively and ALA proportion by 1.2 g 100 g^{-1} ΣFA relative to first growth cuttings (**Table 4.5.**). There was, however, an unexpected increase in ΣFA and ALA content and ALA proportion from first growth to regrowth cuttings of PAST samples at the BRF location in 2013.

4.4.4. Species

On average, sudangrass was slightly higher in ALA and ΣFA content than pearl millet, though slightly lower in ALA proportion (**Table 4.7.**). The ALA and ΣFA content distinction between species was driven by large decreases in regrowth pearl millet ALA and ΣFA content at both locations in 2014, with a smaller decrease in sudangrass samples

from the HREC location, and unanticipated increases in ALA and ΣFA content in regrowth sudangrass in the 2013 BRF site-year samples (**Figure 4.5.**).

4.4.5. HREC 2013 regrowth

A separate analysis of the regrowth samples harvested from the HREC location in 2013 showed similar results to the other site years (**Table C.1.**). In sudangrass, ΣFA and ALA content was 7.2 and 6.1 g kg^{-1} DM lower in the CONS than PAST maturity samples, while in pearl millet the differences were not statistically significant (**Table C.4.**). ALA proportion was similar to ALA and ΣFA content, with PAST samples 13.5 g 100 g^{-1} ΣFA higher than CONS samples for sudangrass and pearl millet differences not being different (**Table C.4.**). Unlike the BRF location samples from the same year (**Figure 4.4.**), there was a slight increase in ΣFA content in the highest N fertility, treatment relative to the 79 kg N ha^{-1} treatment (2.6 g kg^{-1} DM), though all other comparisons were not different (**Table C.5.**).

4.5. Discussion

The largest differences in ALA content, and therefore ΣFA content, are derived from factors affecting the ratio of cellular photosynthetic and metabolic components relative to structural components (Dewhurst *et al*., 2001; Boufaïed *et al*., 2003; Dias *et al*., 2017; Goossen *et al*., 2018b). In this study, that ratio is approximated by a measure of lamina mass ratio (LMR; DM weight of laminae relative to the total DM weight of laminae and pseudostem). While the underlying properties in effect are likely germane to the FA content and composition of all grass species, the tall "stemmy" architecture of summer annual species allows a clear investigation of this relationship.

4.5.1. Plant maturity

The advanced maturity and greater elongation of a summer annual grass harvested as conserved feed impacts the ALA and ΣFA content in two related manners; I) older plant cells have had more time to build structural components (*e.g.*, cell wall), in effect diluting the photosynthetic/metabolic cellular components which contain the vast majority of ALA and all FA on a DM basis (Bracher and Mosimann, 2016), and II) elongated tillers have greater proportions of pseudostem (leaf sheath, culm, and/or jointed stem) relative to laminae (leaf blades). As pseudostem is largely structural, the ratio of photosynthetic and metabolic cellular components relative to structural components is greatly reduced in these fractions (Boufaïed *et al*., 2003; Dewhurst *et al*., 2001; Dias *et al*., 2017; Goossen *et al*., 2018b). A warm season grass of later maturity and greater elongation therefore has laminae fractions which have a reduced ALA and ΣFA content, and a lower LMR relative to an un-elongated stage (**Figure 4.3., panel D; Table 4.4.**). Other research with warm season grasses suggests that the increased ratio of pseudostem components in elongated tillers may have a greater effect upon the FA composition and content of later maturity specimens than the impact of cell wall accumulation alone (Dias *et al*., 2017, Goossen *et al*., 2018b).

4.5.2. Regrowth & Species

Our findings suggest that the decrease in FA content observed in samples from the regrowth cutting is species dependent. The FA decline from the first growth to the regrowth cutting in 2014 was more distinct in pearl millet than sudangrass (**Figure 4.5., panels A, B, C**). This is likely resultant from pearl millet LMR falling from 0.83 in the

first growth to 0.63 in the regrowth, whereas sudangrass LMR remained nearly identical (0.54 – 0.55). The decrease in pearl millet LMR was particularly marked for CONS samples which declined from 0.78 to 0.51 compared to a decline of 0.87 to 0.74 for PAST samples, while the sudangrass, LMR was unchanged from the first growth to the regrowth for both PAST and CONS plots. These results are consistent with observations of Teutsch (2002) that millets often have smaller stems and are generally leafier than sorghum species.

4.5.3. Nitrogen fertility

Higher N fertility levels led to greater ALA and therefore ΣFA content in PAST samples at both locations in 2014, despite the PAST plots at the BRF location not receiving a re-application of the N fertility treatments after first growth samples were harvested that year (**Figure 4.4., panels A, B**). This may, at least in part, explain the lack of FA differences between PAST and CONS samples for that site-year however. The greater range of N response seen at the HREC location in 2014 is likely a result of edaphic conditions, as that site is a very free draining sandy soil without the native fertility capacity of the loamy BRF location soil. N fertility effects were not significant in 2013, with the exception of a small increase in ΣFA content in regrowth samples at the HREC location, which may be explained, at least in part, by the unusually high amount of rain early in 2013 negatively impacting soil N levels (**Figure 4.4.**). This may also explain why PAST regrowth samples from the BRF location had higher FA content than first growth samples, though this increase from first growth to regrowth was only seen in sudangrass samples (**Figures 4.3., 4.5.**).

In this study, increasing levels of N fertility typically reduced LMR at the time of sampling (**Table 4.6.**), possibly resultant from increased growth rates, as Muchow (1988) found in response to N in maize and sorghum. Because there was less lamina component, which has a higher FA content, the increase in FA content associated with higher N fertility is likely to derive from I) increased chloroplast quantity within laminae, and/or II) increased chloroplast size within laminae, or possibly, III) increased grana size and/or quantity within the chloroplasts. We found a chlorophyll response to N fertility amendments with SPAD meter readings increasing from 30 to 43.7 between the lowest and highest fertility treatments in the 2nd cut of 2013, and from 27.7 to 40.8 in the same treatments in 2014. Both increased chloroplast size and grana size and quantity are associated with N nutrition (Hall *et al*., 1972; Laza *et al*., 1993). This conclusion is in agreement with the results of a study of N effects on FA content in timothy by Boufaïed *et al*. (2003).

The effects of N fertility upon ALA and ΣFA content are likely multifaceted and possibly counter-effectual. Higher N may increase size, and to some extent quantity of ALA-rich leaves in warm season grasses (Muchow, 1988), it may also increase pseudostem biomass to a greater degree than lamina biomass as shown in two cool season grasses by (Gatti *et al*., 2015), and limiting N fertility has shown a decreased LMR in *Poa* and *Bromus* species (Muller and Gamier, 1990; Arendonk *et al*., 1997).

CONS and PAST means of ALA content and proportion in pearl millet and sudangrass samples in this study ranged from 4.7 – 12.7 g kg^{-1} forage DM and 40.4 – 57.9 g 100g^{-1} ΣFA, respectively. While similar to warm season perennial grass findings

of Khan *et al*. (2015), ranging from 2.3 – 13.8 g ALA kg^{-1} forage DM and 30.0 – 60.3 g ALA $100g^{-1}$ ΣFA, these measures of ALA proportion are drastically higher than those reported for perennial tropical grass species in a study, utilizing similar sample preservation methods, by O'Kelly and Reich (1976) ranging from 12.8 to 36.1 g $100g^{-1}$ ΣFA, and measures of ALA content in a study of perennial tropical grasses by Mojica-Rodríguez *et al*. (2017) ranging from 0.07 – 1.22 g kg^{-1} forage DM. As described in Goossen *et al*. (2018a), forced hot air drying alone, as used in this study, can lead to a preservation loss of ALA and therefore ΣFA content within forage samples, and to a lesser extent the proportion of ALA relative to ΣFA. Additionally, a lack of DM correction of dried ground samples may underrepresent the content of individual and ΣFA, though without altering the proportion of any individual FA.

When sample preservation methodological concerns were taken into account in Goossen *et al*. (2018b), the ALA content and proportion of pearl millet and a sorghum x. sudangrass hybrid at early and late maturities were shown to have higher maximum values, ranging from 9.2 – 20.2 g kg^{-1} forage DM and 49.0 – 61.8 g $100g^{-1}$ ΣFA. These results are much closer to the findings of Dias *et al*. (2017) for perennial elephant grass (*Pennisetum purpureum*) ranging in ALA proportions from 48.7 - 64.7 g $100g^{-1}$ ΣFA, despite having also utilized forced hot air drying.

It is of note that many of these warm season grass results fall largely within the ALA content and proportion ranges (6.9 – 23.8 g kg^{-1} forage DM and 43.1 – 68.4 g $100g^{-1}$ ΣFA) of six studies on the much lower growing cool season Italian ryegrass (*Lolium multiflorum* L.) discussed in Goossen *et al*., (2018a), suggesting that differences between

studies (whether from treatment or methodology) may be nearly as great as, or greater than, differences between cool season and warm season grasses, though highest attainable ALA content and proportion may still only be found in cool season species. A higher potential ALA content and proportion top range was described for a similarly tall growing, but cool season, annual triticale (× *Triticale rimpaui* (Wittm.) Muntz) in Clapham *et al*. (2005) ranging from 13.2 – 30.0 g kg^{-1} forage DM and 65 - 69 g 100 g^{-1} ΣFA, respectively. These higher ALA values in triticale may be due, in part, to optimal sample preservation and handling.

Differences between the findings of the above warm season grass studies may be due to temperature differences during growth (Dias *et al*., 2017; Falcone *et al*., 2004; Larkindale and Huang, 2004; Narayanan *et al*., 2016), uncertain effectiveness of methodologies of sample preservation (Goossen *et al., 2018a*), the species investigated, or other factors. Further research into FA content and composition of warm season (C_4) grasses is therefore crucial for a more thorough understanding, and must incorporate a diversity of species, maturities, and concomitantly the different LMR created by these combinations. Further research would ideally contain a difference of temperature, and be performed with great care to sample preservation/preparation methodology to eliminate ALA losses.

An unavoidable limitation of the present study was that regrowth harvests could not begin at the same date for PAST and CONS treatments as different first growth sampling dates were inherent to the treatments. Additionally, unusually heavy rains in early 2013 impaired normal plant growth to the point of severe chlorosis in the first

growth at the HREC location. These rare conditions likely affected the comparability of results between years. Potential limitations of sample preservation method are described in greater detail in Goossen *et al*., 2018a.

4.6. Conclusion

Though there were small effects of N fertility, and differences between species, the greatest impact upon ALA and ΣFA content was the maturity stage of plants at harvest. Managing for high proportion of laminae to produce forage with greater ALA and ΣFA content may be of greater importance in these tall growing summer annual species.

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4.10. Figures

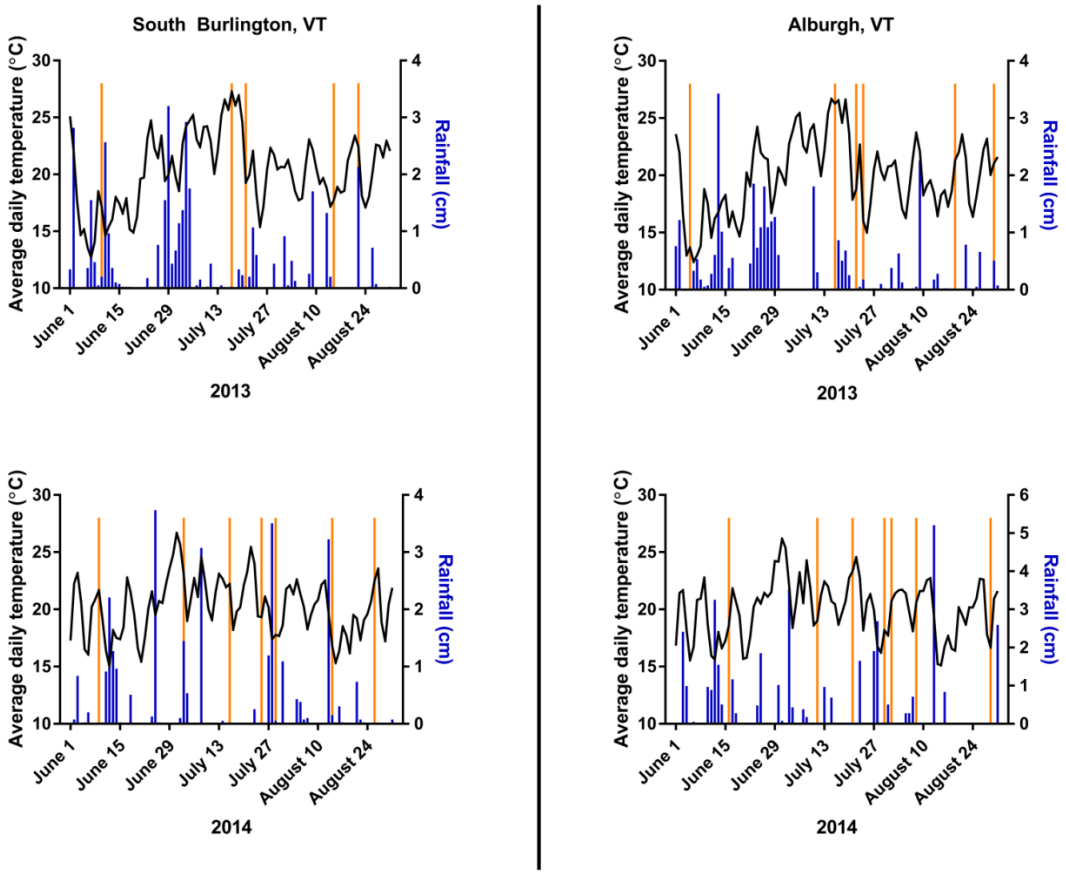

Figure 4.1. Rainfall (right Y axis, blue) and average daily temperature (left Y axis, black) at the HREC (South Burlington, VT) location and BRF (Alburgh, VT) location for duration of experiment in 2013 and 2014. Orange lines indicate timing of seeding, harvests, etc., as indicated in the text.

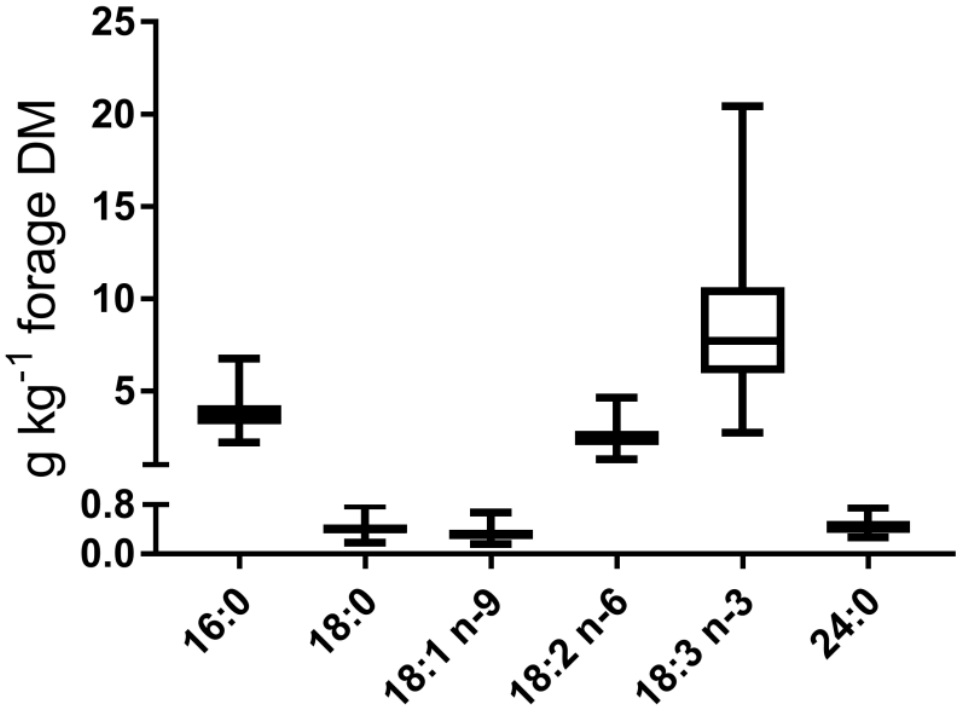

Figure 4.2. Box and whiskers plot of fatty acid (FA) content range of individual FA with a maximum > 0.5 g kg-1 forage, of all samples combined (pearl millet (*Pennisetum glaucum* L. cv. 'Wonderleaf') and sudangrass (*Sorghum* X *drummondii* (Nees ex Steud.) Millsp. & Chase cv. 'Hayking'). Whiskers show minimum and maximum values, box encapsulates 25th to 75th percentile, and center line (where visible) shows median value.

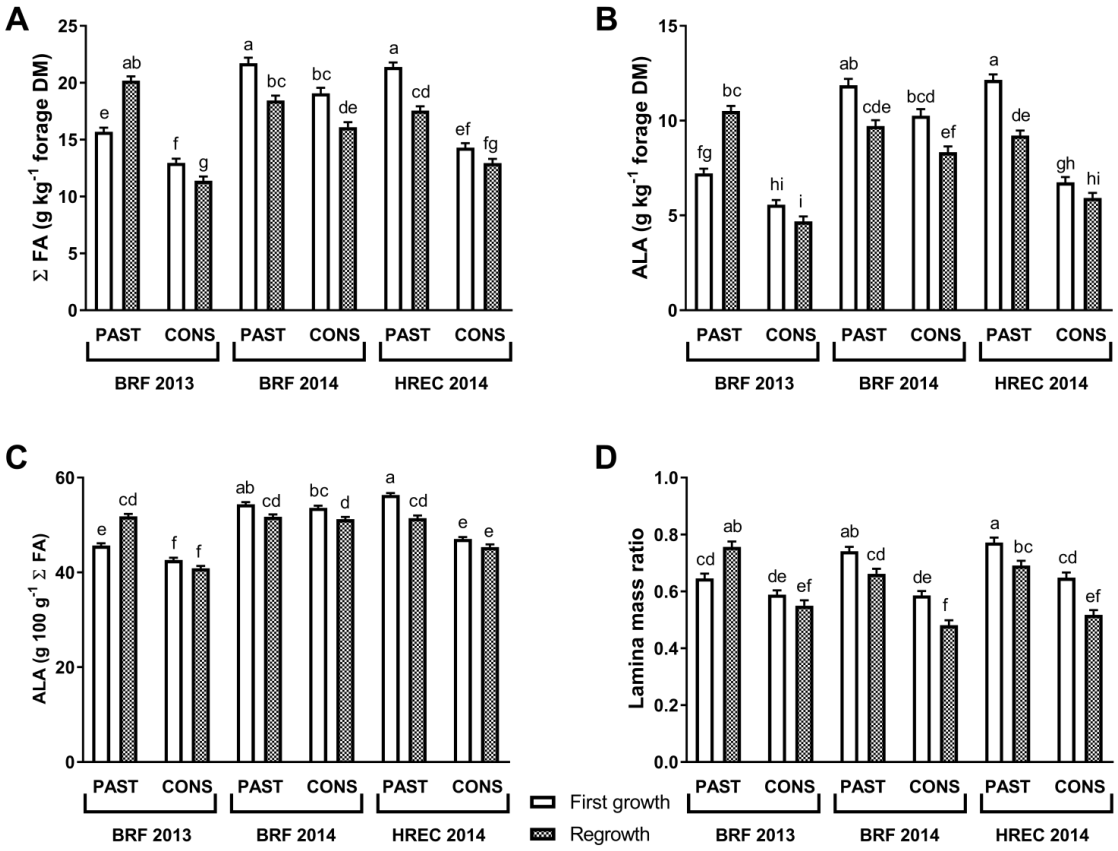

Figure 4.3. Plant maturity (PAST = pasture maturity, CONS = conservation maturity) by cutting (white bars = first growth, grey bars = regrowth) by site-year effects on least squares means of total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio (LMR), and their standard error of means in panels A, B, C, and D, respectively. Least squares means without a common letter differ significantly; $P < 0.05$ (Tukey's HSD).

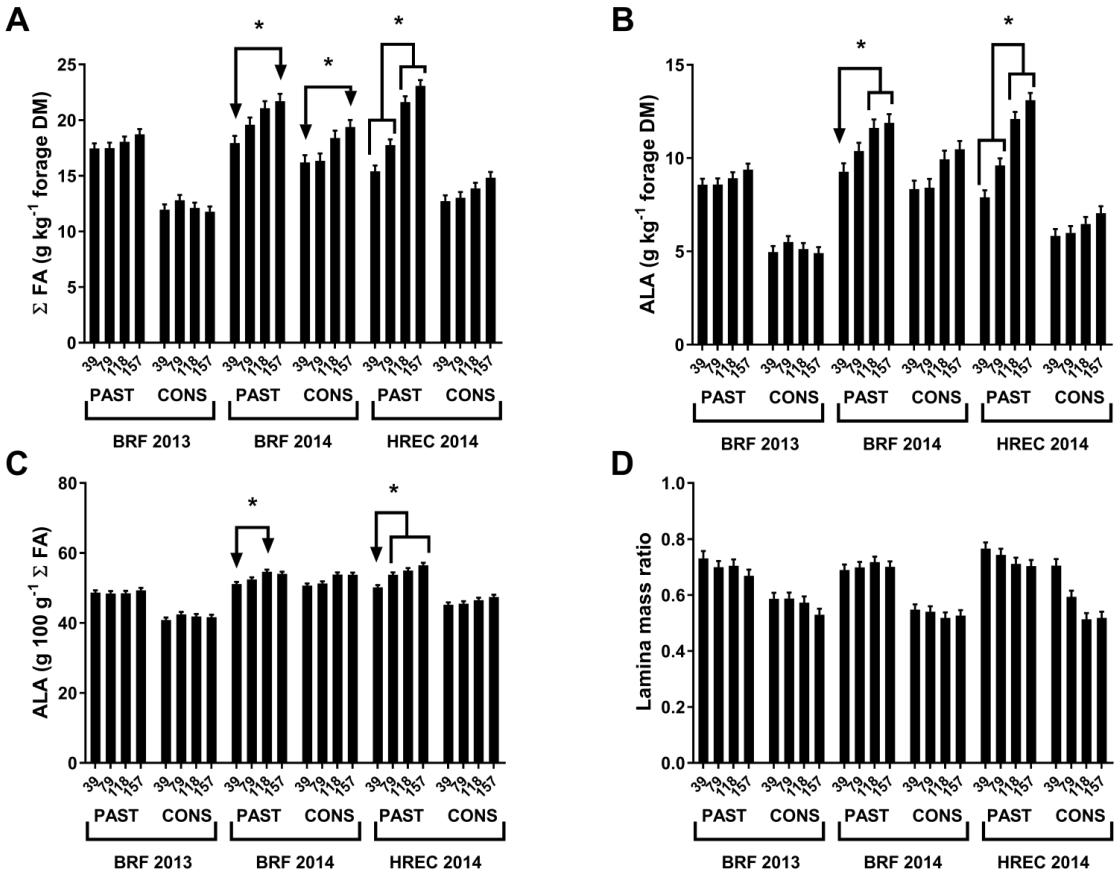

Figure 4.4. Plant maturity (PAST = pasture maturity, CONS = conservation maturity) by nitrogen fertility (kg ha^{-1}) by site-year effects on least squares means of total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio (LMR), and their standard error of means in panels A, B, C, and D, respectively. Individual means (▼) or groups of means (within Π bracket) denoted with * differ significantly; *P* < 0.05 (Tukey's HSD).

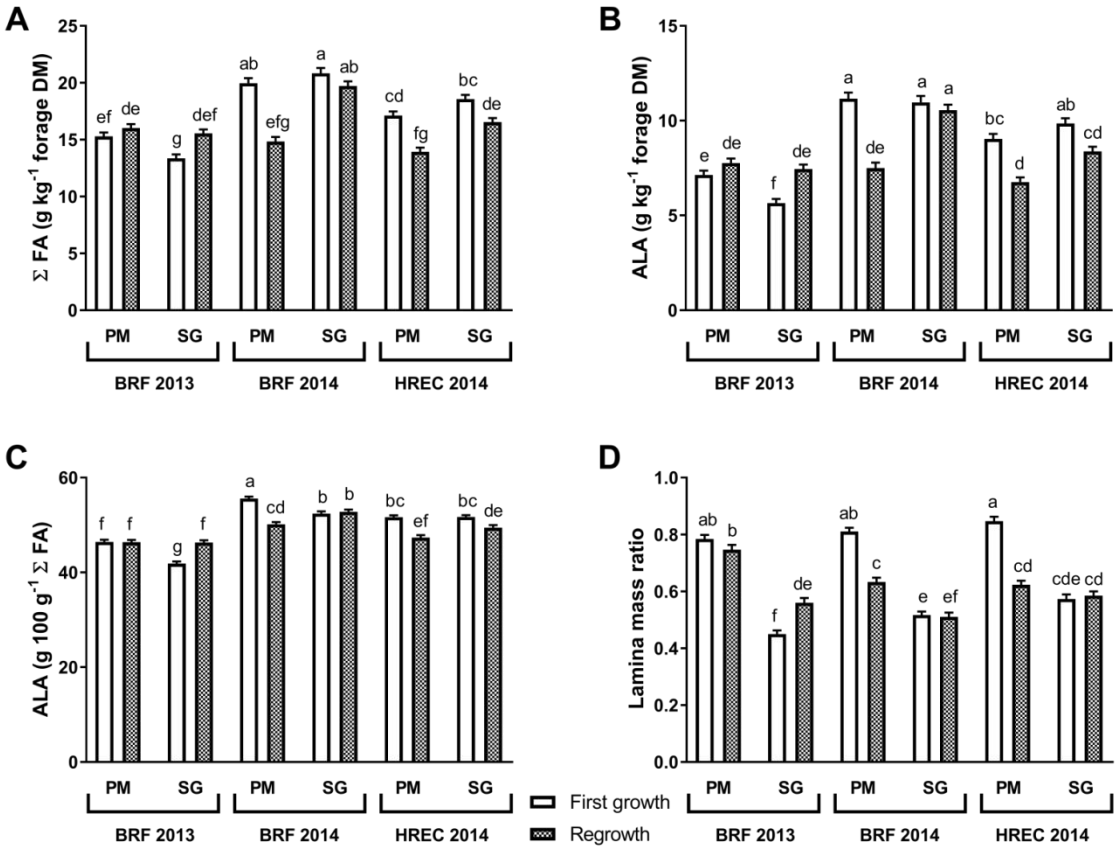

Figure 4.5. Plant species (PM = pearl millet, SG = sudangrass) by cutting (white bars = first growth, grey bars = regrowth) by site-year effects on least squares means of total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio (LMR), and their standard error of means in panels A, B, C, and D, respectively. Least squares means without a common letter differ significantly; $P < 0.05$ (Tukey's HSD).

Table 4.1. Seeding, fertilizing, and harvest dates

Year	Site	Cutting	Maturity	Seeding Date	N Application Date	Harvest Date	Average Canopy Height (cm)		
							Pearl Millet	Sudangrass	Average
2013	**HREC**	**First**	**Pasture**	6/10	6/9	n/a	n/a	n/a	n/a
			Conserved	6/10	6/9	n/a	n/a	n/a	n/a
		Regrowth	**Pasture**	6/10	7/21	8/15	51	57	54
			Conserved	6/10	7/21	8/22	62	70	66
	BRF	**First**	**Pasture**	6/5	6/5	7/16	44	66	55
			Conserved	6/5	6/5	7/22	70	117	93
		Regrowth	**Pasture**	6/5	7/24	8/19	55	69	62
			Conserved	6/5	7/24	8/30	80	105	93
2014	**HREC**	**First**	**Pasture**	6/9	7/3	7/16	42	53	47
			Conserved	6/9	7/3	7/25	57	68	62
		Regrowth	**Pasture**	6/9	7/29	8/14	48	47	47
			Conserved	6/9	7/29	8/26	90	68	79
	BRF	**First**	**Pasture**	6/16	7/11	7/21	45	68	57
			Conserved	6/16	7/11	7/30	76	108	92
		Regrowth	**Pasture**	6/16	n/a	8/8	65	71	68
			Conserved	6/16	8/1	8/29	94	107	101

Table 4.2. Effects of plant maturity, species (Sp), nitrogen fertility (N), cutting (Cut), site-year, and their interactions on total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio.

Effect	ΣFA(g kg^{-1} forage DM[a])		ALA (g kg^{-1} forage DM)		ALA (g 100 g^{-1} ΣFA)		Lamina mass ratio	
	F-Value	*P*-value	F-Value	*P*-value	F-Value	*P*-value	F-Value	*P*-value
Rep	2.0	*ns*	2.4	*ns*	1.8	*ns*	1.1	*ns*
Maturity	313.2	<.0001	286.4	<.0001	256.4	<.0001	152.0	<.0001
Sp	35.1	<.0001	15.8	0.0001	3.9	0.05	825.6	<.0001
Maturity*Sp	7.0	0.01	4.0	0.05	0.0	*ns*	0.1	*ns*
N	40.8	<.0001	37.7	<.0001	22.3	<.0001	13.8	<.0001
Maturity*N	8.0	<.0001	7.7	<.0001	1.1	*ns*	3.4	0.02
Sp*N	0.1	*ns*	0.0	*ns*	0.4	*ns*	2.3	*ns*
Maturity*Sp*N	3.0	0.03	2.7	*ns*	1.3	*ns*	0.5	*ns*
Cut	51.3	<.0001	44.7	<.0001	26.0	<.0001	56.2	<.0001
Maturity*Cut	7.3	0.01	4.7	0.03	9.4	<0.01	27.3	<.0001
Sp*Cut	28.6	<.0001	37.4	<.0001	70.1	<.0001	167.9	<.0001
Maturity*Sp*Cut	0.0	*ns*	1.2	*ns*	5.8	0.02	55.3	<.0001
N*Cut	1.0	*ns*	0.9	*ns*	4.3	<0.01	1.1	*ns*
Maturity*N*Cut	1.8	*ns*	2.1	*ns*	2.1	*ns*	0.1	*ns*
Sp*N*Cut	0.0	*ns*	0.1	*ns*	0.1	*ns*	0.7	*ns*
Maturity*Sp*N*Cut	0.6	*ns*	0.6	*ns*	1.8	*ns*	0.4	*ns*
Site-year	65.7	<.0001	87.4	<.0001	188.7	<.0001	3.6	0.05
Maturity*Site-year	15.1	0.0001	18.3	<.0001	51.0	<.0001	0.8	*ns*
Sp*Site-year	40.3	<.0001	31.5	<.0001	14.2	<.0001	15.3	<.0001
Maturity*Sp*Site-year	2.2	*ns*	2.9	*ns*	1.3	*ns*	0.1	*ns*
N*Site-year	10.7	<.0001	9.6	<.0001	4.3	<0.001	6.1	<.0001
Maturity*N*Site-year	4.1	<0.001	4.0	0.001	2.2	0.05	1.6	*ns*
Sp*N*Site-year	1.6	*ns*	1.6	*ns*	1.1	*ns*	1.0	*ns*
Maturity*Sp*N*Site-year	1.4	*ns*	1.5	*ns*	1.4	*ns*	0.6	*ns*
Cut*Site-year	51.9	<.0001	61.5	<.0001	47.7	<.0001	41.6	<.0001
Maturity*Cut*Site-year	43.5	<.0001	52.0	<.0001	44.6	<.0001	7.1	<0.01
Sp*Cut*Site-year	4.4	0.01	6.1	<0.01	4.9	<0.01	3.3	0.04
Maturity*Sp*Cut*Site-year	2.8	*ns*	4.0	0.02	7.0	0.001	0.0	*ns*
N*Cut*Site-year	1.3	*ns*	1.4	*ns*	2.1	*ns*	1.5	*ns*
Maturity*N*Cut*Site-year	2.0	*ns*	1.9	*ns*	0.9	*ns*	0.6	*ns*
Sp*N*Cut*Site-year	0.7	*ns*	0.5	*ns*	1.0	*ns*	3.5	<0.01
Maturity*Sp*N*Cut*Site-year	1.6	*ns*	1.2	*ns*	1.0	*ns*	1.2	*ns*

[a]DM = dry matter

[b]*ns* = non-significant

Table 4.3. Site-year least squares means of total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio (LMR), and their SEM[a].

Site-year	BRF 2013		BRF 2014		HREC 2014	
ΣFA (g kg^{-1} DM[b])	15.1 c	0.2	18.8 a	0.3	16.5 b	0.2
ALA (g kg^{-1} DM)	7.0 c	0.1	10.0 a	0.2	8.5 b	0.2
ALA (g 100 g^{-1} ΣFA)	45.2 c	0.3	52.8 a	0.3	50.0 b	0.3
LMR	0.64 ab	0.01	0.62 b	0.01	0.66 a	0.01

Least squares means without a common letter differ significantly within a row; $P < 0.05$ (Tukey's HSD)

[a]Standard error of means

[b]DM = dry matter

Table 4.4. Maturity stage least squares means of total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio (LMR), and their SEM[a].

Maturity	Pasture		Conserved	
ΣFA (g kg^{-1} DM[b])	19.2 a	0.2	14.5 b	0.2
ALA (g kg^{-1} DM)	10.1 a	0.1	6.9 b	0.1
ALA (g 100 g^{-1} ΣFA)	51.9 a	0.2	46.8 b	0.2
LMR	0.71 a	0.01	0.56 b	0.01

Least squares means without a common letter differ significantly within a row; $P < 0.05$ (Tukey's HSD)

[a]Standard error of means

[b]DM = dry matter

Table 4.5. Cutting cycle least squares means of total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio (LMR), and their SEM[a].

Cutting	First		Regrowth	
ΣFA (g kg^{-1} DM[b])	17.5 a	0.2	16.1 b	0.2
ALA (g kg^{-1} DM)	9.0 a	0.1	8.1 b	0.1
ALA (g 100 g^{-1} ΣFA)	49.9 a	0.2	48.7 b	0.2
LMR	0.66 a	0.01	0.61 b	0.01

Least squares means without a common letter differ significantly within a row; $P < 0.05$ (Tukey's HSD)

[a]Standard error of means

[b]DM = dry matter

Table 4.6. Nitrogen fertility effects on least squares means of total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio (LMR), and their SEM[a].

Nitrogen (kg ha^{-1})	39		79		118		157	
ΣFA (g kg^{-1} DM[b])	15.3 c	0.2	16.2 b	0.2	17.5 a	0.2	18.3 a	0.2
ALA (g kg^{-1} DM)	7.5 c	0.2	8.1 b	0.2	9.0 a	0.2	9.5 a	0.2
ALA (g 100 g^{-1} ΣFA)	47.8 c	0.3	49.0 b	0.3	50.1 a	0.3	50.5 a	0.3
LMR	0.67 a	0.01	0.64 b	0.01	0.62 bc	0.01	0.61 c	0.01

Least squares means without a common letter differ significantly within a row; $P < 0.05$ (Tukey's HSD)

[a]Standard error of means

[b]DM = dry matter

Table 4.7. Species least squares means of total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio (LMR), and their SEM[a].

Species	Pearl Millet		Sudangrass	
ΣFA (g kg^{-1} DM[b])	16.2 b	0.2	17.4 a	0.2
ALA (g kg^{-1} DM)	8.2 b	0.1	8.8 a	0.1
ALA (g 100 g^{-1} ΣFA)	49.6 a	0.2	49.1 b	0.2
LMR	0.74 a	0.01	0.53 b	0.01

Least squares means without a common letter differ significantly within a row; $P < 0.05$ (Tukey's HSD)

[a]Standard error of means

[b]DM = dry matter

CHAPTER 5: SWATH WIDTH AND TIME-OF-HARVEST EFFECTS UPON REED CANARYGRASS FATTY ACID CONTENT AND PROFILE

C.P. Goossen[a], J. Kraft[b], H.M. Darby[c], and S.C. Bosworth[ac*]

[a]Department of Plant and Soil Science, The University of Vermont, Burlington, VT, 05405, USA

[b]Department of Animal and Veterinary Sciences, The University of Vermont, Burlington, VT, 05405, USA

[c]Extension System, The University of Vermont, Burlington, VT, 05405, USA

[*]**Corresponding author**

Email: sid.bosworth@uvm.edu

Keywords

Wide swath, forage harvest management, alpha-linolenic acid, afternoon cutting

5.1. Abstract

Increased wilting times during forage conservation are associated with losses in alpha-linolenic acid (ALA) and total fatty acid (ΣFA) content. This study compared the FA content and ALA proportion of reed canarygrass (*Phalaris arundinacea* L.) mown in the evening (PM) and following morning (AM) in both wide and narrow swaths at three cuttings in 2015 and again in 2016. Differences across the season in both years, and resultant from initial wilting periods were the most pronounced and consistent result found in this study. There was little evidence that the swath widths used in this study produced forages with any difference in FA content or composition. AM mowing may allow for higher content or proportion of ALA, and ΣFA content up to 2 g kg^{-1} DM greater than PM mowing, though the effect was small enough to only be discernible with increased statistical power. We found that ensiling reduces ALA content and/or proportion of ΣFA beyond that of the initial wilting period preceding ensiling. In conclusion, harvest management strategies such as wide vs. narrow swathing, or AM vs. PM mowing, may have a smaller role for reed canarygrass in optimizing FA content of feed than other production practices.

5.2. Introduction

Wilting forages for conservation (*i.e.*, making silage or hay) has been shown to reduce their content of the polyunsaturated fatty acid (FA) alpha-linolenic acid (ALA; 18:3 n-3) which is both the primary FA in forages and considered desirable as a feedstuff component to promote a healthy FA profile in ruminant animal products (Dewhurst and King, 1998; Boufaïed *et al*., 2003; Elgersma *et al*., 2003a, 2003b; Glasser *et al*., 2013).

This may explain, at least partially, why FA beneficial to human health (*e.g.*, rumenic acid) in ruminant-derived products are found at their maxima during grazing months (Benbrook *et al.*, 2013).

ALA in forage plants is primarily found in thylakoid membranes, and is subject to continual turnover and replacement (Falcone *et al.*, 2004). In addition to enzyme activity facilitating this persistent turnover, ALA is highly susceptible to lipoxygenase enzymes in response to stress, *e.g.*, wounding, to produce metabolites such as jasmonates and green leaf volatiles (Dar *et* al., 2015; Venkatesan 2015; Sofo *et al.*, 2016). As such, enzymatic degradation of polyunsaturated FA (PUFA) begins immediately following mowing, and continues until forage dry matter (DM) is high enough that appreciable plant enzyme activity ceases (above 60 g DM 100 g^{-1} fresh weight; Rotz and Muck, 1994) or oxidation is prevented by the anaerobic conditions of ensiling. Because of the greater enzymatic degradation potential of ALA, its content and proportion of ΣFA are the most evident consequence of FA losses during conservation.

Increasing swath width at mowing, relative to the mower width, has been shown to hasten wilting of forages (Jones and Harris, 1980; Wright *et al.*, 1997; Kung *et al.*, 2010). Conversely, choosing to harvest at the end of the photosynthetic day (PM), with the goal of capturing a greater content of non-structural carbohydrates, can extend forage wilting overnight and may subsequently decrease ALA and ΣFA content of the conserved forage relative to a morning (AM) mowing. Therefore, we hypothesized that management choices that influence wilting duration would alter the ALA and ΣFA content available to ruminant livestock from conserved forages.

In this study, we sought to: I) evaluate FA changes in reed canarygrass (*Phalaris arundanacea* L.) over two seasons, II) test the impact of AM vs. PM cutting times on ALA and ΣFA content, and ALA proportion of reed canarygrass harvested at two target DM content levels (45 and ≥60 g DM 100 g^{-1} fresh weight), III) test the impact of wide or narrow swathing on ALA and ΣFA content, and ALA proportion of reed canarygrass harvested at two target DM content levels (45 and 60+ g DM 100 g^{-1} fresh weight), and IV) test the impact of ensiling on ALA and ΣFA content, and ALA proportion of reed canarygrass.

5.3. Materials and methods

5.3.1. Experiemental design

The experiment was conducted on an existing hayfield at the Borderview Research Farm in Alburgh, Vermont (45°0'N; 73°18'W), consisting primarily of reed canarygrass (*Phalaris arundinacea* L.) on well drained Nellis silt loam with a 3% - 8% slope (coarse-loamy, mixed, superactive, mesic Typic Eutrudepts; Soil Survey Staff, 2018). Harvests were performed at three cutting dates per year in 2015 and 2016 (**Table 5.1.**). The study utilized a split-split-plot design with four replications. The whole plot treatment was time of mowing (morning, AM vs. evening, PM) and the split-plot treatment was swath width (narrow, 40% of mower width vs. wide, 70% of mower width) using a New Holland 415 discbine. The split-split-plot was wilt stage (WS_0, WS_1, and WS_2). AM harvests always followed PM harvests (Table 5.1). Main plots were 14.4 m wide (two mower passes) and 30.5 m long at the first harvest, but shortened to 22.9 m for all the subsequent harvests. Split-plots were two mower widths (half of the main plot).

Split-split-plots were one mower width (half of the split-plot). Replicate plots were separated by 10.8 m buffer strips mowed immediately preceding harvests. Nitrogen (N) fertility was supplied at a rate of 54 kg N ha^{-1} in the form of Chilean nitrate ($NaNO_3$), on June 6 2015, and again on June 7 2016 at a rate of 103 kg N ha^{-1} in the form of urea ($CO(NH_2)_2$). After the wilting period of each cutting, forage was chopped with a John Deere 3940 forage harvester and blown into a feed wagon outfitted with weigh cells to record harvest yields.

Fresh forage samples (WS_0) were collected by hand at the time of mowing, by three composited hand grab samples from the length of each WS_2 subplot. Representative hand grab samples of wilted forage were collected from the chopped material at target DM contents of 45 g DM 100 g^{-1} fresh weight, approximating a typical ensiled forage harvest (WS_1), and ≥60 g DM 100 g^{-1} fresh weight when respiration had ceased (Barnes *et al*., 2003) and enzymatic activity was minimized (Rotz and Muck, 1994; Van Ranst *et al*., 2009a), approximating a hay harvest (WS_2). Harvest cutting and sampling times are shown in **Table 5.1.**, though specific times were lost for the first cutting of 2015. WS_1 samples were split into quarters with one subsample dried for analysis, and the remaining three subsamples, packed in vacuum sealed plastic bags and ensiled out of the light for 40 days, at which time the three subsamples were composited and a representative sample dried for analysis. All samples, save the first cutting 2015 PM WS_0 samples, received a microwave pretreatment prior to forced hot air drying for 24 hours at 38 °C, to halt enzymatic degradation of non-structural carbohydrates (Pelletier *et al*., 2010) and FA (Goossen *et al*., 2018). In the first cutting of 2015, target sample fresh

weights were 400 g, and samples received 1 min microwave pretreatment prior to forced hot air drying. As initial results of Goossen *et al*. (2018) became apparent, samples from the second cutting of 2015 (target: 400 g fresh weight) received 2 min microwave pretreatment prior to drying. As further findings of Goossen *et al*. (2018) were recognized, samples from the third cutting of 2015 and all three cuttings of 2016 (target: 100 - 150 g fresh weight) received 1 min microwave pretreatment prior to drying.

5.3.2. Fatty acid analysis

Fatty acid methyl esters (FAME) were prepared from the dried and ground forage samples using a modified one step transesterification method of Sukhija and Palmquist (1988) as described in Goossen *et al*. (2018).

5.3.3. Statistical analysis

Statistical analysis of all FA measures was performed with the MIXED procedure in SAS version 9.4 (SAS Institute, Cary, NC, USA) for each year, with season (cuttings 1, 2, and 3) as a repeated measure, whole plot as subject, and assuming a compound symmetry covariance matrix. Denominator degrees of freedom were computed using the Kenward-Roger approximation. Sample DM was included as a covariate to control for any unintended DM differences within wilt stages. There were no four-way interactions so interactions were limited to three terms, and multiple comparisons were made upon least squares means with Tukey HSD *P*-value adjustments and the PDMIX800 macro for SAS (Saxton, 1998). Differences were considered significant with an adjusted $P<0.05$. Ensiled WS_1 samples were compared with unensiled WS_1 samples as above, with the

substitution of the term “ensiling” in place of the “wilt stage” term and an “ensiling by season” interaction as a repeat measure.

5.4. Results

5.4.1. WS_0, un-ensiled WS_1, and WS_2 samples

In both 2015 and 2016, the effects of season, wilt stage, and their interaction explained the majority of variation in ALA and ΣFA content (**Table 5.2.**). The same was true for ALA proportion of ΣFA in 2015, though the simple effect of season was not significant in 2016, despite the recurrent interaction between season and wilt stage. There were no simple effects of time of mowing or swath width in either year, however, they were included in some interactions in 2015 (**Appendix D**).

5.4.2. Effect of season

The ΣFA content of WS_0 samples did not differ in 2015 samples, but was greater in the third cutting of 2016 than the first and second cuttings (**Figure 5.2.; panel A**). The ALA content of WS_0 samples was greater in the third cutting than the first in 2015, and greater in the third than the first and second cuttings in 2016 (**Figure 5.2.; panel B**). The ALA proportion of WS_0 samples was greater in the third cutting than the first in both 2015 and 2016, but the third cutting only differed from the second cutting in 2015 which was also greater than the first cutting (**Figure 5.2.; panel C**).

Unensiled WS_1 samples did not differ in ALA or ΣFA content between cuttings in 2015, although ALA content was higher in the third cutting than the second cutting of 2016, and ΣFA content was lower in the second cutting than either the first or third cuttings (**Figure 5.2.; panels A, B**). ALA proportion was greater in the second cutting

than the first cutting of 2015, however, unchanged throughout the three cuttings in 2016 (**Figure 5.2., panel C**).

WS_2 samples had greater contents of ALA and ΣFA, and a higher proportion of ALA in the third cutting of 2015 than both the first and second cuttings, and lower contents of ALA and ΣFA, and a lower proportion of ALA in third cutting of 2016 than the first cutting (**Figure 5.2.**).

5.4.3. Effect of wilting

Unensiled WS_1 samples were lower in ALA and ΣFA content and ALA proportion than WS_0 samples in the third cutting of 2015, and in all three cuttings of 2016 (**Figure 5.2.**). WS_2 samples were only statistically different from WS_0 and unensiled WS_1 samples in ALA and ΣFA content in the third cutting of 2016.

5.4.4. Ensiled and un-ensiled WS_1 samples

Comparing ensiled and unensiled WS_1 samples revealed an effect of ensiling, decreasing ALA content and proportion in the third cutting of 2015 and across all cuttings in 2016 (**Table 5.3., Figure 5.3.**). By limiting the dataset to WS_1 samples and adding ensiled samples, the number of total WS_1 samples (ensiled and unensiled) available for statistical analysis was concomitantly doubled. With this greater statistical power, time of mowing had an effect on ALA and ΣFA content in WS_1 samples in the first and third cuttings of 2015 (**Figure 5.4.**), and PM mown samples had a reduction in ALA proportion relative to AM mown samples across all cuttings of 2016, from 55.2 to 54.2 g $100g^{-1}$ ΣFA. In 2015, ΣFA content was consistent across the season in wide swath samples, but narrow swath samples were greater in the first cutting than the third (**Figure**

D.4.). The season by time of cutting by swath width interaction in 2015 (**Figure D.5.**) shows that AM mown wide swath samples were higher in ALA proportion than narrow swath and all PM samples in the first cutting.

5.5. Discussion

Results from 2015 and 2016 were not compared against each other statistically, however, the greater ALA and ΣFA content of WS_0 samples in 2016 is readily apparent (**Figure 5.2.**). This is possibly representative of I) a greater amount of labile ALA being preserved by the microwave pretreatment methodology of Goossen *et al*. (2018) being fully refined and employed only for the third cutting of 2015 and beyond, and II) impacts of higher applied N fertility in 2016.

5.5.1. Effect of season

While the impact of season on ALA and ΣFA content can be difficult to parse from its constituent/concomitant factors (*i.e*., forage maturity, day length, temperature, etc.) a meta-analysis by Glasser *et al*. (2013) showed a distinct trend among published studies of mid-season minima for ΣFA content and ALA proportion. The first and second cutting of the present study coincide with these minima, as well as the third cutting coinciding with the beginning of autumnal increases in ALA proportion and ΣFA content as reported by Glasser *et al*. (2013). While evolving sample preservation methodology in the present study may have accounted for some of the seasonal variation in 2015, the impact of methodology is likely more evident in the interactions involving season in 2015, that were not present in 2016.

5.5.2. Effect of wilting (wilt stage)

Unensiled WS_1 samples were lower in ALA and ΣFA content than in unwilted WS_0 samples in the third cutting of 2015 and all three cuttings of 2016, which coincides with our adoption of the small sample fresh weight microwave pretreatment preservation method for FA analysis described in Goossen *et al*. (2018). WS_2 samples were only statistically lower than both unensiled WS_1 and WS_0 samples in the third cutting of 2016. This is despite a visibly distinct downward trend in the least squares means of progressing wilt stages at all cuttings except WS_2 in the third cutting of 2015 (**Figure 5.2.**). The difference in WS_2 ALA and ΣFA content in the third cutting of 2016 may be due a culmination of several factors that contributed to a greater initial ALA and ΣFA content in unwilted WS_0 samples; I) a potentially greater supply of N fertility from the greater fertilizer application rate in 2016, II) a greater content of ALA in the late season (effect of season).

Earlier investigations into wilting losses of ALA and ΣFA in perennial ryegrass (*Lolium perenne* L.) showed reductions after extended wilting periods (Dewhurst and King, 1998; Dewhurst *et al*., 2002; Elgersma *et al*., 2003; Van Ranst *et al*., 2009a; Warren *et al*., 2002), and Khan *et al*. (2011) found the proportion of ALA decreased primarily during an initial wilting phase (up to ~ 45 g DM 100 g^{-1} fresh weight) and that ΣFA content did not continue to decrease in long-term controlled lab wilting beyond that point; however, field cured samples dried more quickly and were of a much greater DM content (67 g DM 100 g^{-1} fresh weight) when they reached a similar minimum of ΣFA content. This suggests that the field-cured samples may have reached a DM content at which lipolytic enzyme activity was greatly reduced while there was still labile FA

available to be lost when overnight re-wetting increased lipolytic activity, whereas lab-cured samples took longer to reach a critical DM content for reduced lipolytic activity and readily available pools of FA had already degraded. In the present study, DM contents similar to the potentially critical ~ 45 g DM 100 g^{-1} fresh weight shown by Khan *et al*. (2011) were achieved between 2.25 and 5.5 hours for AM mown WS_1 samples, and between 16.25 and 19.5 hours for PM mown WS_1 samples, which likely explains the difference between AM and PM mowing shown in the comparison of ensiled and unensiled WS_1 samples (**Figure 5.4.**). The potential significance of this DM point is further corroborated by the findings of Van Ranst *et al*. (2009a), demonstrating that lipolytic enzyme activity is greatly reduced in clovers (*Trifolium* spp. L.) as they wilted to 40 - 50 g DM 100 g^{-1} fresh weight.

Similar studies of timothy (*Phleum pretense* L.) are less congruous than those of perennial ryegrass, as Boufaïed *et al.* (2003) and Lee *et al*. (2006) found a drop in ALA and ΣFA content in an initial wilt, but only marginal further reduction in extended drying to hay, while Shingfield *et al*. (2005) found little change with a 6 hour wilt, but reductions after extended curing to hay, and Arvidsson *et al*. (2009a) found no effect on ALA or ΣFA content when wilting to 33.6 or 35.0 g DM 100 g^{-1} fresh weight.

5.5.3. Effect of ensiling

ALA content and proportion of ΣFA in WS_1 samples decreased with ensiling in the third cutting of 2015, and across all cuttings of 2016 (**Figure 5.3., Panels B and C**), again coinciding with adoption of improved sample preservation methodology. These ensiling decreases averaged 0.51 g kg^{-1} DM and 2.4 g 100 g^{-1} ΣFA in 2016 for ALA

content and proportion of ΣFA, respectively. It is difficult to directly compare these findings with published studies, as results have been mixed and many studies are comparing non-wilted fresh samples with wilted ensiled samples; therefore, the impact of ensiling is confounded with the impact of wilting. The meta-analysis of Glasser *et al*. (2013) found average reductions of ALA in silage samples that were very similar to what we have reported here for WS_1 samples, however, the results of those studies included effects of wilting in addition to ensiling. Of the studies that sampled both after wilting and again after ensiling, Arvidsson *et al*. (2009a) and Dewhurst and King (1998) found no effect of ensiling on ΣFA content or ALA proportion, while Boufaïed *et al*. (2003) found increases in both ΣFA and ALA content. Of studies that compared unwilted forage before and after ensiling, Alves *et al*. (2011) and Boufaïed *et al*. (2003) both reported increases in ΣFA content, though only the latter found an increase in ALA content, while Liu *et al*. (2018) reported a decrease in ALA proportion, however, with no change in ΣFA content. In studies comparing fresh forage with silages made from wilted material Vanhatalo *et al*. (2007) reported mixed results for ΣPUFA proportion – decreasing in grass and mature clover silages but increasing in young clover silages – otherwise, reductions in ALA proportion were universal: Chow *et al*. (2004) and Van Ranst *et al*. (2009a) reported increases in ΣFA content, Whiting *et al*. (2004) reported decreases in ALA and ΣFA content, and Ding *et al*. (2013) and Elgersma *et al*. (2003) found decreases in both ALA proportion and ΣFA content, though Ding *et al*. (2013) reported varying degrees of ensiling decreases in both ALA proportion and ΣFA content, pursuant to applied pre-ensiling treatments.

We found no effect of ensiling upon ΣFA content (**Figure 5.3., Panel A**), however, increases in ΣFA content are perhaps the most paradoxical and intriguing result of ensiling reported by several other studies (Alves *et al*., 2011; Boufaïed *et al*., 2003; Chow *et al*., 2004; Van Ranst *et al*., 2009a). Increases in ΣFA content are typically suggested to be the result of DM losses associated with ensiling, such as effluent loss or respiratory/fermentative losses, essentially concentrating the remaining DM components, including FA. In at least one example (Liu *et al*., 2018) DM content decreased 15.6 g DM 100 g^{-1} fresh weight, possibly off-setting the reported ALA decrease, as ΣFA content of the resulting silage was not different than the fresh forage it was made from. In our study, ensiled WS_1 samples had a lower DM content than unensiled WS_1 samples in 2015, but not in 2016, which may explain in part why ALA content was lower in all ensiled 2016 samples, but only in the third cutting of 2015. This would not, however, explain by itself the lack of effects on ΣFA content resultant from ensiling in both years, or the decreases in ALA proportion.

It was posited by Elgersma *et al*. (2003) that ensiling changes in FA composition may be resultant from endogenous plant lipolytic enzyme activity in addition to microbial lipolytic enzyme activity. The examination of alfalfa (*Medicago sativa* L.) silage by Ding *et al*. (2013) confirms that both endogenous plant enzymes and microbial actors reduce ALA content and proportion, and ΣFA content. If the two effectors can be assumed additive, endogenous plant enzymes were responsible for approximately 28 g 100 g^{-1} of the overall 40 g 100 g^{-1} ensiling reduction in ΣFA content found by Ding *et al*. (2013). The need for further research into relative rates of endogenous plant lipolytic activity

between subtribes and individual species is apparent when the lack of ensiling effects upon ΣFA content in reed canarygrass in this study are considered with the above results of Ding *et al*. (2013). Van Ranst *et al*. (2009a) found that white and red clover have 2 - 3 fold greater lipase activity *in silo* than perennial ryegrass, however, with methodological limitations to comparability between species, and no correlation between lipase activity and overall lipolysis. Even the inconclusive findings of relative lipolysis rates between clovers and perennial ryegrass at lower DM contents in Van Ranst *et al*. (2009b) suggest a need for further study. It may be that grasses have a reduced rate of endogenous plant lipolytic enzyme activity, relative to legumes, lowering ΣFA content. Further, some grass subtribes (*e.g.*, Phalaridinae, Phleinae) may have a reduced lipolytic enzyme activity relative to others (*e.g*., Loliinae). This could help to explain why treatment differences that affected wilt durations in the present study had minimal impacts upon ALA and ΣFA content, and why Arvidsson *et al*. found little effect of wilting upon the ALA and ΣFA content of timothy (2009a) and minimal treatment differences between different sample preservation methods, again with timothy (2009b).

5.5.4. Effect of time of mowing

In this study, the effect of time of mowing was expected to influence ALA and overall ΣFA content by providing longer wilting intervals for PM mown samples. This was suggested by higher ALA and ΣFA content in some AM mown samples in 2015 (**Figures 5.4., D.3. Panel A**) and in ALA proportion (**Figures D.3. Panel B, D.5.).** However, the time of mowing difference also seen in some WS_0 samples in 2015 (**Figure D.2.**) may be resultant of an underlying diurnal variation in ALA and concomitantly ΣFA

content on a DM basis. The same trend has been reported previously (Avondo *et al*., 2008; Doreau *et al*., 2007; Vibart *et al*., 2017), and is largely attributed to photosynthetic increases of nonstructural carbohydrates, and subsequently DM, throughout the day diluting FA content. However, Gregorini *et al*. (2008) reported no diurnal changes and an opposite diurnal effect has also been reported by Vasta *et al*. (2012) and Scollan *et al*. (2003) – however, the differences reported in Scollan *et al*. (2003) are likely a result of genetic differences more than diurnal effects. This opposite diurnal trend is perhaps best explained by the work of Browse *et al*. (1981) and corroborated by the work of Ekman *et al*. (2007), which displayed light-dependent synthesis of oleic acid, diluting the proportion of ALA as the photosynthetic day progressed, and light-independent desaturation activity overnight increasing ALA proportion and concomitantly decreasing oleic acid proportion, however, their examples may only be practically applicable to emerging leaves where FA synthesis is greatest (Hawke *et al*., 1974).

The inconsistency of the time of mowing effects seen in this study is exacerbated by the fact that the first and second cutting of 2016 had one hour longer wilting time between the PM harvest and the AM harvest, yet, the only time of mowing effect seen was in ALA proportion, and not ALA and ΣFA content as was sometimes shown in 2015. These differences may once again be resultant of improved sample preservation methodology in 2016, and subsequently reduced variability relative to the 2015 results. Furthermore, the analysis of simple main effects other than ensiling, in ensiled and un-ensiled WS_1 samples may arguably be considered as utilizing pseudo-replication of the

same split-split-plots. As such, great caution should be exercised in interpreting the time of mowing effect shown here.

5.5.5. Effect of swath width

In this study, there was no consistent effect of swath width upon ALA and ΣFA content and ALA proportion of ΣFA, however, drying times to wilt stage were similar between swath widths, and it has been recommended that full benefits of wide-swathing may only be realized with a swath width that is above 80% (Hay and Forage Grower, 2017) or even 90% of the mowing width for best effect (Cherney and Cherney 2006).

Time of mowing and swath width effects may have a greater impact on species with high lipolytic enzyme activity, longer wilting requirements, and if a forage crop's maximum ALA content potential has been reached through optimal N fertility. Additionally, wide swathing may be most impactful in late season harvests when ALA content is typically greater, and when prime wilting/drying time is in short supply (*i.e.*, shorter day length in late season, weaker sun angle). Conversely, time of mowing and swath width may have less impact in June, July and August when days are longer and initial ALA and ΣFA content may already be lower.

5.6. Conclusion

The swath widths investigated in this study had no consistent effect upon the FA content of conserved reed canarygrass. There may be potential to increase the ALA and ΣFA content of forages by mowing as early in the day as possible.

5.7. Acknowledgements

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5.9. Figures

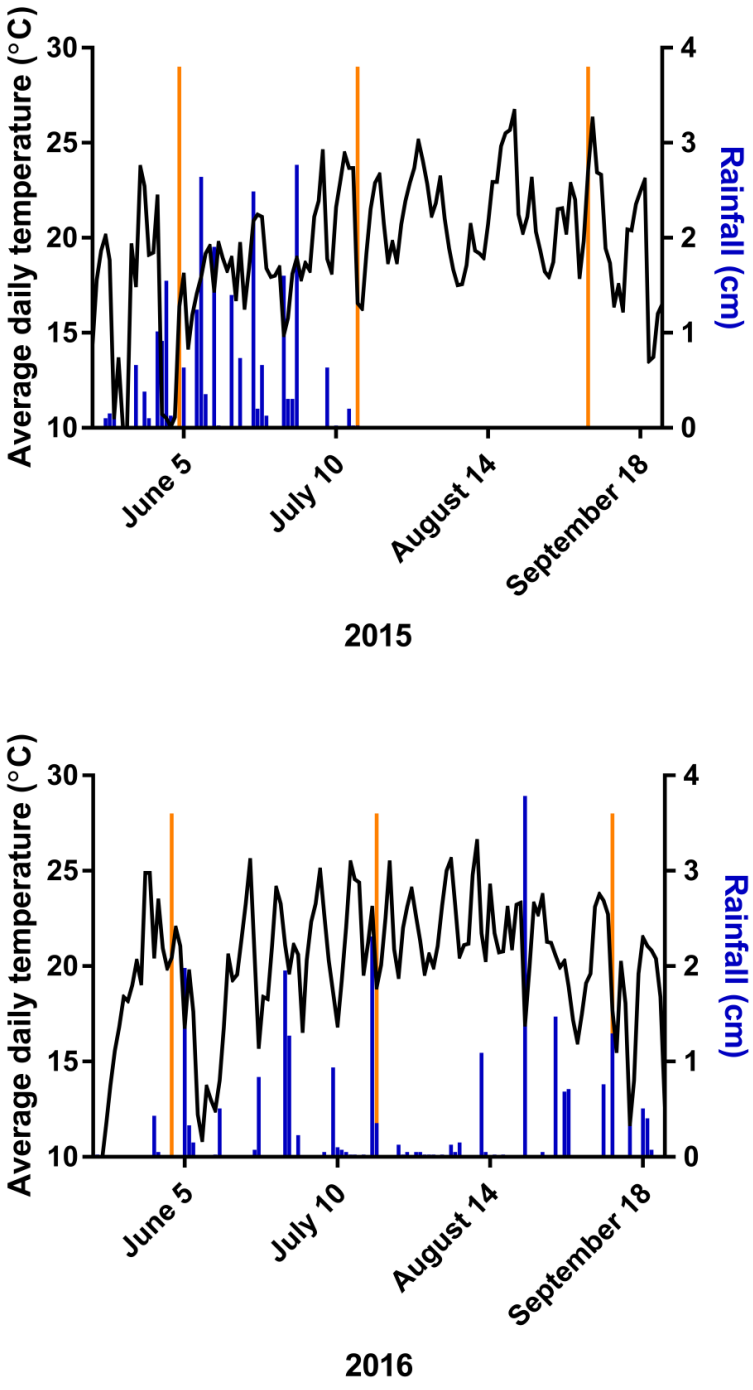

Figure 5.1. Average daily temperature (left Y axis, black line) and rainfall (right Y axis, blue line) at experiment site (Alburgh, VT) in 2015 and 2016. Orange lines indicate commencement of harvests.

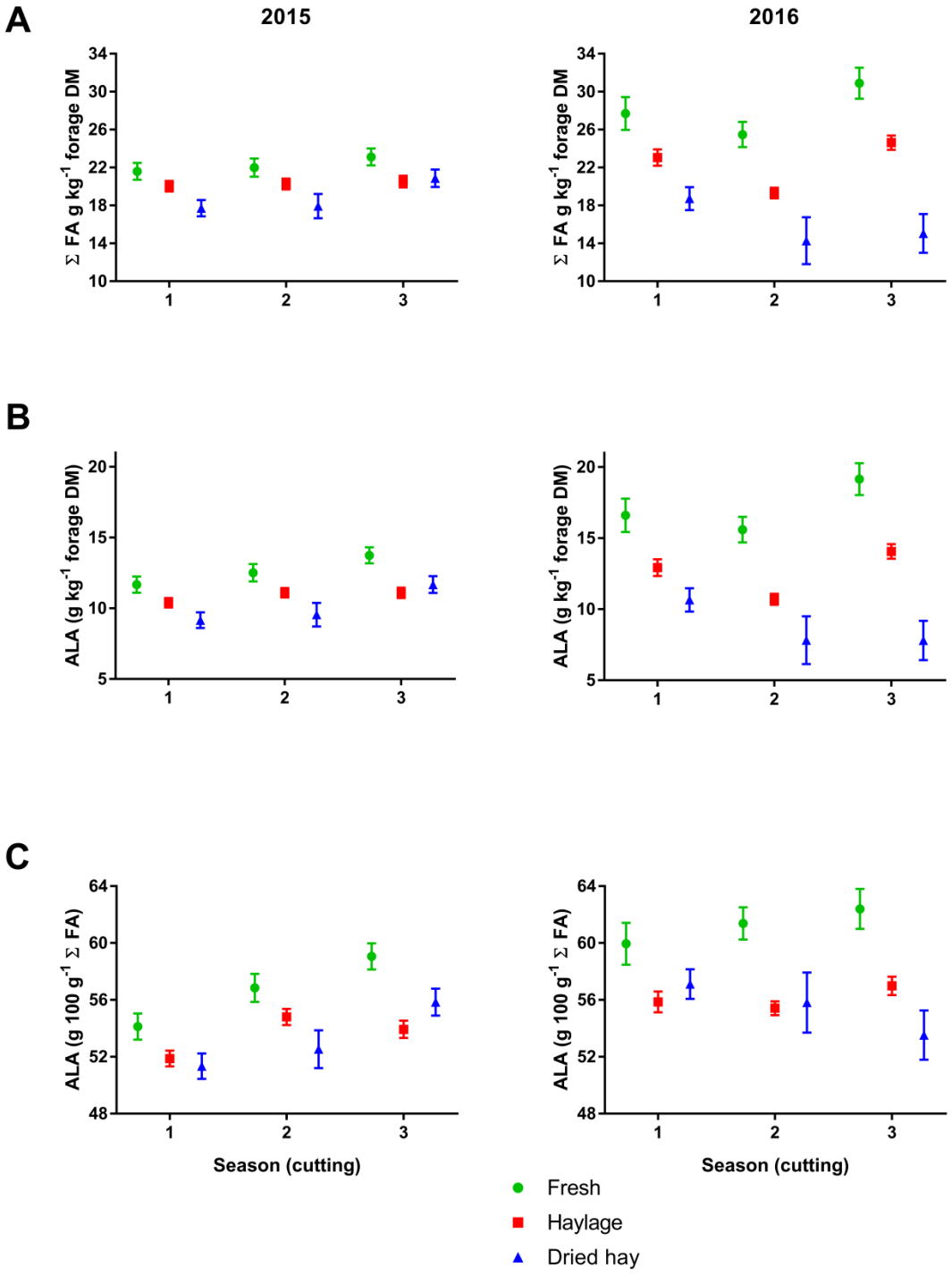

Figure 5.2. Least-squares means and standard error of means, of total fatty acid (ΣFA) content (panel A), alpha-linolenic acid (ALA) content (panel B), and ALA proportion (panel C) in unensiled WS0 (green circles), WS1 (red squares) and WS2 (blue triangles) reed canarygrass samples in 2015 and 2016.

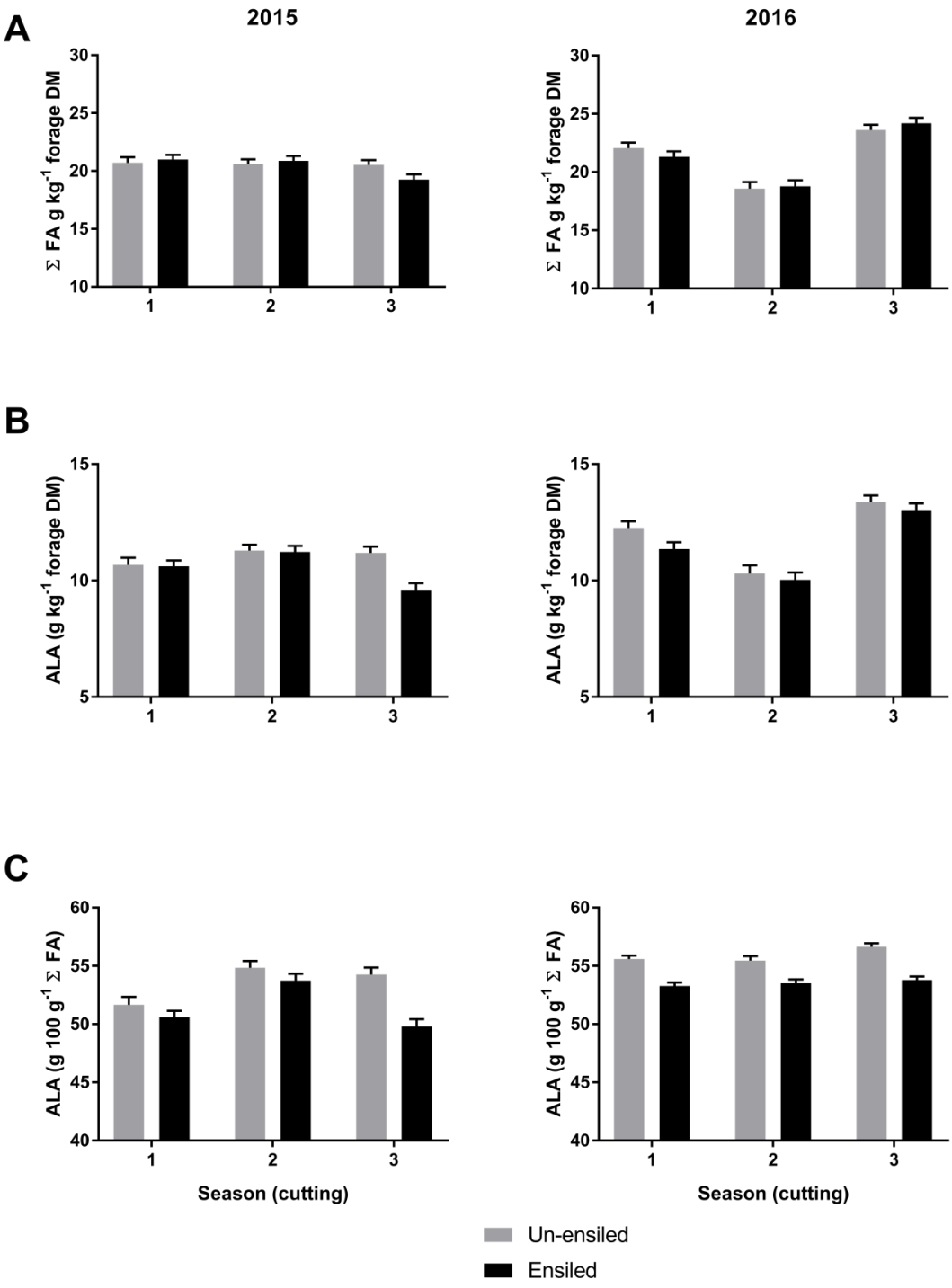

Figure 5.3. Least-squares means and standard error of means, of total fatty acid (ΣFA) content (panel A), alpha-linolenic acid (ALA) content (panel B), and ALA proportion (panel C) in unensiled WS1 (grey bars) and ensiled WS1 (black bars) samples of reed canarygrass in 2015 and 2016.

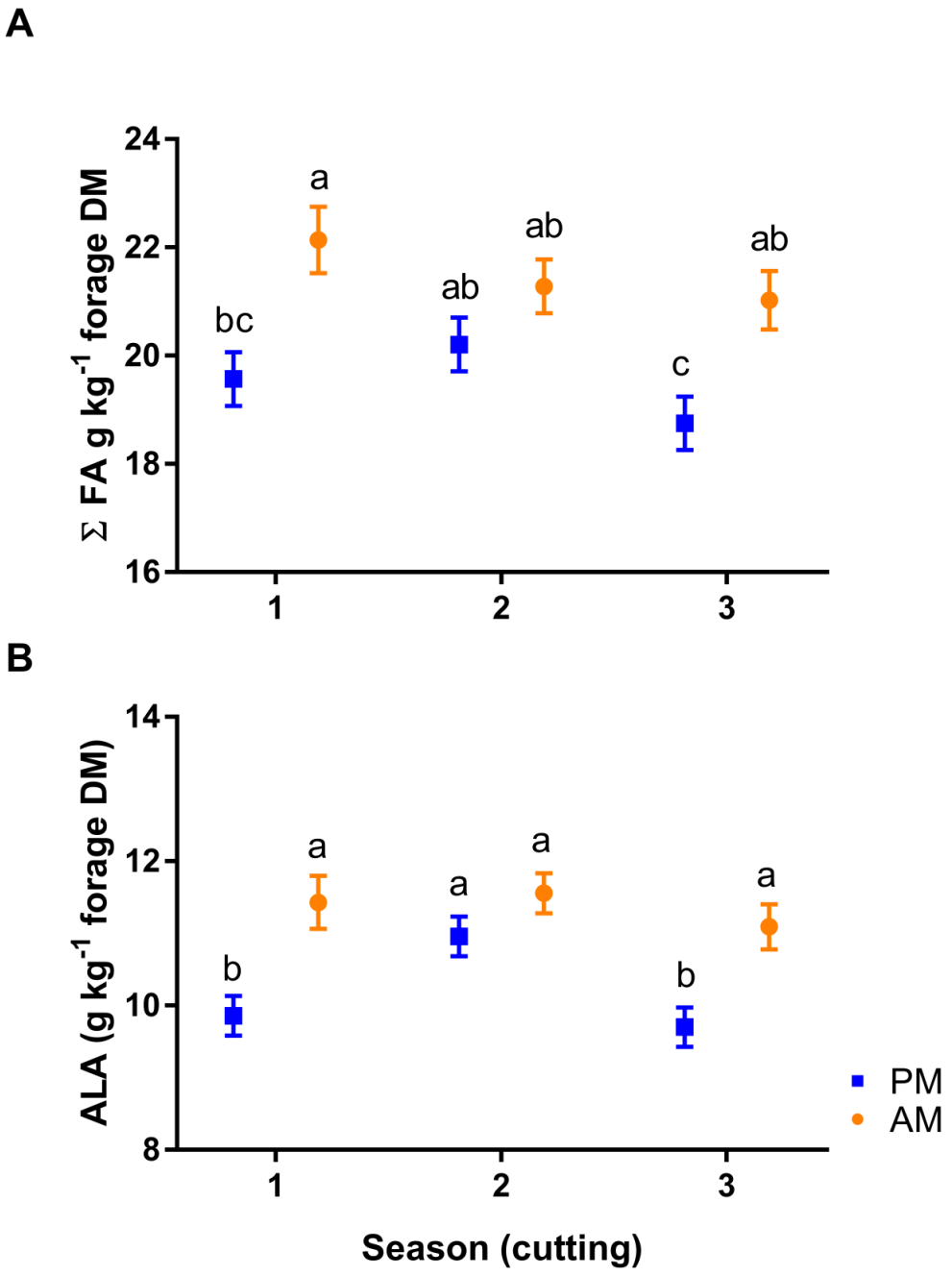

Figure 5.4. Least-squares means and standard error of means, of total fatty acid (ΣFA) content (panel A), and alpha-linolenic acid (ALA) content (panel B) across unensiled and ensiled WS1 samples from PM mown (blue square) and AM mown (orange circle) treatments of reed canarygrass in 2015. Least squares means without a common letter differ significantly; P < 0.05 (Tukey's HSD).

Table 5.1. Harvest dates and sampling times.

2015	1st Cutting				2nd Cutting				3rd Cutting			
	Date	Hour	Hrs wilt	DM[a]	Date	Hour	Hrs wilt	DM	Date	Hour	Hrs wilt	DM
PM mowing	6/4/2015	19:45		27	7/15/2015	20:00		26	9/6/2015	17:45		27.6
Sunset		20:35				20:37				19:22		
Sunrise	6/5/2015	5:08			7/16/2015	5:22			9/7/2015	6:23		
AM mowing		11:30		27.8		10:45		26.5		10:30		27.6
PM wide silage				43.1		13:00	17.00	41.2		11:15	17.50	42.6
AM wide silage				47.1		14:45	4.00	48.2		12:45	2.25	41.3
PM narrow silage				43.3		14:45	18.75	44.0		13:00	19.25	41.2
AM narrow silage				57.3		15:30	5.50	43.2		13:00	3.00	36.7
PM wide hay				56.0	7/17/2015	16:00	44.00	80.3		16:15	22.50	69.1
AM wide hay	6/7/2015			64.5		16:00	29.25	79.7		16:00	6.00	68.2
PM narrow hay				61.9		16:00	44.00	78.2		16:15	22.50	67.2
AM narrow hay				68.8		16:00	29.25	77.5		16:00	6.00	64.9

2016	1st Cutting				2nd Cutting				3rd Cutting			
	Date	Hour	Hrs wilt	DM	Date	Hour	Hrs wilt	DM	Date	Hour	Hrs wilt	DM
PM mowing	6/2/2016	19:00		21.4	7/19/2016	19:00		29.0	9/11/2016	18:30		22.1
Sunset		20:34				20:33				19:11		
Sunrise	6/3/2016	5:09			7/20/2016	5:26			9/12/2016	6:30		
AM mowing		9:00		23.6		9:45		28.4		11:00		25.7
PM wide silage		11:15	16.25	33.8		12:15	17.25	43.2		13:30	19.00	39.7
AM wide silage		11:30	2.50	32.5		12:30	2.75	39.7		13:45	2.75	37.3
PM narrow silage		13:15	18.25	38.3		13:45	18.75	54.7		14:00	19.50	42.3
AM narrow silage		13:30	4.50	38.4		13:45	4.00	47.0		14:00	3.00	34.0
PM wide hay		15:45	20.75	63.9		17:00	22.00	72.2	9/13/2016	12:30	42.00	73.3
AM wide hay		16:00	7.00	62.2		17:00	7.25	70.5		12:30	25.50	72.3
PM narrow hay		17:30	22.50	57.6	7/21/2016	16:00	45.00	85.4		12:45	42.25	72.4
AM narrow hay		17:45	8.75	56.5		15:30	29.75	84.6		12:45	25.75	69.9

[a]DM = dry matter (g DM 100 g^{-1} fresh weight)

Table 5.2. Effect of season, time of harvest, swath width, wilting stage and their interactions (up to three terms) on total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, and ALA proportion in unwilted WS0, unensiled WS1 and WS2 samples of reed canarygrass.

2015	ΣFA (g kg^{-1} forage DM[a])		ALA (g kg^{-1} forage DM)		ALA (g 100 g^{-1} ΣFA)	
Effect	**F-Value**	***P*-value**	**F-Value**	***P*-value**	**F-Value**	***P*-value**
Dry matter content	2.0	*ns*[b]	2.2	*ns*	0.9	*ns*
Season (cutting)	12.7	<.0001	27.1	<.0001	49.3	<.0001
Time of mowing (am vs pm)	6.3	*ns*	7.6	*ns*	2.4	*ns*
Season*time of mowing	1.6	*ns*	2.4	*ns*	4.0	0.0212
Swath width (40% vs. 70%)	0.0	*ns*	0.0	*ns*	0.1	*ns*
Season*swath width	4.7	0.0119	3.2	0.047	1.5	*ns*
Time of mowing*swath	2.5	*ns*	1.9	*ns*	0.3	*ns*
Season*time*swath	1.4	*ns*	3.3	0.0416	6.9	0.0016
Wilt stage (silage vs. hay)	4.2	0.0182	9.9	0.0001	16.5	<.0001
Season*wilt stage	3.3	0.0141	4.4	0.0028	6.4	0.0001
Time of mowing*wilt stage	1.1	*ns*	0.5	*ns*	0.8	*ns*
Season*time*wilt	1.0	*ns*	1.2	*ns*	0.7	*ns*
Swath*wilt stage	0.2	*ns*	0.6	*ns*	2.1	*ns*
Season*swath*wilt stage	0.2	*ns*	0.1	*ns*	0.3	*ns*
Time*swath*wilt stage	4.4	0.0156	4.9	0.0095	1.6	*ns*
2016	**ΣFA (g kg^{-1} forage DM)**		**ALA (g kg^{-1} forage DM)**		**ALA (g 100 g^{-1} ΣFA)**	
	F-Value	***P*-value**	**F-Value**	***P*-value**	**F-Value**	***P*-value**
Dry matter content	4.5	0.0353	3.7	*ns*	0.1	*ns*
Season (cutting)	21.4	<.0001	16.9	<.0001	0.0	*ns*
Time of mowing (am vs pm)	1.6	*ns*	0.9	*ns*	0.2	*ns*
Season*time of mowing	0.3	*ns*	0.2	*ns*	0.1	*ns*
Swath width (40% vs. 70%)	0.0	*ns*	0.0	*ns*	0.2	*ns*
Season*swath width	2.0	*ns*	1.9	*ns*	1.3	*ns*
Time of mowing*swath	0.7	*ns*	0.6	*ns*	0.1	*ns*
Season*time*swath	0.8	*ns*	1.1	*ns*	0.7	*ns*
Wilt stage (silage vs. hay)	16.7	<.0001	31.1	<.0001	54.4	<.0001
Season*wilt stage	10.3	<.0001	13.3	<.0001	10.1	<.0001
Time of mowing*wilt stage	0.7	*ns*	0.4	*ns*	0.5	*ns*
Season*time*wilt	0.5	*ns*	0.6	*ns*	0.5	*ns*
Swath*wilt stage	0.9	*ns*	0.6	*ns*	0.1	*ns*
Season*swath*wilt stage	0.8	*ns*	0.7	*ns*	0.4	*ns*
Time*swath*wilt stage	0.1	*ns*	0.1	*ns*	0.1	*ns*

[a]DM = dry matter

[b]*ns* = non-significant

Table 5.3. Effect of season, time of harvest, swath width, ensiling and their interactions (up to three terms) on total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, and ALA proportion in ensiled and unensiled WS1 samples of reed canarygrass, controlling for dry matter content.

2015	ΣFA (g kg^{-1} forage DM[a])		ALA (g kg^{-1} forage DM)		ALA (g 100 g^{-1} ΣFA)	
Effect	**F-Value**	***P*-value**	**F-Value**	***P*-value**	**F-Value**	***P*-value**
Dry matter content	8.0	0.0062	3.1	*ns*	0.3	*ns*
Season (cutting)	3.5	0.0349	9.4	0.0003	27.5	<.0001
Time of mowing (am vs pm)	10.3	0.0158	19.1	0.0137	1.8	*ns*
Season*time of mowing	3.4	0.0410	3.3	0.0417	1.0	*ns*
Swath width (40% vs. 70%)	0.1	*ns*[b]	0.7	*ns*	2.2	*ns*
Season*swath width	4.6	0.0135	1.4	*ns*	1.8	*ns*
Time of mowing*swath	1.5	*ns*	2.3	*ns*	2.7	*ns*
Season*time*swath	0.1	*ns*	1.2	*ns*	3.5	0.0373
Ensiling	0.7	*ns*	9.0	0.0037	30.4	<.0001
Season*ensiling	4.1	0.0218	9.2	0.0003	9.5	0.0002
Time of mowing*ensiling	0.2	*ns*	0.0	*ns*	0.3	*ns*
Season*time*ensiling	0.6	*ns*	0.2	*ns*	0.1	*ns*
Swath*ensiling	0.1	*ns*	0.6	*ns*	0.9	*ns*
Season*swath*ensiling	0.7	*ns*	0.9	*ns*	0.2	*ns*
Time*swath*ensiling	0.6	*ns*	1.0	*ns*	0.5	*ns*
2016	**ΣFA (g kg^{-1} forage DM)**		**ALA (g kg^{-1} forage DM)**		**ALA (g 100 g^{-1} ΣFA)**	
	F-Value	***P*-value**	**F-Value**	***P*-value**	**F-Value**	***P*-value**
Dry matter content	4.5	0.0380	2.8	*ns*	0.9	*ns*
Season (cutting)	55.4	<.0001	53.1	<.0001	5.7	0.0050
Time of mowing (am vs pm)	3.9	*ns*	0.7	*ns*	6.0	0.0326
Season*time of mowing	0.4	*ns*	0.4	*ns*	0.8	*ns*
Swath width (40% vs. 70%)	1.2	*ns*	0.5	*ns*	1.1	*ns*
Season*swath width	0.8	*ns*	0.4	*ns*	1.2	*ns*
Time of mowing*swath	0.7	*ns*	0.4	*ns*	0.1	*ns*
Season*time*swath	1.9	*ns*	1.9	*ns*	2.7	*ns*
Ensiling	0.0	*ns*	8.2	0.0055	153.3	<.0001
Season*ensiling	1.8	*ns*	1.3	*ns*	1.9	*ns*
Time of mowing*ensiling	0.1	*ns*	0.1	*ns*	0.1	*ns*
Season*time*ensiling	0.1	*ns*	0.1	*ns*	0.3	*ns*
Swath*ensiling	0.3	*ns*	0.7	*ns*	1.3	*ns*
Season*swath*ensiling	0.0	*ns*	0.1	*ns*	1.6	*ns*
Time*swath*ensiling	0.1	*ns*	0.2	*ns*	0.1	*ns*

[a]DM = dry matter

[b]*ns* = non-significant

CHAPTER 6: SUMMARY, LIMITATIONS, AND CONCLUSIONS

6.1. Research summary

The research presented in this dissertation contributes to a limited scientific body of knowledge regarding the fatty acid (FA) content of forage crops, and emphasizes modes of inquiry and methodologies that improve the ease and accuracy of future scientific investigations (**Figure 6.1.**).

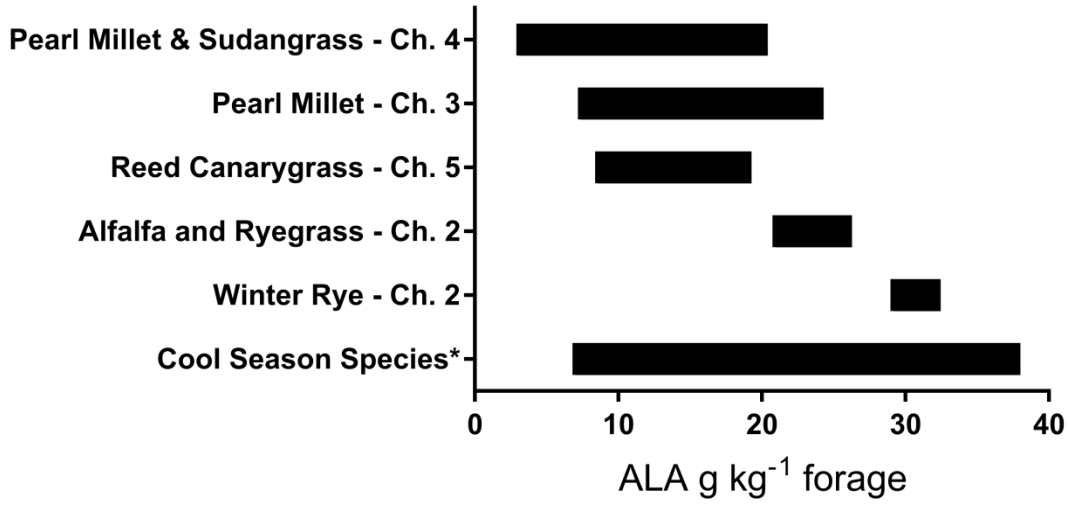

Figure 6.1. Comparison of forage ALA content ranges from this dissertation and from published studies of cool season species. *Adapted from Clapham et al. (2005) and Dierking et al. (2010).

Chapters 3 and 4 present FA content data for representatives of two genera of annual warm season forage grasses, of which only pearl millet has been described previously, in only one growth condition (Bainbridge *et al*., 2017). These studies have shown the impact of advancing maturity in these species, and highlighted the role which a decreasing lamina mass ratio (LMR; lamina DM / lamina + pseudostem DM) plays in associated alpha-linolenic acid (ALA) and ΣFA content declines. Additionally, the role in

which nitrogen (N) fertility may ameliorate some of the FA losses associated with decreasing LMR was investigated in chapter 4.

The comparison of individual plant fractions in chapter 3 (*i.e.*, laminae and pseudostems) moves away from more customary whole-plant analysis. This highlights the difference between the FA of these two fractions, and the manner in which their ratio affects overall whole-plant FA content and composition, which will be important in future considerations of these species, and others with a similarly tall growing architecture.

Evaluating the impacts of several forage conservation practices helps to inform production. The results of chapter 5 suggest that impacts of two easily approached practices (*i.e.*, swath width, and time of harvest) are relatively minimal on the final FA content and composition, at least when optimal drying conditions are present.

The examination of forage sample preservation methodologies in chapter 2 reinforces that what might otherwise be a typical agronomic research practice (*i.e.*, forced hot air drying of relatively large fresh weight samples) should not be considered consistently accurate or dependable enough for FA analysis. A simple and inexpensive alternative preservation strategy was presented that appears to work as well as lyophilization. Additionally, oxidative losses of ALA in ground dried forage samples were shown to be a valid concern when considering long term storage, as losses could be greater than 2 percentage points of ΣFA after 72 weeks, though preferred preservation methods (*i.e.*, lyophilization or microwave pretreatment) had lesser ALA losses.

6.2. Research limitations

The examination of maturity and N fertility effects upon the FA content of sudangrass and pearl millet in chapter 4 took place before the sample preservation method study in chapter 2. Though samples were preserved in accordance with a published method that purported to be "as good or better" than freeze drying (Arvidsson *et al*., 2009b), the slow to dry nature of the species that we investigated may have underestimated the true ALA and ΣFA content at harvest. While the relative differences between treatments that we identified are likely indicative of actual treatment differences, we may have lost signal definition that could have otherwise aided clear interpretation of our data. An unavoidable limitation in chapter 4 was that regrowth harvests could not begin at the same date for both plant maturity treatments, as different first growth sampling dates were inherent to the treatments. Additionally, unusually heavy rains impaired normal plant growth to the point of severe chlorosis in the first growth harvest of 2013 at the South Burlington location with extremely sandy soil.

The sample preservation method study (chapter 2) had an evolving methodology as it progressed, which unfortunately did not allow for a direct comparison of first growth and regrowth harvests. Additionally, limitations of funding and time practicalities restricted the possibilities of comparing further combinations of species, microwave pretreatment durations, and countless other variables (*e.g.*, vegetative maturity stage, drying temperature, air flow). Another limitation inherent to re-sampling the same bagged sample for the oxidation loss investigation, was introduction of fresh air at each sampling time point. The results, however, do not appear to indicate a greater impact

from a higher rate of sampling than from time elapsed, as later time points were fewer and further between.

In the examination of forage conservation practices in chapter 5, harvest date selection was pursued in accordance with standard agricultural practice, and therefore optimal drying conditions were sought out. Wide swathing may hasten primary drying enough to better preserve ALA and ΣFA content than narrow swathing in situations with less optimal drying conditions (*e.g.*, cloudier days, higher humidity, intermittent rain). Additionally, a wide swath that represents a greater percentage of mower width may be more effective. A mixed hayfield was selected for the study, however, stand composition was primarily reed canarygrass. Differences in wilting time may be more readily apparent in stands dominated by other species (*e.g.*, alfalfa), that may take longer to dry, or possibly have greater lipolytic enzyme activity.

6.3. Future perspectives

Warm season summer annuals are often planted for supplemental grazing in mid-summer months. They are also utilized as an emergency planting for producing silage when maize plantings have failed. Considering their natural desiccation resistance, warm season annual species may require a long wilting duration, and subsequently be susceptible to significant wilting losses of ALA and ΣFA content. As such, research into harvest strategies for their conservation is likely warranted. Swath width may have a greater effect on the FA content and composition of wilted warm season grass silages.

A future avenue of FA research may be the description of the FA content and composition of brassica forage varieties. The majority of forage FA research is performed

in grass and legume species, and as such, the FA standards used to identify chromatographic peaks may not include hexadecatrienoic acid. If significant quantities of hexadecatrienoic acid can be found in a moderately high brassica diet, a further investigation into its fate in the rumen would be justified. Being highly unsaturated, hexadecatrienoic acid is likely bacteriostatic in the same manner that ALA is. Biohydrogenation of hexadecatrienoic acid by rumen bacteria would likely form biohydrogenation intermediate isomers that would end up in milk and meat, which may include 16 carbon analogues of the 18 carbon biohydrogenation intermediates RA and VA. Investigation into the effect of these isomers upon consumption by humans may be of great interest as such FA have likely rarely occurred in the human diet.

6.4. General conclusions

The research presented in this dissertation reaffirms the importance of forage maturity in dictating FA content and composition, while highlighting the role that the ratio of lamina and pseudostem plays in FA declines associated with advancing maturity. The positive impact of N fertility was also shown, confirming that increased chloroplast content can off-set FA losses that might otherwise be expected from a reduced lamina mass ratio. Both factors can be summarized as the maximization of ALA-rich thylakoid membrane on an overall forage DM basis. Warm season annual grasses were found to fall generally within ranges of ALA and ΣFA content of the better studied cool season species.

Forage conservation practices that involve wilting will likely always result in a reduction of ALA and ΣFA content, as the lipoxygenation of ALA is a major stress

response mechanism in plants. Simple management changes such as changing swath width or time of harvest seem to have limited potential for reducing those conservation losses of FA. This research has also contributed to an unsettled inquiry into small diurnal fluctuations of FA content, and reinforced observed autumnal increases in FA content.

The analysis of research sample preservation method shows ample evidence of the insufficient performance of forced hot air drying alone to preserve accurate forage FA content and composition. A simple and inexpensive microwave pretreatment before forced hot air drying was proposed as a new method for preserving forage samples for FA analysis. Additionally, ground dried samples were shown to decreased in ALA content.

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APPENDIX A

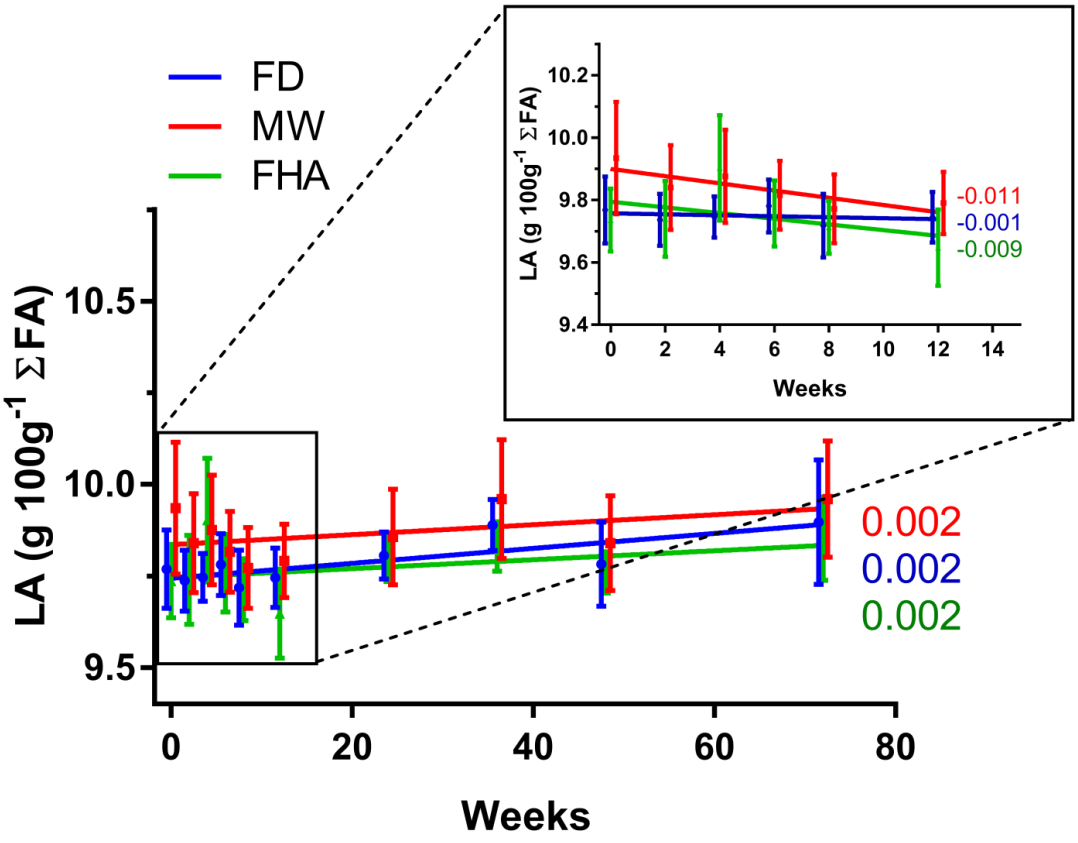

Figure A.1. Linoleic acid (LA) proportion of total fatty acids (ΣFA) over time (*n*=8).
Slope of linear regressions reported in corresponding color to right of regression line.
FD (blue) = Freeze-dried, MW (red) = microwave pre-treatment prior to forced hot air drying, FHA (green) = forced hot air drying alone.

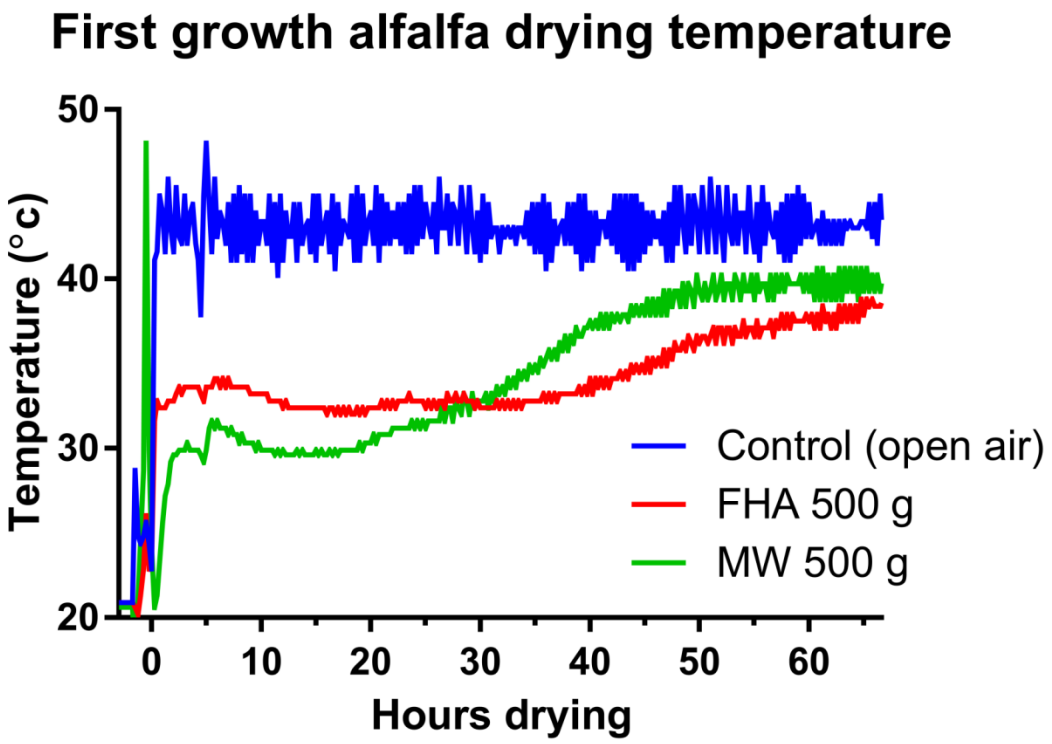

Figure A.2. Experiment 1 first growth sampling drying room temperature data from loggers: exposed (blue), in a large FHA sample bag (red), and in a large 1 min MW sample bag (green)

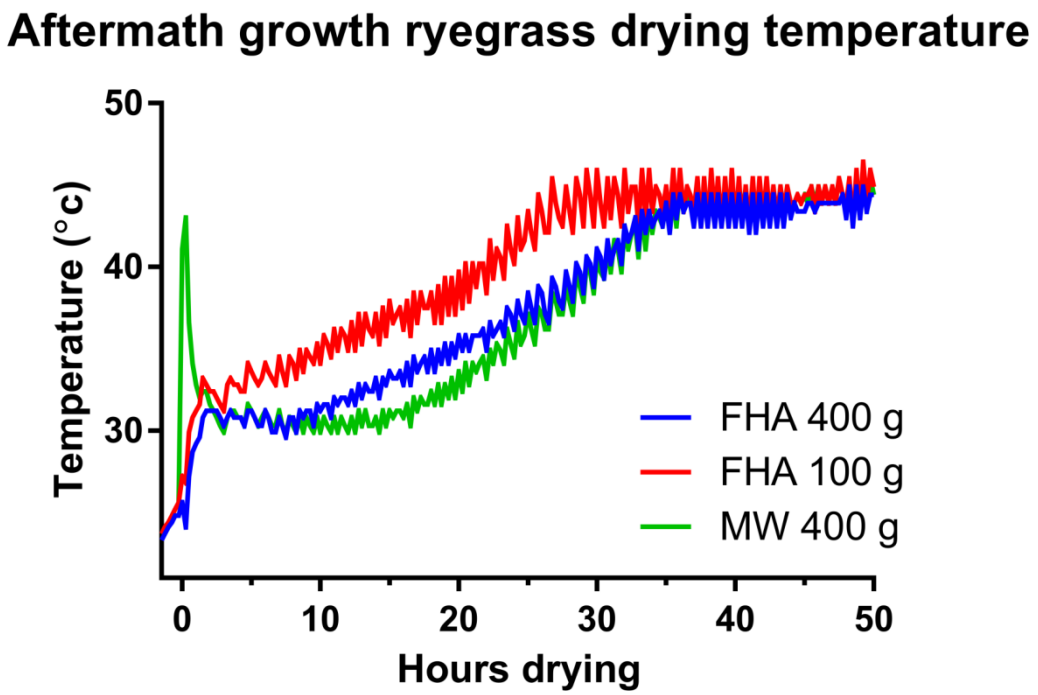

Figure A.3. Experiment 1 aftermath sampling drying room temperature data from data loggers: in a large FHA sample bag (blue), in a small FHA sample bag (red), and in a large 2 min MW sample bag (green)

APPENDIX B

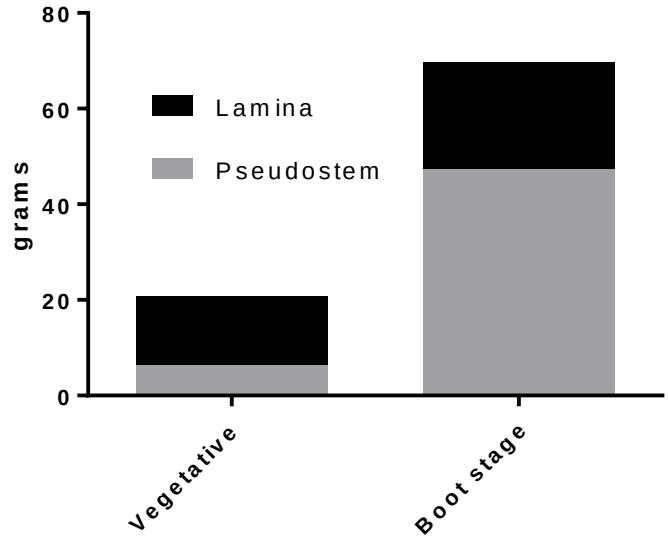

Figure B.1. Sample dry weight mean, by plant fraction of sorghum-sudangrass in Experiment 1

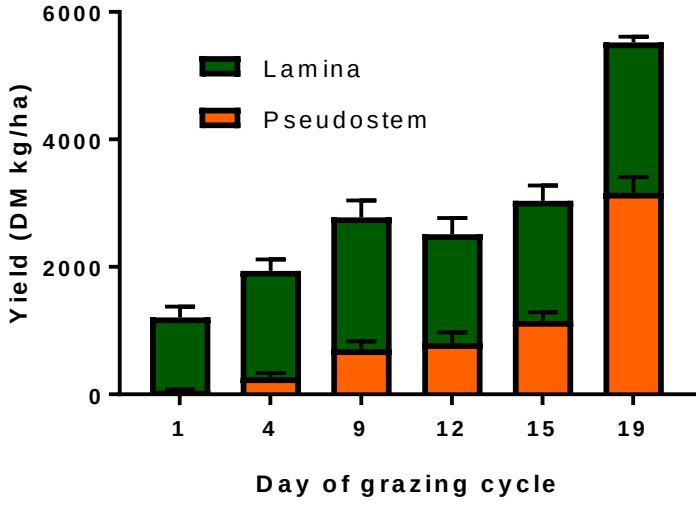

Figure B.2. Mean dry matter yield, by plant fraction, of pearl millet in Experiment 2

Table B.1. Pearl millet yield and nutritive quality

Day of grazing	Height (in)	Component	DM Yield (lb/acre)	Crude Protein (%)	NDF (%)	dNDF48 (%)	NDFD48 (%)	Sugars (%)
1 7/18/16	21	**Entire plant**	**1078**	**18.4**	**57.3**	**40.9**	**73.4**	**6.0**
		Leaf 95.5%	1025	18.8	57.0	40.7	73.5	5.9
		Stem 4.5%	53	8.0	63.5	44.6	71.7	7.8
4 7/21/16	29	**Entire plant**	**1727**	**16.4**	**56.9**	**39.3**	**72.1**	**7.5**
		Leaf 86.7%	1487	17.5	56.3	39.0	72.4	7.4
		Stem 13.3%	240	9.3	60.8	41.3	70.4	7.9
9 7/26/16	39	**Entire plant**	**2480**	**17.1**	**62.0**	**40.5**	**69.1**	**4.8**
		Leaf 75%	1849	19.0	60.1	40.1	70.1	4.7
		Stem 25%	631	11.4	67.5	41.9	66.1	5.0
12 7/29/16	42	**Entire plant**	**2240**	**14.7**	**64.1**	**41.8**	**68.7**	**4.3**
		Leaf 68.8%	1525	17.5	61.3	41.3	69.9	4.2
		Stem 31.2%	715	8.6	70.2	43.2	66.2	4.6
15 8/1/16	48	**Entire plant**	**2712**	**12.0**	**66.7**	**43.8**	**69.5**	**6.2**
		Leaf 62%	1684	15.0	63.4	42.5	69.5	5.8
		Stem 38%	1027	7.1	72.2	45.9	69.4	6.7
19 8/5/16	55	**Entire plant**	**4926**	**9.0**	**69.3**	**44.3**	**67.5**	**7.4**
		Leaf 43%	2106	14.4	64.5	42.1	68.2	5.8
		Stem 57%	2820	4.9	73.0	45.9	67.0	8.7

APPENDIX C

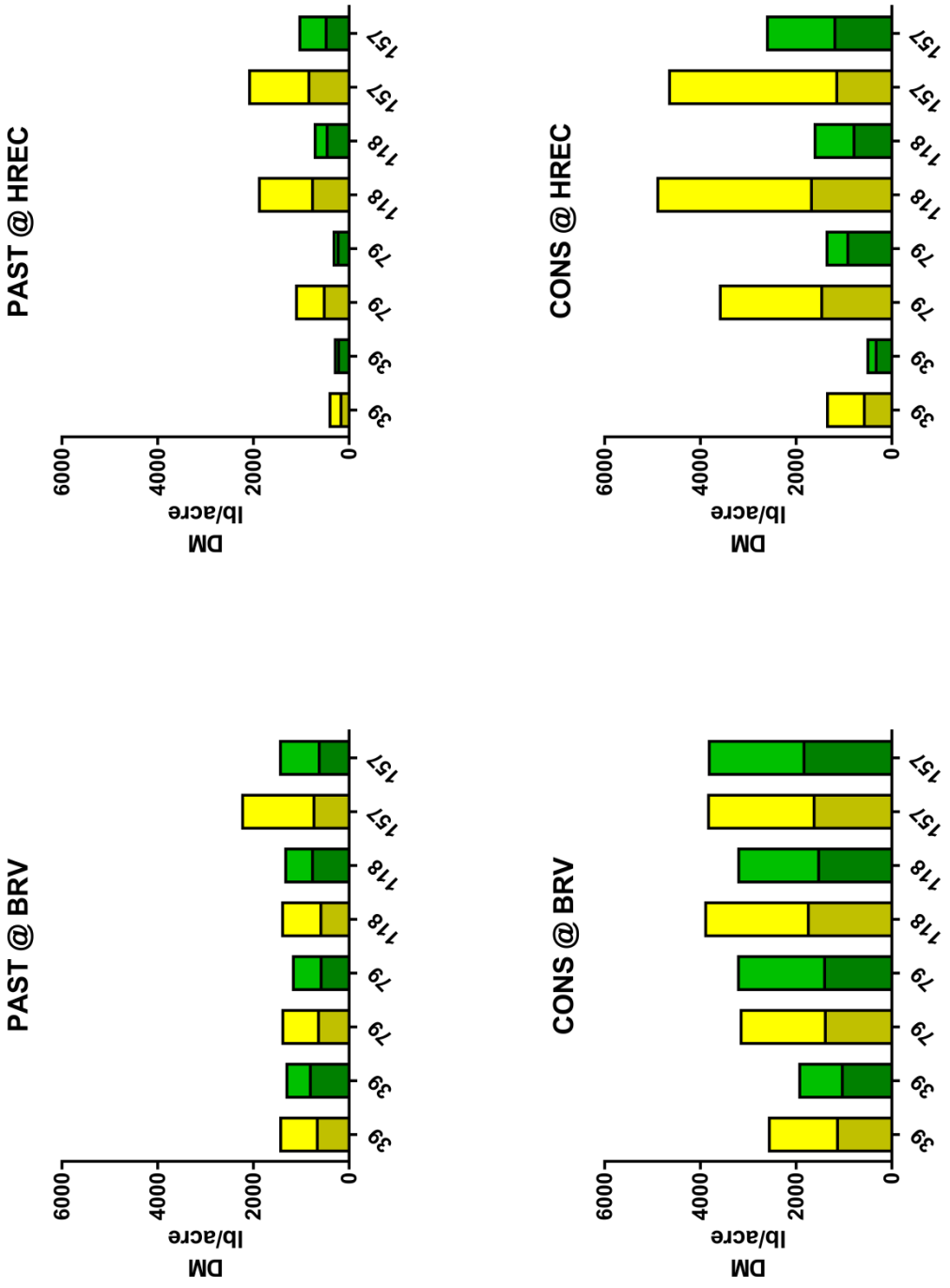

Figure C.1. Dry weight yields of pearl millet (yellow) and sudangrass (green) in the first (shaded) and second (unshaded) harvests of 2014.

Table C.1. Effects of plant maturity, species (Sp), nitrogen fertility (N), and their interactions on total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio in 2013 HREC regrowth samples.

Effect	ΣFA(g kg^{-1} forage DM[a])		ALA (g kg^{-1} forage DM)		ALA (g 100 g^{-1} ΣFA)		Lamina mass ratio	
	F-Value	*P*-value	F-Value	*P*-value	F-Value	*P*-value	F-Value	*P*-value
Maturity	44.16	0.0006	74.43	0.0001	106.84	<.0001	35.16	0.0010
Sp	73.96	<.0001	106.16	<.0001	121.51	<.0001	103.17	<.0001
Maturity*Sp	20.04	<.0001	48.37	<.0001	108.62	<.0001	5.68	0.0218
N	3.00	0.0412	2.44	0.0774	1.24	0.3074	33.05	<.0001
Maturity*N	0.20	0.8975	0.12	0.9506	0.53	0.6620	1.95	0.1368
Sp*N	0.63	0.6023	0.68	0.5673	1.47	0.2369	3.98	0.0139
Maturity*Sp*N	0.27	0.8449	0.49	0.6885	2.10	0.1148	1.77	0.1681

[a]DM = dry matter

Table C.2. Maturity stage least squares means of total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio (LMR), and their SEM[a] in 2013 HREC regrowth samples.

Maturity	Pasture		Conserved	
ΣFA (g kg^{-1} DM[b])	17.5 a	0.5	13.1 b	0.5
ALA (g kg^{-1} DM)	9.3 a	0.3	5.8 b	0.3
ALA (g 100 g^{-1} ΣFA)	51.7 a	0.5	44.0 b	0.5
LMR	0.84 a	0.01	0.73 b	0.01

Least squares means without a common letter differ significantly within a row; $P < 0.05$ (Tukey's HSD)

[a]Standard error of means

[b]DM = dry matter

Table C.3. Species least squares means of total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio (LMR), and their SEM[a] in 2013 HREC regrowth samples.

Species	Pearl Millet		Sudangrass	
ΣFA (g kg^{-1} DM[b])	12.6 b	0.5	18.0 a	0.5
ALA (g kg^{-1} DM)	5.7 b	0.3	9.4 a	0.3
ALA (g 100 g^{-1} ΣFA)	44.8 b	0.5	50.8 a	0.5
LMR	0.86 a	0.01	0.70 b	0.01

Least squares means without a common letter differ significantly within a row; $P < 0.05$ (Tukey's HSD)

[a]Standard error of means

[b]DM = dry matter

Table C.4. Maturity stage by species least squares means of total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio (LMR), and their SEM[a] in 2013 HREC regrowth samples.

Species	Pearl Millet				Sudangrass			
Maturity	**PAST**		**CONS**		**PAST**		**CONS**	
ΣFA (g kg^{-1} DM[b])	13.4 bc	0.6	11.8 c	0.6	21.6 a	0.6	14.4 b	0.6
ALA (g kg^{-1} DM)	6.2 b	0.4	5.2 b	0.4	12.5 a	0.4	6.4 b	0.4
ALA (g 100 g^{-1} ΣFA)	45.9 b	0.7	43.8 b	0.7	57.6 a	0.7	44.1 b	0.7
LMR	0.90 a	0.01	0.83 b	0.01	0.77 b	0.01	0.62 c	0.01

Least squares means without a common letter differ significantly within a row; $P < 0.05$ (Tukey's HSD)

[a]Standard error of means

[b]DM = dry matter

Table C.5. Nitrogen fertility effects on least squares means of total fatty acid (ΣFA) content, alpha-linolenic acid (ALA) content, ALA proportion, and lamina mass ratio (LMR), and their SEM[a] in 2013 HREC regrowth samples.

Nitrogen (kg ha^{-1})	39		79		118		157	
ΣFA (g kg^{-1} DM[b])	14.9 ab	0.6	14.3 b	0.6	15.1 ab	0.6	16.9 a	0.6
ALA (g kg^{-1} DM)	7.4	0.4	7.1	0.4	7.3	0.4	8.4	0.4
ALA (g 100 g^{-1} ΣFA)	48.5	0.6	47.6	0.6	47.1	0.6	48.2	0.6
LMR	0.92 a	0.01	0.78 b	0.01	0.72 bc	0.01	0.71 c	0.01

Least squares means without a common letter differ significantly within a row; $P < 0.05$ (Tukey's HSD)

[a]Standard error of means

[b]DM = dry matter

APPENDIX D

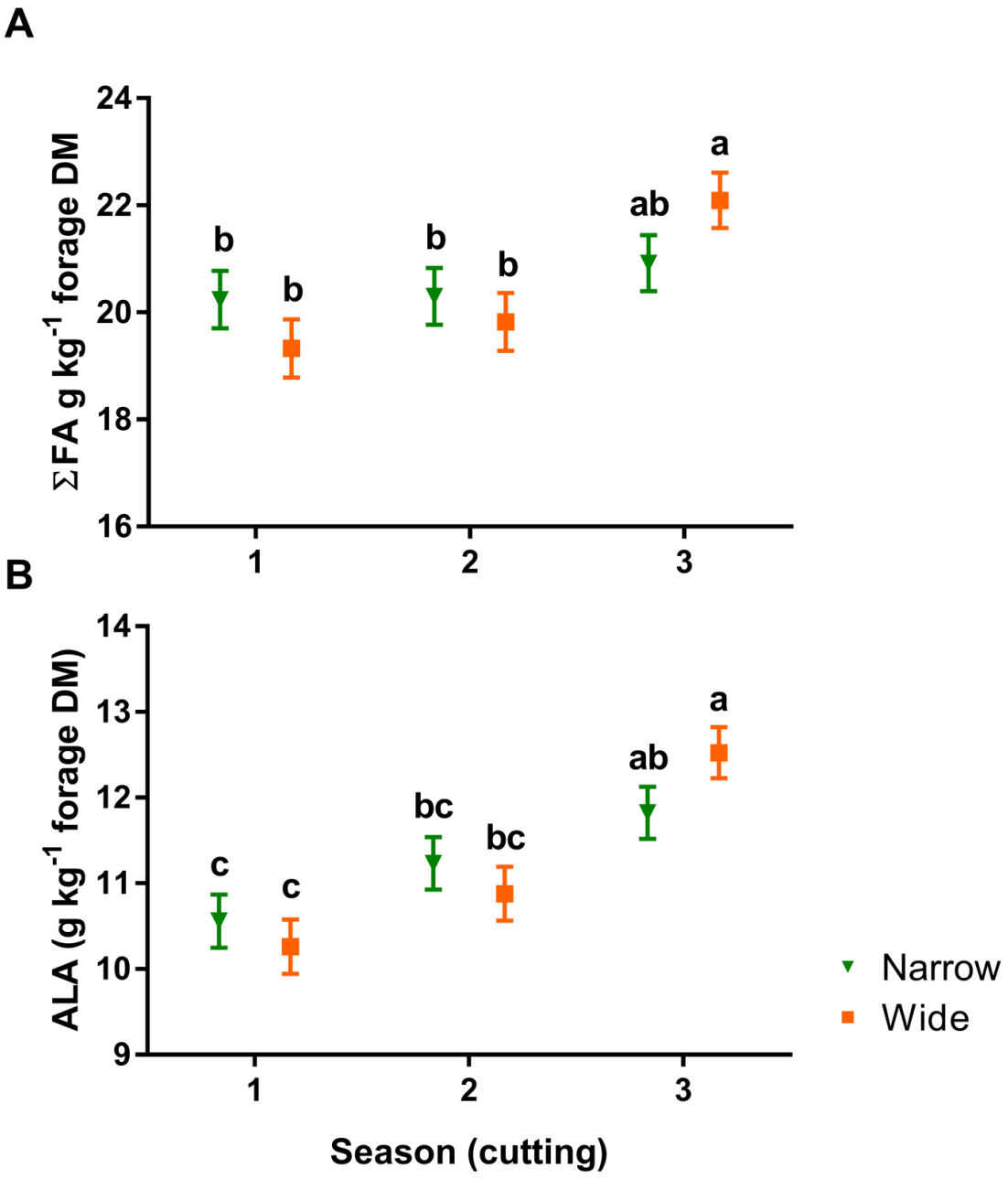

Figure D.1. Least-squares means and standard error of means, of total fatty acid (ΣFA) content (panel A), and alpha-linolenic acid (ALA) content (panel B) across unensiled WS0, WS1 and WS2 samples from narrow (green triangle) and wide(orange square) swath treatments of reed canarygrass in 2015. Least squares means without a common letter differ significantly; P < 0.05 (Tukey's HSD).

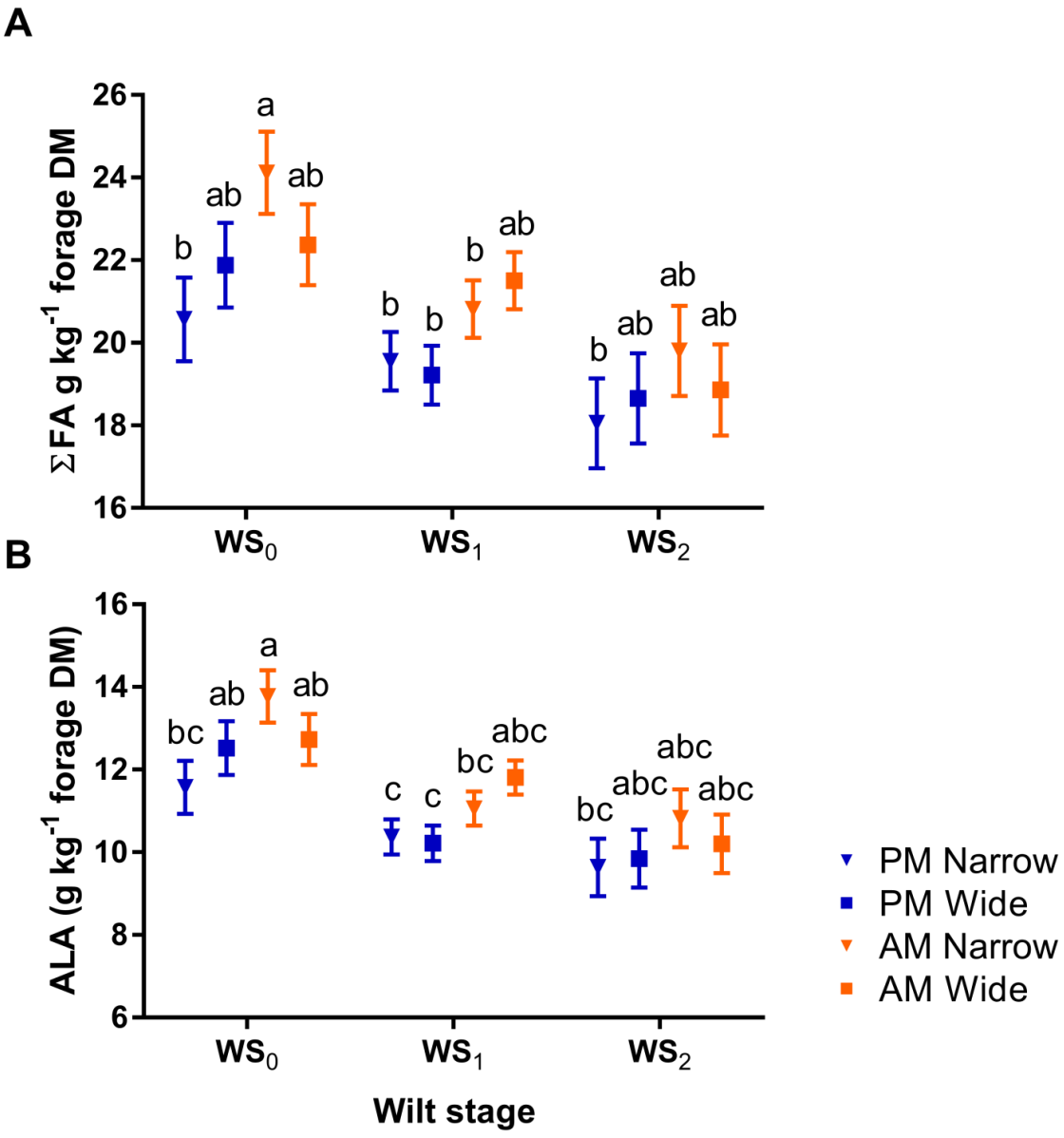

Figure D.2. Least-squares means and standard error of means, of total fatty acid (ΣFA) content (panel A), and alpha-linolenic acid (ALA) content (panel B) in unensiled WS0, WS1 and WS2 samples from narrow (triangle) and wide(square) swath treatments mown in the PM (blue) and the following AM (orange) across three cuttings of reed canarygrass in 2015. Least squares means without a common letter differ significantly; P < 0.05 (Tukey's HSD).

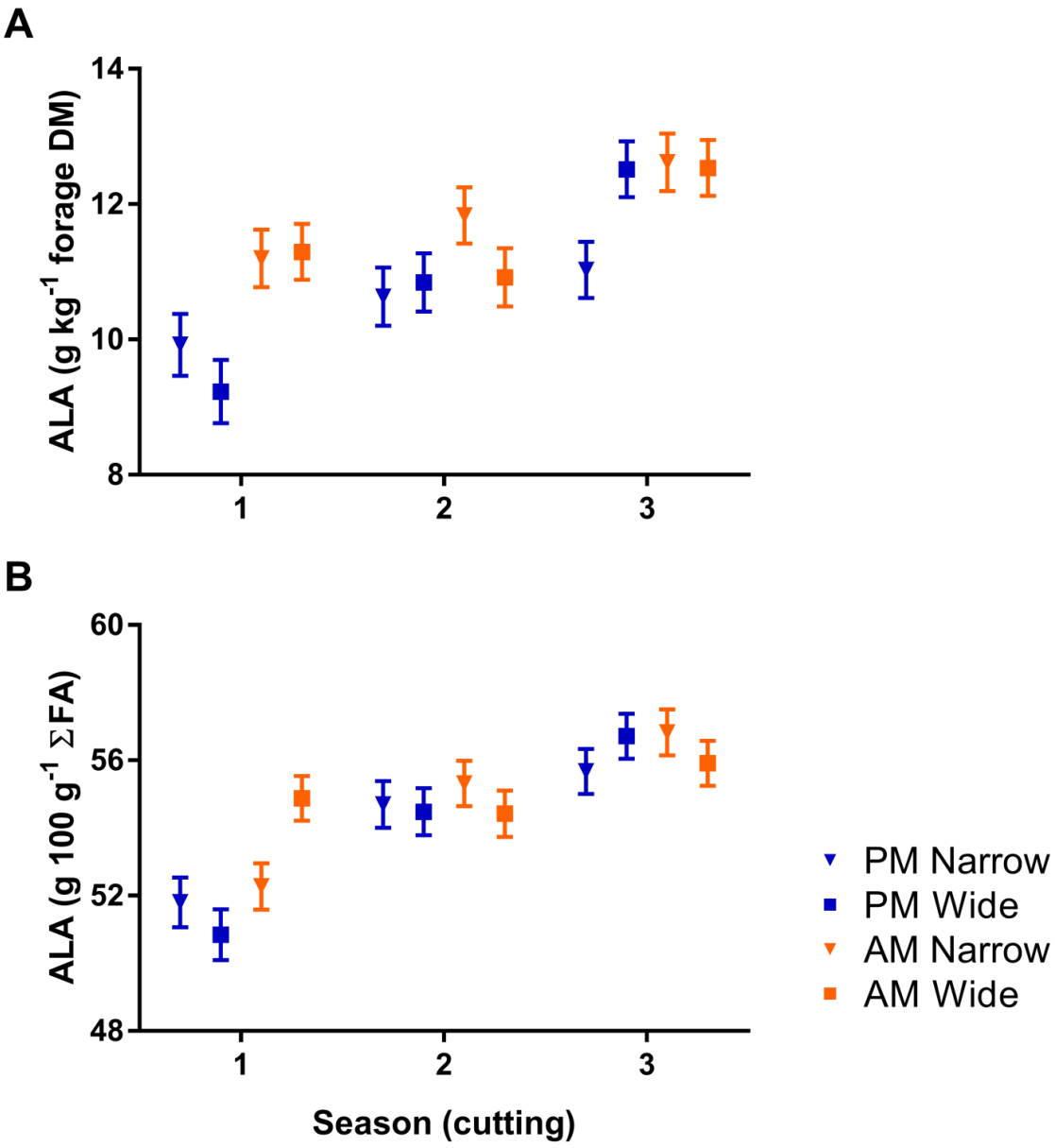

Figure D.3. Least-squares means and standard error of means, of alpha-linolenic acid (ALA) content (panel A), and ALA proportion (panel B) across unensiled WS0, WS1 and WS2 samples from narrow (triangle) and wide (square) swath treatments of reed canarygrass mown in the PM (blue) and following AM (orange) across three cuttings in 2015.

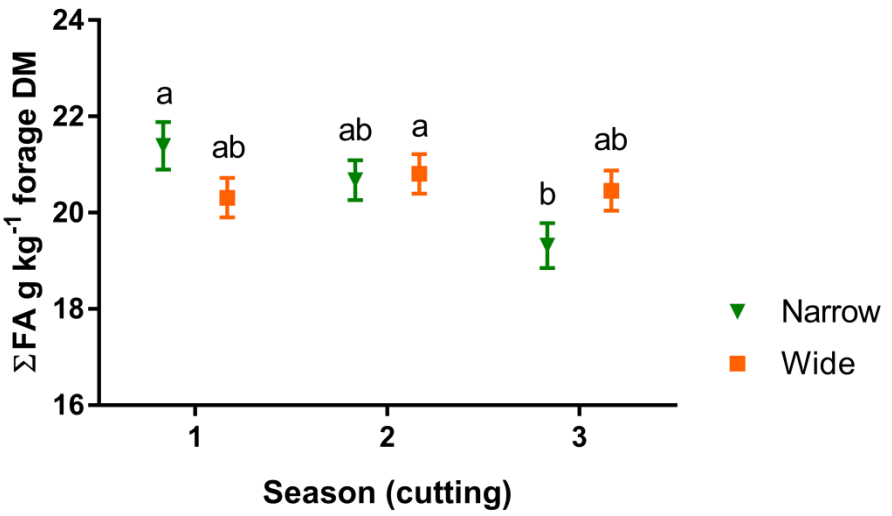

Figure D.4. Least-squares means and standard error of means, of total fatty acid (ΣFA) content across unensiled and ensiled WS1 samples from narrow (green triangle) and wide(orange square) swath treatments of reed canarygrass in 2015. Least squares means without a common letter differ significantly; P < 0.05 (Tukey's HSD).

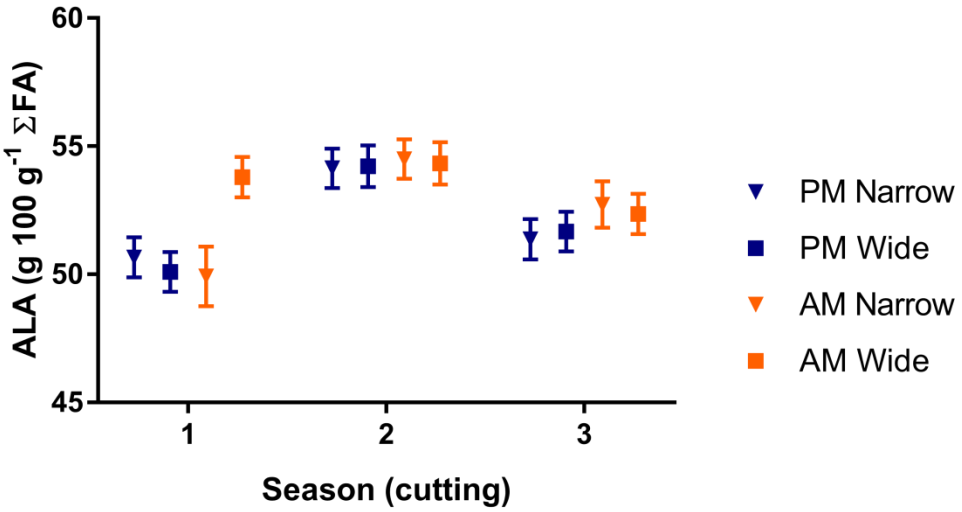

Figure D.5. Least-squares means and standard error of means, of alpha-linolenic acid (ALA) proportion across unensiled and ensiled WS1 samples from narrow (triangle) and wide (square) swath treatments mown in the PM (blue) and following AM (orange) across three cuttings of reed canarygrass in 2015.