The Effect of Maturity and Frost Killing of Forages on Degradation Kinetics and Escape Protein Concentration

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Summary

Two consecutive *in situ* studies were conducted to determine the effects of maturity and frost killing of forages (alfalfa and berseem clover) on degradation kinetics and escape protein concentrations. Four maturities (3, 5, 7, and 9 weeks after second harvest) of forages collected from three locations were used to determine the effects of maturity. Four weeks after a killing frost (-2° C), berseem clover was harvested from the same locations previously sampled. To evaluate maturity, 336 Dacron® bags containing all maturities of either alfalfa or berseem clover were placed into the rumen of two fistulated steers fed alfalfa-grass hay. Frost killing effects of berseem clover were compared with mature-cut berseem clover by placing Dacron® bags into the rumen of one fistulated steer fed alfalfa hay. Bags were incubated for periods of 0 to 48 hours. With increasing maturity, the proportion of non-degradable protein (NDP) and the rate of crude protein (CP) degradation increased in both forages. While the rate of neutral detergent fiber (NDF) degradation and potentially degradable protein proportion (PDP) increased with increasing maturity in alfalfa, the rate of NDF degradation and PDP proportion decreased and proportion of water soluble protein (WSP) increased in berseem clover. The proportion of protein escaping rumen degradation (PEP) was greater in berseem clover than alfalfa, but was not affected by maturity. Frost killing of mature berseem clover decreased WSP proportion and increased PDP proportion compared to mature berseem clover harvested live. Even though ADIN concentration was higher for frost-killed berseem clover, PEP and total escape protein concentration (CEP) was also higher for frost-killed berseem clover than mature berseem clover harvested live, due to decreases in the rate of ruminal N degradation with frost-killing.

Introduction

High-quality forages such as alfalfa and clovers are the major source of dietary protein for lactating and growing beef cows in some regions of the United States. Forages usually have higher soluble nitrogen compared to grains, which is almost completely converted to ammonia by rumen bacteria. This higher soluble nitrogen content usually exceeds the nitrogen required for microbial protein synthesis in the rumen and is wasted as ammonia. A lot of effort has been spent to identify the forage species which resist microbial degradation in the rumen. Forages greatly vary in their resistance to microbial degradation in the rumen because of their differences in structure. This diversity among forages results in different ruminal digestion kinetics in forages. Much of the nitrogen present in forages is encased in rigid, ligno-cellulosic structures; its exposure to the digestive action of the rumen is largely dependent on cell-wall rupture. With maturity, cell-wall content of forages increases, which may result in a greater resistance to microbial degradation and escape of protein.

Mechanical disruption of forage is the major cause for the release of the cellular soluble protein. Susceptibility of forages to mechanical damage varies; bloat-safe legumes tend to be more resistant than bloat-causing species. It has been reported that bloat-safe legumes usually contain greater escape protein concentrations than bloat-causing legumes because soluble proteins have been implicated as the foaming agents largely responsible for pasture bloat. This low level of soluble proteins in bloat-safe legumes may indicate the presence of a higher potentially degradable protein fraction, which is a major contributor to escape protein.

Berseem clover is a relatively new clover in Iowa, introduced as a rotational crop. Limited information is available about the use of berseem clover as forage for livestock production in Iowa. Berseem clover has a high level of CP, as do other legumes, and our previous study with stockpiled berseem clover also showed that berseem clover had two times higher percentage of protein escaping ruminal degradation than alfalfa hay. It has been reported that berseem clover did not produce bloat in grazing cows. All this evidence indicates that berseem clover may have greater escape protein than alfalfa.

Even though the ruminal digestion kinetics of alfalfa hay has been relatively well studied, there is a lack of information regarding the ruminal degradation kinetics of berseem clover. Therefore, our objectives in this study were to determine digestion kinetics and escape protein of alfalfa and berseem clover at maturity. Because our stockpiled berseem clover study was done after frost killing in a previous study, our second objective was to determine the effect of frost killing on ruminal digestion kinetics and escape protein of frost-killed berseem clover.

Materials and Methods

Alfalfa and berseem clover used in this study were second-cut materials harvested at four stages of maturity: 3, 5, 7, and 9 wk. Both alfalfa and berseem clover were harvested at the same time to obtain the same maturity at three locations from each field; then those locations were protected with cages for sample collections. Forages were hand-harvested and stored at -20°C until they were freeze-dried. Four weeks after a killing frost (-2°C), berseem clover was harvested from the same locations previously sampled.

To determine the effects of maturity on chemical compositions of forages, freeze-dried samples of alfalfa and berseem clover were ground through a 1-mm screen and then analyzed for NDF, ADF, CP, and ADIN concentrations.

To estimate *in situ* degradation kinetics and fractions of N and cell wall (CW), freeze-dried samples of alfalfa and berseem clover forages collected 3, 5, 7, and 9 wk after second cut and four weeks after killing (berseem clover) were ground through a 2-mm screen. Approximately 3 g of one of the forage samples was weighed into a bag with internal dimensions of 12 x 10 cm; therefore, the ratio of sample weight to exposed bag surface area was approximately 12.5 mg/cm2. Bags used were constructed of Dacron® polyester with an average pore size of 50 microns. Bags were suspended in the rumen by stringing the looped portion of the bag onto a ring of tygon tubing filled with .63 cm steel hunting shot. Four bags were affixed to each ring.

Two mature fistulated steers fed alfalfa-grass hay as large round bales were used for incubation of samples in Dacron® bags in a maturity study. One mature fistulated steer fed alfalfa hay twice a day was used for incubation of samples in a frost killing study, however, this experiment was repeated twice to obtain replication for laboratory analysis. Dacron® bags were placed in the ventral portion of the rumen and incubated for periods of 0, 3, 6, 12, 24, 32, and 48 h. Two bags of samples for each maturity and forage species were inserted for each incubation time except at 12 h. At 12 h, four bags of samples--two for estimation of *in situ* N degradation and two for estimation of the percentage of escape protein were inserted. Bags were inserted into the rumen in reverse order of incubation times, allowing all bags to be removed simultaneously and minimizing variation associated with the washing process that followed. Bags were washed under running water three times and placed in ice water overnight. The following morning, bags were washed under running water until rinsates were clear. After washing, all bags were dried for 48 h at 65°C, and DM recovery was determined. Undigested forage residues were analyzed for nitrogen by the micro-Kjeldahl procedure (AOAC, 1980) and NDF (Goering and Van Soest, 1970).

Kinetic parameters associated with the disappearance of N from bags were estimated from a one-pool version of Mertens' (1977) discrete lag model of CW digestion. Modifications of the model by Wechsler (1981), which allows estimation of both digestion and lag functions from a single formula, were also incorporated. Model estimates of the pool size, rate constant (k), and discrete lag time of the potentially digestible N in each sample were obtained by fitting recovery data to the model, using nonlinear regression analysis (SAS, 1982).

Table 1. Changes in composition of forages with maturity.

	Forages										
	Alfalfa				Berseem clover[a]				SE	Significance[b]	
Item	EIM	IM	M	VM	EIM	IM	M	VM	M	F	
Proportion of DM, %											
NDF	36.62	41.70	42.15	50.86	45.88	47.59	49.45	53.90	.43	.01	.01
ADF	27.31	32.18	33.00	39.75	32.95	35.08	41.30	43.68	.63	.01	.01
CP	22.55	19.23	16.81	16.02	22.92	18.80	16.68	14.30	.39	.01	.08
Proportion of N, %											
ADIN	1.34	1.83	2.05	2.24	1.74	1.63	1.83	1.77	.08	.03	.01

[a]EIM=early immature (3 week mature), IM=immature (5 week mature), M=mature (7 week mature), VM=very mature (9 week mature)

[b]M=maturity, F=forage species.

Loss of DM from bags caused by exposure of substrates to the digestive action of the rumen and the washing process that followed resulted in the partitioning of CW and N in each of the maturity-treatment combinations into three fractions: 1) soluble fractions of CW (WSCW) and N (WSP) were determined as the differences between initial CW and N content and amounts of CW and N recovered in 0 time-incubation; 2) potentially digestible fractions of CW (PDCW) and N (PDP) were determined as 100 - (non-digestible fraction and water soluble fractions of CW and N); 3) non-digestible fractions of CW (NDCW) and N (NDP) were determined as the differences between initial CW and N content and amounts of CW and N recovered after 48 h incubations of samples in the rumen.

A modified technique reported by Mullahey et al. (1992), as adapted from the *in situ* technique reported by Anderson et al. (1988), was used to determine the percentage of forage protein that escaped ruminal degradation.

The proportion and concentration of total protein which escaped ruminal digestion were calculated as total residual N remaining following 12-h incubation, adjusted for the indigestible N (ADIN) using the following equations:
Escape Protein Percentage, % of total protein = (Total residual N - ADIN of total residue)/ (Total plant-N - ADIN of total plant) x 100
Escape protein concentrations, % of DM = 6.25 x (Total residual N - ADIN of total residue)

Results and Discussion

Berseem clover had higher concentrations of NDF and ADF than alfalfa. Both NDF and ADF concentrations increased in both forages, presumably because of an increase in lignin concentration of forages with maturity. Alfalfa tended to have a higher CP concentration than berseem clover; however, CP concentrations decreased with maturity in alfalfa and berseem clover. Overall ADIN concentrations, except 3- week-mature alfalfa, were higher in alfalfa than in berseem clover, and increased with maturity in alfalfa (Table 1).

Table 2. Estimates of *in situ* rate of disappearance of potentially digestible N fractions, digestion lag times, digestible pool size of N, and proportion and concentration of protein escaping rumen digestion by varying maturities in alfalfa and berseem clover.

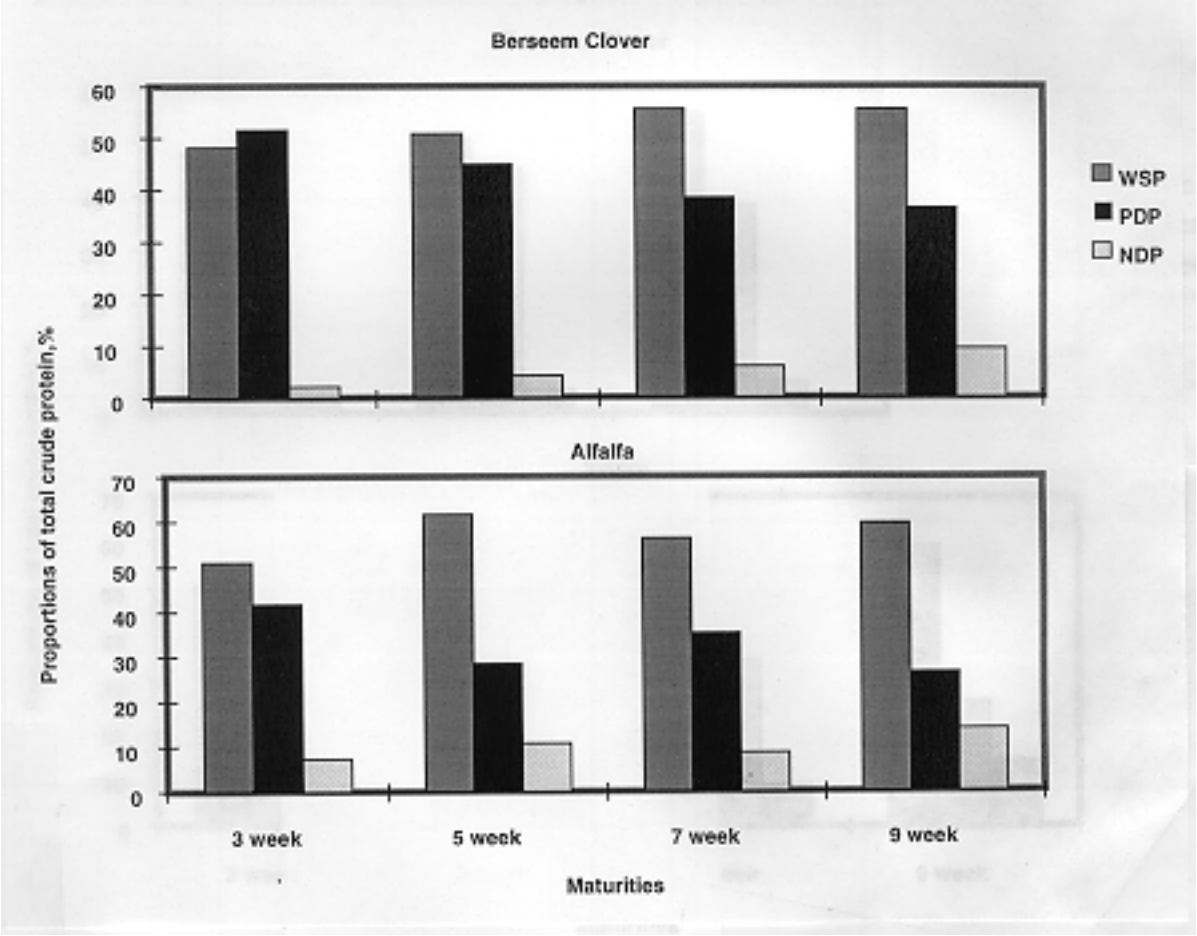
	Forage species[a]									Significance[b]	
	Alfalfa				Berseem clover				SE		
Item[c]	EIM	IM	M	VM	EIM	IM	M	VM	M	M	F
Nitrogen											
-k, h-1	.219	.331	.439	.439	.380	.236	.270	.332	.038	.06	.07
Lag.58	1.02	2.00	1.99	1.88	2.27	1.60	1.98	.345	.20	.04	
PDN	75.70	63.34	77.53	77.53	93.53	90.12	83.68	76.24	1.28	.01	.01
PEP	26.88	32.09	27.11	25.95	27.29	30.02	32.04	31.13	2.16	.51	.04
CEP	5.70	5.58	3.79	3.78	6.21	5.16	4.76	4.15	.369	.01	.16

[a]EIM=early immature (3 week mature), IM=immature (5 week mature), M=mature (7 week mature), VM=very mature (9 week mature)
[b]M=maturity, F=forage species
[c]DNP=digestible N pool size, PEP=proportion of protein escaping rumen digestion, CEP=concentration of protein escaping rumen digestion.

The rate of N degradation increased in both forages with maturity; however, the highest rate of N degradation in berseem clover was observed with 3-week-mature berseem clover. The rate of N degradation tended to be slower in berseem clover, except at 3-week maturity, than in alfalfa. The lag time increased with maturity in alfalfa; however, maturity did not affect the lag time in berseem clover. The lag time for N degradation was shorter in alfalfa than berseem clover. The potentially digestible N pool, determined by a kinetics model, was higher in berseem clover, except at 9-week-maturity than in alfalfa. It decreased linearly with maturity in berseem clover but was not affected in alfalfa. The percent of protein escaping ruminal degradation increased with maturity in berseem clover but fluctuated in alfalfa; it was higher in berseem clover than in alfalfa. The concentration of protein escaping ruminal degradation decreased with maturity in both forages and was similar in both forages (Table 2). Even though CP concentrations of alfalfa in 7- and 9-week maturities were similar and higher respectively, berseem clover tended to have greater escape protein concentrations due to the fact that berseem clover had lower ADIN concentrations and a lower rate of N degradation than alfalfa.

The proportions of WSP and NDP increased and the proportion of PDP decreased with maturity in both forages, indicating a possible inefficient utilization by ruminant animals with forage maturity. While the proportions of WSP and NDP were higher in alfalfa, the proportion of PDP was lower in alfalfa than berseem clover (Figure 1). This evidence suggests that berseem clover may be better utilized by ruminant animals than alfalfa is.

Figure 1. Changes in nitrogen distribution of forages with maturity.



While the rate of CW degradation tended to decrease in berseem clover with 3-, 7-, and 9-week maturities, it seemed to increase in alfalfa with maturity. Berseem clover had a slower rate of CW degradation than did alfalfa. The lag time for CW degradation was not affected with maturity in berseem clover; however, it increased in alfalfa. The pattern of lag time observed was similar for both N and CW degradation, indicating that rumen microbes prefer very immature alfalfa over both mature alfalfa and berseem clover. The potentially degradable CW pool, determined by a kinetics model, linearly decreased in both forages, indicating increased lignification and decreased degradability of forages with maturity. Berseem clover had a greater DCWP than alfalfa (Table 3), suggesting a possible structural difference between these forage species; even though berseem clover had a greater ADF concentration (Table 1), an indicator of digestibility in feedstuffs, berseem clover had greater ruminal degradation than alfalfa.

Table 3. Estimates of *in situ* rate of disappearance of potentially digestible CW fractions, digestion lag times, and digestible pool size of CW by varying maturities in alfalfa and berseem clover.

	Forage species[a]										
	Alfalfa				Berseem clover				SE	Significance[b]	
Item[c]	EIM	IM	M	VM	EIM	IM	M	VM	M	F	
Cell wall											
-k, h-1	.194	.146	.201	.308	.190	.153	.102	.131	.034	.19	.02
Lag.00	1.00	1.17	2.20	1.15	1.77	.86	1.371	.382	.04	.47	
DCWP	65.77	60.74	55.50	44.33	84.99	76.53	69.04	61.37	1.97	.01	.01

[a]EIM=early immature (3 week mature), IM=immature (5 week mature), M=mature (7 week mature), VM=very mature (9 week mature).
[b]F=forage species, M=maturity.
[c]DCWP=digestible cell wall pool size.

Similar to protein distribution, the proportions of WSCW and NDCW increased and the proportion of PDCW decreased with maturity in both forages. The proportion of potentially degradable cell walls decreased because lignin concentrations of forages increase with maturity, which makes CW unavailable to rumen microbes for degradation. Berseem clover had higher proportions of WSCW and PDCW and a lower proportion of NDCW (Figure 2). These data also suggest that CW of berseem clover is better utilized in ruminant animals than alfalfa is.

Frost killing of berseem clover increased the ADIN concentration but did not affect the concentrations of NDF, ADF, or CP relative to mature berseem clover harvested live (Table 4). Frost killing of forage may cause an increase in the binding of nitrogen to lignin content and make it unavailable for digestion.

The rates of N and CW degradations, PDNP, and PDCW were similar in both frost-killed and live-harvested berseem clover; however, PEP and CEP of frost-killed berseem clover were two times higher than berseem clover harvested live (Table 5), confirming our previous study with stockpiled berseem clover in which we had two times higher PEP with stockpiled berseem clover after a frost than with alfalfa hay. We do not know exactly what causes higher escape protein in frost-killed berseem, but hypotheses are; 1) As soon as the plant is cut, rupture of plant membranes begins releasing the proteases from the vacuoles and enzymatic and membrane proteins from plant organelles. Activation of these proteases accelerates the process of autolysis. Autolysis results in high levels of non-protein nitrogen (soluble protein), which is rapidly degraded to ammonia and almost does not contribute to PEP. However, when forages are frost-killed, autolysis does not exist and no breakdown of protein takes place. The presence of low levels of WSP in frost-killed berseem clover supports this theory; 2) Frost-killing of forages may change the bond between protein and fiber content of forage and increase binding of protein to fiber content of forage, even converting some WSP into PDP. This theory is supported by higher ADIN, PDP, and NDP in frost-killed berseem clover than berseem clover harvested live; 3) A combination of 1 and 2.

Figure 2. Changes in proportions of cell wall of forages with maturity.

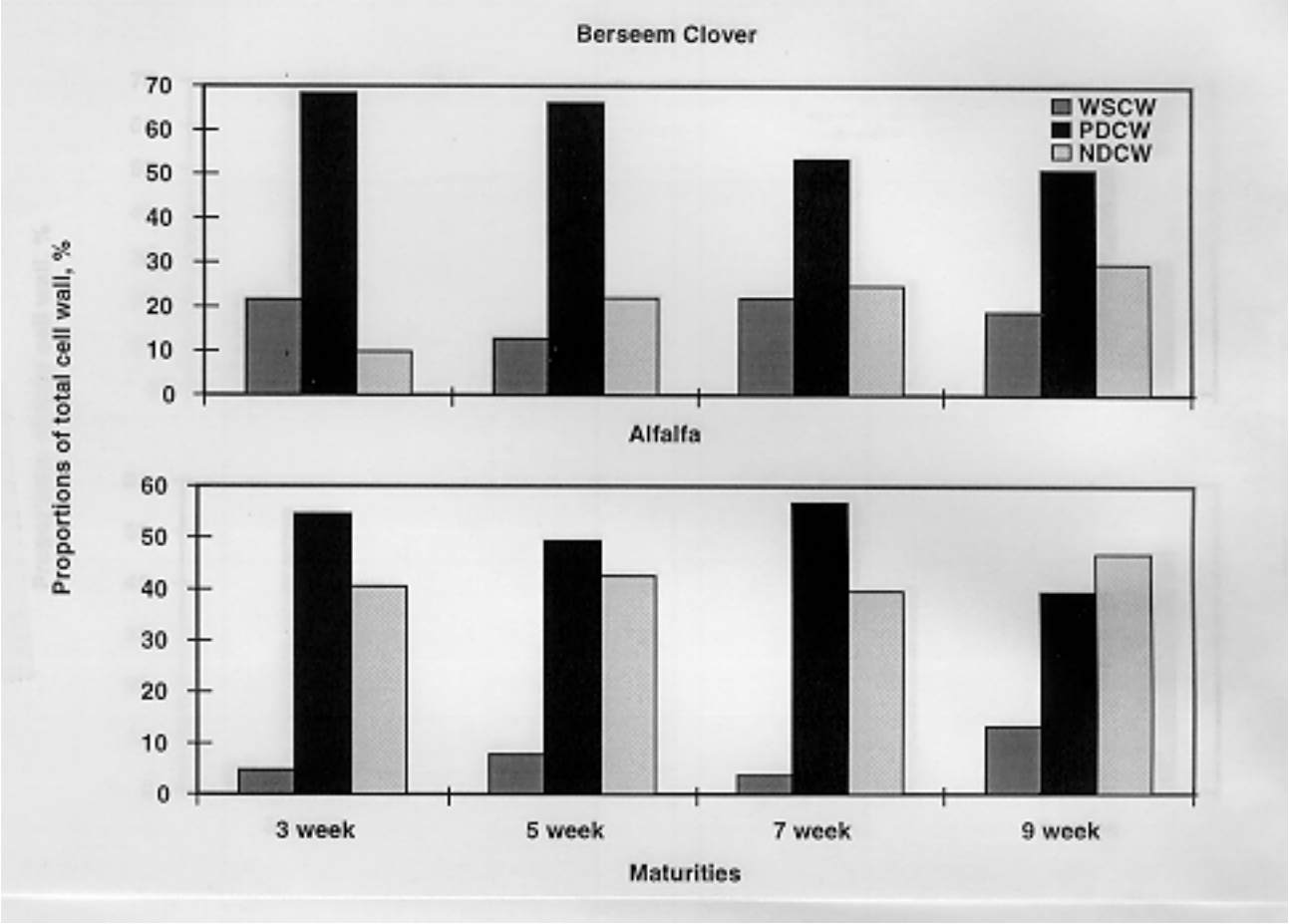


Table 4. The effects of frost killing on chemical composition of berseem clover.

Items[a]	Forage		Significance
	Late mature-cut	Frost-killed	
Proportion of DM, %			
NDF	52.87	54.32	.54
ADF	39.75	42.11	.43
CP 14.30	14.86	.56	
Proportion of N, %			
ADIN	1.77	7.80	.01

Table 5. Estimates of *in situ* digestible pool size of N and CW, rate of disappearance of potentially digestible N and CW fractions, digestion lag times, and proportion and concentration of protein escaping rumen digestion with frost-killing and late maturity in berseem clover.

Items[a]	Forage		Significance
	Late mature-cut	Frost-killed	
Nitrogen			
-k, h^{-1}	.452	.350	.53
Lag, h	5.21	2.09	.06
PDNP,%	73.70	76.24	.86
PEP,%	32.45	64.37	.01
CEP,%	4.2	7.89	.01
Cell wall			
-k, h^{-1}	.131	.141	.35
Lag, h	1.38	4.15	.01
DCWP,%	51.18	56.18	.21

[a]DNP=digestible N pool size, PEP=proportion of protein escaping rumen digestion, CEP=concentration of protein (% of DM) escaping rumen digestion, DCWP=digestible cell wall pool size.

Berseem clover harvested live had a greater proportion of WSP and WSCW and lower proportions of PDP, NDP, and NDCW than frost-killed berseem clover (Figures 3 and 4). The higher PDP may be caused by conversion of WSP into PDP by frost killing, as mentioned earlier. The higher NDP is mainly caused by a higher ADIN concentration in frost-killed berseem clover.

Implications

With maturity, utilization of both forages by ruminant animals decreased, due to increases in water soluble protein and non-degradable protein and decreases in potentially degradable and escape protein concentration. It seems that five-week maturity is best for both forages if high digestibility and escape protein concentrations per unit of forage are the goals.

Frost-killing of forages holds some promise in terms of achieving better utilization of forage protein by ruminant animals; however, further research is needed to determine the mechanism or mechanisms which causes this effect.

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Figure 3. Effect of frost killing on proportions of total nitrogen of berseem clover.

Figure 4. Effect of frost killing on proportions of total cell wall of berseem clover.