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Abstract. A three-year study was carried out to assess the effects of different grass-legume mixtures on: (i) dietary CP and NE_L (Net Energy for milk production) concentrations and milk urea level; and (ii) the relationship between milk urea and several dietary variables. Replicate groups of Sardinian sheep in early-mid lactation (January-May) rotationally grazed plots of three grass-legume mixtures consisting of common grass (*Lolium rigidum*, Gaudin) and different legumes, namely: *Medicago polymorpha* L. (burr medic, treatment BM), *Trifolium subterraneum* L. (subclover, treatment SC) and *Hedysarum coronarium* L. (sulla, treatment SU). Herbage dry matter intake and diet composition were estimated by the n-alkane method. Across the three grazing seasons the dietary CP and milk urea concentrations were consistently higher in BM than in the other treatment groups ($P < 0.05$). Dietary NE_L content was similar between treatments. Pooling all data ($N = 72$), a linear relationship between milk urea concentration (MUC, mg/100 ml) and dietary CP (% DM) was found: MUC = 2.012 CP + 1.8411, $R^2 = 0.55$, RMSE = 6.73. Significant but weaker linear relationships were also found between MUC and CP/NE_L (g CP/Mcal NE_L, $r = 0.673$, $P < 0.001$) and tannic phenols (TP, % DM, $r = -0.506$, $P < 0.001$). After stepwise regression analysis, besides CP, dietary TP concentration was kept in the model ($R^2 = 0.60$, RMSE = 6.39). To conclude, the grass-legume mixture exerted a clear effect on milk urea concentration with higher values in groups having higher CP and lower tannic phenols concentrations in their diet.

Keywords. Milk – Urea – Legume – Grazing – Sheep.

L'urée du lait : Un indicateur nutritionnel pour la brebis laitière au pâturage sur différentes associations légumineuses-graminées

Résumé. *Une étude de trois ans a été menée pour évaluer les effets de différentes associations légumineuses-graminées sur : (i) la concentration des matières azotées totales (MAT), l'énergie nette pour le lait (EN_L) de la ration et le taux d'urée dans le lait ; et (ii) l'influence entre la teneur en urée du lait et la composition chimique de la ration. Des lots de brebis ($n = 3$) ont pâturé trois types de parcelles composées de graminée commune (*Lolium rigidum*, Gaudin) et de différentes légumineuses, notamment :* Medicago polymorpha *L. (Luzerne polymorphe, lot BM),* Trifolium subterraneum *L. (Trèfle souterrain, lot SC) et* Hedysarum coronarium *L. (Sainfoin d'Espagne, lot SU). La matière sèche d'herbe consommée et la composition de la ration ont été estimées par la méthode des n-alcanes. Pendant ces trois années, la teneur en MAT de la ration ingérée et la teneur en urée du lait ont été plus élevées dans le lot BM ($P < 0,05$). La teneur en EN_L a été similaire pour les trois différentes associations légumineuses-graminées. Ce travail a permis de mettre en évidence une relation linéaire entre le taux d'urée du lait (MUC, mg/100 ml) et la teneur en MAT de la ration : MUC = 2,012 MAT + 1,8411, $R^2 = 0,55$, RMSE = 6,73. Une relation linéaire a été aussi trouvée entre MUC et MAT/NE_L (g MAT/Mcal NE_L, $r = 0,673$, $P < 0,001$) ou avec les tannins phénoliques (TP, % MS, $r = -0,506$, $P < 0,001$). Après analyse des données par la méthode "stepwise regression analysis" MAT et TP ont été inclus dans le modèle, qui a amélioré sa prédiction ($R^2 = 0,60$, RMSE = 6,39). Pour conclure, les pâturages associant graminées et légumineuses fourragères affectent le taux d'urée du lait, qui est plus élevé chez les brebis soumises à une ration riche en MAT et pauvre en TP.*

Mots-clés. *Lait – Urée – Légumineuses – Pâturage – Brebis.*

I – Introduction

The nutrition of grazing ruminants is still a concern for the difficulty of adequately balancing requirements and allowances of nutrients. In Mediterranean pastures, excess of protein is commonly experienced by lactating sheep grazing herbaceous pastures at vegetative stage. Under these conditions, grazed herbage is rich in NPN and soluble protein while it is often relatively low in non-fibre carbohydrates (NFC; Molle *et al.*, 2007). The excess of dietary protein and the asynchronous pattern of protein and carbohydrates degradation in the rumen result in extensive uptake of ammonia by rumen wall. This in turn entails an energy cost for ammonia detoxification in the liver, a raise of urea in blood and milk, and hence higher N losses, particularly through urine.

Sustainable grazing systems often encompass the use of forage legumes with the aim of reducing input of fertilizers and supplements, thanks to their high nutritive and feeding value. However, the incorporation of legumes at high proportion in sheep diet can exacerbate the unbalance between energy and protein and hence increases the release of N to the environment.

The following study is part of a wide-framed multidisciplinary EU project (LEGGRAZE, www.univ-perp.fr/newsite/leggraze) aimed, among others, at evaluating the effect of grazing different legume-based grazing systems on N utilisation at animal and system scales. This report is focussed on the assessment of the effect on dietary content of nutrients and milk urea. The underlying hypothesis is that legume-based pastures differing for agronomic features and chemical composition are able to affect diet composition and in turn milk urea concentration. Moreover, this study aimed at confirming under grazing conditions the putative role of milk urea as sensor of dietary CP and CP unbalances already found in stall fed sheep (Cannas *et al.*, 1998).

II – Materials and methods

The study was carried out at Bonassai research station (NW Sardinia, 41°N latitude, mean annual rainfall 582 mm). Three experiments were run in three grazing seasons: 2003 (January-May), 2004 (January-May) and 2005 (February-May). A randomized-block design was used with replicate plots or groups as experimental units.

The pasture consisted of three replicate plots (7500 m^2 each) of three grass-legume binary mixtures consisting of a common grass (*Lolium rigidum*, Gaudin, ecotype Nurra) and different legumes, namely: *Medicago polymorpha* L. (burr medic, cultivar Anglona, treatment BM), *Trifolium subterraneum* L. (subclover, cultivar Antas, treatment SC) and *Hedysarum coronarium* L. (sulla, cultivar Grimaldi, treatment SU). The pasture plots were established twice: in autumn 2002 and 2004 using the same seed rate for all legumes (35 kg/ha) but different seed rates for the grass (14 and 10 kg/ha in 2002 and 2004, respectively). In both occasions the plots were fertilised with 92 kg/ha of P_2O_5 before seeding. Prior to the beginning of the first grazing experiment each plot was subdivided by metallic fences into two homogeneous sub-plots. A minimum of three "core" adult Sarda ewes at an early lactation stage (on average at 50-54 days in milk), were allocated to each treatment and replicate at the beginning of each experiment on the basis of live weight (mean ± SEM, 43.1 ± 0.6 kg, 45.6 ± 0.8 kg, and 44.1 ± 1.2 kg in Experiment 1, 2 and 3, respectively), and milk yield (1904 ± 19 ml, 2200 ± 20 ml and 1849 ± 53 ml) as measured immediately prior to each experiment. These nine groups rotationally grazed the corresponding sub-plots with 2 week duration. The time access to pasture was 22 h/d. Spare sheep were used to adjust stocking density to pasture growth using compressed sward height as management criterion. The sheep were machine milked twice daily. Herbage mass and its botanical composition were monitored as described in the companion paper (Molle *et al.*, 2009). Three "core" sheep per replicate per treatment were used for intake measurements. Different animals were used in each year. Intake, botanical and chemical composition and digestibility of diet were all measured using the n-alkane method (Dove and Mayes, 1991) in three (years 2003 and 2004, January, March and end of April) or two periods (year 2005, March and end of April) per grazing season (i.e. early, mid and late vegetative phases of pasture). On each occasion the 3 "core" ewes in each group were dosed with a controlled release intra-ruminal bolus (CAPTEC, Auckland, New Zealand) releasing C_{32} and C_{36} alkanes at a constant rate. Feces samples were collected from rectum twice daily after milking commencing on the day 8 and ending on day 12 after dosing. Hand-plucked samples were also taken with a 36 hour lead time relative to faeces collection.

Herbage (grass and legume) and faeces samples were freeze-dried, ground, bulked across collection days within each sheep, and sub-sampled for determination of C_{27}-C_{36} n-alkanes. Sub-samples of bulked freeze-dried herbage were also analysed for CP (Kjeldhal), fiber fractions (NDF, ADF and ADL; van Soest *et al.*, 1991), non protein nitrogen (NPN, fraction A), buffer soluble protein, neutral detergent and acid detergent insoluble protein in order to determine fractions B1, B2, B3 and C (Licitra *et al.*, 1996). Tannic phenols (Folin-Ciocalteu method) were determined according to Makkar *et al.* (1993). The dietary content of NE for milk production (NE_L) was calculated according to NRC (1994) on the basis of alkane estimates of diet digestibility. Individual milk yields were measured at morning and afternoon milking once a week during each intake measurement period and samples were taken for milk urea content (MUC) determination using an enzymatic-colorimetric assay (Chem Spec 150, Bentley Instruments, Inc., 4004 Peavey Road, Chaska, MN 55318, USA).

The data were analysed within experiment by a general linear model which included the random effect of replicate (block) and the fixed effects of period within grazing season, treatment and their interaction. Treatment means were separated by Tukey test. Data were then pooled across experiments and treatments to assess the relationships between MUC and several dietary variables using linear and quadratic regression models. Quadratic components were never significant. Stepwise regression analysis was also performed to select the models with the best goodness of fit using coefficient of determination and predicted residual sum of squares as selection criteria.

III – Results and discussion

The herbage mass was not limiting while the legume mass was very low in the second grazing experiment when SU was the only treatment that showed a detectable proportion of legume in the pasture (Molle *et al.*, 2009). Nutrient contents of the diets are reported in Table 1.

The average concentration of CP in ewes' diet was always higher than the optimal range for a lactating sheep weighing 50 kg and producing from 1 to 2 kg/d of fat (6.5%) and protein (5.5%) corrected milk: ca. CP 16-17% DM (Cannas, 2002). In particular dietary CP content was higher in BM than in the other treatments in all years. This occurred despite in the Experiment 2 – second year for sulla and first self-regeneration year for the other legumes – grass was the main dietary component in all treatments [95 (BM), 93 (SC) and 84% DM (SU), $P > 0.05$]. This apparent oddness is probably explained by the carry over effect of burr medic – the higher yielding legume in year 1 – on ryegrass CP content in year 2 [263 (BM) *vs* 253 (SC) and 246 g/kg DM, (SU), $P < 0.05$ between BM and SU].

In the establishment years (Experiment 1, year 2003 and 3, year 2005), SU groups showed lower B1 and a lower proportion of soluble N (A + B1, $P < 0.05$, not shown) than the counterparts. SU treatment also displayed a trend to higher B2, although difference between treatment groups varied with years. Also C fraction (the unavailable protein component) was higher in SU, but only in Experiment 3. Overall N partitioning was in favour of a slower degradation and possibly a lower rumen digestion of protein in sheep grazing SU than counterparts grazing BM or SC. In Experiment 2, although differences in protein fractioning were evident, their explanation is difficult because also grass chemical composition was affected by treatment.

The non-fibre carbohydrates (calculated NFC) tended to be higher in SU in both establishment years, thanks to the relatively high levels of these components in sulla (e.g. Molle *et al.*, 2007). Fibre carbohydrates were less sensitive to treatment effect, with exception of ADL which was higher in SU for the high level of ADL in sulla. ADL in sulla probably includes, apart from lignin, some amount of poly-phenols. Indeed SU groups showed a higher average content of tannic phenols (Table 1).

A trend to higher NEL in BM than counterparts was hardly detectable ($P < 0.07$, in Experiment 1). The effect of treatment on CP/NEL was significant in year 2005 and close to be significant ($P < 0.06$) in year 2003. Overall the period within grazing season exerted a clear effect on fibre carbohydrate content (particularly NDF), which increased along the seasons and on dietary NEL content and CP/NEL, which decreased. The interaction between treatment and period was evident in year 2 and 3 with reference to dietary CP content. This can be due to a relatively faster decay of dietary CP content in SC than SU and BM treatment groups (not shown).

Table 1. Dietary nutrient content in milked sheep grazing grass-legume binary mixtures differing for the legume component (BM, burr medic, SC, subclover, SU, sulla). Least square means

	Treatment			Level of significance		
	BM	**SC**	**SU**	**Treat.**	**Period**	**T. × P.**
Experiment 1 (2003)						
CP (g/kg DM)	234 a	193 b	195 b	***	NS	NS
A (% CP)	12.7 a	16.8 b	13.4 a	*	NS	NS
B1 (% CP)	16.7 a	16.0 a	11.1 b	***	NS	NS
B2 (% CP)	52.3 a	48.5 b	54.3 a	**	NS	NS
B3 (% CP)	11.3 a	14.1 b	14.4 b	***	**	**
C (% CP)	7.0 a	4.7 b	6.7 a	***	***	NS
NFC† (g/kg DM)	292 a	315 ab	353 b	**	NS	NS
NDF (g/kg DM)	343	355	321	NS	**	NS
ADF (g/kg DM)	176	175	173	NS	NS	NS
ADL (g/kg DM)	16 a	14 a	31 b	***	NS	NS
TP (g/kg DM)	2.0 a	4.5 b	10.5 c	***	NS	NS
NE_L (Mcal/kg DM)	1.61	1.51	1.51	NS	***	NS
CP/NE_L (g/Mcal NE)	140	124	124	NS	*	NS
Experiment 2 (2004)						
CP (g/kg DM)	263 a	249 b	249 b	**	***	**
A (% CP)	15.4	14.5	11.4	NS	*	NS
B1 (% CP)	19.6 a	30.2 b	27.4 b	***	NS	NS
B2 (% CP)	37.1 a	32.6 b	33.0 b	**	NS	NS
B3 (% CP)	10.2 b	6.8 a	17.4 c	***	NS	NS
C (% CP)	17.6 a	15.8 b	10.8 c	***	***	NS
NFC† (g/kg DM)	135	150	155	NS	***	NS
NDF (g/kg DM)	449	452	439	NS	***	NS
ADF (g/kg DM)	261	258	256	NS	***	NS
ADL (g/kg DM)	37	37	45	NS	NS	*
TP (g/kg DM)	2.2 a	2.6 a	7.1 b	***	*	*
NE_L (g/kg DM)	1.51	1.47	1.53	NS	**	NS
CP/NE_L (g/Mcal NE)	174	169	163	NS	***	NS
Experiment 3 (2005)						
CP (g/kg DM)	259 a	214 b	224 b	***	**	*
A (% CP)	17.3	12.6	10.8	NS	NS	NS
B1 (% CP)	26.4 ab	27.7 a	23.2 b	*	**	***
B2 (% CP)	32.7 a	36.9 ab	40.0 b	***	**	NS
B3 (% CP)	13.3	12.1	13.7	NS	NS	**
C (% CP)	10.4 a	10.7 a	12.3 b	***	***	***
NFC† (g/kg DM)	277 a	284 a	325 b	*	***	NS
NDF (g/kg DM)	341	364	332	NS	***	NS
ADF (g/kg DM)	185	195	205	NS	***	NS
ADL (g/kg DM)	19 a	19 a	51 b	***	NS	NS
TP (g/kg DM)	2.4 a	5.5 b	12.3 c	***	NS	NS
NE_L (g/kg DM)	1.56	1.47	1.47	NS	NS	NS
CP/NE_L (g/Mcal NE)	167 a	145 b	153 ab	*	**	NS

†Calculated as follows: NFC = 100-(NDF-NDIP)-CP-EE-ash.
*P < 0.05; **P < 0.01; ***P < 0.001; NS = not significant.
a, b, c: means in rows with different letters differ at P < 0.05.

Milk urea average concentration (mg/100 ml) was relatively high in all years and treatments with higher levels in BM than in the other treatment groups (Table 2). High levels of milk urea had been already found by Molle *et al.* (2002) in milked sheep grazing burr medic as compared with annual ryegrass monocultures and by Landau *et al.* (2005) in burr medic – as compared to chicory – or safflower – based mixed pastures grazed by late-lactating sheep. A decreasing MUC was evident in all treatments and years ($P < 0.01$ for the period).

Table 2. Milk urea concentration (MUC, mg/100 ml) in sheep grazing the different pasture mixtures

	Treatment†			P<		
	BM	**SC**	**SU**	**Treat.**	**Period**	**T. × P.**
MUC – Exp. 1 – 2003	50.02 a	43.02 ab	36.53 b	***	**	NS
MUC – Exp. 2 – 2004	55.59 a	49.45 b	50.15 ab	*	***	NS
MUC – Exp. 3 – 2005	60.72 a	48.17 b	44.04 b	**	**	NS

†Legume in the grass-legume binary mixture BM = burr medic; SC = subclover; SU = sulla.
*P < 0.05; **P < 0.01; ***P < 0.001; NS = not significant.
a, b: means in rows with different letters differ at P < 0.05.

Pooling all the data of the study, an exploratory correlation analysis showed that the best single predictor of milk urea concentration was dietary CP content ($r = 0.74$, $P < 0.001$), followed by CP/NE_L ($r = 0.67$, $P < 0.001$), NFC ($r = -0.52$, $P < 0.001$), TP ($r = -0.51$, $P < 0.001$), fraction B1 (% DM) ($r = 0.50$, $P < 0.001$) and fraction A (% DM) ($r = 0.49$, $P < 0.001$).

The regression of dietary CP (% DM) on MUC (mg/100 ml) resulted in the following equation: MUC = 2.012 CP (±0.218, $P < 0.001$) + 1.8411 (±5.096, $P > 0.05$), $R^2 = 0.55$, RMSE = 6.73. When including all the above independent variables in a stepwise regression analysis with MUC as dependent variable, only CP and tannic phenol contents were retained in the final model. The equation was: MUC (mg/100 ml) = -11.21 (±5.78, $P < 0.056$) + 1.74 (±0.23, $P < 0.001$) × CP (% DM) – 5.89 (±1.99, $P < 0.01$) × TP (% DM); $R^2 = 0.60$, RMSE = 6.39, $P < 0.001$. Although TP was kept in the model, its proportional contribute to variance explanation was limited (0.05 unit of the determination coefficient out of 0.60).

Cannas *et al.* (1998) reviewing several studies on stall fed ewes calculated a different equation using either blood (BUC) or milk urea concentrations [BUC or MUC, (mg/100 ml) = -38.89 + 4.49 × CP (% DM), $R^2 = 0.82$]. The diets were virtually tannin-free. A proper comparison between the above equations cannot be run on the whole dataset used in this study since the range of dietary CP proportions investigated is different (CP 8-21% DM, in Cannas *et al.*, 1998 and CP 16-32% DM, in this study). A data sub-set falling in the CP range of the former study was therefore used (n = 20). The predicted MUC based on the two equations averaged 45.75 ± 4.63 and 40.22 ± 2.97 mg/100 ml, respectively, being the actual average MUC 38.08 ± 7.26. The predicted residual sum of square were 1250 and 714, for the former and the latter equations, respectively.

Although this study partially back up the use of MUC as a sensor for estimating dietary CP, a relevant portion of variance is unexplained by the factors under study. In a recent study, Giovanetti (2007), reviewing several studies on stall-fed dairy sheep found that MUC is more strongly correlated with CP/NE_L ratio than dietary CP content. Interestingly, swapping independent with dependent variables in the regression of CP/NE_L on MUC, we obtained a slope similar to that found by Giovanetti (2007) (1.61 in the current study *vs* 1.69 in Giovanetti's paper). However, the intercept was much higher in the former (74.8) than in the latter study (36.5). This similarity in regression slopes tends to support the hypothesis, already confirmed in dairy cattle (e.g. Nousianen *et al.*, 2004) that MUC should be primarily used to estimate the balance between protein and energy in the diet rather than dietary CP content or CP intake. Apart from tannic phenols, other factors affecting protein and energy degradation and utilisation in the rumen play probably a role in modulating this relationship.

IV – Conclusions

Milked sheep grazing different legume species in grass-legume Mediterranean mixtures have diets that differ for CP, carbohydrate concentration and their fractions as well as for the content of plant secondary metabolites such as tannins. The difference in diet composition in turn results in different MUC. The content of MUC in milk increases with the dietary CP content and decrease with the level of dietary tannic phenols. MUC is confirmedly envisaged as a gauge of dietary CP and CP/NE_L ratio but other factors, such as tannins in our study, modulate this relationship.

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