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# Protein and carbohydrate profiles of a diploid and a tetraploid red clover cultivar

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Red clover (*Trifolium pratense* L.) is characterized by high dry matter (DM) yield and a high nutritive value. It has a higher concentration of soluble carbohydrate than lucerne and its protein is less degradable in the rumen than lucerne protein. The aim of this study was to quantify the main crude protein (CP) and carbohydrate (CHO) fractions of a tetraploid (4n, *cv* K-32) and a diploid (2n, *cv* K-39) red clover cultivar harvested at three stages of development in the spring growth. Results showed that the cultivar and stage of development had a large impact on chemical composition and the protein and carbohydrate fractions determined by the Cornell Net Carbohydrate and Protein System (CNCPS). The rapidly degradable protein fraction (PA) was significantly higher in the tetraploid (272 to 287 g kg$^{-1}$ CP with advancing plant development) than the diploid red clover cultivar (214 to 268 g kg$^{-1}$ CP). Rumen degradable carbohydrate concentration was not affected by cultivar but it was significantly influenced by the stage of development. Concentration of rumen degradable protein decreased from 726 to 655 g kg$^{-1}$ CP with advancing plant development in the diploid red clover cultivar. In conclusion, the tetraploid red clover cultivar was higher in CP, its protein was more rapidly degradable, and it was characterized by lower concentrations of lignin and unavailable carbohydrate fractions than the diploid cultivar.

*Key words:* carbohydrate fractionation, protein degradability, *Trifolium pratense* L.

## Introduction

Red clover (*Trifolium pratense* L.) is a very important perennial forage legume characterized by a high productivity (Murray et al. 2007, Mustonen et al. 2018) and a high nutritive value (Asci 2012), and it is used as hay, haylage and silage in animal nutrition. It is able to fix atmospheric nitrogen and grows well on different types of soil (Leto et al. 2004). In temperate areas, red clover DM yields can be equal to or even exceed lucerne yields if soil moisture is adequate (Topp and Doyle 2004). The quality of forage plants depends on many factors, including plant growth and development, environmental conditions, cutting system, fertilization, and variety. Red clover cultivars vary in quality based on the stage of development at harvest and on whether they are diploid or tetraploid. Tetraploid red clover cultivars are characterized by higher concentrations of protein, water-soluble carbohydrate, potassium, and phosphorus but lower fibre concentration than diploid cultivars (Drobna and Jančovič 2006).

There is an increased interest in studying forage legume species that reduce proteolytic processes during ensiling and reduce CP degradation in the rumen. Leguminous species may indeed contain compounds that can reduce CP degradability (Krawutchke et al. 2013). Leguminous species that contain CP and tannin complexes, such as birdsfoot trefoil (*Lotus corniculatus* L.) for example, have CP that is less degradable, compared to species that do not contain tannins, such as lucerne (Julier et al. 2003). In contrast to birdsfoot trefoil, red clover has a very low concentration of condensed tannins (Grabber 2009), but the proportion of degradable CP is lower compared to lucerne. Lower protein degradability of red clover is due to the presence and the activity of polyphenol-oxidase in the plant (Broderick et al. 2001, 2004).

It is critical to establish the correct content of structural and non-structural CHO and CP in ruminant diet to avoid unnecessary nutrient losses. According to Yu et al. (2004), the place of digestion and the ratio of volatile fatty acids and lactic acid generated in the rumen determine the breakdown of nutrients and the features of the fermentation process. Both of these characteristics are important because they have a direct impact on type of nutrients available to the animals.

Degradability and utilization of CP and CHO fractions, as well as parameters related to the rumen degradability process, are critical for adequate estimation of the nutritional value of forages and the evaluation of the

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animal production performance. The Cornell Net Carbohydrate and Protein System (CNCPS, Lanzas et al. 2007), established at Cornell University, USA, is based on the passage and degradation rates of each CP and CHO fraction. The aim of this study was to investigate the effect of the stage of development (SD) on nutritive attributes, including various CP and CHO fractions, of the spring growth of a diploid red clover cultivar (DRCC) and a tetraploid red clover cultivar (TRCC).

# Material and methods

## Field trial

This experiment was carried out in the experimental field of the Institute for Forage Crops Kruševac, R Serbia (43° 34′ 58′′ N, 21° 19′ 35′′ E). The study area was located 166 m above sea level in South-East Serbia. According to its chemical properties, the soil belonged to the group of slightly acid soils (pH of 6.5 in aqueous solution and 5.7 in N KCl). In the humus-accumulating layer, the humus content was 2.6% and it belonged to the group of medium-rich soils. The total nitrogen content of 0.16% classified this soil also in the group of medium-rich soils. The soil had a very poor content of available phosphorus (4.9 mg $P_2O_5$/100 g of soil), and a potassium content of 23.1 mg $K_2O$/100 g of soil.

The climate of Kruševac has a temperate-continental character, with warmer autumns than spring in the transitional seasons. Cyclonic activity from the Atlantic Ocean and the Mediterranean Sea, as well as the so-called winter Siberian anticyclone, influence meteorological conditions throughout the winter. The area of Kruševac and its surrounding is frequently under the influence of anticyclones in the summer, due to the movement of the subtropical high pressure zone towards the north, with rather steady conditions, and occasionally brief severe rains of local character. In the year of research (2016), from January to June, average monthly temperatures were –0.3, 8.6, 8.0, 14.2, 15.6, and 21.5° C, respectively, while average precipitation was 89.5, 48.6, 86.0, 63.2, 144.7, and 77.4 mm, respectively.

The DRCC - K 39 (2n) was developed from a large number of domestic populations through numerous cycles of phenotypic recurrent selection for productivity and biological-morphological characteristics (FMA 2001). It is drought and low temperature tolerant, as well as resistant to the most economically significant diseases. It makes an excellent bee pasture and has a strong seed output potential due to its long flowering cycle. The TRCC - K 32 (4n) was made by artificially doubling (by colchicine) the chromosomes of three separate varieties: K 17, Changins, and Viola (FMA 1993). It is characterized by big leaves and vigorous plants. This red clover cultivar is resistant to economically important diseases and pests, as well as to low temperatures, however, it is drought sensitive. It produces high DM yields in years with sufficient precipitation and irrigation. It has high protein and low fibre concentrations due to its high proportion of leaves.

Sowing was done manually in April 2015, with a row spacing of 20 cm. The basic plot was 2 $m^2$ in size (2 × 1 m). The plots were 60 cm apart, and the blocks were 1.5 m apart. The amount of seeds used for sowing was 2 g $m^{-2}$ for the diploid cultivar and 3.2 g $m^{-2}$ for the tetraploid cultivar. Red clover and forage maize were the previous crops. The compound fertilizer NPK 15:15:15 in the quantity of 300 kg $ha^{-1}$ was used in the pre-sowing preparation. The DM biomass was estimated after fresh biomass was weighed and herbage samples were air-dried at 65° C for 48 hours.

## Cutting system of red clover

The experiment was set up as a randomized complete block with three replications and in a 2 × 3 arrangement of treatments (2 cultivars and 3 stages of development). Two cultivars of red clover (*cv* K 39 and *cv* K 32) were evaluated at three stages of development in the spring growth, in the first post-seeding year (2016). Plants from a pure stand were cut manually with scissors at about 5–7 cm above the soil surface. The first studied stage of development was mid bud (MB) where plants were harvested after a growing period of 62 days (7 May), the second was early bloom (EBL) at around 10–15% of flowering where plants were harvested after a growing period of 77 days (21 May), and the third was mid bloom (MBL) at around 50% of flowering where plants were harvested after a growing period of 85 days (29 May). Forage samples were dried to constant weight at 65 °C for 48 h and then ground using a hammer grinding laboratory mill PX-MFC 90 D (KINEMATICA, Luzern, Switzerland) through a screen size of 1 mm. All analyses were done in duplicate and component concentrations were expressed on a 105 °C DM basis.

## Assessing leaf to stem ratio

Each plot was sampled at the three studied stages of development (MB, EBL, and MBL) to determine the forage leaf to stem ratio. The stems were manually separated from the leaves and these subsamples were dried at 65 °C in a forced-air oven. For each sample, the weight of the leaf and stem components was measured.

## Protein and carbohydrate fractions

Concentration of the different CP and CHO fractions was determined according to the CNCPS (Fox et al. 2004, Lanzas et al. 2007, Tylutki et al. 2008). The CP concentration of samples was determined using the Kjeldahl method (AOAC 1990). The non-protein nitrogen (NPN), neutral detergent insoluble crude protein (NDICP), acid detergent insoluble crude protein (ADICP), soluble protein (SolP), true protein (TP), and insoluble protein (IP) were determined according to Licitra et al. (1996). The CNCPS protein fractions of the samples (PA, $PB_1$, $PB_2$, $PB_3$, and PC) were calculated based on CP, NPN, SolCP, NDICP, and ADICP concentrations according to Fox et al. (2004): PA = NPN; $PB_1$ = SolCP – NPN; $PB_2$ = CP – SolCP – NDICP; $PB_3$ = NDICP – ADICP; and PC = ADICP.

The PA fraction, or non-protein nitrogen compounds, consists of nitrogen that dissolves in the borate-phosphate buffer solution and does not precipitate with reagents such as trichloroacetic acid. These compounds hydrolyze to ammonia very quickly in the rumen. The PB fraction is the true protein and this nitrogen compound is further divided into three subfractions ($PB_1$, $PB_2$, and $PB_3$) that are believed to have different rates of degradation in the rumen (Sniffen et al. 1992). The $PB_1$ fraction is soluble in a borate-phosphate buffer solution (pH of this buffer corresponds to the pH of the rumen) and does not precipitate with trichloro-acetic acid. It is considered to degrade very quickly in the rumen, with a degradation rate of over 100% $h^{-1}$. The $PB_2$ fraction represents proteins obtained as the difference between buffer insoluble proteins and proteins insoluble in the neutral detergent solution. Degradation rate of this fraction is 3–16% $h^{-1}$. The $PB_3$ fraction degrades slowly in the rumen (< 2% $h^{-1}$) and it is mainly composed of proteins bound to the cell wall. Fraction C consists of proteins associated with lignin and tannin complexes and these proteins cannot be broken down in the rumen, so they are unavailable to the animals.

Total CHO [CHO = 1000 – (CP + ash + ether extract)] and non-fibre CHO (NFC = 1000 – [aNDF + CP + ash + ether extract]) were calculated according to NRC (2001). Neutral detergent fibre was determined according to the method of Mertens (2002). Acid detergent fibre and acid detergent lignin were determined according to the method AOAC 973.18 (AOAC 1997). Concentration of total ethanol soluble CHO (TESC – monosaccharides and disaccharides) and starch were determined according to procedures described by Hall et al. (1999). Total CHO are divided into five fractions: instantaneously degradable CHO mainly composed of simple sugars (CA, g $kg^{-1}$ CHO); rapidly degradable CHO mainly composed of starch ($CB_1$, g $kg^{-1}$ CHO); intermediately degradable CHO mainly composed of soluble fibres such as pectic polysaccharides, ß-glucans, and fructans ($CB_2$, i.e. NFC – NSC – starch, g $kg^{-1}$ CHO); slowly degradable CHO composed of available NDF ($CB_3$, i.e. aNDF – NDICP × CP / 1000 - CC, g $kg^{-1}$ CHO) and undegradable CHO (CC, i.e. aNDF × (Lignin / aNDF) × 2.4, g $kg^{-1}$ CHO) composed of completely undegradable NDF (Lanzas et al. 2007).

## Protein and carbohydrate degradabilities

Rumen-degradable proteins (RDP) and rumen-degradable carbohydrates (RDC) were calculated based on the CNCPS subfractions using fractional rate of degradation (Kd) values given for legume pasture (Table 1; Lanzas et al. 2007).

Rumen-undegradable proteins (RUP) and rumen-undegradable carbohydrates (RUCHO) were calculated by subtracting RDP and RDCHO from total CP and total CHO concentrations, respectively. Intestine-digestible rumen-undegradable proteins (IDRUP) and intestine-digestible rumen-undegradable carbohydrates (IDRUCHO) were calculated by subtracting the completely undegradable proteins (PC) and completely undegradable carbohydrates (CC) fraction from RUP and RUCHO, respectively (Aufrère and Guérin 1996). The nitrogen:carbohydrate (N:CHO) attribute was calculated as a N concentration expressed in terms of g N per 1000 g of CHO. The PA:CA attribute was calculated as the amount of nitrogen representing the PA fraction relative to 1000 g of easily degradable CHO; it is expressed in g of N per 1000 g of CA fraction. The PB:CB attribute was calculated as the amount of nitrogen representing the PB fraction relative to 1000 g of the intermediately degradable CHO; it is expressed in g of N per 1000 g of CB fraction. The PC:CC attribute was calculated as the amount of nitrogen representing the PC fraction relative to 1000 g of the unavailable CHO; it is expressed in g of N per 1000 g of CC fraction.

Table 1. Calculation and ruminal rate of degradation (Kd) of protein and carbohydrate fractions

| Protein and carbohydrate fractions | Calculations | Kd ($h^{-1}$) |
|---|---|---|
| PA | NPN | 2.00 |
| $PB_1$ | SolCP – PA | 0.20 |
| $PB_2$ | 100 – (PA + $PB_1$ + $PB_3$ + PC) | 0.15 |
| $PB_3$ | NDICP – PC | 0.08 |
| PC | ADICP | – |
| CA | CHO – $CB_1$ – $CB_3$ - CC | 2.00 |
| $CB_1$ | Starch | 0.45 |
| $CB_2$ | NFC-CA-$CB_1$ | 0.35 |
| $CB_3$ | aNDF-NDICP × CP/1000 –CC | 0.08 |
| CC | aNDF × Lignin × 2.4/1000 | – |

NPN = Non-Protein Nitrogen; SolCP = Soluble CP; NDICP = Neutral Detergent Insoluble Crude Protein; ADICP = Acid Detergent Insoluble Crude Protein; NFC = Non-Fibre Carbohydrates; aNDF = amylase treated Neutral Detergent Fibre; PA = instantaneously solubilised protein; $PB_1$ = rapidly degradable protein; $PB_2$ = intermediately degradable protein; $PB_3$ = slowly degradable protein; PC = completely undegradable protein; CA = instantaneously degradable carbohydrates; $CB_1$ = Starch; $CB_2$ = intermediately degradable carbohydrates; $CB_3$ = slowly degradable carbohydrates; CC = unavailable cell wall

## Statistical analysis

The experiment was established as a randomized complete block design with three replications and a factorial arrangement of six treatments (two red clover cultivars and three stages of development, n = 18). The data were subjected to an analysis of variance using the SAS MIXED procedure (version 9.4, SAS Institute Inc., Cary, NC, USA). Red clover cultivar and stage of development, as well as their interactions were added as fixed effects in the model. Block was added as a random effect. Differences among treatments were tested using Tukey´s test. The results were considered statistically significant when $p< 0.01$.

# Results

## Leaf to stem ratio, DM concentration, and DM yield

Table 2 shows the impacts of red clover cultivar and stage of development on leaf to stem ratio, DM concentration, and DM yield. Averaged leaf to stem ratio of the TRCC was higher ($p< 0.01$) than the DRCC. The forage leaf to stem ratio decreased with advancing maturity in both red clover cultivars, with average values ranging from 40.4 to 32.6% in the DRCC, and from 45.1 to 38.1% in the TRCC at MB to MBL stage of development , respectively ($p< 0.01$).

There were significant differences between red clover cultivars in DM concentration and DM yield, although with plant growth and development, DM yield increased from 5.03 to 8.46 t $ha^{-1}$ in the DRCC and from 5.03 to 8.60 t $ha^{-1}$ in the TRCC, respectively.

Table 2. Forage leaf to stem ratio, DM yield, and DM concentration of a DRCC and a TRCC harvested at three stages of development in a spring growth

| Item | DRCC | | | SEM | TRCC | | | SEM | *p-value*[1] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | MB | EBL | MBL | | MB | EBL | MBL | | C | SD | C × SD |
| Leaf to stem ratio, % | 40.4 | 34.9 | 32.6 | 1.23 | 45.1 | 40.6 | 38.1 | 1.09 | < 0.001 | < 0.001 | 0.798 |
| DM yield, Mg $ha^{-1}$ | $5.03^c$ | $5.56^b$ | $8.46^a$ | 0.535 | $5.03^c$ | $6.83^b$ | $8.60^a$ | 0.521 | 0.001 | < 0.001 | 0.001 |
| DM concentration, g $kg^{-1}$ | $175^c$ | $189^b$ | $196^a$ | 3.1 | $221^a$ | $199^c$ | $213^b$ | 3.2 | 0.001 | < 0.001 | 0.001 |

DRCC = Diploid red clover cultivar, K 39; TRCC = Tetraploid red clover cultivar, K 32; MB = Mid Bud stage of development; EBL = Early Bloom stage of development (10% of flowering); MBL = Mid Bloom stage of development (40–50% of flowering); SEM = Standard error of the mean; ; C = Cultivar; SD = Stage of development; [1]Means in the same row with different superscript letters are significantly different ($p < 0.01$)

## Protein and carbohydrate fractions of the tetraploid and the diploid red clover cultivars

Significant differences ($p < 0.01$) were found in CP concentration of the red clover cultivars (Table 3). The TRCC had a higher CP concentration than the DRCC (Table 3). The forage CP concentration was 12.5%, 8.7%, and 10.4% greater in the tetraploid than the diploid cultivar at the first, the second, and the third stage of development, respectively. Although the TRCC had a higher CP concentration, it also had higher NPN and SolP concentration ($p< 0.01$) than the DRCC.

The NDICP and ADICP concentrations, on the other hand, were higher in the DRCC than in the TRCC. With advancing plant growth and development, the NDICP concentration increased from 110 to 238 g $kg^{-1}$ CP in the TRCC, and the ADICP concentration increased from 110 to 203 g $kg^{-1}$ CP in the DRCC and from 79.4 to 123 g $kg^{-1}$ CP in the TRCC, respectively. The maximum TP concentration was observed at the MB stage of development in the DRCC, whereas the highest IP concentration was observed at the MB stage of development in both red clover cultivars (747 g $kg^{-1}$ CP in the DRCC and 728 g $kg^{-1}$ CP in the DRCC, respectively).

The CP fraction concentrations of the studied red clover cultivars were significantly influenced by cultivar, stage of development and their interactions ($p< 0.01$). The largest CP fraction in all investigated stages of development was $PB_2$, and this CP fraction was greatest at the MB stage of development (602 g $kg^{-1}$ CP in the DRCC and 618 g $kg^{-1}$ CP in the TRCC, respectively). Concentration of PA protein fraction was the highest at the EBL stage of development (Table 3). The PC fraction increased from the MB to the MBL stage of development (110 to 203 g $kg^{-1}$ CP in the DRCC and from 79.4 to 123 g $kg^{-1}$ CP in the TRCC, respectively).

Table 3. Forage concentrations of primary and CNCPS protein fractions of a DRCC and a TRCC harvested at three stages of development in a spring growth

| Item | DRCC | | | SEM[6] | TRCC | | | SEM[6] | *p-value*[1] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | MB | EBL | MBL | | MB | EBL | MBL | | C | SD | C × SD |
| CP, g $kg^{-1}$ DM | 160 | 149 | 135 | 3.6 | 180 | 162 | 149 | 4.5 | < 0.001 | < 0.001 | 0.034 |
| NPN, g $kg^{-1}$ DM | $214^c$ | $357^a$ | $268^b$ | 20.8 | $449^a$ | $368^b$ | $287^c$ | 23.4 | < 0.001 | < 0.001 | < 0.001 |
| SolP, g $kg^{-1}$ DM | $252^c$ | $283^a$ | $275^b$ | 4.6 | $272^c$ | $393^a$ | $339^b$ | 17.6 | < 0.001 | < 0.001 | < 0.001 |
| NPN, g $kg^{-1}$ SolP | $847^b$ | $1000^a$ | $978^a$ | 24.1 | $1000^a$ | $935^b$ | $847^c$ | 22.2 | 0.011 | < 0.001 | < 0.001 |
| TP, g $kg^{-1}$ DM | $786^a$ | $642^c$ | $732^b$ | 20.9 | $551^c$ | $635^b$ | $713^a$ | 23.4 | < 0.001 | < 0.001 | < 0.001 |
| IP, g $kg^{-1}$ DM | $747^a$ | $718^c$ | $725^b$ | 4.5 | $728^a$ | $613^c$ | $661^b$ | 16.6 | < 0.001 | < 0.001 | < 0.001 |
| ADICP, g $kg^{-1}$ DM | 110 | 118 | 203 | 14.9 | 79.4 | 86.6 | 123 | 77.0 | 0.697 | 0.536 | 0.319 |
| NDICP, g $kg^{-1}$ DM | $145^c$ | $320^a$ | $280^b$ | 26.5 | $110^c$ | $168^b$ | $238^a$ | 18.5 | < 0.001 | < 0.001 | < 0.001 |
| PA, g $kg^{-1}$ CP | $214^c$ | $286^a$ | $268^b$ | 10.8 | $272^c$ | $365^a$ | $287^b$ | 14.5 | < 0.001 | < 0.001 | < 0.001 |
| $PB_1$, g $kg^{-1}$ CP | $38.6^a$ | $0.00^b$ | $0.63^b$ | 6.401 | $0.00^c$ | $25.3^b$ | $51.9^a$ | 7.521 | < 0.001 | < 0.001 | < 0.001 |
| $PB_2$, g $kg^{-1}$ CP | $602^a$ | $397^c$ | $445^b$ | 30.9 | $618^a$ | $445^b$ | $423^b$ | 30.8 | < 0.001 | < 0.001 | < 0.001 |
| $PB_3$, g $kg^{-1}$ CP | $35.0^c$ | $199^a$ | $76.9^b$ | 24.59 | $30.8^c$ | $72.1^b$ | $115^a$ | 12.13 | < 0.001 | < 0.001 | < 0.001 |
| PC, g $kg^{-1}$ CP | $110^b$ | $118^b$ | $203^a$ | 14.9 | $79.4^c$ | $92.6^b$ | $123^a$ | 6.5 | < 0.001 | < 0.001 | < 0.001 |

DRCC = Diploid red clover cultivar, K 39; TRCC = Tetraploid red clover cultivar, K 32; MB = Mid Bud stage of development; EBL = Early Bloom stage of development (10% of flowering); MBL = Mid Bloom stage of development (40–50% of flowering); SEM = Standard error of the mean; CP = Crude Protein; NPN = Non-Protein Nitrogen (N× 6.25); SolP = Soluble Protein; TP = True Protein; IP = Insoluble Protein; ADICP = Acid Detergent Insoluble Crude Protein; NDICP = Neutral Detergent Insoluble Crude Protein; PA = instantaneously solubilised protein; $PB_1$ = rapidly degradable protein; $PB_2$ = intermediately degradable protein; $PB_3$= slowly degradable protein; PC = completely undegradable protein; C = Cultivar; SD = Stage of Development; [1]Means in the same row with different superscript letters are significantly different ($p < 0.01$)

The DRCC had a greater ($p< 0.01$) CHO concentration than the TRCC (700 *vs* 674 g $kg^{-1}$ DM at the MB stage of development, 723 *vs* 715 g $kg^{-1}$ DM at the EBL stage of development, and 738 *vs* 724 g $kg^{-1}$ DM at the MBL stage of development). Results of this study showed that NFC and aNDF concentrations increased from MB to MBL stage of development in both studied red clover cultivars, but no significant cultivar × stage of development interactions were observed (Table 4).

Table 4. Forage concentrations of primary and CNCPS carbohydrate fractions of a DRCC and a TRCC harvested at three stages of development in a spring growth

| Item | DRCC | | | SEM | TRCC | | | SEM | *p-value*[1] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | MB | EBL | MBL | | MB | EBL | MBL | | C | SD | C × SD |
| CHO, g kg$^{-1}$ DM | $700^{c}$ | $723^{b}$ | $738^{a}$ | 5.6 | $674^{c}$ | $715^{b}$ | $724^{a}$ | 7.7 | < 0.001 | < 0.001 | < 0.001 |
| TESC, g kg$^{-1}$ DM | $75.5^{c}$ | $133^{a}$ | $120^{b}$ | 8.85 | $99.7^{c}$ | $121^{b}$ | $130^{a}$ | 4.64 | 0.001 | < 0.001 | < 0.001 |
| NFC, g kg$^{-1}$ DM | 301 | 332 | 327 | 5.4 | 272 | 303 | 320 | 7.1 | < 0.001 | < 0.001 | 0.035 |
| aNDF, g kg$^{-1}$ DM | 422 | 438 | 449 | 4.0 | 422 | 438 | 440 | 3.1 | 0.237 | < 0.001 | 0.221 |
| ADF, g kg$^{-1}$ DM | $315^{b}$ | $319^{b}$ | $355^{a}$ | 6.4 | $286^{b}$ | $327^{a}$ | $329^{a}$ | 6.9 | < 0.001 | < 0.001 | < 0.001 |
| Hemicellulose, g kg$^{-1}$ DM | $107^{b}$ | $118^{a}$ | $93.6^{c}$ | 4.26 | $135^{a}$ | $111^{b}$ | $111^{b}$ | 4.18 | < 0.001 | < 0.001 | 0.001 |
| Lignin, g kg$^{-1}$ DM | $47.9^{b}$ | $44.8^{c}$ | $55.9^{a}$ | 1.77 | $35.1^{b}$ | $52.1^{a}$ | $54.3^{a}$ | 3.12 | 0.047 | < 0.001 | < 0.001 |
| CA, g kg$^{-1}$ CHO | $108^{c}$ | $184^{a}$ | $163^{b}$ | 11.5 | $148^{c}$ | $170^{b}$ | $180^{a}$ | 4.9 | < 0.001 | < 0.001 | < 0.001 |
| $CB_1$, g kg$^{-1}$ CHO | 48.2 | 38.2 | 36.6 | 2.36 | 41.5 | 36.2 | 36.1 | 1.87 | 0.187 | 0.055 | 0.649 |
| $CB_2$, g kg$^{-1}$ CHO | $273^{a}$ | $237^{b}$ | $243^{b}$ | 6.4 | $214^{c}$ | $218^{b}$ | $225^{a}$ | 2.1 | < 0.001 | 0.018 | 0.001 |
| $CB_3$, g kg$^{-1}$ CHO | $406^{a}$ | $391^{b}$ | $375^{c}$ | 4.7 | $472^{a}$ | $401^{b}$ | $378^{c}$ | 14.4 | < 0.001 | < 0.001 | < 0.001 |
| CC, g kg$^{-1}$ CHO | $164^{b}$ | $149^{c}$ | $182^{a}$ | 4.9 | $125^{b}$ | $175^{a}$ | $180^{a}$ | 9.1 | 0.110 | < 0.001 | < 0.001 |

DRCC = Diploid red clover cultivar, K 39; TRCC = Tetraploid red clover cultivar, K 32; MB = Mid Bud stage of development; EBL = Early Bloom stage of development (10% of flowering); MBL = Mid Bloom stage of development (40–50% of flowering); SEM = Standard error of the mean; CHO = Total Carbohydrates; TESC = Total Ethanol Soluble Carbohydrates; NFC = Non-Fibre Carbohydrates; aNDF = amylase treated Neutral Detergent Fibre; ADF = Acid Detergent Fibre; CA = instantaneously degradable carbohydrates; $CB_1$ = Starch; $CB_2$ = intermediately degradable carbohydrates; $CB_3$ = available cell wall; CC = unavailable cell wall; [1]Means in the same row with different superscript letters are significantly different ($p < 0.01$); C = Cultivar; SD = Stage of Development

The TRCC was higher in rapidly degradable CHO fraction of soluble sugars (CA) than the DRCC except at the EBL stage of development where it was the opposite. The $CB_1$ concentration was similar at all stages of development, and no significant difference existed between the two cultivars. Available cell wall ($CB_3$) was the largest CHO fraction in this investigation, followed by the slowly degradable ($CB_2$) CHO fraction. As the stages of development progressed, concentrations of CA and CC fractions increased, whereas those of $CB_1$ and $CB_3$ decreased (Table 4).

## Protein and carbohydrate degradability profile

Despite the fact that the TRCC had a greater CP concentration due to a larger leaf to stem ratio, its CP was more degradable in the rumen than that of the DRCC (Table 5). The DRCC had a higher IDRUP score at the MB and EBL stages of development, but a lower IDRUCHO score at the MB and MBL stages of development ($p< 0.01$) than the TRCC (Table 5).

Table 5. Concentrations of degradable and undegradable proteins and carbohydrates of a DRCC and a TRCC harvested at three stages of development in a spring growth

| Item | DRCC | | | SEM | TRCC | | | SEM | *p-value*[1] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | MB | EBL | MBL | | MB | EBL | MBL | | C | SD | C × SD |
| RDCHO, g kg$^{-1}$ CHO | $639^{b}$ | $659^{a}$ | $632^{c}$ | 4.2 | $661^{a}$ | $632^{b}$ | $631^{b}$ | 4.9 | 0.274 | < 0.001 | < 0.001 |
| RUCHO, g kg$^{-1}$ CHO | $360^{b}$ | $344^{c}$ | $368^{a}$ | 3.7 | $339^{b}$ | $368^{a}$ | $369^{a}$ | 4.9 | 0.605 | < 0.001 | < 0.001 |
| RDP, g kg$^{-1}$ CP | $726^{a}$ | $713^{b}$ | $655^{c}$ | 11.0 | $761^{a}$ | $767^{a}$ | $722^{b}$ | 7.1 | < 0.001 | < 0.001 | < 0.001 |
| RUP, g kg$^{-1}$ CP | $274^{c}$ | $287^{b}$ | $345^{a}$ | 11.0 | $239^{b}$ | $233^{b}$ | $278^{a}$ | 7.1 | < 0.001 | < 0.001 | < 0.001 |
| IDRUCHO, g kg$^{-1}$ RUCHO | $197^{a}$ | $194^{b}$ | $186^{c}$ | 1.7 | $214^{a}$ | $194^{b}$ | $188^{c}$ | 3.9 | < 0.001 | < 0.001 | < 0.001 |
| IDRUP, g kg$^{-1}$ RUP | $166^{b}$ | $180^{a}$ | $146^{c}$ | 4,9 | $162^{a}$ | $147^{b}$ | $161^{a}$ | 2.5 | < 0.001 | < 0.001 | < 0.001 |
| N:CHO, g N kg$^{-1}$ CHO | $36.6^{a}$ | $33.4^{b}$ | $29.3^{c}$ | 1.06 | $42.7^{a}$ | $36.9^{b}$ | $32.9^{c}$ | 1.42 | < 0.001 | < 0.001 | < 0.001 |
| PA:CA, g N kg$^{-1}$ CA | $72.8^{a}$ | $51.8^{b}$ | $48.2^{b}$ | 3.95 | $78.5^{a}$ | $79.3^{a}$ | $52.4^{b}$ | 4.44 | < 0.001 | < 0.001 | < 0.001 |
| PB:CB, g N kg$^{-1}$ CB | $33.9^{a}$ | $29.9^{b}$ | $23.6^{c}$ | 1.51 | $38.0^{a}$ | $30.6^{b}$ | $30.3^{b}$ | 1.27 | < 0.001 | < 0.001 | < 0.001 |
| PC:CC, g N kg$^{-1}$ CC | $24.4^{c}$ | $25.9^{b}$ | $32.7^{a}$ | 1.28 | $27.1^{a}$ | $19.0^{c}$ | $22.5^{b}$ | 1.23 | < 0.001 | < 0.001 | < 0.001 |

DRCC = Diploid red clover cultivar, K 39; TRCC = Tetraploid red clover cultivar, K 32; MB = Mid Bud stage of development; EBL = Early Bloom stage of development (10% of flowering); MBL = Mid Bloom stage of development (40–50% of flowering); SEM = Standard error of the mean; RDCHO = Rumen Degradable Carbohydrates; RUCHO = Rumen Undegradable Carbohydrates; RDP = Rumen Degradable Protein; RUP = Rumen Undegradable Protein; IDRUCHO = Intestine Degradable Rumen Undegradable Carbohydrates; IDRUP = Intestine Degradable Rumen Undegradable Protein; N:CHO = Nitrogen:carbohydrate attribute; PA:CA = the soluble rapidly degradable N:CHO attribute; PB:CB = the insoluble slowly degradable N:CHO attribute; PC:CC = unavailable and completely undegradable N:CHO attribute; C = Cultivar; SD = Stage of Development; [1]Means in the same row with different superscript letters are significantly different ($p < 0.01$);

The RDP decreased and the RUP increased in the DRCC as the plant matured. On the other hand, the RDCHO and IDRUCHO decreased in the TRCC as the plant matured, whereas the concentration of RUCHO increased. The DRCC had a higher undegradable PC:CC concentration than the TRCC, while the TRCC had a higher total N:CHO, rapidly degradable N:CHO (PA:CA), and insoluble potentially degradable PB:CB concentrations ($p < 0.01$) than the DRCC (Table 5).

## Discussion

Leto et al. (2004) found that red clover cultivars grown at higher altitudes achieved higher DM yields, primarily due to more moderate temperatures, higher rainfall and better rainfall distribution during the year. When red clover harvested at the same stage of development, plants may differ in nutritive value due to climatic, seasonal and geographical conditions (Buxton 1996). Vasiljević et al. (2011) also indicated that genetic and seasonal variation in fibre content is one of the main factors that influences quality variation. Studying the content of structural CHO fractions, Vasiljević et al. (2008) found statistically significant differences in five red clover varieties harvested in different seasons, while there were no differences between varieties.

Higher CP, NPN and SolP concentrations in TRCC suggest that its CP would be more rapidly degraded in the rumen. Forage concentrations of PA and $PB_2$ fractions reported in the current study were higher and that of $PB_3$ fraction was lower than concentrations reported by Krawutschke et al. (2013). These authors also indicated that red clover plants with a high proportion of leaves contained the smallest PA fraction. It is probably because the proteolytic activity in leaves increases with advancing plant maturity. We assume that non-protein nitrogen compounds in young red clover leaves are immediately converted to proteins, so these compounds do not belong to the PA protein fraction.

The results obtained in the current study are generally in agreement with those reported by Homalka et al. (2012) for cell wall components of red clover cultivars at various stage of development. Balancing the appropriate level and type of non-structural and structural carbohydrates is a major challenge in formulating ruminant diets. Diet ingredients differ in the amount and composition of structural and non-structural CHO, and the CHO fractions differ in the rate of fermentation and fermentation end-products (Hall and Hejrek 2001), so for these reasons, the amount of those CHO fractions in diets greatly affects animal performance. The increasing of RUCHO concentration could be explained by an increase in forage concentration of structural CHO and lignin, as well as a decrease in the leaf to stem ratio, as the plant matures.

The N:CHO attributes (PA:CA, PB:CB, and PC:CC) in both red clover cultivars may have a considerable benefit in animal feeding, minimizing N excretion into the environment and reducing energy expenditure for ammonia to urea conversion, according to Jonker et al. (2010). Current findings demonstrate that red clover has excessive N per unit of CHO, as reported by Yu et al. (2004) and Jonker et al. (2011) for lucerne CP and CHO profile. Red clover has to be cut at a late stage of maturity, at the MBL stage of maturity, in order to improve N utilization and limit N excretion into the environment.

Other researchers (Hoover and Stokes 1991, Stern et al. 1994) found that ruminal degradable CHO and CP are required for microbial protein production. According to Yari et al. (2012), the ideal N:CHO ratio for microbial development is roughly 32 g N $kg^{-1}$ CHO. A lower N:CHO ratio will impact microbial development (protein synthesis), while a greater N:CHO ratio will affect the deamination of excess CP into energy and ammonia (Van Duinkerken et al. 2005). Red clover has a larger proportion of RUP and a better fibre digestibility than lucerne, according to Broderick et al. (2001), although milk production from dairy cows fed red clover are typically lower than expected.

Forage quality should be improved so that animals can be fed adequately during the long winter periods. Nutrient deficiencies in forages affect the growth of microorganisms and fermentation in the rumen, and as a result have lower animal productivity (Assefa and Ledin 2001). Therefore, the choice of superior genotypes in terms of yield and quality is very important for designing diets, which significantly contribute to high meat and milk production (Tavlas et al. 2009).

## Conclusions

Our results indicate that cultivar and stage of development had a significantly influence on the CP and CHO profile of investigated red clover cultivars. TRCC was higher in leaf to stem ratio, DM yield, DM concentration, CP, NPN, SolP, PA, $CB_3$, RDP and IDRUCHO concentration, as well as N:CHO, PA:CA and PB:CB attributes than DRCC at all stages of development. With advancing stage of development leaf to stem ratio, CP, $CB_1$, $CB_3$ and IDRUCHO concentration and N:CHO and PB:CB attributes decreased, whereas DM yield, ADICP, PC, CHO, aNDF and ADF concentration increased in both investigated red clover cultivars. CP of TRCC was characterized by higher degradability in the rumen. Both red clover cultivars are characterized by high nutritive value and could be successfully used in ruminant nutrition. However, further research is required to determine CP and CHO profiles of red clover across different seasons and environments.

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