

# Leaf morphogenesis influences nutritive-value dynamics of tall fescue (*Lolium arundinaceum*) cultivars of different leaf softness

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**Abstract.** The objective of this study was to compare the dynamics of neutral detergent fibre (NDF), and the 24-h *in-vitro* digestibility of NDF (NDFD) and dry matter (DMD) in leaf blades of two tall fescue (*Lolium arundinaceum* (Schreb.) Darbysh.) cultivars of different leaf softness: a soft- and a tough-leaved cultivar. The experiment was conducted during the summer regrowth of three replicated, dense mini-swards per cultivar arranged in a completely randomised design, all grown under non-limiting water, nitrogen and phosphorus. Cultivars were harvested eight times over 14 weeks to measure morphogenetic traits and nutritive value in six predefined leaf-age categories (from growing to complete senescence). The leaf lifespan and leaf length of the first three successive leaves were measured on 30 marked tillers throughout the experiment. Following analysis of variance, linear regression models were fitted to describe variations of NDF, NDFD and DMD with increasing leaf age and leaf length. Similar leaf NDF contents were found for the two cultivars, which remained stable throughout the leaf lifespan and increased markedly during leaf senescence. Leaf NDFD and leaf DMD both declined with increasing leaf age and length for the two cultivars. However, owing to shorter leaf lifespan of the soft-leaved cultivar, this decline in leaf NDFD and leaf DMD was faster for the soft- than for the tough-leaved cultivar. These results suggest that the soft-leaved cultivar will require more frequent defoliations than the tough-leaved cultivar to prevent decreases in nutritive value.

**Additional keywords:** defoliation, leaf development, leaf morphology, leaf stage, leaf turnover, temperate grasses.

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## Introduction

One of the main obstacles for more widespread use of tall fescue (*Lolium arundinaceum* (Schreb.) S.J. Darbyshire (syn *Festuca arundinacea*)) in dairy and livestock systems is the low nutritive value and acceptability of traditional cultivars (Burns 2009, and literature therein), commonly described as ‘tough-leaved’. Hence, alternative soft-leaved cultivars have recently been selected in an effort to improve forage digestibility and consequently to achieve improved animal intake and performance (Hopkins *et al.* 2009). However, to exploit these expected superior attributes further, the relationships between plant morphogenesis, leaf traits and forage nutritive value must be investigated.

The nutritive value of tall fescue and other forage grasses has been described extensively in terms of neutral detergent fibre (NDF) and dry matter digestibility (DMD) at the whole-sward level (e.g. Burns *et al.* 2002; Callow *et al.* 2003; Fulkerson *et al.* 2007; Donaghy *et al.* 2008; Raeside *et al.* 2012; Scheneiter *et al.* 2016). Conversely, only a few mechanistic studies have documented the effect of leaf morphogenesis on the evolution

of the NDF, DMD and NDF digestibility (NDFD) of successive leaves developed during a regrowth period (Groot and Neuteboom 1997; Duru and Ducrocq 2002; Avila *et al.* 2010; Agnusdei *et al.* 2011; Di Marco *et al.* 2013). Study of the alterations in forage nutritive value at the leaf level not only takes into account the main grazable fraction of plants, but also allows a direct separation of the effect of leaf turnover from several pasture traits that influence forage nutritive value, such vegetative–reproductive development, pasture herbage mass, and its morphological composition. Additionally, compared with measurements of NDF and DMD alone, the analysis of leaf NDFD could be considered a better indicator of changes in forages nutritive value, because NDFD is not affected by changes in cell soluble contents (Oba and Allen 1999), a leaf trait that can markedly vary within days, between days and between seasons.

On the other hand, leaf senescence is the main pathway for decline in forage nutritive value during a given vegetative pasture regrowth cycle. The onset of this crucial process can be monitored easily and accurately on the basis of the tiller’s ‘leaf

stage' (Fulkerson and Donaghy 2001), which is defined by the interval required to reach the maximum genotypic number of live leaves per tiller (NLL) that a given grass species or cultivar is able to retain (Lemaire and Chapman 1996). Accordingly, the leaf-stage defoliation-management approach (Fulkerson and Donaghy 2001) can be implemented to define the maximum defoliation interval required to optimise the forage production and nutritive value of grazed plants. This defoliation interval also corresponds to the leaf lifespan (LLS) of the species, expressed in thermal time, which is another relatively stable, plant-related indicator to synchronise grazing frequency with leaf tissue turnover (Lemaire *et al.* 2009). The leaf-stage management concept was first developed for perennial ryegrass (*Lolium perenne*) by Fulkerson and Slack (1994). Thereafter, the concept was further extended to the management of a wider range of plant species including kikuyu grass (*Pennisetum clandestinum*; Reeves *et al.* 1996; Fulkerson *et al.* 1999), prairie grass (*Bromus willdenowii*; Fulkerson *et al.* 2000; Slack *et al.* 2000; Turner *et al.* 2006b), cocksfoot (*Dactylis glomerata*; Rawnsley *et al.* 2002; Turner *et al.* 2006a) and tall fescue (Donaghy *et al.* 2008; Raeside *et al.* 2012). The assumption of the leaf-stage management concept is that forage nutritive value does not change appreciably before leaf senescence (Fulkerson and Donaghy 2001). However, it has been demonstrated in different temperate (Groot and Neuteboom 1997; Di Marco *et al.* 2013) and tropical grasses (Avila *et al.* 2010; Agnusdei *et al.* 2011) that the NDFD can decrease significantly during the LLS of leaves. This phenomenon has not been quantified for tall fescue, including soft-leaved tall fescue cultivars selected for lower leaf-tissue tensile strength.

In order to improve understanding of the underlying mechanisms that govern changes in forage nutritive value, information at a whole-sward level must be integrated with detailed investigations that effectively separate the direct effects of morphological changes of leaves during pasture regrowth from those inherent to ageing. Likewise, information is lacking on the pattern of leaf nutritive-value variation with leaf stage in successive leaves produced on tall fescue tillers during regrowth. Information is also lacking on comparisons of plant morphogenesis and leaf nutritive value between tough- and soft-leaved tall fescue cultivars that can confirm the superior forage attributes of cultivars selected for low leaf-tensile strength (Fribourg and Milne 2009; Hopkins *et al.* 2009).

The present study was performed first to compare the leaf nutritive value dynamics of two continental tall fescue cultivars differing in leaf softness, and second to analyse the relationship between leaf turnover and forage nutritive value of tall fescue cultivars. If differences between cultivars were detected, a third aim was to evaluate whether the defoliation regime that results in similar forage nutritive values for both cultivars is shorter than the minimum two-leaf stage reported as adequate for this species (Donaghy *et al.* 2008). To this end, the NDF, 24-h *in-vitro* NDFD, and DMD of leaf blades for the first three, successively appearing leaves were evaluated at different leaf stages during the summer regrowth of a soft- and a tough-leaved tall fescue cultivar. The findings of the study should contribute to knowledge of leaf morphogenesis effects on nutritive value dynamics of vegetative grasses.

## Materials and methods

### Experimental conditions

The study was carried out from 8 December 2009 to 15 March 2010 (vegetative summer growth) at an experimental site established in Balcarce, province of Buenos Aires, Argentina (37°45 S, 58°18 W, 130 m a.s.l.). The soil was a typical Argiudol (Soil Survey Staff 2014) with a top A horizon 25 cm deep, 62 g kg<sup>-1</sup> of organic matter content and pH 6.2. The study included two continental tall fescue cultivars differing in leaf softness, both grown under experimental conditions of water and natural ambient temperature (daily average of 21°C ± 2.8°C, Fig. 1). The tough-leaved cultivar was cv. El Palenque Plus<sup>INTA</sup> (referred to hereafter as 'tough-leaved'). This is a local cultivar developed from the preceding continental cv. El Palenque MAG, and is widely used in the region. The soft-leaved cultivar was cv. Grassland Advance<sup>Gentos</sup> (referred to hereafter as 'soft-leaved'). This cultivar was developed in the 1990s by AgResearch, New Zealand (Easton and Pennell 1994), from the selection of tall fescue plants for low leaf tensile strength (Hopkins *et al.* 2009). This cultivar was chosen for its promising improvement in forage nutritive value (Donaghy *et al.* 2008) and milk production comparable to perennial ryegrass (Milne *et al.* 1997; Chapman *et al.* 2008).

The two cultivars were sown in winter (4 August 2009) in three replicated mini-swards of area 2 m<sup>2</sup> arranged in a completely randomised design. Sowing of cultivars was conducted by using conventional tillage practices and a recommended seeding rate of 25 kg ha<sup>-1</sup>. Applications of 200 kg nitrogen (N) ha<sup>-1</sup> as urea and 100 kg phosphorus (P) ha<sup>-1</sup> as triple superphosphate were applied during sowing. Three weeks after sowing, plants were thinned to a density of ~200 plants m<sup>-2</sup> in order to ensure fast canopy closure. Thereafter, a pre-experimental phase from September to November was used to obtain a dense leafy canopy (~5000 tillers m<sup>-2</sup>) in all mini-swards. During this phase, mini-swards were defoliated three times at regular intervals by clipping all plants to a 40-mm stubble. Once this phase was completed, a last defoliation of plants to a 40-mm stubble was performed

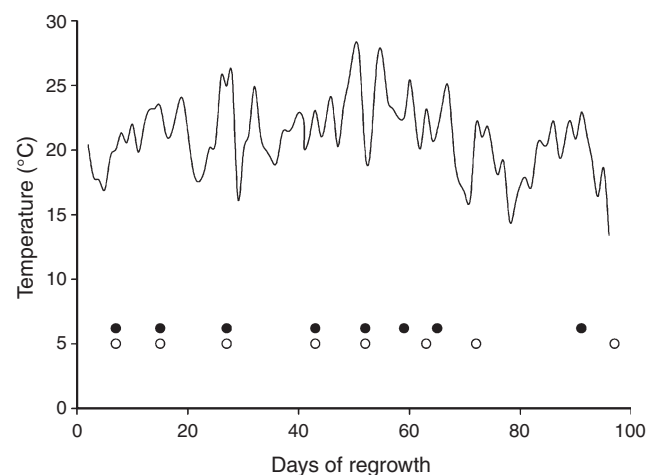


Fig. 1. Daily average temperature for the experimental period 8 December 2009–15 March 2010. Symbols denote successive harvest events for a soft-leaved (●) and a tough-leaved (○) cultivar.

(8 December 2009) to synchronise all mini-swards to the same stubble condition and similar tiller leaf stage.

The experimental phase started on 8 December 2009 and lasted until 15 March 2010. The experiment was planned to ensure non-limiting nutrients and water, so additional N and P fertiliser and irrigation were applied as follows. Urea fertiliser (138 kg N/ha) was split evenly over three equal applications in weeks 0, 5 and 10 of the experimental phase. This split application was decided on to synchronise N supply with the plants ability to use pulses of N, as well as to mitigate the loss of N from volatilisation, runoff or leaching. Triple superphosphate (12 kg P ha<sup>-1</sup>) was applied only once at the beginning of the experimental phase. Irrigation was conducted periodically (2–3 days) to maintain soil moisture at ~0.6 of field capacity.

Temperature was monitored with four Watchdog data loggers (Spectrum Technologies Inc., East-Plainfield, IL, USA), spaced at the centre (two data loggers) and at the opposite outermost points (two data loggers) of the experimental area. Data loggers were placed between tillers at ground level. The accumulated growing degree-days (GDD) were calculated as the sum of the daily average temperature above a base temperature of 4°C (Peacock 1976; Hutchinson *et al.* 2000). The GDD was used to express time of regrowth and leaf age in thermal units.

#### Morphological and structural measurements

Five quadrats 0.2 m by 0.2 m per mini-sward (15 per cultivar) were left uncut to measure morphogenetic and structural traits of leaves during the regrowth period, using methods adapted from Davies (1993). One tiller per uncut quadrat was randomly identified and permanently marked by a loop of coloured, plastic-coated wire. Marked tillers of similar size were selected by measurement of the tiller's sheath tube length. This sampling intensity (15 marked tillers per cultivar) and the methodology to measure morphogenetic traits were consistent with previous studies conducted with tall fescue and other forage grasses (Gastal and Nelson 1994; Berone *et al.* 2007; Agnusdei *et al.* 2011; DiMarco *et al.* 2013). Additionally, a compromise between using a few marked tillers and conducting greater frequency of measurements per tiller was decided to improve further the precision to quantify morphogenetic changes over time (Kuehl 2000). All morphogenetic measurements were conducted three times per week for 14 weeks, on ~seven successively formed leaves per marked tiller. They included measurement

of leaf appearance, leaf blade length, sheath tube length, and the onset of leaf senescence (decrease in green blade length). These data were used to estimate the following morphogenetic traits: leaf elongation rate (LER, mm day<sup>-1</sup>), leaf appearance interval (LAI, degree-days), LLS (degree-days), NLL, and final leaf length (i.e. leaf blade length in mm at ligule appearance). LAI was calculated as the GDD accumulated between two successive leaf appearances, and LLS as the GDD accumulated between leaf appearance and the onset of leaf senescence.

#### Destructive harvests

Destructive harvests were started when, in most tillers, the first leaf to appear (L1) had ~70 mm of leaf-blade length, and concluded when the third leaf to appear (L3) was completely senescent. The frequency of successive harvests was scheduled to coincide with the expansion of each successively formed leaf per tiller (i.e. ligule appearance), hereafter referred to as L1, L2, L3...Lx. The mean interval between successive harvests was 12 ± 6 days, but this varied with the thermal time to emergence of one new leaf (LAI) and the mean daily temperature in each interval. Therefore, because of faster leaf turnover in the soft-leaved cultivar (i.e. faster senescence), the last harvests were performed ~6 days earlier for the soft- than for the tough-leaved cultivar (Fig. 1, Table 1). At each harvest event, the herbage mass of ~1000 tillers from one randomly placed quadrat (0.5 m by 0.5 m) per plot was cut to a tiller base stubble. The collected material was immediately placed in liquid N<sub>2</sub> and then preserved in a freezer at -20°C. In the laboratory, the clipped material was thawed and a subsample of ~300 tillers (or equivalent material sufficient for nutritive value analysis) was randomly selected for further leaf measurements. Leaves of selected tillers were separated into leaf blade and leaf sheath components for measurement of blade and sheath length and blade nutritive value. Leaf blades of L1, L2 and L3 were further classified into six leaf-age categories: (i) growing (leaf blade <70 mm), (ii) just expanded (ligule appearance), (iii) adult (between ligule appearance and pre-senescence), (iv) pre-senescent (leaf blade tip senescent), (v) advanced senescence (between pre-senescence and completely senescence), and (vi) completely senescent (100% of leaf blade senescent) (Table 1). All remaining leaves (L4, L5, L6, L7, L8) were discarded, and all sorted leaf-blade fractions were then returned to the freezer until completion of leaf nutritive value analyses.

**Table 1.** Harvest number, days of regrowth and leaf age categories for the first three successive leaves (L1, L2, L3) produced following cutting of two tall fescue cultivars of different leaf softness

Successive leaves	Harvest number:							
	I	II	III	IV	V	VI	VII	VIII
L1	Growing	Expanded	Adult	Pre-senescent	Advanced senescence	Complete senescence		
L2		Growing	Expanded	Adult	Pre-senescent	Advanced senescence	Complete senescence	
L3			Growing	Expanded	Adult	Pre-senescent	Advanced senescence	Complete senescence
Days of regrowth								
Soft cultivar	7	15	27	43	52	59	65	91
Tough cultivar	7	15	27	43	52	63	72	97

### Leaf nutritive value analysis

Frozen leaf blades were freeze-dried to constant weight (Lyophilizer Rificor L-A-B4, Rificor SH, Buenos Aires, Argentina), ground to pass a 1-mm screen (Cyclotec Sample Mill 1093; Foss TECATOR, Höganäs, Sweden), and uniformly mixed for chemical analysis. The NDF content was determined on a 700-mg (dry matter) DM sample according to Van Soest *et al.* (1991) with a heat-stable  $\alpha$ -amylase and sodium sulfite (ANKOM<sup>200</sup> fibre analyser; ANKOM Technology, Macedon, NY, USA), and expressed inclusive of ash residual. A second 500-mg DM sample was placed in Dacron bags (F1020, ANKOM) and incubated *in vitro* for 24 h in the Daisy<sup>II</sup> apparatus (ANKOM Technology) with ruminal liquor from a donor steer fed a diet comprising (kg<sup>-1</sup> DM): 360 g lucerne hay, 360 g grass hay, 190 g ground maize grain and 90 g sunflower meal. After incubation, bags were boiled in neutral detergent solution (ANKOM<sup>200</sup> fibre analyser) to measure residual NDF (NDF<sub>r</sub>). The 24-h NDFD ((NDF × weight of incubated DM – NDF<sub>r</sub>)/(NDF × weight of incubated DM)) was estimated (Goering and Van Soest 1970) and expressed as percentage of DM. The 24-h apparent DMD was estimated by subtracting the metabolic factor 119 mg kg<sup>-1</sup> (Van Soest 1994) from the 24-h true DMD (1 – (NDF<sub>r</sub>/weight of incubated DM)) (Goering and Van Soest 1970). The determination of 24-h *in-vitro* digestibility (rather than 48-h digestibility) was decided as better representing the mean retention time of leaf grass particles in the rumen (Poppi *et al.* 1981) and the true *in-vivo* digestibility of forages typically reported for high-producing dairy cows at production intake level (Hoffman *et al.* 2003; Oba and Allen 2005; Di Marco *et al.* 2009).

Nutritive value traits of leaf blades (NV.leaf), including NDF, NDFD and DMD, were used to estimate the nutritive value of the pool of successively formed leaves per tiller (NV.pool) during a regrowth cycle. This NV.pool of NDF (NDFpool), NDFD (NDFDpool) and DMD (DMDpool) was calculated as follows:

$$\text{NV.pool}_j = \sum_{i=1}^3 (\text{NV.leaf}_{ij} (\text{LL}_{ij}/\text{TL}_{ij}))$$

where NV.leaf is NDF, NDFD or DMD of the individual leaf blades, LL is leaf blade length, TL is total LL per tiller, *i* is successive leaves (L1, L2, L3), and *j* is thermal time (GDD) from L1 emergence.

### Statistical analyses

Morphogenetic and structural data determined by monitoring of marked tillers were evaluated by using repeated-measures analysis. Final leaf blade and sheath length were compared between cultivars and among successive leaves (L1, L2, L3) by analysis of variance (ANOVA), using the MIXED procedure of SAS System 2000 (SAS Institute Inc., Cary, NC, USA) for a completely randomised design (*n* = 3). Data on nutritive value traits were compared between cultivars and successive leaves and among leaf-age categories by ANOVA, using the MIXED procedure of SAS. When significant effects were detected (*P* < 0.05), means were separated using a Tukey test at *P* < 0.05. In the text, mean values are presented ± standard error of the mean. Thereafter, linear regressions were fitted by using the GLM procedure of SAS to assess likely relationships

between NDF, NDFD or DMD, and increasing leaf age, leaf blade length or thermal time of regrowth. Regression models also considered the dummy variables cultivar (soft- or tough-leaved) or leaf number (L1, L2, L3) to test convergence (null hypothesis: intercepts are equal) and parallelism (null hypothesis: slopes are equal) between cultivars or among successive leaves. For these tests, the significance (*P* < 0.05) and root-mean-square error (RMSE) of models were determined.

## Results

### Leaf morphogenesis

There were no significant differences in LER or LAi (Table 2) or final leaf length between cultivars (Table 3). However, the soft-leaved cultivar had significantly lower LLS and NLL than the tough-leaved cultivar (Table 2). The low variability observed in morphogenetic traits was similar to (coefficient of variation 16% for LAi) or lower than (coefficient of variation 4% for LLS and 10% for LER) previous reports (Davies 1993; Pérez-Harguindeguy *et al.* 2013).

The final leaf blade and sheath length both increased for the first three consecutively formed leaves (Table 3). This association was properly described by a linear regression of final leaf blade length on leaf-sheath tube length:  $y = 0.27 \pm 0.02x + 3.10 \pm 4.10$ ,  $R^2 = 0.95$ ,  $P < 0.0001$  (*n* = 18).

### Leaf-blade nutritive value

There was no main effect of cultivar on leaf blade NDF, NDFD or DMD of the first three emerged leaves (Table 3). However, leaf blade NDFD and DMD both decreased in successive leaves (Table 3) and decreased with successive leaf-age category (from growing to completely senescent; Table 4). NDF content did not vary among leaves (Table 3), and increased only in the senescent leaf blades (Table 4).

The variation of leaf blade NDF, NDFD and DMD with increasing leaf-age category, expressed in thermal units (GDD), is shown in Fig. 2. NDF content was similar between cultivars (*P* > 0.05), and was relatively stable throughout the LLS (54.5% ± 3%), before increasing (*P* < 0.05) throughout senescence to reach 64.9% ± 0.8% (Fig. 2a). Conversely, leaf blade NDFD and DMD both continuously declined with the increase in leaf age, in particular throughout senescence (Fig. 2b, c). Rates of NDFD and DMD decline were higher for the soft- than the tough-leaved cultivar throughout LLS (parallelism *P* < 0.001, RMSE = 6.4% for NDFD and 3.8% for DMD), but no significant difference between cultivars was detected during senescence

**Table 2.** Leaf lifespan (LLS), number of live leaves per tiller (NLL), leaf elongation rate (LER) and leaf appearance interval (LAI) of two tall fescue cultivars of different leaf softness

Within a row, means followed by different letters are significantly different (*P* < 0.05); s.e., standard error

Morphogenetic and structural traits	Cultivar		Mean	s.e.	<i>P</i> -value
	Soft	Tough			
LLS (degree-days)	490b	632a		16	<0.001
NLL (leaves tiller <sup>-1</sup> )	2.5b	3.3a		0.2	0.025
LER (mm day <sup>-1</sup> tiller <sup>-1</sup> )	18	16	17	1.8	0.407
LAI (degree-days)	189	192	191	17	0.911

**Table 3.** Final length of leaf blade and sheath, neutral detergent fibre (NDF) content, and 24-h *in-vitro* digestibility of NDF (NDFD) and dry matter (DMD) for the first three leaves (L1, L2, L3) produced following cutting of two tall fescue cultivars (C) of different leaf softness

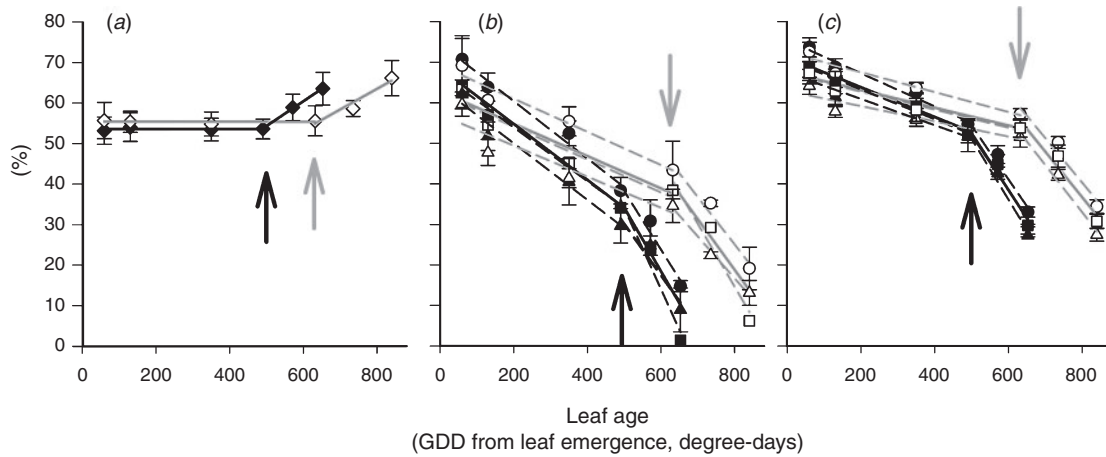
Within parameters, means followed by different letters are significantly different ( $P < 0.05$ ); s.e., standard error

Parameter and leaf	Cultivar		Mean	s.e.	C	P-value L	C × L
	Soft	Tough					
Final leaf blade length (mm)				7.1	0.177	<0.0001	0.147
L1	142	136	139c				
L2	255	222	239b				
L3	354	360	357a				
Mean	250	240	245				
Sheath length (mm)				2.8	0.301	<0.0001	0.270
L1	41	37	39c				
L2	73	64	69b				
L3	99	102	100a				
Mean	71	68	69				
NDF (%)				0.46	0.150	0.068	0.831
L1	55.8	56.6	56.2				
L2	55.3	56.5	55.9				
L3	57.3	59.5	58.4				
Mean	56.1	57.6	56.9				
NDFD (%)				0.82	0.123	<0.0001	0.641
L1	45.2	47.2	46.2a				
L2	37.7	41.4	39.5b				
L3	36.3	36.5	36.4c				
Mean	39.7	41.2	40.5				
DMD (%)				0.35	0.873	<0.0001	0.116
L1	56.7	57.5	57.1a				
L2	53.2	53.3	53.3b				
L3	51.0	49.9	50.4c				
Mean	53.6	53.6	53.6				

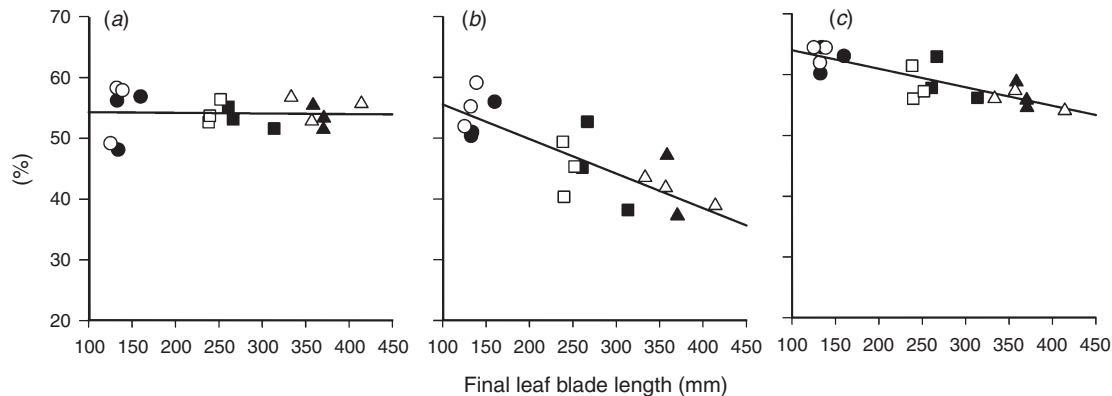
**Table 4.** Neutral detergent fibre (NDF) content, and 24-h *in-vitro* digestibility of NDF (NDFD) and dry matter (DMD) for the first three leaves produced following cutting of two tall fescue cultivars (C) of different leaf softness in six different leaf age categories (A)

Within parameters, means followed by different letters are significantly different ( $P < 0.05$ ); s.e., standard error

Parameter and age category	Cultivar		Mean	s.e.	C	P-value A	C × A
	Soft	Tough					
NDF (%)				0.79	0.071	<0.0001	0.817
Growing	53.2	55.7	54.4c				
Just expanded	54.1	55.4	54.7c				
Adult	53.5	54.8	54.2c				
Pre-senescent	53.6	55.6	54.6c				
Advanced senescence	58.9	58.6	58.8b				
Complete senescence	63.6	66.2	64.9a				
Mean	56.1	57.6	56.9				
NDFD (%)				1.45	0.218	<0.0001	0.250
Growing	65.7	64.0	64.9a				
Just expanded	57.4	54.3	55.9b				
Adult	46.1	47.3	46.7c				
Pre-senescent	34.0	38.8	36.4d				
Advanced senescence	26.5	29.0	27.7e				
Complete senescence	8.3	12.8	11.0f				
Mean	39.7	41.2	40.5				
DMD (%)				0.83	0.924	<0.0001	0.422
Growing	69.8	68.0	68.9a				
Just expanded	65.1	62.7	63.9b				
Adult	59.3	59.2	59.3c				
Pre-senescent	52.8	54.2	53.5d				
Advanced senescence	44.9	46.5	45.7e				
Complete senescence	30.0	30.9	30.5f				
Mean	53.6	53.6	53.6				



**Fig. 2.** Relationship between leaf age and (a) leaf neutral detergent fibre (NDF) content, and 24-h *in-vitro* digestibility of (b) leaf NDF and (c) leaf dry matter. Leaf age (expressed in growing degree-days, GDD, from emergence) is for the first three leaves produced following cutting of two tall fescue cultivars of different leaf softness: soft-leaved (black symbols and lines) and tough-leaved (grey symbols and lines). Arrows denote the onset of leaf senescence. Error bars indicate the corresponding standard deviations. In (a) symbols denote the average value for the three successive leaves to appear and lines describe the linear functions for the whole dataset. In (b) and (c) symbols denote each successive leaf (L1: ●○, L2: ■□, L3: ▲△), and lines describe the linear functions for either the whole dataset (solid lines) or for each successive leaf (dashed lines).



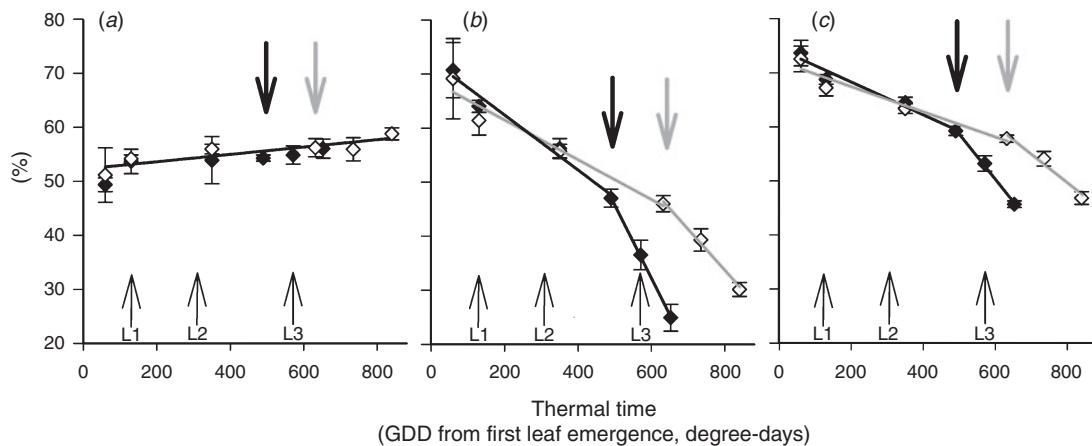
**Fig. 3.** Relationship between increasing final leaf blade length and (a) leaf neutral detergent fibre (NDF) content, and 24-h *in-vitro* digestibility of (b) leaf NDF and (c) leaf dry matter. Increasing final leaf blade length is for the first three leaves produced following cutting of two tall fescue cultivars of different leaf softness: soft-leaved (solid symbols) and tough-leaved (open symbols). Symbols denote each of the first three successive leaves (L1: ●○, L2: ■□, L3: ▲△).

(parallelism  $P > 0.05$ , RMSE = 6.0% for NDFD and 3.8% for DMD). Rates of NDFD and DMD decline with leaf age did not differ between successively formed leaves (parallelism  $P > 0.05$ , RMSE = 4.8% for NDFD and 2.5% for DMD), whereas the intercepts terms declined ( $P < 0.05$ ) with successive leaves (Fig. 2b).

The increment of final leaf blade length in successive leaves did not have any effect on leaf-blade NDF content, remaining stable ( $54.4\% \pm 2\%$ ) along the range of final leaf blade lengths (NDF =  $-0.0011 \pm 0.007x + 54.4 \pm 2.0$ ,  $R^2 = 0.001$ , RMSE = 3.0,  $P > 0.05$ ; Fig. 3a). By contrast, leaf blade NDFD and DMD both decreased with increasing final leaf blade length,  $\sim 0.6$  and  $\sim 0.3$  percentage points per 10 mm leaf blade, respectively

(NDFD =  $-0.057 \pm 0.01x + 61.2 \pm 2.7$ ,  $R^2 = 0.67$ , RMSE = 4.1,  $P < 0.0001$ ; DMD =  $-0.031 \pm 0.01x + 67.1 \pm 1.4$ ,  $R^2 = 0.70$ , RMSE = 2.1,  $P < 0.0001$ ; Fig. 3b, c).

The variables NDFpool, NDFDpool and DMDpool correspond to the nutritive value for the whole pool of leaf blades that appear during regrowth (Fig. 4). Therefore, these values consider the evolution of sward nutritive value from emergence until full senescence of L1 ( $\sim$ three leaves accumulated). During this thermal time, NDFpool increased slowly from  $\sim 50\%$  to  $55\%$  (NDF =  $-0.008 \pm 0.002x + 51.4 \pm 0.7$ ,  $R^2 = 0.43$ , RMSE = 2.4,  $P < 0.0001$ ; Fig. 4a). On the other hand, NDFDpool and DMDpool declined. For the soft-leaved cultivar, equations were: NDFD =  $-0.051 \pm 0.01x + 72.7 \pm 1.6$ ,  $R^2 = 0.91$ , RMSE = 3.0,



**Fig. 4.** Relationship between increasing thermal time of regrowth and (a) leaf neutral detergent fibre (NDF) content, and 24 h *in-vitro* digestibility of (b) leaf NDF (NDFD) and (c) leaf dry matter (DMD). Increasing thermal time of regrowth expressed in growing degree-days (GDD). Values represent the mean for the pool of leaf blades of the first three successive leaves (NDFpool, NDFDpool and DMDpool) produced following cutting of two tall fescue cultivars of different leaf softness: soft-leaved (black symbols and lines) and tough-leaved (grey symbols and lines). Downwards arrows denote the respective leaf lifespan (LLS) of cultivars: soft-leaved (black) and tough-leaved (grey). Upward arrows denote the leaf stage for one (L1), two (L2) and three (L3) leaves and correspond to the moment when each of the leaves had just expanded (visible ligule). Error bars indicate standard deviations.

$P < 0.0001$ ; and  $DMD = -0.030 \pm 0.003x + 74.4 \pm 0.9$ ,  $R^2 = 0.92$ ,  $RMSE = 1.7$ ,  $P < 0.0001$ . For the tough-leaved cultivar, equations were:  $NDFD = -0.037 \pm 0.01x + 68.9 \pm 2.0$ ,  $R^2 = 0.83$ ,  $RMSE = 4.2$ ,  $P < 0.0001$ ; and  $DMD = -0.023 \pm 0.003x + 72.1 \pm 1.0$ ,  $R^2 = 0.89$ ,  $RMSE = 2.0$ ,  $P < 0.0001$  (Fig. 4b, c). This decline in the NDFpool and DMDpool with increasing thermal time was similar to the decline in leaf blade NDFD and DMD observed for individual leaves (Fig. 2b, c). Further, the decline in the NDFDpool was greater than that of the DMDpool for both cultivars.

## Discussion

Tall fescue cultivars are being selected for leaf softness in an effort to improve forage digestibility and animal intake and performance when they are used as a forage source for beef and dairy cattle (Hopkins *et al.* 2009). However, the present study of *in-vitro* leaf nutritive value did not find any evidence of an association between divergent leaf softness in tall fescue cultivars and either leaf blade fibre content or 24 h *in-vitro* digestibility (i.e. NDF, NDFD, DMD) of successive leaves when compared in the same leaf-age category. The methodology used in this study was designed to separate the effects of leaf morphology (leaf length) and leaf age on leaf nutritive value, thereby allowing a more comprehensive and unconfounded comparison of cultivars. Only two such detailed studies examining morphogenetic effects at the leaf level have been reported for grasses (Groot and Neuteboom 1997; Agnusdei *et al.* 2011).

### Variation of nutritive value with leaf age

The increase in leaf blade NDF (from ~55% to 65%) and pronounced decreases in leaf blade NDFD (from ~36% to 11%) and DMD (from ~54% to 31%) observed with increasing leaf senescence in both cultivars (pre-senescent to completely senescent categories, Table 4) fully support

the generality of principles behind the leaf-stage concept. These principles indicate a need to set a maximum defoliation interval by the onset of senescence in order to avoid increasing fibre content and sharp losses in forage digestibility (Fulkerson and Donaghy 2001). It is noteworthy that the increase in NDF throughout leaf senescence stages does not reflect the production of new fibre in leaf tissue, but a relative increase in fibre content due to the export of cell contents towards other growing leaves during the senescence process (Robson and Deacon 1978). Further, the sharper reduction in digestibility (NDFD and DMD) during senescence is a result of the progressive increase in the proportion of senescent relative to live leaf tissue from the beginning of senescence until the leaf is completely dead.

In addition, and contrary to our expectations, the soft-leaved cultivar accumulated a lower NLL (2.5 live leaves per tiller) than the tough-leaved cultivar (3.3 live leaves per tiller). This difference in leaf stage occurred because the LLS of the former cultivar was ~145 GDD shorter (Table 2). Hence, according to Lemaire and Chapman (1996), the lower NLL in the soft-leaved cultivar results from the combined effect of two morphogenetic traits: (i) a similar leaf-appearance interval for both cultivars (Table 2), and (ii) a shorter LLS for the soft-leaved cultivar ( $NLL = LLS/LA_i$ ). This difference in tissue turnover between cultivars is somewhat surprising. Although the LLS is a genetically determined morphogenetic trait that can vary substantially among grasses (Lemaire and Chapman 1996; Lemaire and Agnusdei 2000; Agnusdei *et al.* 2007; Lemaire *et al.* 2009), there are very few reports of variations in LLS between cultivars of the same grass species (Silveira *et al.* 2010). Studies in the field of functional ecology have proposed variations in LLS as an adaptive strategy of plants to survive severe environments that are determined by genetically based physiological processes and, consequently, would explain the considerable genetic flexibility for LLS observed within some species (Chabot and Hicks 1982, and literature therein; Reich

*et al.* 1992). In this regard, a plausible reason for the difference in LLS between the soft- and tough-leaved cultivars could be related to a different sensitivity to shading (Reich *et al.* 1992). This different sensitivity can result from a phenotypic selection for more erect plants (e.g. soft-leaved cultivars) or for other cultivar-specific leaf morphological traits (e.g. specific area, thickness) that might be highly correlated with LLS (Ryser and Urbas 2000; Mediavilla *et al.* 2008).

The leaf-stage concept also assumes that leaf nutritive value does not appreciably change before the onset of senescence (Fulkerson and Donaghy 2001). However, the present study (Table 4, Fig. 2), and published data for different forage grasses (Wilson 1976; Groot and Neuteboom 1997; Duru and Ducrocq 2002; Avila *et al.* 2010; Agnusdei *et al.* 2011; Di Marco *et al.* 2013), demonstrate that while NDF is in effect a stable trait before senescence, DMD and/or NDFD can greatly decrease during LLS. These patterns of NDFD and DMD variation with leaf ageing were common for the first three successive leaves that appeared during the regrowth period (parallelism between L1, L2, L3 in Fig. 2*b* and *c*). The relative stability of the NDF content in the live green leaves is due to the fact that deposition of DM, and hence of cell-wall constituents, occurs only in the meristematic zone and only when the leaf blade is growing inside the sheath tube (MacAdam and Nelson 1987; Schnyder *et al.* 1987; Maurice *et al.* 1997). On the other hand, the decrease in NDFD and DMD could be a consequence of physical (Wilson and Mertens 1995) and chemical (Akin 1989; Jung and Allen 1995; Buxton and Redfearn 1997) cell wall changes that reduce fibre digestibility during leaf-tissue ageing. The data from the present study suggest that the decrease in DMD of live leaves is due to the decrease in NDFD rather than an increase in NDF. Further, DMD tended to decrease more slowly (~54% less) than NDFD (Fig. 2*b*, *c*) because of the relative stability of the NDF content.

The lower LLS of the soft-leaved cultivar (Table 2) concurred with the quicker succession of leaf-age categories in this cultivar than the tough-leaved cultivar. Therefore, although the nutritive value of the leaf blades was similar in both cultivars at the same leaf-age category (Table 4), meaning a similar range of values from appearance until the onset of senescence, the rate of decline in forage digestibility over time in the soft-leaved cultivar was ~2-fold that in the tough-leaved cultivar ( $-0.07$  v.  $-0.04$  NDFD percentage points per degree-day and  $-0.04$  v.  $-0.02$  DMD percentage points per degree-day) (Fig. 2*b*, *c*). However, it is important to note that the initial NDFD and DMD were numerically higher (+1.7 and +1.8 percentage points) and the final NDFD and DMD numerically lower ( $-4.8$  and  $-1.4$  percentage points) in the soft-leaved than the tough-leaved cultivar (although not significantly different; Table 4). Hence, these differences in initial and final values, together with the lower LLS in the soft-leaved cultivar, meant a greater range of variation and rate of decline in NDFD and DMD in the soft-leaved than the tough-leaved cultivar. As a result, throughout most of LLS (~55% of the LLS), the NDFD and DMD of leaf blades were lower for the soft-leaved cultivar. These findings therefore highlight the relevance that morphogenetic traits such as LLS could have for easy identification of plants with contrasting dynamics of forage nutritive value (or conservation of nutrients for animals) in plant breeding and selection programs.

#### *Variation of nutritive value with leaf length*

The two cultivars showed the same concurrent increase in final leaf blade length and decrease in NDFD and DMD across successive leaves (Table 3). According to the results of Agnusdei *et al.* (2011) for Rhodes grass (*Chloris gayana* Kunth), leaf blade length is the main determinant of the decrease in leaf nutritive value as successive leaves became larger. However, their data did not allow for a clear separation of the effect of leaf length from the inherent effect of leaf ageing.

In order to analyse and quantify the rate of decrease in leaf nutritive value with leaf blade length in the present study, leaves were analysed at a common leaf age category (i.e. adult, Fig. 3). This evaluation indicates that the lower NDFD and DMD values observed across successively emerged, longer leaves (Table 3) were associated with the increase in leaf blade length (Fig. 3*b*, *c*), as found for a range of forage species (e.g. for DMD: Duru and Ducrocq 2002; Chapman *et al.* 2012; for NDFD and DMD: Groot and Neuteboom 1997; Agnusdei *et al.* 2011; Di Marco *et al.* 2013), and not with changes in NDF content (Fig. 3*a*). This reduction in nutritive value with leaf blade length is also consistent with the progressively lower initial NDFD and DMD values noted across successively formed leaves through the LLS (Fig. 2*b*, *c*).

The decline in NDFD and DMD with increasing leaf blade length (0.6 and 0.3 percentage points per 10 mm, respectively), together with a leaf blade length difference of ~100 mm between successive leaves (Table 3), meant that the average decrease in NDFD and DMD between successive leaves was ~6 and 3 percentage points, respectively. Thus, the present findings reinforce the importance of leaf blade length as a structural variable with remarkable effect on forage nutritive value. It therefore follows from this analysis that the decrease in forage digestibility commonly viewed as a result of the addition of lower digestibility leaves is especially so if the length of these successive leaves increases, such as occurs during spring and summer regrowth. Further analysis of relationships between leaf length and nutritive value in other, cooler seasons, such as autumn, is required to generalise this hypothesis because changes in leaf length across successively formed leaves would not be as marked as in the present summer study (Lemaire and Agnusdei 2000).

#### *Variation in nutritive value of whole leaf pool*

The variation of forage nutritive value related to leaf turnover was evaluated for the whole pool of leaf blades present per tiller (Fig. 4). The NDF for this pool slightly increased with regrowth in both cultivars (Fig. 4*a*), as previously observed by Agnusdei *et al.* (2011) in Rhodes grass. This smaller variation in NDF for the leaf pool than found for individual leaves (Fig. 2*a*) was a consequence of the scarce contribution (<20%, Table 3) of the oldest senescing leaf (L1) to the pool. Furthermore, trends in leaf-blade digestibility for the pool of leaves (Fig. 4*b*, *c*) also declined at a slightly lower rate (~10% less) than observed with each individual leaf (Fig. 2*b*, *c*). This was because the higher digestibility of a newly formed leaf in a given tiller may offset, in part, the negative effect of leaf ageing in older leaves. In other words, although the youngest leaf at the 2- or 3-leaf stage was the longest leaf of the tiller, it was more digestible than all other older



and shorter leaves in that same tiller. This phenomenon may have partially masked cultivar differences in leaf ageing along LLS (compare Figs 4 and 2).

Results for leaf nutritive value at whole leaf-pool level (Fig. 4) indicate that defoliation intervals near the LLS (i.e. 490 and 630 GDD for soft- and tough-leaved cultivars, respectively) would provide similar forage nutritive value in both cultivars (estimated values calculated from equations in Fig. 4: 55.6% NDFpool, 46.6% NDFDpool and 58.4% DMDpool). Furthermore, these desirable maximum defoliation intervals corresponded to a tiller leaf stage (*sensu* Fulkerson and Donaghy 2001) of 2.5 and 3.3 live leaves for the soft- and tough-leaved cultivar, respectively (Table 2). These results therefore confirm the relevance of monitoring the onset of leaf senescence in terms of thermal time (i.e. LLS) or the number of live leaves per tiller (i.e. leaf stage) to gain control of the nutritive value of forages.

#### Controlling nutritive value with grazing management

Farmers and ranchers are often required to shorten defoliation intervals in order to meet specific pasture-management goals, such as improving the nutritive value of pasture when the requirements of the herd increase (Callow *et al.* 2003; Milne 2009; Chapman *et al.* 2012; Raeside *et al.* 2012; Nave *et al.* 2013). In this sense, intervals shorter than the LSS will limit declines in NDFD and DMD, as the present study shows (Fig. 4*b, c*). For example, defoliating the present tall fescue cultivars at the previously recommended 2-leaf stage (Donaghy *et al.* 2008) would improve the NDFDpool by ~11 percentage points (from 46.6% to 57.1%) and the DMDpool by ~7 percentage points (from 58.4% to 64.9%) compared with extended defoliation intervals defined by LLS or the leaf stage of 2.5 and 3.3 leaves for the soft- and tough-leaved cultivars, respectively. However, this proposition must take into account the likely trade-off of increasing forage nutritive value on herbage mass. In this sense, Chapman *et al.* (2012) proposed that a reasonable compromise would be obtained if the use of sufficient post-defoliation leaf area for fast pasture regrowth (i.e. 1500 kg DM ha<sup>-1</sup> or 5 cm stubble) has a small penalty on subsequent herbage mass accumulation. Thus, given the continuous decline in herbage digestibility with increasing herbage mass or leaf age (Fig. 4*b, c*), the present results suggest a complex trade-off between herbage mass and nutritive value that must be considered in interaction with the quantity and quality of post-grazing pasture to inform management priorities. Notwithstanding, evidence in the literature is quite contradictory, supporting (e.g. Nave *et al.* 2013) or denying (e.g. Scheneiter *et al.* 2016) an association between nutritive value and herbage mass. In this sense, additional analysis is needed to verify the association between forage nutritive value and herbage mass observed here in tall fescue pastures as related to the age and length of leaf blades.

Moreover, previous work in temperate (Duru and Ducrocq 2002; Di Marco *et al.* 2013) and tropical (Agnusdei *et al.* 2011) grasses proposed that the management of the initial sheath tube length can significantly affect the subsequent length of leaves and hence their nutritive value. In agreement with these studies, the strong relationship found here between leaf blade and sheath tube length ( $R^2=0.95$ ) suggests that any reduction in residual (post-defoliation) stubble could improve the leaf nutritive value

of pasture by reducing the sheath tube length and hence the negative effect of increasing leaf blade length on NDFD and DMD (Fig. 3*b* and *c*). If this hypothesis is correct, then it is reasonable to assume that any given residual post-grazing pasture containing a dense set of short sheath tubes (4–5 cm) and sufficient leaf area would produce a subsequent regrowth of short and highly digestible leaves that would minimise any unnecessary compromise between nutritive value and herbage mass. This can be achieved by using a consistent defoliation management that targets a leafy, dense and short sward structure (Chapman *et al.* 2012). Further studies of longer duration considering different post-grazing stubble heights and seasonal effects are required to confirm this expected variation in nutritive value and herbage mass as being affected by changes in sward structure, including linkages between concomitant changes in sheath tube and leaf length and herbage nutritive value.

#### Conclusions

Under the present study conditions, the soft-leaved tall fescue cultivar had a faster decline in leaf nutritive value, mainly due to a shorter LLS. This morphogenetic effect suggests that the maximum defoliation interval for the soft-leaved cultivar should be ~140 GDD shorter, or 0.8 less accumulated leaves per tiller (2.5 *v.* 3.3), than for the tough-leaved cultivar. This strategy will prevent faster decline in nutritive value due to leaf ageing and leaf senescence. The senescence process itself was the main driver for rapid declines in leaf NDFD and DMD with increasing leaf ageing. Aside from leaf ageing, an important driver for declines in NDFD and DMD was the increase in leaf blade length across successively formed leaves, a significant effect that was also associated with increases in sheath tube length. Therefore, the use of defoliation intervals that also consider the maintenance of a leafy, dense sward with sheath tubes no longer than 4–5 cm could be an effective measure to improve the nutritive value and production of tall fescue swards further.

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