Functional complementarity in diverse pastures: an agroecological approach for pasture-based dairy systems in temperate climates

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Abstract

The diversification of plant species within intensively managed pastures has been proposed as a potential solution to address climatic-driven issues of pasture-based livestock production. In New Zealand, pasture-based dairy systems are mostly composed of *Lolium perenne* L. with a smaller proportion of *Trifolium repens* L. *Bromus valdivianus* Phil. and *Dactylis glomerata* L. are grass species which have attributes of interest for pasture-based dairy systems, such as great productivity, quality and persistence. Previous studies have shown that these four species present distinct functional traits, such as deep- or shallow-root system and cool- or warm-season activity, among others. This thesis hypothesises their distinct functional traits to be complementary to each other when these four species comprise a diverse pasture. The leaf regrowth stage, plant-based grazing management, is adopted as the defoliation criteria management.

Three studies were developed in the thesis. The first study assessed and validated the hydraulic lift, a new functional trait occurring in the root system C_3 grass species. The second study investigated agronomical parameters and ecological features of a diverse pasture comprising *L. perenne*, *B. valdivianus*, *D. glomerata* and *T. repens*. When grown as a diverse pasture, these four species presented complementary functional traits, including growth asynchrony (seasonal growth activity) and development synchrony (temporal overlapping of their individual optimal LS intervals). Therefore, termed during the thesis as a diverse pasture of complementary species (DPCS). In relation to binary pastures (one grass and one legume component), DPCS presented lowered seasonality of production, annual and seasonal overyielding and a decrease in the ingression of volunteer species. The third study investigated the suitability of DPCS for dairy animals by measuring their nutritional quality, grazing behaviour, and dietary preferences of dairy cows. The animals displayed no preference when offered binary and DPCS simultaneously. Overall, this thesis provides enough evidence to support the advising of diverse pastures comprising *L. perenne, T. repens, B. valdivianus,* and *D. glomerata* defoliated according to the leaf regrowth stage as an alternative for pasture-based dairy systems in temperate humid climates.

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Table of Contents

List of Tables

List of Figures

Chapter 1

GENERAL INTRODUCTION

1.1. OVERVIEW AND THESIS OUTLINE

New Zealand's livestock production systems are predominantly pasture-based, and its dairy systems are mostly composed of *Lolium perenne* L. (perennial ryegrass) with a smaller proportion of *Trifolium repens* L. (white clover) (Manaaki Whenua 2018; Caradus et al. 2021). This combination of species has been the basis of New Zealand dairy systems over the past century (Roche et al. 2017), with pasture-based systems being a key factor for New Zealand's low-cost production and, therefore, international competitiveness (Holmes and Roche 2007). However, more intense and frequent extreme climatic events associated with climate change are challenging the conventional perennial ryegrass-based pasture systems (Lee et al. 2012; Keller et al. 2021). Enhanced seasonality of production, high susceptibility to the ingression of invasive species, low plant population persistence and mismatched pasture supply to cow feed requirements for milk production are among the main issues currently faced by dairy farmers in New Zealand (Briske and Richards 1995; Baudracco et al. 2010; Garcia and Holmes 2010; Hernández-Garay et al. 2010; Lane 2011; Tozer et al. 2011; Chapman et al. 2014).

More recently, there has been an interest in using increased diversification of plant species, such as the inclusion of other grasses, herbs, and legumes into pasture mixes in order to create more resilient, productive and persistent pastures (Cranston et al. 2015; Pembleton et al. 2015; Jaramillo et al. 2021; Stevens et al. 2021). However, the more diverse a system is, the more complex its management becomes due to an increment in the number of interaction levels between soil, several plant species, animal, and resource utilisation (by plants, e.g. water, mineral nutrients, light, etc., or by animals, e.g. plant species, nutrient pool and feed mass). In that sense, it is plausible for farmers to question the suitability of diversification: 'Which species should be included in my system?', 'Do these species fit together?' and 'If so, how to graze them?'. Scientific reviews on the topic of diversification by Sanderson et al. (2007) and Pembleton et al. (2015) suggest there is no simple answer to these questions. Overall, the choice of appropriate species depends on soil type, topography, climate and farming system in which a diverse pasture will be used (Pembleton et al. 2015). Beyond that, once suitable species are selected, grazing management (e.g. rotational or continuous grazing, defoliation criteria, fertilisation, animal category and stocking rate) may dictate the unfolding intra and inter-specific relationships developed among these species (Sanderson et al. 2007). In that sense, it is crucial to research these unfolding relationships as they regulate growth, competition, and persistence in a diverse pastoral ecosystem. Furthermore, only by developing scientific capabilities in the topic of pasture diversification will it be possible to deliver more appropriate grazing management guidelines to assist and guide farmers who intend to utilise diverse pastures as an alternative to cope with problems associated with climate change.

Following these foundational steps by Sanderson et al. (2007) and Pembleton et al. (2015) to enable systematic diversification, this thesis advances the overall scientific knowledge on utilising *L. perenne*, *Bromus valdivianus* Phil., *Dactylis glomerata* L., and *T. repens* to compose a diverse pasture for dairy systems in temperate humid climates such as New Zealand, which already are exposed to and affected by more extreme and frequent climatic events due to climate change (Tubiello et al. 2007; Kalaugher et al. 2017; Keller et al. 2021). More specifically, this thesis advances the experimental knowledge on (i) the underlying capabilities of functional processes within the root system of *L. perenne*, *B. valdivianus* and *D. glomerata*, (ii) the utilisation of the leaf regrowth stage interval, which is a plant-focused grazing decision tool, as a criterion for defoliation of diverse pastures and (iii) the grazing behaviour and acceptance by dairy animals grazing these diverse pastures.

Firstly, the choice of these four species is based on their individual suitability for grazing systems. In New Zealand, *L. perenne* and *T. repens* are the most widely researched and utilised grass and legume species, respectively, due to their easy and fast establishment, ability to produce seeds enabling commercial cultivation, great productivity and nutritional parameters and positive feedback under grazing disturbance (Brougham 1959, 1960; Hunt and Easton 1989; Belgrave et al. 1990; Caradus et al. 1996; Thom et al. 1996; Brock and Hay 2001; Bahmani et al. 2003; Stewart 2006; Lee et al. 2010; Poff et al. 2011; Thom 2011; Lee et al. 2012; Matthew et al. 2012; Dodd et al. 2018; Chapman et al. 2021). *Bromus valdivianus* is a temperate grass, originally from the South of Chile, where it is widely utilised in pasture-based grazing systems and presents great nutritional parameters and productivity, positive feedback under grazing disturbance, high acceptability by dairy animals, and the ability to withstand periods of water restriction (Stewart 1996; López et al. 1997; Balocchi et al. 2000; Turner et al. 2007; López et al. 2013; López et al. 2016; Ordóñez et al. 2017; Descalzi et al. 2018; Calvache et al. 2020a; Calvache et al. 2020b; García-Favre et al. 2021a; García-Favre et al. 2021b; Ordóñez et al. 2021; García-Favre et al. 2022). *Dactylis glomerata* is a temperate grass, with some strains and cultivars endemic to Mediterranean climate areas as well. It presents great productivity and persistence, positive feedback under grazing disturbance, and the ability to withstand periods of water restriction or drought (Volaire 1995; Volaire et al. 1998a; Volaire et al. 1998b; Volaire and Lelièvre 2001; Mills et al. 2006; Turner et al. 2006a, b, c, 2007; Lolicato and Rumball 2010; Turner et al. 2012; Gatti et al. 2016; Cosgrove et al. 2020). Secondarily, the choice of these four species is based on their potential ability to fit as a community due to their functional complementarity and to become a group of species to compose a type of diverse pasture for temperate humid climates.

The thesis is structured by publishable experimental chapters, utilising the ‘PhD by publications’ format and presents:

i. Literature review covering grazing management concepts, plant responses to grazing, animal grazing behaviour, ecological and morpho-physiological concepts behind pasture science, and species complementarity as an ecosystem feature.
ii. The first experimental chapter (study 1) approaches the soil-plant interface and examines and validates new morpho-physiological phenomena occurring in *L. perenne*, *B. valdivianus,* and *D. glomerata* in drought conditions.
iii. The second experimental chapter (study 2) approaches pasture succession (productive, nutritional and persistence parameters) under grazing conditions to determine the suitability of a diverse pasture composed of *L. perenne*, *B. valdivianus*, *D. glomerata*, and *T. repens* defoliated according to the leaf regrowth stage criteria.
iv. The third experimental chapter (study 3) approaches the suitability of the diverse pasture for dairy animals and assesses their preference traits when offered ryegrass-based and diverse pastures simultaneously.

v. General discussion and conclusion link the three experimental chapters and the general hypothesis under theoretical and tacit knowledge reasoning.

1.2. GENERAL HYPOTHESIS AND OBJECTIVES

The general hypothesis of this thesis was that *L. perenne*, *B. valdivianus*, *D. glomerata*, and *T. repens* have complementary functional traits that enable these species to coexist and function as a diverse pasture and, therefore, present high herbage productivity, enhanced short-term persistence, and high-quality nutritional feed resources for pasture-based dairy systems.

The objectives of this thesis were to:

i. Assess and validate the hydraulic lift phenomenon in *L. perenne*, *B. valdivianus* and *D. glomerata* under extreme drought conditions.
ii. Determine whether *L. perenne*, *B. valdivianus*, *D. glomerata*, and *T. repens* present enhanced ecosystem functionality as a diverse pasture due to their complementary functional traits.
iii. Determine the suitability of the leaf regrowth stage interval as a defoliation criterion for a diverse pasture comprised of *L. perenne*, *B. valdivianus, D. glomerata*, and *T. repens*.
iv. Determine whether there is a temporal overlapping of the different species' optimal intervals for defoliation and, if so, whether productivity, seasonality and persistence were affected.
v. Assess and determine the diet preference of dairy animals when offered ryegrass-based and diverse pastures simultaneously.

The practical aim of this thesis is to provide answers for farmers who wish to diversify their pasture systems.

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Chapter 2

LITERATURE REVIEW

“The primary form of food is grass. Grass feeds the ox: the ox nourishes man: man dies and goes to grass again; and so, the tide of life, with everlasting repetition, in continuous circles, moves endlessly on and upward, and in more senses than one, all flesh is grass.”

John James Ingalls (1872), Kansas,

US Senator 1873–1891

2.1. HISTORY AND BACKGROUND

2.1.1. Origin of grasslands biomes, grazing ungulates and pastoralism

The origin and evolutionary history of more open-spread vegetation types, namely grasslands and rangelands, took place in different stages during the Cenozoic geological era (66 to 0 mega-annum; Ma) (Strömberg 2011). However, the grasslands and rangeland only permanently advanced as a biome during the Neogene geologic period (23 to 2.5 Ma). As a consequence of a huge shift in the climate, the global weather became cooler, increasingly seasonal and drier in the mid-latitudes, which favoured grasses (initially C_3, and then, eventually, C_4 photosynthesising grasses) to spread and dominate the largest part of the vegetation on the planet (Saarinen 2019). Consequentially, this led to the co-evolution and dispersal of specialised grazing ungulates and other large herbivores (e.g. *Equidae*, *Equinae*, *Cervidae*, *Giraffidae*, *Bovidae*, *Elephantidae* etc.) (Janis 2008). Beyond the conjoined evolution of large herbivores, another extraordinary fact is believed to have happened in these ecosystems - the assumed cradle for the origin and evolution of early *Hominidae* took place in grassland areas (Squires and Feng 2018).

A multitude of definitions for grasslands are found in the literature; in a wider sense, the definition provided by the Food and Agriculture Organisation of the United Nations (FAO) in the book entitled ‘Grasslands of the World’ defines grasslands as ‘grazing lands’, covering around 52.5 million square kilometres (M km^2) by the year 2000’s. A more detailed definition is given by (White 1983), in which grasslands are described as land areas covered by vascular herbaceous plants with less than 10% of trees and shrubs in their composition (White 1983). Regarding their origin, grasslands can be classified into natural grasslands, seminatural grasslands, and improved (intensive) grasslands (Hejcman et al. 2013). In the Mongolian and Chinese seminatural grasslands, nomadic pastoralism activity (as a wider distribution of pastoral livestock husbandry) has been reported to exist during the Bronze Age [2000 to 700 BC Volkov (1967) as cited in Pfeiffer et al. (2018)]; yet, conclusive pieces of evidence with a specific chronology are difficult to prove, with the most recent evidence of charcoal traces and pollen profiles imprinted on the landscape being dated to not earlier than 900 to 600 years ago [Dinesman et al. (1989) as cited in Pfeiffer et al. (2018). In Europe, the first detailed written records on improved grasslands are dated from the Roman Empire [234 to 149 BC in ‘De Agricultura’ (Hejcman et al. 2013)]. Throughout the history of humanity, the grasslands most importantly contributed to food production. However, the ecosystem services provisioned by grasslands surpass human nutriment - for instance, carbon sequestration and allocation, improvement of water dynamics in the microclimate, habitat for many invertebrates, soil structure development preventing erosion and leaching, and others (Squires and Feng 2018).

2.1.2. Modern agriculture and livestock production: geographical distribution and intensification

The Green Revolution was an outstanding success due to a massive increase in the efficiency of production of crops, horticulture and animal husbandry, evolving from a familial to an industrial scale between the 1940s and the late 1970s. During those decades, the agricultural and livestock systems were focused on increasing their production and profitability while providing nutrition for an ever-increasing global population (Borlaug et al. 1969; Borlaug 1971). Global data from the 1990s shows that managed grazing areas [defined by Asner et al. (2004) as any geographically extensive operation designed to produce animals and animals' secondary bioproducts] covered approximately 34 M km^2, making it the single most extensive form of land use on the planet (Goldewijk 2001). These areas are subdivided into two major production systems: (a) grasslands, which are comprised of sown swards, intensively utilised, frequently ploughed and resown, and highly ecologically unstable, and (b) rangelands (e.g. steppe or savannah), which are seminatural grasslands, extensively utilised and presenting a more stable and resilient species biodiversity (Wesche et al. 2016). In the most recent info-document from FAO (2021), Asia is reported as the continent with the largest grassland area, approximately 11.1 M km^2, followed by Africa with 8.9 M km^2, South America with 4.0 M km^2, North and Central America with 3.7 M km^2, Oceania with 3.4 M km^2 and Europe with 2.3 M km^2.

In grasslands, management intensification is often associated with an increase in the number of animals utilising the pasture area, termed 'carrying capacity', which can be measured as 'stocking rate' (animal units [AU = 450 kg] per hectare; AU ha^{-1}). In that sense, the following figure by Asner et al. (2004) reveals that, even though Europe has the relatively smallest grassland area among the continents, it presents one of the highest levels of intensification - interpreted as stocking rate units (Figure 2.1). High stocking rates can also be found in countries like India due to their spiritual beliefs that often prevent the culling of old, sick or non-productive animals, and Kenya, where cattle represent the fundamental currency in the Maasai pastoralist society (National Geographic Society 2023). Particular attention must be given to New Zealand, which also presents high stocking rates and has a national economy heavily dependent on pasture-based and intensively managed commercial farms (Clark et al. 2010; Johnson et al. 2021; Caradus et al. 2023).

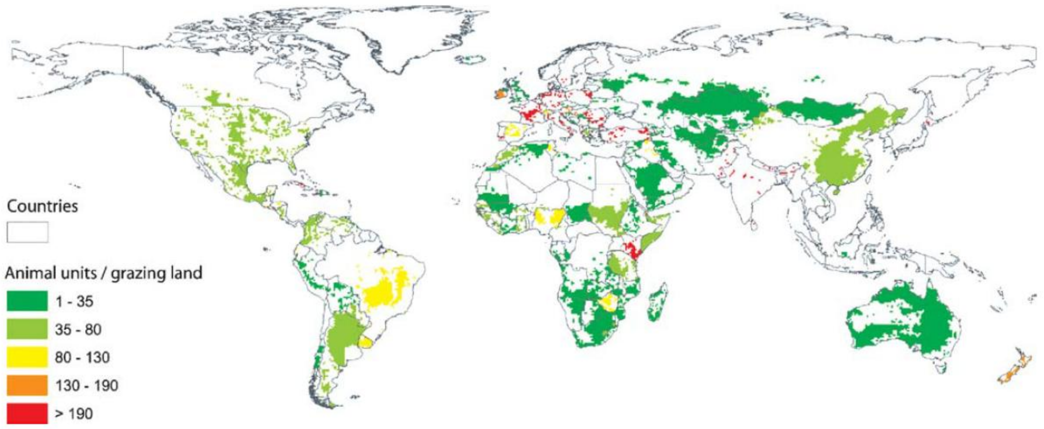

Figure 2-1. The global distribution of grasslands and grazing livestock, where the intensity of production is based on the average stocking rate (animal unit per square kilometre - AU km^{-2}). Source: Asner et al. (2004).

Grasslands and rangelands may comprise several plant clades; however, grasses (Poaceae or Gramineae family) are among the most abundant vascular plants in this ecosystem (Gibson 2009b). The grass family encompasses around 780 genera and over 12,000 species (Christenhusz and Byng 2016) and can be found in every continent, including Antarctica (Peterson 2013). Because grasses cover a wide range of the globe, with different climatic conditions, they developed two different physiological features to transform light, water, and carbon dioxide (CO_2) into chemical energy (Gibson 2009a). The difference between temperate or cool-season grasses (C_3 photosynthetic pathway) and tropical or warm-season grasses (C_4) is their efficiency in converting light into energy. In brief, C_4 plants utilise variations in the usual photosynthetic pathway (C_3 pathway) to use CO_2. The C_4 plants have a four-carbon compound (malate or acetate) as the first stable intermediate product of carboxylation, as opposed to the C_3 plants with a three-carbon compound (3-phosphoglycerate) as the first product. The main outcome of this difference is that C_4 plants transpire less water per molecule of CO_2 utilised, at a typical rate of 250 molecules of water lost for every molecule of CO_2 utilised by photosynthesis (Taiz and Zeiger 2002). This efficiency rate is also interchangeably termed ‘water use efficiency’, which will be more deeply discussed in section 2.2.5.

In the past century, breeding selection programs for temperate grass species have started in the United States, Europe, Australia, and New Zealand. The aim was to find pioneering grass species and varieties with higher agricultural value, which is defined as plant traits that will result in seedling vigour, improved flowering time, disease and cold resistance, persistence under defoliation, improved forage quality and digestibility, and lastly, but not least, increased forage productivity (Kneebone 1960; Rhodes et al. 2021). Nowadays, modern grasslands are basically composed of a few species of agricultural importance. In the temperate areas, *Dactylis glomerata* L., *Lolium perenne* L., *Festuca arundinacea* Schreb., and *Phleum pratense* L. are some of the most important species (Hunt and Easton 1989; Lolicato and Rumball 1994; Sleper and West 1996; Stewart and Ellison 2016); and in the tropical areas, *Brachiaria* spp., *Panicum maximum* Jacq., *Pennisetum purpureum* Schum., *Pennisetum clandestinum* Hochst. and *Cenchrus ciliaris* L. (Do Valle et al. 2001; Hanselka et al. 2004; Jank et al. 2011; Jank et al. 2014).

Even though the composition of modern grasslands has been simplified into species with the best agricultural value and potential performance, several stress factors (both biotic and abiotic) still have a critical influence on the final productivity and persistence of pastures (Caradus et al. 2021). Under natural environmental conditions in all agricultural lands worldwide, combining two or more stressors will often impact plant production and survival (Suzuki et al. 2014). Therefore, understanding plant responses to different constraints is essential to every individual in the agricultural industry (researchers, companies, and farmers). The following sections of this literature review will discuss the different causes of stress, disturbance and competition in pastoral ecosystems and the resulting soil-plant-animal-atmosphere relationships that drive the productivity, persistence, and resiliency in temperate pastures.

2.2. PLANT, SOIL AND ENVIRONMENT

2.2.1. Abiotic and biotic factors controlling plant growth

Environmental conditions often shape plant species' performance, so plants undergo adaptation, acclimation, and speciation processes over thousands of years of evolution (Osmond et al. 1987). Evidence suggests that agricultural systems are limited by environmental constraints (namely abiotic factors) to about a quarter of their potential yield (Boyer 1982; Hodgson 1990). Amongst a wide range of abiotic factors affecting plants, the three most relevant for the current thesis are temperature, irradiance, and water, which will be briefly described in the following subsections. However, it is worth mentioning that soil nutritional status and topography are also major influencers on plant production and persistence (Gastó et al. 1993). Beyond that, biotic factors, such as the choice of species, cultivars, and management approach (fertilisation, irrigation, defoliation frequency, grazing intensity, forage utilisation, and pest and weed control), also greatly influence the pastoral ecosystem. Amongst such a wide range of biotic factors, the plant responses to grazing management (both frequency and intensity) and choice of species will be thoroughly discussed in the following subsections.

2.2.2. The effects of temperature

As previously discussed, plants have developed different pathways to utilise carbon through photosynthesis. The temperature regulates photosynthesis and also mediates plant respiration, membrane fluidity and integrity (Nilsen and Orcutt 2000), such that agronomic performance and productivity are closely linked to fluctuations in temperature. Temperatures above 25 degrees Celsius (°C), which is the upper base temperature (T_B) for most temperate grasses (Duru and Ducrocq 2000; Moreno et al. 2014), can decrease the seasonal yield (tonnes of dry matter per hectare; t DM ha^{-1}) of rainfed *L. perenne* by 91% and of irrigated *L. perenne* by 47% (Rogers et al. 2019; Rogers et al. 2020).

The accumulation of heat from below T_B and above a specific lower base temperature (T_b) is one of the most popular measurements utilised in modern agriculture (Castillo and Ospina 2016); it is expressed as accumulated thermal time or growing degree-days (AGDD) and measured as the sum of daily mean temperatures above the threshold of a given species for a pre-defined period:

$$\sum_{1}^{n=period} = \left[\frac{(T_{max} - T_{min})}{2}\right] - T_b$$

In a review by Hutchinson et al. (2000), the T_b for temperate grasses was reported as approximately 5°C, and for *Trifolium repens* L. as 9 to 10°C.

2.2.3. The effects of irradiance

Light is the main source of plant energy, and plants can absorb a range of radiation (400 to 700 nanometres) via chlorophyll and then transform that solar energy into carbon compounds utilised for survival and growth (Taiz and Zeiger 2002). The conversion process of light into carbohydrates, namely photosynthesis, differs in efficiency from species to species, from component to component of the same plant and from age to age of the same components (Hodgson 1990). However, the majority of the potential conversion efficiency is determined by the quantity of light intercepted by the canopy (Stone et al. 1998), in such a way that access to light is essential for survival, persistence and, lastly, production of all plant species (Taiz and Zeiger 2002).

Moreover, light also triggers an important phenological event in plants, the transition from the vegetative to the reproductive stage. Photoperiodism regulates the flowering in plants as an active response to changes in day length (Taiz and Zeiger 2002). This is a decisive moment for intensively managed pastures when adjustments in the system's grazing intensity and frequency and, potentially, supply of outsourced nutrients for the grazing animals will be required. The effects of the reproductive stage in pasture management are highly important - several authors have reported a decline in the overall pasture nutritive quality during spring, associated with stem elongation, flowering and seeding (Fulkerson et al. 1998; Delagarde et al. 2000; Roche et al. 2009; Cullen et al. 2017).

2.2.4. The effects of water

All resource exchanges between a plant and its environment occur through the medium of water (Nilsen and Orcutt 2000), continuously absorbed from the soil solution by the root system due to a relatively more negative gradient of water potential in the plant tissues (Taiz and Zeiger 2002; Hodgson 1990). In photosynthesis, water is one of the energetic sources to convert CO_2 into carbohydrates that later become cellulose, glucose, fructose, ribose, deoxyribose, lactose, glycogen, sucrose, maltose, lactose, starch, etc. (Nelson and Cox 2013).

Under moderate soil water restriction, plants need to utilise multiple mechanisms to resist the water availability constraints, such as an increase in diffusive resistance, reallocation of carbohydrates, growth of the root system, and reduction in leaf size to decrease losses through transpiration (Siddique et al. 2016). Under extreme and prolonged soil water restriction, some plants may become dormant (Norton et al. 2008; Nie and Norton 2009). However, because of these new morpho-physiological adjustments and the plants' active responses to water restriction, a drop in pasture productivity may occur (Volaire et al. 1998; Bahmani et al. 2003; Poirier et al. 2012; Volaire et al. 2014).

2.2.5. Water use efficiency and plant adaptive strategies under drought

In a simple approach, water use efficiency (WUE) consists of the total DM produced per plant per unit of water use in such a way that a high WUE in plants is associated with an increased biomass yield per unit of water used (Kramer and Boyer 1995). The WUE is related to genetic potential, so that different plant species will diverge in performance under the same environmental conditions and water supply (Sinclair et al. 1984). Research in forage responses to drought has found that plants often present a better WUE after being exposed to drought conditions as a feedback response to stress (Marais et al. 2006; Kunrath et al. 2018), also called compensatory growth. Some plant responses to soil water restriction are related to plant species' ability to resist drought, which may occur as greater survivability of leaves and tillers, deeper root profiles, greater ability to maintain water status, compensatory osmotic adjustment, or dormancy (Barker et al. 1993; Norton et al. 2008).

Different plant adaptive mechanisms and within them, morpho-physiological strategies lead grasses to develop the ability to withstand periods of water restriction. Initially, the ‘drought resistance framework’ developed by Turner (1986) identified some physiological and biochemical characteristics that assisted plants in drought-prone environments. Later on, a review by Volaire (2018) proposed a conceptual framework defining in detail the drought-related terminologies presented by Turner (1986) and also other authors (Figure 2.2). According to Volaire’s review, most perennial grasses can withstand moderate drought and maintain leaf growth (elongation and appearance) and, therefore, forage productivity. These species present mechanisms to resist drought, namely ‘drought resistance’ traits (Volaire 2018). However, under continued or severe drought, only some plants will present features that facilitate survival, and these active responses will primarily occur in meristematic tissues, which are the plant organs capable of surviving severe drought. These species present ‘drought survival’ traits (Volaire 2018). Within these two main and broader adaptive mechanisms, another three strategies associated with short-term morpho-physiological responses to water stress are presented and defined in the conceptual framework. For annual plants (e.g. ruderals), the ‘dehydration escape’ allows the reproductive cycle of these species to be completed before the period or season of water shortage. Following, for perennials under 'moderate' water stress, 'dehydration avoidance' occurs as an attempt to reduce water loss via increased water uptake from the root system and stomatal regulation to optimise water retention. Finally, in the transition from 'moderate' to 'severe' water stress, which is usually above a possible lethal threshold of 30% of water content in plant tissues (Volaire et al. 1998; Volaire and Lelièvre 2000), perennials that have stress-tolerator features will activate 'dehydration tolerance' mechanisms, such as maintenance of turgor in leaves up to endogenous restriction of metabolic activity (dormancy).

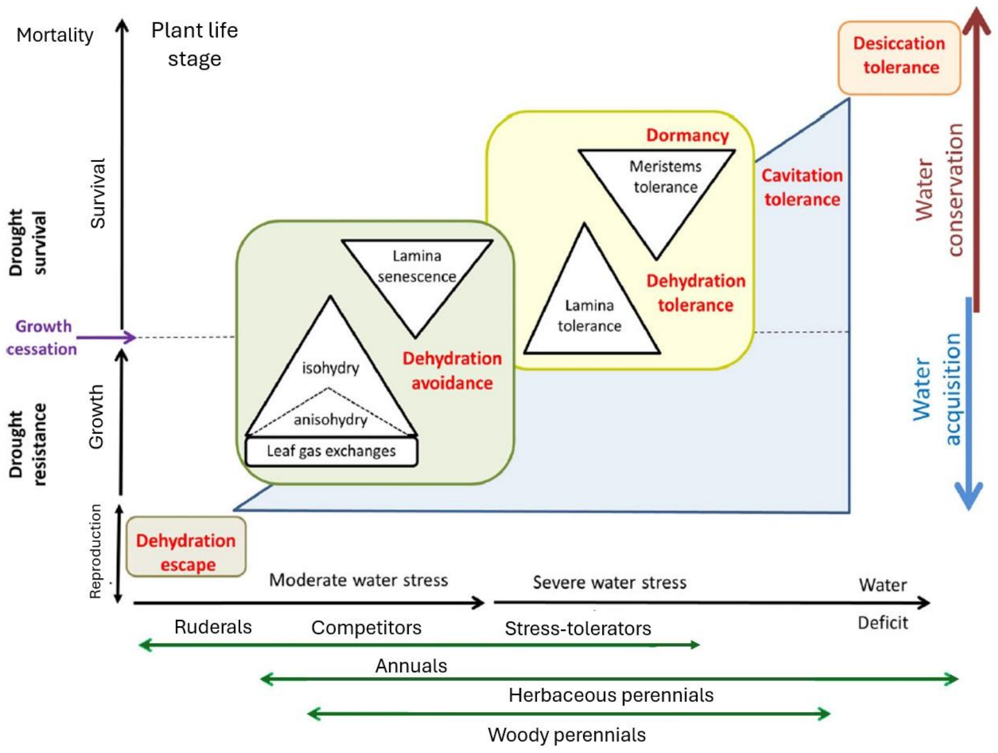

Figure 2-2. Physiological adaptation and strategies to plant responses as drought intensifies. Source: Volaire (2018).

Among some major research needs to enhance traits conferring drought survival in perennial grasses, (Norton et al. 2016) identified the necessity to develop the seed production capability of new cultivars and the development of agronomic management tools to produce stable mixtures combining perennial grasses and legumes. In that sense, it is necessary to re-access the repository of potential species to be used in pasture systems, aiming to find new species or cultivars suitable for areas currently facing more frequent and intensive periods of drought. Furthermore, it is of great importance to also consider areas that are expected to become drought-prone due to climate change. These potential species can be more suited to soil water-restricted periods, either by having longer roots or physiological mechanisms to cope better in these environments while maintaining production and plant species population.

2.2.6. The role of the roots in pastures

Within the first six to eight weeks after seedling appearance, grasses will have developed a fibrous root system, mostly composed of adventitious roots with their greatest proportion located in the upper 12 centimetres (cm) of soil (Beyrouty et al. 1990). Because of its intricate difficulty of assessment, there is a lack of consensus in the literature regarding the root growth and turnover patterns in pastoral ecosystems. Stuckey (1941) suggested that temperate grasses would present either annual (almost fully regenerated within a year) or perennial (maintained root systems with little regrowth) roots. However, recent studies show that root systems are time-, space- and ability-wise multifunctional (Lux and Rost 2012; Hoekstra et al. 2014; Fort 2023; Glass et al. 2023). In a detailed glasshouse hydroponic study regarding the root appearance and growth of *L. perenne*, Robin et al. (2010) found that root development normally occurs simultaneously on several adjacent phytomers over a period of about six leaf appearance intervals, starting on a given phytomer around the same time that the leaf appertaining to that phytomer starts to senesce. Thus, root dynamics is a continuous formation of new roots coordinated between adjacent phytomers and leaf development.

Regarding water uptake, the ability of grasses to source water from different soil profile layers depends on both the root architecture and the distribution and evolution of the hydraulic conductivities among root types and along the root length (Siddique et al. 2016). Grasses concentrate total root biomass in the shallow soil layers (0 to 10 cm), but some can have root lengths deeper than 2 metres (m) (Nippert and Knapp 2007). In that sense, grass species that present deep-rooting characteristics may be of agricultural interest because of the potential improvement in water utilisation in depth and throughout the soil profile, mostly when there is a lack of water in shallow layers due to drought events.

Seeking a better understanding of grass species and their different rooting characteristics, Crush et al. (2005) conducted a pot (1 m depth) experiment with eleven temperate forage grass species (*Agrostis capillaris* L., *Arrhenatherum elatius* L., *Bromus willdenowii* Kunth, *Cynosurus cristatus* L., *D. glomerata*, *Elytrigia repens* Nevski., *Lolium multiflorum* Lam., *L. perenne*, *Phalaris aquatica* L., *Poa pratensis* L., and *F. arundinacea*) and one extra cultivar of *L. perenne* (cv. RG185) bred for high surface root mass. The two ryegrass cultivars showed a different root distribution in depth, with the cultivar RG185 presenting more than 75% of roots within the upper 20 cm of the pots, as opposed to less than 65% from the other *L. perenne* cultivar. A distinctive distribution of the *C. cristatus* roots was found, with over 80% of the roots in the upper 10 cm layer. An accumulation of roots in the bottom of the tubes containing *B. willdenowii*, *L. multiflorum*, *P. aquatica* and *E.*

repens indicates that these four grasses can grow roots to a depth that exceeds 1 m. In a field study by (García-Favre et al. 2022) with *L. perenne* and *Bromus valdivianus* Phil. under sheep grazing in New Zealand, the monocultures of *B. valdivianus* presented 38% more root biomass in depth (31 to 70 cm) than monocultures of *L. perenne*. Beyond root depth and architecture, other root features and functions, such as the hydraulic lift, may be of agricultural interest in continuously pursuing more drought-tolerant grass species.

2.2.7. Hydraulic lift: a new perspective into the function of roots

Commonly associated with the drought tolerance of deep-rooting vascular plant species, the hydraulic lift is a functional property that some species exert by capturing water from deep soil layers and releasing it into shallower soil layers via roots (also cited as water reverse flow elsewhere). In 2022, a literature search on the Web of Science™ database found almost 3,000 publications about deep rooting and trees and over 1,500 publications about deep rooting and grasses. Substantially fewer results were observed when searching for deep rooting and hydraulic lift, with just over 200 publications on the topic. The oldest publication utilising the term ‘hydraulic lift’ to describe the phenomena was authored by (Caldwell and Richards 1989), and from 2004 onwards, a considerable increase in the number of publications (>10 per year) and citations (>200 per year) was observed.

The origin of research around the hydraulic lift dates from a publication by (Shone and Flood 1980), which at that time described the phenomenon as water uptake and loss via roots. Subsequently, (Corak et al. 1987) described the phenomena as an ecologically significant occurrence of water transfer via nongrafted roots. Later, appropriately named and described as ‘hydraulic lift’, the phenomenon was defined as a direct or indirect water redistribution process via a soil-root continuum exerted by vascular plants, initially observed in Artemisia tridentata Nutt. (Richards and Caldwell 1987; Caldwell and Richards 1989). Two research studies by (a) Vetterlein and Marschner (1993) and (b) Dawson (1993) demonstrated two techniques to unveil the hydraulic lift and its consequential water redistribution into different profiles of the soil: (a) indirect measurements using microtensiometers in different soil layers to assess the fluctuations on the soil water potential gradients (Ψs), and (b) direct measurements using standard water relations methods associated to stable isotope (e.g. deuterium; ^{2}H) techniques. After that, the phenomena have been largely modelled and demonstrated in literature for forestry systems and savannah woodland ecosystems due to the more extensive root system size of trees and shrubs that facilitates the hydraulic lift modelling, quantification and validation (Jackson et al. 2000; Ishikawa and Bledsoe 2000; Feddes et al. 2001; Scholz et al. 2008; Domec et al. 2010; Yu and D’Odorico 2014; Ferreira et al. 2018; Asensio et al. 2020; Bayala and Prieto 2019; Lee et al. 2021; Montaldo et al. 2021). The exploration around the hydraulic lift for trees and shrubs has been increasing significantly, up to complex soil-plant-atmosphere continuum models and predictions on commensalistic relationships of plants. Meanwhile, the same level of complexity in studies cannot be found for crops and forages. A few studies have assessed the hydraulic lift in cropping and foraging systems, for instance: Corak et al. (1987) with *Medicago sativa* L., and *Zea mays* L.; Sekiya and Yano (2004) with *Cajanus cajan* L., *Sesbania sesban* L. and *Z. mays*; Doussan et al. (2006) with *Lupinus angustifolius* L.; and Mahindawansha et al. (2019) with *Oryza sativa* L. and *Z. mays*. Independently on the smaller stature and biomass accumulation in relation to trees and shrubs, it is, directly and indirectly, proven that some crop and forage plants can also exert hydraulic lift. Nonetheless, there is still a considerable level of uncertainty about its scale and potential application to farming systems. Concerning the more ecological outcomes and applications of the hydraulic lift of an ecosystem, Bogie et al. (2018) and, subsequently, Singh et al. (2020) raised the concept of 'bioirrigation' into the debate. The bioirrigation hypothesis is based on the deliberate choice of species to be added in diversified agricultural ecosystems, such that the redistributed water by the added species would benefit the neighbouring plant, enhancing its chance of survival or growth during periods of drought.

2.3. PASTURE SCIENCE AND ADVANCES: THE PLANT-ANIMAL INTERFACE

2.3.1. Plant responses to defoliation: morpho-physiological features

Tillers are the basic unit of grass forage shoots and are morphologically formed by a series of phytomers (Nelson 2000). The phytomers grow and rise from the shoot apex and are morphologically formed by the leaf (lamina + sheath), stem (node + internode) and axillary bud. Later, the axillary buds, formed as part of a new phytomer, may develop into a new shoot apex for a lateral branch (daughter tiller), which can subsequently develop its own series of phytomers (Nelson 2000). Pasture above-ground biomass may consist of varying proportions of leaves, stems and inflorescence, with different compositions and ages depending on the season and growth stage (Sheaffer et al. 1998). The proportion of these morphological components, the tiller regrowth rate, and the appearance of new tillers can be manipulated by grazing since defoliation stimulates the plant to mobilise reserves into regrowth for survival (Bullock and Marriot 2000). Grazing also allows light into the canopy of the pasture, which stimulates tillering (Matthew et al. 1995).

In a rotational grazing system, an individual plant has the ‘opportunity to regrow’ from the prior defoliation until the next grazing event. However, the regrowth rate is greatly affected by previous defoliation frequency and intensity, and consequentially, the opportunity to manipulate both grazing variables has been the subject of extensive research, covering the most varied range of forage species ((Bahmani et al. 2003; Brougham 1957; Da Silva et al. 2009; Hoogendoorn 1986; Macdonald and Roche 2023). Figure 2.3a depicts the slow initial phase of plant tissue accumulation after defoliation, dependent on the non-structural carbohydrate (NSC) reserves of the roots and shoot residue after defoliation. A phase of exponential herbage accumulation follows, promoted by the higher abundance of NSC generated by the newly growing leaves, and finally, a plateau phase as the sward approaches ceiling yield. Once defoliated, pastures will present short-term initial responses, such as fluctuations in the carbon:nitrogen ratio (C:N) due to the removal of photosynthetically active plant tissues (shoot), and long-term secondary effects, such as structural adaptions of the plant species, with changes in the proportion of morphological components (Lemaire and Chapman 1996).

The short-term responses are dictated by the shoot regrowth, which is initially influenced by the levels of the NSC [interchangeably referred to as water-soluble carbohydrates (WSC) reserves for C_3 grasses] stored in the residual shoot left after defoliation (Fulkerson and Donaghy 2001); these enable the initial regrowth of the leaf canopy post-defoliation. After that stage, as a result of the initial regrowth, the tiller presents a certain photosynthetically active elongating leaf mass that can supply enough NSC from photosynthesis to sustain and exponentially increase the rate of plant regrowth (White 1973; Thornton et al. 2000). The rate of exponential increase of a given pasture is driven by the energetic efficiency of the new plant tissues and the number and activity of the tillers and/or stolons per ground area (Hodgson 1990). After some days, the NSC produced by the regrown leaves is large enough to allow the plant to allocate NSC into storage rather than strictly growth, which accumulates in the tiller base (bud) for subsequent use in the regrowth cycle following the next defoliation (White 1973; Irving 2015). At a period following the replenishment of plant energy reserves, leaf turnover may begin, a moment in which leaf tissues become more fibrous and senescent, and the net accumulation of herbage starts to decrease (Figure 2.3b). This is a critical moment in terms of pasture management since the substantial turnover of leaves indicates that the pasture should have been defoliated again, avoiding a detrimental decline in the nutritional quality of the herbage due to the increase of complex fibre content (Da Silva et al. 2009; Fulkerson and Donaghy 2001).

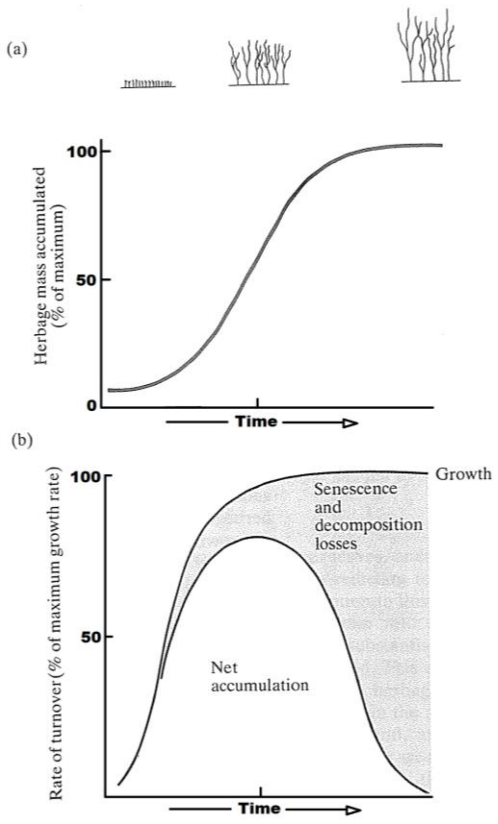

Figure 2-3. The pattern of plant tissue accumulation after defoliation. Source: Lemaire and Agnusdei (2000).

Pasture's regrowth cycle can be numerically observed in a study on *Brachiaria brizantha* Stapf. subjected to three defoliation strategies, two by canopy light interception (LI; target grazing at LI$_{95\%}$ and delayed grazing at LI$_{100\%}$) and one as a fixed period (28-day grazing cycle). The first 5 cm regrowth (initial phase of accumulation - first slope in Figure 2.3a) had a herbage accumulation of approximately 400 kilograms of dry matter per hectare (kg DM ha^{-1}), while the following 5 cm regrowth (exponential phase of accumulation) corresponded to approximately 1300 kg DM ha^{-1} during summer and spring of two consecutive years (Pedreira et al. 2017). To understand plant morphogenesis and its implications on grazing swards and defoliation strategies, three morpho-physiological features are presented as the basis for different growth responses after defoliation and can be utilised in the assessment and measurement of grass herbage mass accumulation [see Figure 2.4; Lemaire and Agnusdei (2000)]:

a) The leaf elongation rate (LER), which measures the daily increase in length of the individual leaf in millimetres per day (mm day^{-1}).
b) The leaf appearance rate (LAR), which measures the number of leaves appearing per unit of time.
c) The leaf lifespan (LLS), which measures the period in which a given portion of newly appearing leaf tissue remains green.

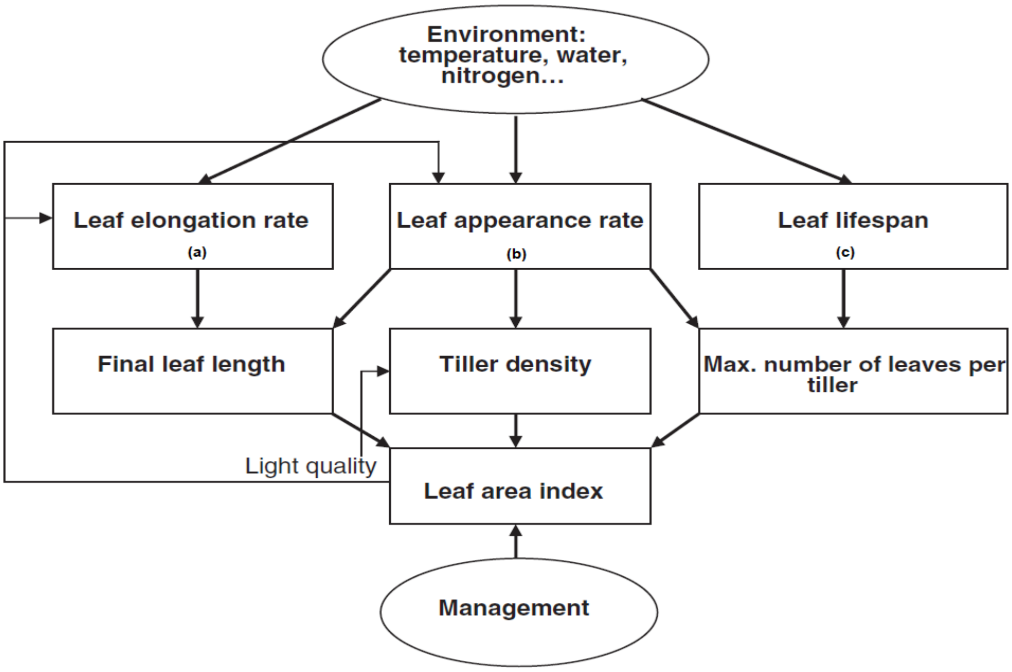

Figure 2-4. Relationship between morphogenetic variables and sward structural characteristics. Source: Lemaire and Agnusdei (2000); Lemaire et al. (2009).

The relationship between these three variables determines the regrowth dynamics in a pasture after defoliation, and compensatory effects seen as different regrowth strategies may change among different grass species (Matthew et al. 1995). Even though different regrowth strategies can still result in similar herbage accumulation in a given period. The utilisation of accumulated forage is the key aim of grazing management (Hodgson 1990), and this is because the consumption of pastures and crops is the main driver of animal and financial performance for pastoral systems (Neal et al. 2018; Beca 2020). Parsons and Penning (1988) indicated that the efficient use of herbage produced is the array between optimised light interception by the canopy and defoliation before senescence. Such a causal relationship has been previously characterised by Brougham (1958), who proposed that the optimum moment to defoliate a pasture is at around $LI_{95\%}$, measured as the quantity of irradiance that the leaves are intercepting and the amount of this that reaches the soil surface. In that sense, several studies by R. W. Brougham assessed the suitability of the targeted light interception rates by the pasture sward as criteria to better define the optimal moment to graze. These methodologies' scientific and practical suitability are further discussed in the following subsections.

2.3.2. Principles of rotational grazing management

Within rotational grazing systems, several different criteria have been proposed as the optimal time to decide when to defoliate pastures, based on visual indicators or a fixed rotation based on ‘n’ day intervals (Hoogendoorn 1986; John and Bird 2000). These types of assessments were, and still are, very popular among farmers once they are easily applicable and primarily envisage feed allocation according to animal intake or herbage allowance (Donaghy et al. 2021; Holmes et al. 1993; Brock and Hay 1993). As previously discussed, $LI_{95\%}$ has also been proposed as a method to define grazing frequency. However, it has never been widely employed on commercial farms due to the necessity for specialised devices in its assessment. Notwithstanding, its development highlighted the potential to be gained by parametrising rotation in grazing systems according to pasture structural features (Brougham 1957; Heitschmidt 1993; McCall and Sheath 1993). With the origins based on light interception, secondary practical tools, such as pre-grazing height targets and the rising plate-metre, accounting for both pasture density and sward height (Hodgson 1985; Barthram 1986), were developed to be practical and applicable to farming systems (Latinga et al. 2004; Da Silva et al. 2009; Macdonald and Roche 2023). These different management decision criteria provided farmers with a more accurate range of possibilities to manipulate rotational grazing. Conversely, as is expected from any type of technology, each one of these practical tools presents pros and cons.

From a plant science perspective, tools prioritising animal intake rather than plant physiology may negatively affect pasture persistence. For instance, in one of the experiments with *L. perenne* and *T. repens* pastures presented in (Holmes et al. 1993), a significantly higher stem and dead material proportion and a lower clover presence and digestibility content were found in the 'high mass' treatment, which was defoliated in a fixed grazing frequency of 90 days. Animals grazing the 'high mass' treatment presented significantly lower milk production and quality (fat and protein content). Among these detrimental consequences of applying fixed grazing dates, when intervals between grazing events are relatively long, the pasture nutritive parameters may no longer be optimal for animal nutritional requirements due to the onset of senescence and the consequent loss of quality, as discussed in section 2.3.1. Alternatively, when intervals between grazing are relatively short, plants might not yet be physiologically ready to be grazed, and within repeated grazing events, the pasture will experience a drop in tiller population and eventually persistence (Turner et al. 2007). In a review by Donaghy et al. (2021), these and other negative unfolding consequences of animal-focused grazing criteria are discussed in depth, raising the question of the suitability of these management practices for future intensive and rotational grazing systems. As a proposition to optimise grazing decisions, Donaghy et al. (2021) recommend utilising the leaf regrowth stage (LS) to determine grazing rotation, a criterion that considers plant morpho-physiological development.

2.3.3. Leaf regrowth stage: plant-focused grazing criteria

The development and growth of grasses are characterised by the repeated formation, expansion, and subsequent senescence of phytomers (Nelson and Moore 2020). The interval between these repeated and similar growth stages of successive leaves in the same phytomers is termed the phyllochron (Wilhelm and McMaster 1995), which, in practical terms, is assessed as the time between consecutive ligule (collar) appearances above the whorl of older sheaths (Nelson and Moore 2020). Plant genetics has a determining role in the phyllochron of each species, and even though more detailed genetic evaluation is necessary (Bayoumi 2005), it is well-established that the phyllochron is not a phenotypically plastic feature within the same grass genotype (Nelson 2000), and under even and favourable growing conditions, it remains relatively constant (Robson 1973; Moore and Moser 1995). Most of the variation in the phyllochron value of a given plant species occurs as an active response to changes in temperature and can be assessed by a thermal measure of time, such as AGDD (Rickman and Klepper 1995). To a much smaller extent, variation may also occur as a response to other environmental factors (Wilhelm and McMaster 1995), for instance, water stress tended to lengthen the phyllochron of *L. perenne* tillers (Barker et al. 1985; Volaire et al. 1998).

From a practical perspective, the phyllochron has been utilised as a basis to define the moment to apply certain agricultural practices based on the morpho-physiological development of grass crops, such as *O. sativa* (Nemoto et al. 1995), *Z. mays* (Hanway 1963), *Triticum aestivum* L. (Haun 1973) and *Sorghum bicolor* L. (Vanderlip and Reeves 1972). In perennial grasses, the rate at which new leaves are produced on a tiller can be measured by the phyllochron, which is inversely proportional to the LAR (Phyllochron = LAR^{-1}), as long as it is measured within the same accumulated thermal rate (i.e. AGDD) (Lemaire and Agnusdei 2000). Along those lines, a wide body of research has been undertaken regarding factors affecting LAR (and, interchangeably, the phyllochron) of the several varied perennial grass species (Moore and Moser 1995; Gastal and Lemaire 2015).

From the aforementioned research with its basis on grass development, a practical application was developed, termed the leaf regrowth stage (LS), which allows pasture management to be conditioned to morpho-physiological stages of grass plants as affected by the wide range of environmental factors occurring at a paddock-level (Fulkerson and Slack 1994). For example, *L. perenne* is termed a '3-leaf' plant because each tiller maintains around 3 live leaves at a time, with the oldest leaf (first to emerge) beginning to senesce as the youngest fourth leaf starts to appear (Robson 1973; Fulkerson and Slack 1994). Considering that, Fulkerson and Slack (1994) found a strong correlation between the leaf regrowth stages of *L. perenne* and two different morpho-physiological phenomena of importance for perennial grasses of agricultural value: (a) the rate replenishment of WSC reserves in the tiller base, and (b) the onset of oldest leaf senescence at around 3.5 LS.

These two events are crucial in terms of pasture defoliation management and can be applied as a criterion to define an optimal interval of defoliation in *L. perenne*-based pastures (Fulkerson and Donaghy 2001). The importance of the lower limit of the interval (replenishment of WSC reserves) is associated with a reduction in the persistence of the tiller population within the pasture, as WSC reserves will not yet be replenished for the next regrowth cycle. On the other extreme, the importance of the upper limit of the interval (onset of senescence) is associated with a reduction in pasture nutritive quality. Further in-field (Fulkerson and Donaghy 2001) and on-farm research (Clarke et al. 2021) established the practical interval for optimal defoliation of *L. perenne* ranging from 2.5 LS to 3.0 LS (Figure 2.5).

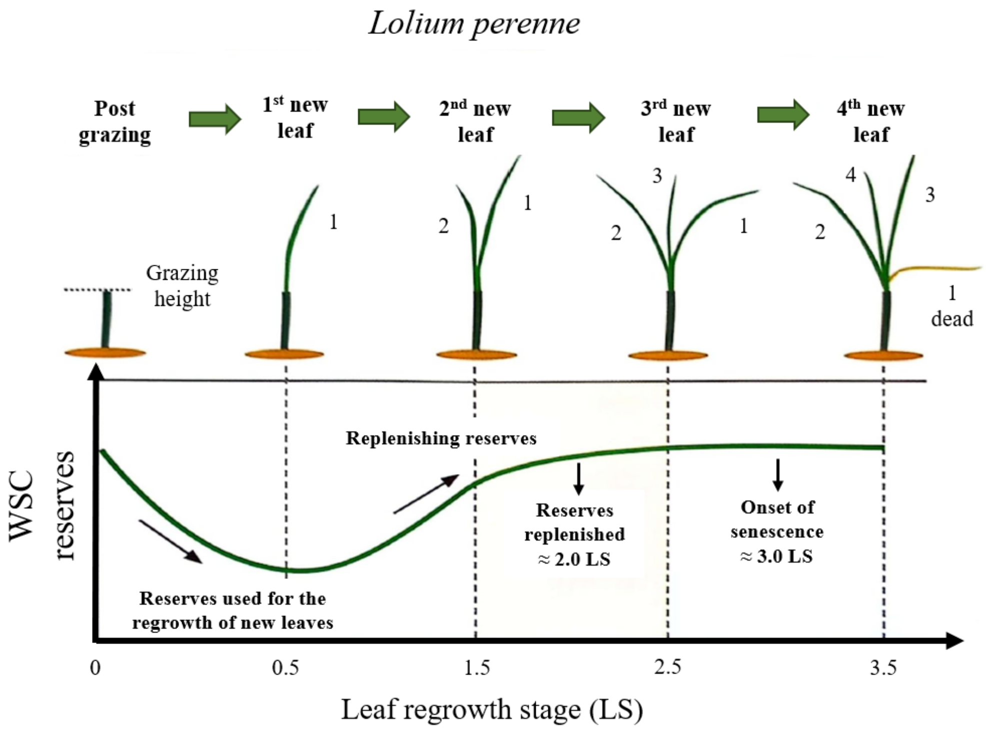

Figure 2-5. Schematic representation of the tiller energy status and correlated leaf-regrowth stage in *L. perenne* regrowth after defoliation. Adapted from: (Barenbrug New Zealand 2023; DairyNZ 2023).

Along the same lines, it is also possible to define the optimal defoliation interval for other grass species. However, because different species will present different phyllochron and genetic capabilities for maintaining a given number of live leaves per tiller, their LS will also be dissimilar. For example, *B. valdivianus* can maintain up to six live leaves per tiller (Ordóñez et al. 2017), and the replenishment of WSC reserves is around 4 to 5 live leaves, equivalent to 3.5 LS (Ordóñez et al. 2021). Further field research by García-Favre et al. (2022) confirmed the practical interval for optimal defoliation of *B. valdivianus* ranging from 3.5 LS to 4.0 LS. Additional grass species have also had their WSC replenishment assessed in relation to their LS - for instance, in glasshouse conditions, *D. glomerata*, *B. willdenowii*, *Bromus stamineus* Desv., and *Bromus coloratus* Steud. at around 4.0 LS (Turner et al. 2006a, b, c, 2007) and under field conditions, *D. glomerata* and *B. willdenowii* at around 4.0 LS (Gatti et al. 2016). To date, García-Favre et al. (2022) was the only study to have tested the LS of mixed swards grazed by sheep in a field experiment under an optimal grazing interval of 2.5-3.0 LS for *L. perenne* pastures and 3.5-4.0 LS for *B. valdivianus* pastures.

2.3.4. Foraging hierarchy, animal behaviour and preferential grazing

The temporal and spatial dynamics of the grazing process involve complex interactions among different trophic levels, and understanding such complexity is essential to optimise management choices in livestock production systems (Stuth 1991). The herbivore animal and the forage plants are the two main characters within these dynamics, and when an animal decides to graze a plant, a series of hierarchical instinctive responses drives their behavioural actions, from choice to prehension and then consumption of a given component across and within the pasture [Figure 2.6;

(Stuth 1991)]. The process of diet selection can be separated into (a) spatial choice, which occurs when the animal is first introduced into the pasture and makes recognition of physical boundaries, and (b) species choice as a secondary process that is guided by the animal's objective to purely meet its nutritional requirements by grazing the most 'palatable' and readily accessible plant community or component in a given area (Hodgson 1990; Stuth 1991). Evidence suggests that within the plant community, species composition, plant density, abundance, structure and size, and even soil moisture holding capacity can influence the animal's choice (Stuth 1991). Further, the grazing animal can develop knowledge around habitats and plant species in such a way as to optimise its grazing abilities and, therefore, energy conversion (Senft 1989).

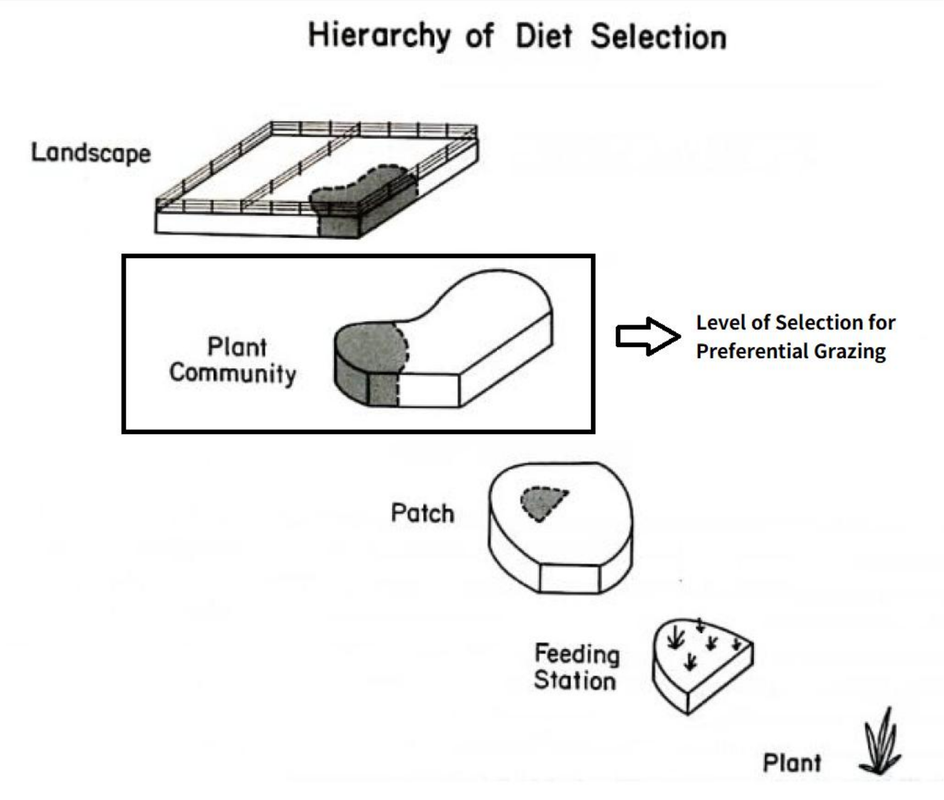

Figure 2-6. The hierarchical levels in diet selection of grazing animals. Adapted from: Stuth (1991).

Assessment of grazing behaviour can be based on the assumption that the longer an animal harvests the same site, the more nutrients are harvested, and therefore, the more that particular plant species or community is capable of ‘affording’ nutrition to the animal (Senft et al. 1987). In that sense, this understanding originated the idea of plant communities ‘profitability’ or grazing preference, which can be measured as the ratio of grazing time per grazing area or the animal’s potential DM intake rate (Senft et al. 1987). In that sense, the greater the density of high-nutrition forage, the longer the animal would spend grazing the same area when compared to other communities with a low nutritive quality (Senft et al. 1987). However, such a causal relationship (plant nutritional offer and grazing preference) does not properly describe the rather complex dynamic occurring between grazing animals and forage plants. Pasture growth accumulation (e.g. pre-grazing herbage mass), morphology (e.g. lamina:stem ratio), structural characteristics (e.g. pre-grazing sward height) and nutritional parameters (e.g. energy content and fibre) constitute a large array of plant-related factors that can affect dairy cows’ preferences (Hodgson 1990; Poli 1998). Further research has widened the scope in an attempt to cover more accurately and explain the animal preferences and selectivity as a relationship between intake per bite, biting rate and grazing time (Hodgson 1986). These dynamics have since been developed and pertain to an area of study, namely grazing allometry, that brings new aspects to animal behaviour, such as the ‘dwell time’ parameter, mechanistic grazing models and others (Poli 1998; Baumont et al. 2004; Pauler et al. 2020).

Within the plant community hierarchical level, the behavioural activities of a dairy cow can be defined as grazing (actively grazing or selecting forage with its head down), ruminating (standing up or lying down), and idle (not grazing or ruminating)

(Penning et al. 1984; Sheahan et al. 2013). Once the animal stops walking, lowers its head and bites a plant, a new level within the hierarchy is established: the feeding station (Stuth 1991). At this level, the ‘grazing’ behaviour activity is composed of (i) search or browsing time, (ii) biting while at a feeding station, (iii) time spent travelling to the next point of the feeding station (Stuth and Searcy 1997), and overall, the animal tends to intensify its grazing activity within the ‘leafy’ layers in the sward (Hodgson 1990). Studies have suggested that dairy animals have seasonal grazing strategies that change according to the plant phenological stage (Stuth et al. 1987), structure and the botanical composition of the sward canopy (Hodgson 1990). Combined, these impact the potential dry matter herbage intake of grazing animals (Hodgson 1990).

During spring, for instance, when sward height is generally greater, animals tend to increase their time searching between feeding stations and decrease the time spent selecting plant species, focusing their selection on the maximum amount of free forage per bite (bite size) (Stuth, 1991; Cave et al. 2015). On the other extreme, during summer, for instance, if forage becomes limited, and the amount of senescent or dead material in the sward increases, animals tend to reduce time searching between feeding stations and increase selection time at each feeding station, more fully exploiting each feeding station. In fact, once the animal ceased grazing in a specific feeding station, most of the available green forage probably had been fully consumed. During these periods, intraspecific (animal-animal) competition becomes more critical with respect to the nutritional well-being of the individuals (Forbes and Hodgson 1985; Stuth 1991).

2.4. PASTURE ECOLOGY

2.4.1. Principles of pasture ecology

Although being in different climatic zones, which implies the most varied range of environmental circumstances, natural grasslands are always characterised by the presence of a wide plant genetic pool - no natural grassland ecosystems are composed of one or two plant species. The resilience of these ecosystems rests on their ability to readjust the presence and proportion of each plant species as an active response to disturbances caused by grazing ungulates, stresses caused by abiotic factors, and competition originating from intra- and inter-specific plant relationships. Elton (1958) proposed the diversity-stability hypothesis, which assumes a positive linear relationship, namely ecosystem processes, between the number of species and the ecosystem stability, in such a way that as the genetic pool (number of species) is widened, the probability of the ecosystem to survive through environmental constraints increases, consequently, the stability also increases. This hypothesis established that the stability of an ecosystem depends on each individual species' presence or absence. Later, Walker (1992) and Walker (1995) proposed the redundancy hypothesis, in which the pasture survival probability is driven by the fulfilment of all functional groups to maintain the ecosystem processes. A functional group is defined by Tilman (2001) as “a set of species that have similar traits and that thus are likely to be similar in their effects on ecosystem functioning”. Both hypotheses, by Charles Elton and by Brian Walker, explain the relevance of species diversity in providing resilience to a pastoral ecosystem.

From an ecological perspective, conceptualising productive pastures as an ecosystem is the first principle to be acknowledged (Dyksterhuis 1958). Secondarily, it is important to perceive individual functions of the species within the ecosystem since they will direct and indirectly influence one or more aspects of the community functionality (i.e., dynamics, stability, productivity, nutrient balance) (Tilman 2001). Many authors have proposed theoretical models to qualify the different types of functions of species in grasslands and rangelands. Ramensky (1938) as cited in During (1979) with violents (aggressive species), patients (tolerant species) and explerents (non-competitive species); Dyksterhuis (1949) with the rangeland condition and the decreasers, increasers and invaders, Grime (1974) with the competitor, stress-tolerators and ruderals model, Tilman (1985) with R* value, Westoby et al. (1989) with the state-and-transition model, among others.

Dyksterhuis (1958) pointed out the necessity of classifying rangeland conditions based on quantitative ecology in order to be more assertive when defining extensive management practices to be utilised in those types of ecosystems. Using a similar approach, Gastó et al. (1993) determined the degree of ‘healthiness’ in more intensively managed pastures. The ‘rangeland condition’ by Dyksterhuis (1949) was originated from in-site observations, defined as “the percentage of the present vegetation which is original vegetation for the site”, and differences in its condition are recognised by comparing present vegetation with the climax vegetation. For interpretation of the degeneration and/or improvement processes, a new grouping of range plants was proposed (decreasers, increasers and invaders), as shown in the diagram (Figure 2.7). This classification was one of the first guidelines to understand and apply ecological principles of competition and succession in pastoral management. The objective of the rangeland condition model was to apply the proper stocking rate that allows a long-term equilibrium between grazing pressure and the natural successional tendency to the climax (Westoby et al. 1989).

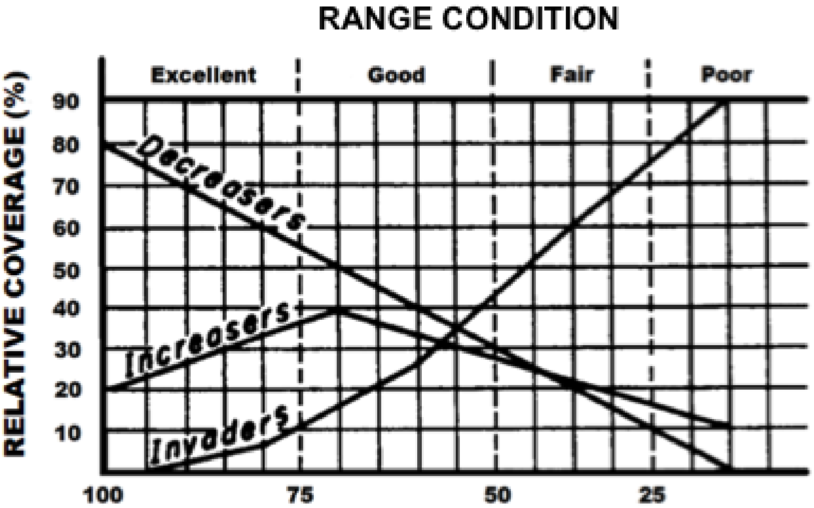

Figure 2-7. Quantitative basis for determining range condition due to relative coverage of decreasers, increasers and invaders. Source: Dyksterhuis (1949).

Further on, Grime (1974) proposed the Competition (Ic%) – Stress (Is%) - Disturbance (Id%) ecological model, highlighting the presence of the competitors (C), stress-tolerators (S), and ruderals (R) in grasslands (Figure 2.8). The model recognises the major adaptive primary and secondary functional strategies of different plant species and relates them with the role of each species in grasslands, namely functional roles (Figure 2.8). Grime et al. (1988) defined competitors as species that can monopolise resources, with 'foraging' of roots and shoots that more rapidly access and utilise water, light or nutrients (e.g., *Phalaris arundinacea* L., *Urtica dioica* L.). The stress-tolerators have mechanisms to cope with abiotic stresses, such as low rates of mineral nutrient supply or soil water restriction, and also a smaller extent of biotic stress, such as herbivory disturbance (e.g., *Festuca ovina* L. - rock and scree slope, lead-mine heaps and quarry spoil, *Avenula pratensis* L. - dry calcareous habitats, *Succisa pratensis* Moench. - lowland damp grasslands).

The ruderals have a short lifespan and the tendency to rapidly invest captured resources in reproductive growth (e.g., *Poa annua* L., *Polygonum aviculare* L., *Bromus hordeaceus* L.). Intermediate positions within the triangular model are also proposed for the species that adjust their strategies within different environmental conditions, creating combined strategies (C-R, S-R, S-C and C-S-R) to cope with stress, disturbance, and competition.

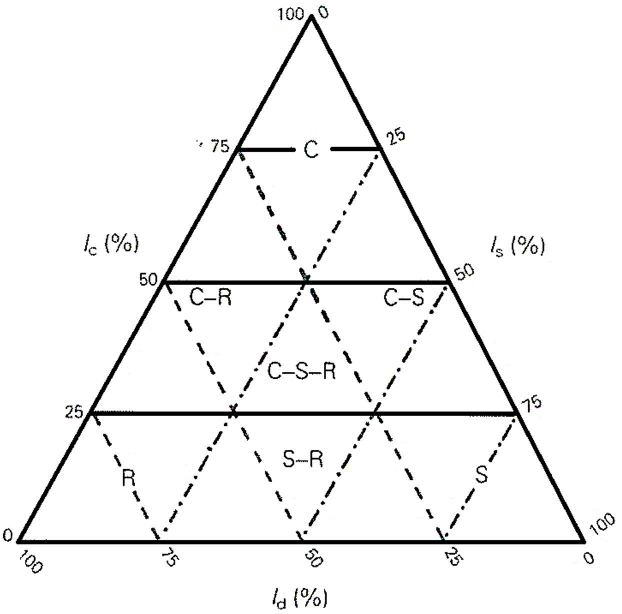

Figure 2-8. Model describing the various equilibria between competition, stress and disturbance in vegetation and the location of primary and secondary strategies. Source: Grime et al. (1988).

The main advantage of Grime’s model is that it includes the variation in adaptive responses by considering the relationship between the maximum potential productivity in a given environment (climax vegetation as per Dyskterhuis) and the frequency and intensity of biomass ‘destruction’, namely ‘disturbance’. This approach brings Grime’s theory closer to the current reality of commercial farming systems, in which pastures are often harvested and/or grazed (disturbance), not working exclusively under climax conditions. Beyond that, in the current scenario of climate change and the necessity to diversify systems to cope with climatic instability (Cranston et al. 2020), Grime's theory also bounds the ecological background of his theory with the paddock-scale intra- and inter-specific relationships (competition) and the increased exposure of plants to more extreme environmental constraints (stress), composed of different types of herbs, grasses, legumes and forages.

2.4.2. Community interactions and succession in biodiverse ecosystems

Ecologists have widely described competition; however, it is differently contextualised in research, which raises the possibility of misguided interpretations. In this thesis, competition will be utilised as per Begon et al. (2006), corroborating with Tilman (1994) and Grime et al. (1988), where competition is "an interaction between individuals, brought about by a shared requirement for a resource, and leading to a reduction in the survivorship, growth and/or reproduction of at least some of the competing individuals concerned".

In a diverse ecosystem with high species richness, which implies a higher level of competition due to a greater number of species (Begon et al., 2006), the trait-mediated responses of individual species to competition occur as morpho-physiological features (above- and below-ground) that confer an advantage in the uptake or utilisation of resources. For example, the individual that greater competes can either rapidly pre-empty and deplete a resource or can present growth rates under resource-depleted zones (Tow and Lazenby 2001). Usually, below-ground competition is greater than above-ground (Milthorpe 1961) and will be more determinant in the competitive balance between species, namely ecosystem equilibrium (Begon et al. 2006). The ecosystem equilibrium, or in other terms, the balanced coexistence of species, is given by the fact that species will occupy the same ecosystem but explore different - however overlapping – niches (Tow and Lazenby 2001). The ‘ecosystem equilibrium’ theory agrees with the redundancy theory (Walker 1992, 1995). In these lines, as long as the dominance does not become too severe, resulting in one species prevailing over another, there is a tendency towards an ecosystem equilibrium.

Some ecologists may interpret such equilibrium as stability or proportionality in terms of botanical composition (Wit 1962). Often, though, this association is subject to revision, with ecologists not agreeing on whether diversity necessarily increases ecosystem stability (McNaughton 1977; Walker 1992, 1995; Hairston et al. 1968). Arguably, the stability of botanical composition in a diverse pasture cannot mean an even proportion of species. In fact, a more appropriate, or at least less misguiding, approach is termed ‘succession’ - where climatic conditions change over time, the course of competition may also change, resulting in fluctuations in the botanical composition (Westoby et al. 1989; Tow and Lazenby 2001). Following the same line, vegetation may also change due to grazing disturbance. Succession in pasture ecosystems is, therefore, the result of an interaction between competition, stress and disturbance (Grime et al. 1988; Gastó et al. 1993; Tilman 1993, 2001).

The utilisation of ecological background related to succession was applied by Chapman (2001) in an attempt to re-create botanically diverse grassland communities via extensification (defined as “cessation of fertilisation and management”) of intensively managed pasture monocultures. However, it had little to no results in the improvement of community diversity, associated with the residual fertility maintaining productivity and competition at undesired levels and slow rates of successional change due to limitations in the recruitment rate of additional species into the extensified community. Alternatively, it is possible to achieve diversity by simply ‘creating’ man-made diverse pastures, resowing pastures with desired species to compose a whole new ecosystem, focusing on its productivity and suitability but also considering succession and persistence of the different plant species within the community (Descalzi et al. 2019). Achieving sustainable and diverse pastoral communities is related to the possibility of manipulating some limiting factors for succession [germination site, light, water, CO_2, mineral nutrients, disturbance, predation, mutualistic relations, etc., as defined by Tilman (1993)], which will determine the presence, abundance or disappearance of a given plant species within that ecosystem. However, the choice of desired species is a complex challenge, with some diverse pastures often culminating on a common ‘drift’ over time, losing many of the intended species (Sanderson et al. 2007), and, in other words, resulting in a somewhat unwanted succession flow.

Appropriate monitoring tools linked to management recommendations are required for the long-term maintenance of a diverse pasture. For this reason, a guideline that presents suggestions to establish diverse pastures for intensive production was proposed by Sanderson et al. (2007), adapted from Hobbs and Morton (1999) (Figure 2.9). In the guideline, the choice of species is advised through the selection and association of different functional groups and seeks to avoid over-diversified systems, bringing it to a valuable and resilient combination of plant species that are feasible to be established and maintained by farmers. The benefits associated with increased plant species types within a pasture have been measured in different studies (e.g. overyielding, better responses to abiotic stress, increased resilience, and greater resistance to weed invasion, among others (Tracy and Sanderson 2004; Brophy et al. 2017; Isbell et al. 2017; Finn et al. 2018; Cranston et al. 2020; Loreau et al. 2021; Lüscher et al. 2022).

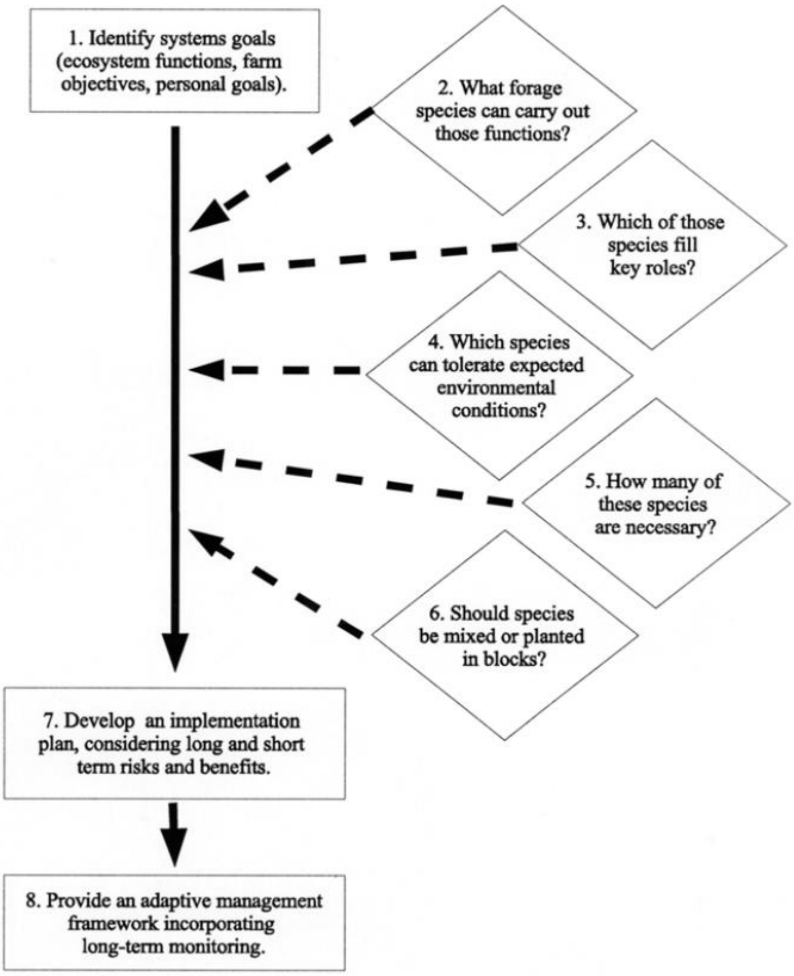

Figure 2-9. Steps in the design and management of diverse, multifunctional pasture communities. Source: Sanderson et al. (2007).

2.4.3. Current utilisation and systems suitability

The role of diversity in promoting resistance and resilience of ecosystem function in the face of environmental disturbance is well-established in ecosystems and experiments based on semi-natural grasslands (López 2000; Craven et al. 2016; Descalzi et al. 2019; Lüscher et al. 2022). However, there is still a considerable fallback in the transference of ecological knowledge into productive and intensively managed pasture-based systems (Cranston et al. 2020; Suter et al. 2021). In order to achieve the so-called functional diversity in productive grasslands, the collaboration between ecologists, eco-physiologists and agronomists is required in an 'agroecological' approach to identify and enhance suitable pasture species composition and desired plant strategies to provide rules that will allow the enhancement of functional complementarity and limit competition within productive, diverse pastures (Volaire 2018).

The principles related to complementarity will be discussed in the following section. Here, the focus is to present studies around species diversity in pastoral ecosystems and how they have become more assertive within the past few years in the continuous attempt to adapt functional ecology seen in natural or semi-natural grasslands into intensively managed production systems. One of the main expected outcomes of increased biodiversity is the so-called overyielding. Wilson (1988) and Garnier et al. (1997) have rejected the hypothesis that species richness is, in fact, resulting in a statistically greater production of the mixtures in relation to the monocultures, and these findings are consistent with what Crawley (1984) calls a 'rule of logic': if "one species is less productive than the other, replacing a more productive individual by a less productive one is bound to reduce yield". For instance, Garnier et al. (1997) discussed an experiment by Austin and Austin (1980), carried out in a glasshouse, where five grass species (the perennials, *D. glomerata*, *F. ovina* and *L. perenne*, and the annuals *P. annua* and *Vulpia membranacea* L.) were grown either as a monoculture in pot, or as a mix of the five. In this study, the mixture produced the same as the most productive monoculture (*D. glomerata*) and more than all the other monocultures. However, according to Garnier et al. (1997), a correction parameter should be applied to these results since the plant density in a mixture is greater than in a monoculture.

On the other hand, field studies testing the increment in biodiversity in the field have shown mixtures overyielding in relation to productive monocultures. Kirwan et al. (2007), in a study to quantify diversity-function relationships in 28 sites around Europe with a common experimental design that implied two grass species and two legume species combination, showed that the four-species mixture often yielded more than the highest-yielding monoculture. One of the grasses and one of the legumes were fast establishing, while the other two were slow establishing, being the species selection due to the different geographical areas. In another experiment, a five-species mixture containing *Cichorium intybus* L., *P. pratensis*, *D. glomerata*, *L. perenne* and *T. repens* had greater yields under varied levels of soil moisture drought compared with a binary pasture of *T. repens* and *P. pratensis* (Skinner et al. 2004). In another study, the pasture with the most species had a negative relationship with weed abundance. This was better explained due to the forage evenness at which species are distributed within mixed pastures, suggesting that adding many species to the system does not necessarily mean greater yields or weed control (Tracy and Sanderson 2004).

The contrasting findings in research are of great importance for the ecological and agronomical understanding of how diverse pastures may or may not present productive and persistent advantages over monocultures. A missing link in the studies that did not (or argued not to) find overyielding by mixtures is the lack of complementarity, or at least productive-prone traits, among the species chosen to compose the diverse pasture. In conclusion, not all grass species are suitable to compose a diverse pasture, for example, the choice of utilising *P. annua* by Austin & Austin (1980), which is considered a weed in New Zealand.

2.5. DIVERSE PASTURE COMPOSED OF COMPLEMENTARY SPECIES

2.5.1. Principles of complementarity

One explanation behind the understanding that biodiversity enhances ecosystem functioning is the so-called ‘functional complementarity’, often interchangeably used to explain the cause of enhanced ecosystem functioning simultaneously as it is presented as the main outcome of increased ecosystem diversity (Loreau 2000). In a review of the topic, Barry et al. (2019) discussed the types of ‘complementarities’ and disentangle causes from consequences. ‘Functional complementarity’ can cause enhanced ecosystem functioning is driven by three major sets of processes within the ecosystem: (i) resource partitioning, (ii) abiotic facilitation and (iii) biotic feedback, which result in different consequences that may occur within a diverse pasture (Barry et al. 2019).

i. Resource partitioning may occur when species use several portions of the available resource pool by either exploiting different layers of above- and below-ground [e.g. deep- or shallow-rooted plants; Mommer et al. (2010); García-Favre et al. (2022)] or primarily utilising different chemical forms of a mineral [e.g. inorganic N or glycine or serine; Weigelt et al. (2005);

Kahmen et al. (2006)]. As a result of resource partitioning, the resource pool is more completely explored and used in more diverse communities as opposed to monocultures or single-grass pastures.

ii. Abiotic facilitation may occur when one individual species benefits another plant species by either enriching the resource pool of the environment [e.g. biological N fixation; Roscher et al. (2010); Gubsch et al. (2011)] or positive changes in the environmental stress gradients [mediation of heat-stress; Steudel et al. (2012); Milcu et al. (2016)]. As a result of abiotic facilitation, the ecosystems' resilience to abiotic stressors may be enhanced.

iii. Biotic feedback may occur and cause complementarity by enhancing the interactions between the different plant species and other trophic levels. Because this case involves many trophic levels, the complete understanding of their interactions is yet to be fully unfolded for productive pastoral systems. However, it has been better elucidated, for example, in the relation between the number of trees and herbivores in tropical forests by Janzen (1970) or the relationship between plants and fungal pathogens diversity and the infection rate per plant (Rottstock et al. 2014).

Complementarity effects, either perceived as a cause or a consequence, have been previously observed in pastoral systems. Probably the most overlooked yet the most common situation is the association of *L. perenne* and *T. repens* in pastures. In this case, complementarity occurs as abiotic facilitation, when mostly over springtime in Temperate Humid climates, *T. repens* biologically fixes N, which becomes available to be utilised by *L. perenne* (Kemp et al., 1999).

These highly productive artificial pastures (e.g. *L. perenne* and *T. repens*) normally present a low level of species richness [low ‘n’, e.g. n=1 for pasture with only *L. perenne*, n=2 for binary pastures with *L. perenne* and *T. repens*; species richness defined as the number of species present in a pasture community (Begon et al., 2006)]. On the other hand, a natural grassland community will present a high level of species richness (Descalzi et al., 2019). The general goal of a diverse pasture for grazing systems is simply to reach a higher ‘n’ than usual found in artificial pastures (Nobilly et al. 2013). The present research line of which this thesis is part aims to increase the ‘n’ within the pasture community by adding species in a systematic and controlled manner, in such a way as to at least maintain high levels of production but also to increase the system stability due to a potential complementarity of functional traits within the pool of species chosen to compose the diverse pasture.

The general hypothesis of this thesis (see Chapter 1. Section 1.2. General hypothesis and objectives) emerges from the potential functional complementarity among *L. perenne*, *B. valdivianus*, *D. glomerata* and *T. repens*. Beyond that, it is expected that the choice of defoliation criteria based on the plant morpho-physiology parameters facilitates the co-existence of the species within diverse pastures, enhancing functionality. The cause of complementarity between these three grasses and the legume may be driven by plant traits that result in resource partitioning (growth asynchrony, root systems exploitation), abiotic facilitation (hydraulic lift and biological N fixation), and biotic feedback (grazing preference). However, these causes are yet to be fully understood and evaluated. The expected consequences of this complementarity are enhanced productivity (overyielding), persistence and the provision of high-quality nutritional resources for pasture-based dairy systems.

2.5.2. *Lolium perenne* L.

Lolium perenne has been cultivated for over 300 years, with its later strains (or cultivars) being the most desirable pasture plant species in temperate, moist environments due to high productivity, great nutritional parameters, excellent persistence under grazing (Hubbard 1978), and great seed production for further propagation in commercial farms. In New Zealand, *L. perenne* is the most utilised plant species and has been the topic of numerous studies and research about breeding, productivity, persistence, management and nutritional parameters (Hunt and Easton 1989; Belgrave et al. 1990; Bahmani et al. 2003; Chapman et al. 2021). *Lolium perenne* presents green, shiny, and hairless leaves, smooth sheaths and a basal node that is usually pinkish when young. The ligules are membranous (up to 2 mm long) and sometimes present small, narrow projections (auricles) at the base, and flowering starts rather late in spring (Figure 2.10) (Hubbard 1978). *Lolium perenne* presents a basic diploid chromosome (n=14). However, it is possible to double this number artificially by exposing the germinating seed to colchicine, and this results in the production of so-called tetraploids (Langer 1973).

Figure 2-10. Botanical illustration of *Lolium perenne* L.

Historically, the plant population persistence of *L. perenne* under intensively managed rotational systems has been great, mostly due to breeding research (Caradus et al. 2021; Lee et al. 2012; Hunt & Easton 1989) and management research (Brougham 1959, 1960; Thom 1996, 2011; Lee et al. 2010; Hernandez Garay et al. 1997; da Silva et al. 2004). However, recently, this persistence has been decreasing (Matthew et al. 2012; Dodd et al. 2018), mostly in regions where rainfall is lower than 700 mm or where fertilisation supply is limited (Waller and Sale 2001). This decrease in persistence may be associated with its root structural distribution. *Lolium perenne* is considered a shallow-rooted species compared to other species utilised in productive grasslands (Komainda et al. 2020; García-Favre et al. 2022). It can present between 60 and 80% of its root distribution within the first 20 cm of the soil (Crush et al. 2010; Crush et al. 2005).

2.5.3. ***Bromus valdivianus* Phil.**

Bromus valdivianus is a native species of southern Chile and a perennial forage plant around 30 to 80 cm high. *Bromus valdivianus* presents leaves with sheaths and light green hairy laminas (10 to 30 cm long by 2 to 5 mm wide), ligule hyaline, oblong and somewhat denticulate (2 to 2.5 mm long; Figure 2.11) (Muñoz 1980; Balocchi et al. 2000). It may present a lot of variation in flowering dates and has an inflorescence corresponding to a lax panicle (10 cm long) that is 'palatable' for dairy cows (López et al. 2016). *Bromus* spp. are moderately deep rooting grasses with high tolerance to drought in good soils but are poorly adapted to soils with waterlogging problems or that have a heavy texture, are very acidic, or have moderate to high levels of aluminium (Stewart 1996; López et al. 1997; López et al. 2013). In New Zealand pasture systems, *B. valdivianus* has been found to be a deep-rooted species and presented 38% more root biomass in-depth compared to *L. perenne* (García-Favre et al. 2022).

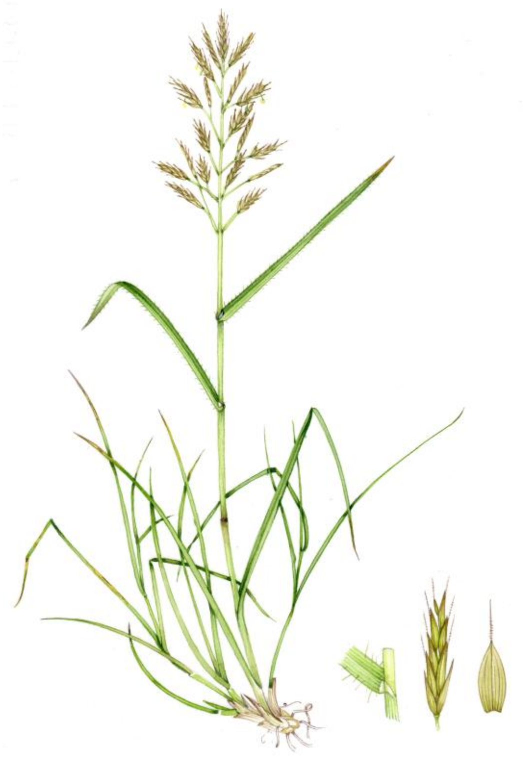

Figure 2-11. Botanical illustration of *B. valdivianus* Phil.

Bromus valdivianus produces high-quality and 'palatable' forage (Balocchi et al. 2000) and, from an agricultural perspective, is considered less winter active than other pasture species (i.e. *L. perenne*) (Stewart 1996). In mixtures of *L. perenne* and *B. valdivianus*, the species co-dominate in spring (September and October), but as water restriction increases in summer (January and February), *L. perenne* population declines and *B. valdivianus* dominates (Descalzi 2011; García-Favre et al. 2022).

2.5.4. *Dactylis glomerata* L.

Dactylis glomerata is a very important pasture grass, with numerous strains naturally occurring all around Europe, parts of Asia, north of Africa, and most importantly, some varieties growing in Mediterraneum dry and warm-exposed places (Hubbard 1978; Lolicato and Rumball 1994). *Dactylis glomerata* presents dull green or greyish-green leaves, a membranous ligule (12 to 20 mm long), and ovate and sharply pointed laminas (Figure 2.12) (Hubbard 1978). *Dactylis glomerata* is a tufted perennial with flattened tillers, which presents a lot of variation in flowering dates and seasonal spread tillering growth (Langer 1973), and its establishment is slower than *L. perenne* and *B. valdivianus* (Turner et al. 2007). Nie et al. (2008) found that its rooting depth may range from 0.81 to 0.93 m, and Crush et al. (2005), in a glasshouse study, found that it can be comparatively long-rooted when compared to some cultivars of *L. perenne*. *Dactylis glomerata* is well adapted to moderate fertility and low soil moisture (Rumball 1982; Stevens et al. 1992).

Figure 2-12. Botanical illustration of *Dactylis glomerata* L.

Dactylis glomerata pastures must be kept under more severe or more frequent grazing. Otherwise, it tends to become rather coarse and highly tufted (Langer 1973; Lolicato and Rumball 1994). Reports about the nutritive value of *D. glomerata* often describe the species as less ‘palatable’ (Oram 1991; Horadagoda et al. 2009), and its crude protein, metabolisable energy, and digestibility content have been found to decrease if it is allowed to mature, mostly in spring (Cullen et al. 2017). In New Zealand, *D. glomerata* is the second most sown grass species, being an important component in dryland environments (Mills et al. 2006). Most of the recent commercially successful *D. glomerata* cultivars are an intermediate between Mediterranean and northern European types, which confer them active growth during winter and also summer while still being drought-tolerant and having an early to mid-spring flowering (Lolicato and Rumball 1994).

2.5.5. *Trifolium repens* L.

Of all pasture legumes, *Trifolium repens* clover is by far the most important and widely grown and has two main ecosystem functions: nitrogen fixation and the provision of high quality (Brock and Hay 2001). In New Zealand, the association of *L. perenne* and *T. repens* as a pasture is the basis of pastoral farming, and the *T. repens*’ biological nitrogen [ranging from approximately 80 to 350 kg N ha^{-1} annually, depending on the proportion of *T. repens* in the sward (Ball et al. 1979; Hoglund et al. 1979)] is one of the key factors for New Zealand’s successful farming (Caradus 2007; Ball et al. 1979). *Trifolium repens* is a hairless legume prostrate in habit, with many stems running along the ground and growing adventitious roots from each node. *Trifolium repens* presents oval leaflets that most frequently have white crescent marks on their upper surface, head-shaped white flowers, and heart-shaped golden yellow seeds (Figure 2.13). There are three main strains of *T. repens*: small-leaved wild types, intermediate-sized leaf types, and large-leaved types (Smetham 1973).

Figure 2-13. Botanical illustration of *Trifolium repens* L.

Within the first establishment year, *T. repens* presents a tap root, which will die as soon as the first step in fragmentation of the plant seedling to form clonal plants starts; this transition into a clonal population in its majority may affect *T. repens*' characteristics and performance, markedly as smaller, but more numerous plants that are more vulnerable to biotic and abiotic stress (Caradus and Williams 1989; Caradus et al. 2021). *Trifolium repens* presents an overall low root:shoot ratio (Forde et al. 1989). It is particularly vulnerable in seasonally dry regions, where summer rainfall and summer management limit its performance and persistence (Brock and Hay 2001). It presents a quicker and greater regrowth after grazing compared to other clovers, more rapid regrowth of roots, and faster turnover of nodules and new nodules after grazing (Brougham 1959). The production peaks may happen during summer due to the growth rate being related to high temperatures (Brougham 1959), and when mixed with *L. perenne*, it may also happen in autumn (Smetham 1973).

2.6. CONCLUSION

The proposed study intends to cover the soil-plant-animal continuum of pastures composed of *L. perenne*, *B. valdivianus*, *D. glomerata*, and *T. repens* grazed by dairy cows in temperate humid climate conditions. The interactive approach between functional complementarity of plant traits to enhance survival and performance is novel for New Zealand pasture-based production systems. Those studies are developed to gather more information about the agronomic performance, competition ability, persistence and dairy cows' grazing preference of single-grass and diverse pastures subjected to the leaf regrowth stage criterion for defoliation.

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Chapter 3

^{18}O isotopic labelling and soil water content fluctuations validate the hydraulic lift phenomena for C_3 grass species in drought conditions

Plant Stress, Research Article, Published.

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3.1. ABSTRACT

Hydraulic lift is a functional characteristic observed in some plant species, often associated with their ability to withstand drought conditions. It involves capturing water from deep soil layers and redistributing it to shallower soil layers through the plant's roots. *Bromus valdivianus* Phil., *Dactylis glomerata* L., and *Lolium perenne* L. may perform hydraulic lift at varying rates. Using both direct (isotopic labelling - $\delta^{18}O$) and indirect (soil water content sensors) techniques, the study assessed and validated the hydraulic lift under extreme drought conditions on the soil top layer (below permanent wilting point), maintaining the bottom layer at high (20-25% field capacity [FC]) and low (80-85% FC) levels of soil water restriction. Above- and below-ground biomass growth and morpho-physiological responses were evaluated. All species displayed some degree of hydraulic lift, with significant differences observed in the isotopic analysis and soil water content ($p<0.05$). This illustrates that water was redistributed from the deep to shallower soil layer and validates that the hydraulic lift phenomenon is occurring in these C_3 grasses. *Bromus valdivianus* presented the highest $\delta^{18}O$ values (25.05‰) and highest increases in soil water content ($\mu = 0.00626$ m^3 m^{-3}; five events). *Bromus valdivianus* had a dry matter ratio of approximately 4:1 (0-20 cm:20-40 cm). In contrast, *L. perenne* and *D. glomerata* had approximately 6:1 and 5:1, respectively. This difference in root morphology may explain the higher rate of hydraulic lift observed in *B. valdivianus* relative to *L. perenne* and *D. glomerata*. This paper validates the occurrence and provides initial insights into the hydraulic lift process occurrence of temperature grass species.

3.2. GRAPHIC ABSTRACT

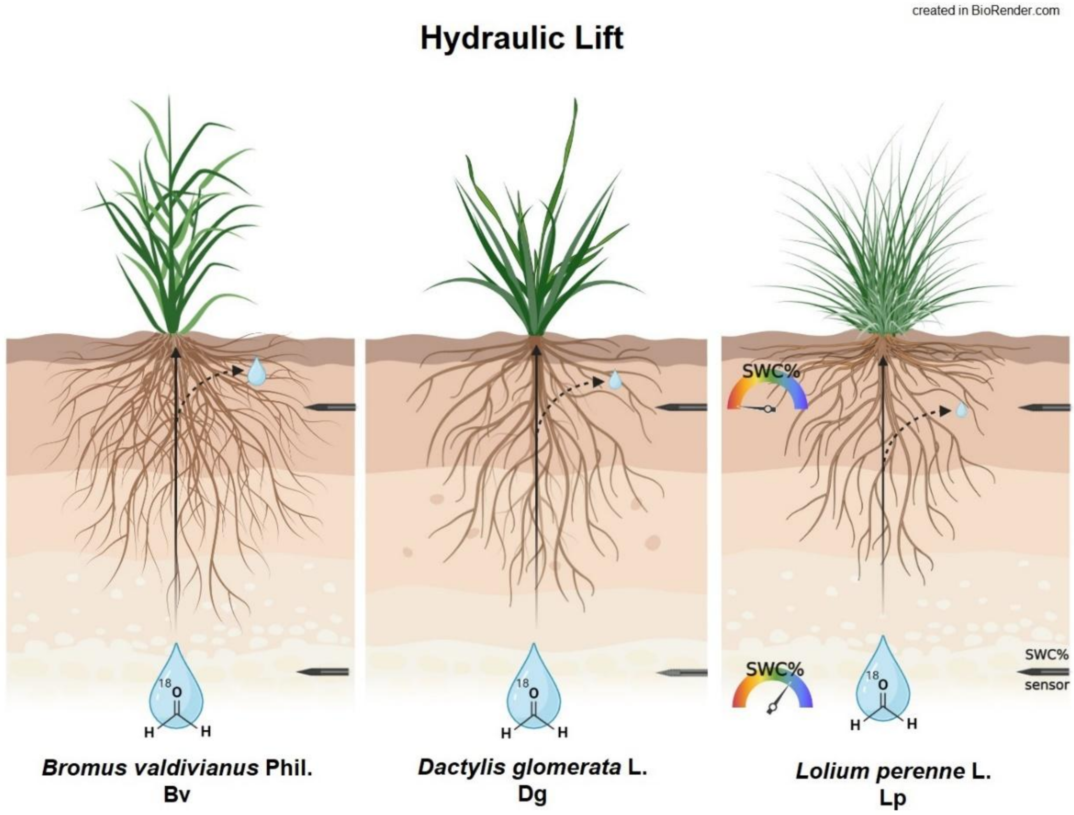

Figure 3-1. Graphic abstract.

3.3. KEYWORDS

Hydraulic lift; perennial ryegrass; pasture brome; cocksfoot; drought resilience

3.4. INTRODUCTION

Commonly associated with the drought tolerance of deep-rooting plant species, the hydraulic lift is a functional property that some species exhibit by capturing water from deep soil layers and releasing it into shallower soil layers via roots (Richards and Caldwell 1987; Caldwell and Richards 1989). The hydraulic lift phenomenon was initially described as water uptake and loss via roots (Shone and Flood 1980) and defined also as water redistribution process via the soil-root continuum exhibited by vascular plants (Richards and Caldwell 1987; Caldwell and Richards 1989; Volpe et al. 2013). However, the demonstration of the functional importance of deep rooting and its relationship with soil water dynamics presents some inherent difficulties (Maeght et al. 2013; Pierret et al. 2016), mainly associated with the necessity for non-destructive research to assess hydrological phenomena (Schymanski et al. 2008; Lux and Rost 2012; Hayat et al. 2020; Cai et al. 2022).

Techniques to reveal the hydraulic lift of plant species and its consequential water redistribution into different soil layers initially consisted of indirect measurements using micro tensiometers at different soil depths to assess the fluctuations in the soil water potential gradients (Ψs) (Vetterlein and Marschner 1993) and direct measurements using standard water relation methods associated to isotopic labelled water techniques (Dawson 1993). The phenomena have been modelled and demonstrated for forestry systems and savanna ecosystems due to the extensive root size of trees and shrubs. This facilitates the hydraulic lift validation, quantification up to complex soil-plant-atmosphere continuum models and predictions on commensalistic relationships of plants (Scholz et al. 2008; Domec et al. 2010; Yu and D’Odorico 2014; Ferreira et al. 2018; Bayala and Prieto 2019; Lee et al. 2021).

Even though crop and forage plants have a relatively smaller stature and a less extensive root system than trees, it is, directly and indirectly, proven that some crop and forage species can also exhibit hydraulic lift. Studies have assessed the hydraulic lift in cropping and foraging systems, for instance: *Medicago sativa* L. and *Zea mays* L. (Corak et al. 1987); *Cajanus cajan* L., *Sesbania sesban* L. and *Z. mays* (Sekiya and Yano 2004); *Lupinus angustifolius* L. (Doussan et al. 2006); *Oryza sativa* L. and

Z. mays (Mahindawansha et al. 2019). Nonetheless, considerable uncertainty remains about its scale and possible applications to farming systems.

Regarding the potential positive outcomes on an ecosystem resulting from hydraulic lift, Bogie et al. (2018) and subsequently, Singh et al. (2020) introduced the concept of ‘bioirrigation’ into the debate. These authors’ hypothesis is based on the deliberate inclusion of deep-rooted species into monocultures, such that the redistributed water by the added species benefits the neighbouring plant species, enhancing their chance of survival or growth during drought periods. A study based on a soil water budget from a 15-year data set in a marine Mediterranean landscape found that *Olea europea* L. and *Quercus suber* L. trees subsidised deep water (below fractured-basalt layer) for neighbouring grass species during spring (Montaldo et al. 2021). In a study carried out on intercropped areas of *Panicum virgatum* L. (> ten years old) and adjacent shallow-rooted annual crops within the southern great plains of North America (climate ranging from continental humid to semi-arid), it was found that the soil under *P. virgatum* had consistently higher soil water content (margin of 15-100%) than nearby soil under shallow-rooted annual crops using stable hydrogen and oxygen isotopes (deuterium [δ^2H] and oxygen-18 [δ^{18}O] stable isotope delta values) (Oerter et al. 2021).

The hydraulic lift brings a new perspective on how the combination of functional species (e.g., different root system architecture) benefits the ecosystem, whereby commensalism allows one species to cope with periods of water restriction or drought that can negatively affect the ecosystem’s stability. It is often conceptualised as the plant with the greater stature (tree or shrub) being the ‘donator’ and the smaller plant the ‘recipient’; however, this does not encompass interactions in pasturelands, where the botanical composition of the ecosystems is based on small-sized plants (grasses, legumes, and herbs). Studies carried out on the hydraulic lift of *Bromus tectorum* L., *Lolium multiflorum* Lam., and *P. virgatum* proved that grasses are also capable of exhibiting hydraulic lift, to a certain extent defined by the aptitude of their root system (Leffler et al. 2005; Meunier et al. 2017; Oerter et al. 2021). The present study hypothesises that the temperate perennial grasses *Bromus valdivianus* Phil. (Bv), *Dactylis glomerata* L. (Dg) and *Lolium perenne* L. (Lp) perform the hydraulic lift when subjected to water stress, and the phenomena occurrence is related to their root size and distribution. It was verified whether the three species exhibit the hydraulic lift or not, and if so, under which conditions of soil water restriction. The above- and below-ground biomass growth and morpho-physiological responses of the three species under two levels of water restriction were also evaluated to assess their relationship to the hydraulic lift phenomenon.

3.5. MATERIAL AND METHODS

3.5.1. Environmental conditions and experiment setup

The study was conducted in a glasshouse at Massey University’s Plant Growth Unit, Palmerston North, New Zealand, between October 2020 and April 2021. The glasshouse was fully exposed to natural light conditions, and when air temperature rose above 30°C, inbuilt fans were activated for two hours, and air humidity regulators were activated for twenty minutes. The glasshouse environmental conditions were registered from March to April 2021, when the experimental data was collected. (Figure 3.2). The accumulated growing degree days were calculated according to Calvache et al. (2020), utilising a base temperature of 5^{o}C.

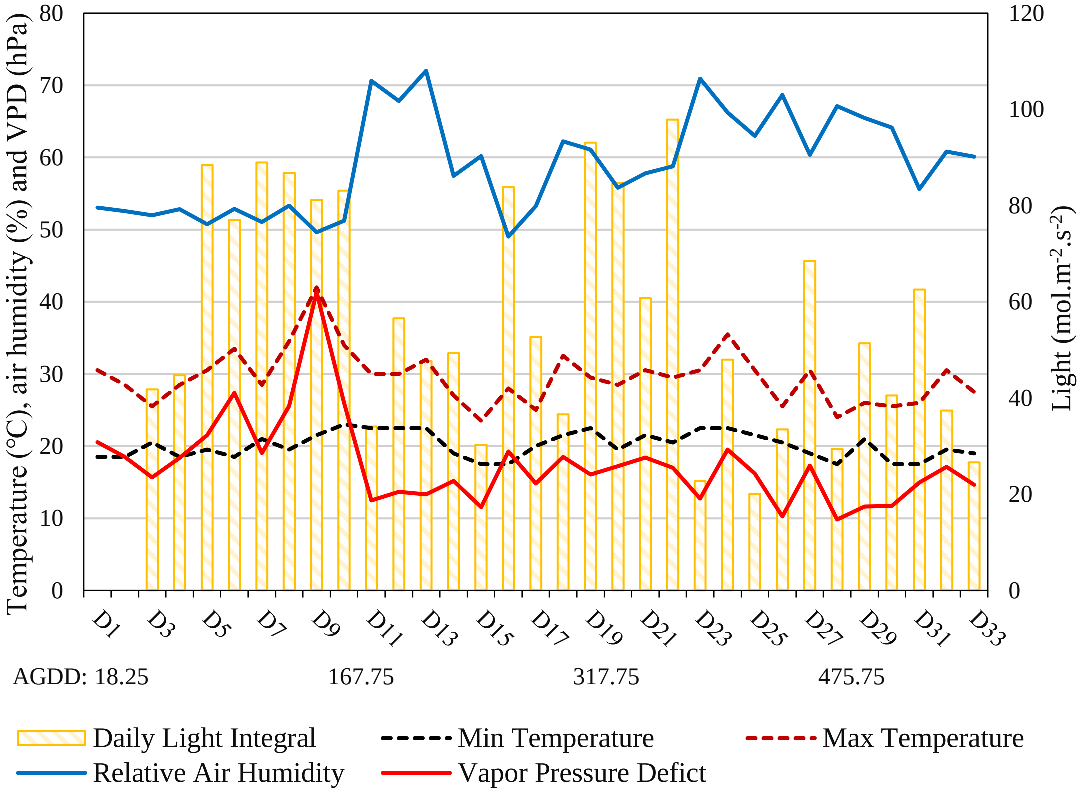

Figure 3-2. Environmental conditions: daily light integral (moles of light per day – mol m^{-2} day^{-1}), minimum and maximum temperature (Celsius degree - ºC), relative air humidity (percentage - %), vapour pressure deficit (VPD – hPa) and in the glasshouse during 19 March 2021 (D1), 27 March 2021 (D9), 04 April 2021 (D17), 12 April 2021 (D25) and 20 April 2021 (D33).

The study used twenty-four pots designed and built, as shown in Figure 3.3. The pot had a mesh at the bottom, two irrigation pipes placed from outside, three windows sealed with translucent plastic to appreciate the root development and two gaps for soil water content (SWC%) sensors at the top and bottom layer. Each pot constituted one experimental unit. The experimental design was a randomised complete block design with three blocks (n=3) and a 3 x 2 factorial arrangement of treatments. The main factors were the three grass species (Spp = Lp, Bv and Dg) and two levels of water restriction (WR) applied in the bottom soil layer (low level of water restriction [LR] – 600 mL/week, and high level of water restriction [HR] – 300 mL/week) for approximately 600 growing degree days (Calvache et al. 2020), equivalent to thirtyfour days in the current glasshouse environment. One pot of each experimental treatment (Spp x WR) was randomly allocated within each of the three blocks (each block: 1 m x 0.8 m). The irrigation values were calculated to keep the bottom layers of the pots respectively at 90-100% field capacity (FC) and 20-30% FC, depending on the irrigation treatment, until the end of the trial. During the evaluation period, the top layer was never irrigated to simulate field conditions during the dry season. The bottom layer was irrigated through the outside pipes twice weekly according to the two water restriction levels.

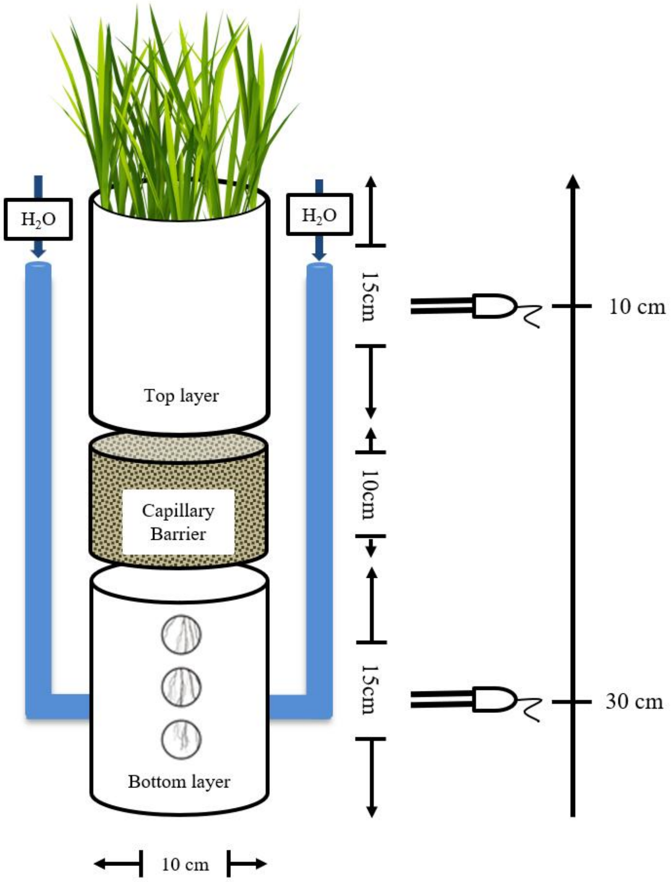

Figure 3-3. Experimental pots diagram indicating pots' dimensions (10 cm x 40 cm), pots' layers heights (top layer - 15 cm; capillary barrier - 10 cm; bottom layer – 15 cm), irrigation tubes position from outside the pot, SWC% sensors position (at 10 cm and 30 cm depth) and windows position for root monitoring.

The soil substrate used in the experimental pots was a mix of 50% allophanic soil (volcanic soil from Taranaki-Wanganui region in New Zealand) and 50% sand, fertilised with a combination of a slow-release (3-4 month Osmocote ®; 19% N, 9% P, 10% K + 2% MgO + trace), a fast-release fertiliser (8-10 week Osmoform ®; (22% N, 3% P, 11% K, 2% MgO + trace) and dolomite at a rate of 4 g L^{-1}, 0.5 g L^{-1} and 1.5g L^{-1}, respectively. The soil physical attributes were determined at Landcare Research – Manaaki Whenua, Palmerston North, New Zealand, with six disturbed soil samples and nine undisturbed soil samples of 54.26 cm^3 cores ($h = 3.00$ cm; $\phi = 4.80$ cm). The sixteen cores were utilised to determine the water retention points (Ordóñez et al. 2018) at -330 hPa, -1,000 hPa, -3,000 and -15,000 hPa. With the addition of the calculated soil saturation, it was possible to parameterise the points and to generate the soil water retention curve (van Genuchten 1980; van Genuchten et al. 1991). The soil FC was assessed at -330 hPa, and the permanent wilting point (PWP) at -15,000 hPa (Figure 3.4). Finally, the samples were dried in a forced ventilation oven at 106°C and weighed again to determine other physical attributes. The soil had a dry bulk density of 1.34 g cm^{-3}, particle density of 2.65 g cm^{-3}, porosity of 49%, and was categorised as a sandy-loam soil texture.

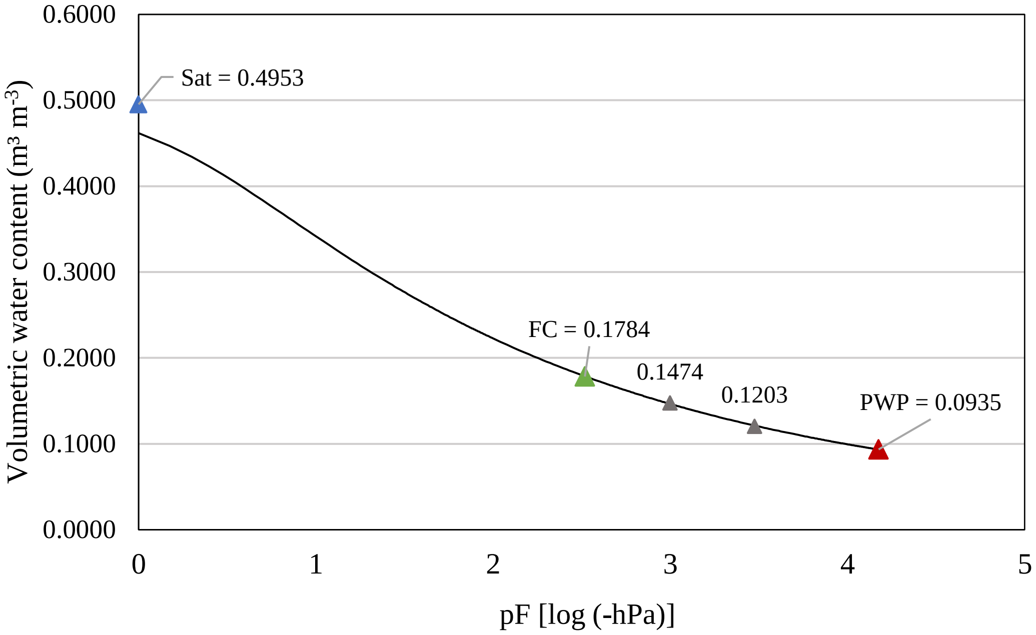

Figure 3-4. Soil water retention curve: the relationship between volumetric water content (m^3 m^{-3}) and log of the soil water matric potential (hPa) of the substrate utilised in the study. The curve indicates the saturation point (Sat in blue), field capacity (FC in green; -330 hPa), two intermediary points (-1,000 hPa and -3,000 hPa) and the permanent wilting point (PWP in red; -15,000 hPa).

On 19 November 2020, adult plants of *L. perenne* cv. Maxsyn, *B. valdivianus* cv. Bareno and *D. glomerata* cv. Greenly II were collected from a three-year-old pasture field and planted in spare pots to stimulate growth and tillering. Liquid fertiliser and defoliations down to 5 cm height were performed on 24 November 2020 and 19 December 2020. The fertilisation rate was based on a nitrogen (N) application equivalent to 20 kg N ha^{-1}, with water-soluble fertiliser (Peters Professional “Allrounder”; 20% N, 8.7% P, 16.6% K) plus trace elements (B, Cu, Fe, Mn, Mo, Zn).

On 20 January 2021, fifteen tillers and roots of each grass species were transplanted into the experimental pots. Each group of fifteen joint tillers was detached from the original grass patch, and the roots were carefully washed. Each group of tillers was held with its roots hanging down in its final position, reaching the bottom layer of the pot. Then, the soil substrate (2.1 kg) was settled as the 15 cm bottom layer, followed by a mix of thick sand (1 to 2 mm) and thin gravel (10-20 mm) to complete the 10 cm capillary barrier, and another 15 cm soil substrate was added as the top layer. The SWC% sensors were placed at 10 and 30 cm depth. Six extra pots of bare soil were built up with SWC% sensors; the capillary barrier was present in three bare soil pots and not in the other three. Two bare soil pots of each type were added to each block. These pots were utilised to validate the barrier as a soil capillarity breaker, preventing the water rising flow (i.e., capillarity flow), thus “rewetting” the upper soil layer (Dawson 1993; Vetterlein and Marschner 1993).

The plants were irrigated once a day, ensuring no stress due to soil water restriction or soil waterlogging. On 01 February 2021, the plants were defoliated to 5 cm height and received the second application of water-soluble fertiliser to stimulate root growth. The experimental period started on 15 March 2021, when all pots presented roots in the bottom layer, visible through their windows (Figure 3.3). For three days, the irrigation stopped to induce all the pots to reach the PWP in the top and bottom layer so that at the start of the experimental measurement period, all the pots had similar conditions below PWP. On 18 March 2021 (day 0), the plants were defoliated to 5 cm height, and the two levels of water restriction were imposed.

3.5.2. Below-ground measurements

Soil water content sensors (ECH2O series EC-5 VWC – METER Group, Pullman, Washington, USA) were placed in all experimental pots (bare soil and with plants) at two depths to determine the SWC%: top layer (10 cm) and bottom layer (30 cm) (Figure 3.3). These sensors were connected to a measurement and control data logger

(CR1000 model – Campbell Scientific, Logan, Utah, USA) and a multiplexer (16/32B model – Campbell Sci., Logan, Utah, USA). The SWC% of plant and bare soil pots were assessed every five minutes, and an average was recorded every fifteen minutes. The data collected by the soil moisture sensors and registered in the data logger were calibrated and corrected according to the soil substrate's physical properties and presented as volumetric water content ($m^3\ m^{-3}$).

The hydraulic lift was assessed using enriched labelled water with stable isotopes of oxygen (Water-18O 10atom%, Tayo Nippon Sanso, Tokyo, JPN) diluted in tap water and presented as oxygen-18 stable isotope standard delta notation value ($\delta^{18}O$). On 19 April 2021 (day 32), the labelled water enriched to 0.51% oxygen-18 ($\delta^{18}O$ = 400‰) was applied in the bottom layer at the same rates as the previous irrigations with tap water for the two water restriction treatments. The bare soil pots also received the ^{18}O-labelled water at the same level as the LR treatments. Two soil sub-samples across each top layer were collected the following day (day 33), between 11 AM and 2 PM, when the top SWC% sensors indicated that hydraulic lift was occurring. An auger was utilised to collect two 20 g samples per pot. The soil sub-samples were immediately placed in plastic containers and immersed in liquid nitrogen to stop root activity and release of exudates.

The sub-samples were utilised for isotopic abundance analysis, presented as in $\delta^{18}O$ values, which is the ratio of stable isotopes oxygen-18 and oxygen-16 samples. The soil sub-samples were placed inside a glove bag (Polyethylene glove bag, Thermo Scientific™, Waltham, Massachusetts, USA) filled with dry air, where the remaining small pieces of fine root material were thoroughly screened and manually removed.

This process ensured that only the water from the soil material was extracted through cryogenic distillation. Approximately 0.30 mL of water was extracted from each sub-sample, and distillation was carried out until no more water residual was present in the soil samples. Later, the two sub-samples of each treatment were bulked to compose one water sample per pot, aiming for the minimum quantity of 0.50 mL necessary for the isotopic determination. A total of 24 samples were generated after the soil distillation – a factorial combination of Lp, Bv, Dg and 20-25% FC, 80-85% FC plus bare soil no-barrier and bare soil with-barrier pots samples, in three blocks.

Oxygen isotope ratios of water were determined by the method of equilibration with CO_2, the CO_2 being measured by isotope ratio mass spectrometry (IRMS) at the University of Otago’s Chemistry Department - Isotrace NZ Ltd laboratories, Dunedin, New Zealand. A water sample of 0.5 mL was equilibrated with 12 mL of 0.3 % CO_2 in helium on a Gasbench II (Thermo Scientific, Bremen, DEU) preparation unit for 18 hours at 25.0 ± 0.1°C. Ten repeated injections of the equilibrated gas were measured with a Thermo Advantage isotope ratio mass spectrometer in continuous-flow mode (CF-IRMS). The ten results were filtered by removing more than one standard deviation value from the average. The filtered average was corrected to the international VSMOW-SLAP isotope scale using a three-point calibration provided by three laboratory standards analysed before and after every batch of 18 samples. In addition, a control sample, chosen to be similar to the experimental samples, was measured at every 12th position to correct for instrumental drift. If applied, instrumental drift correction was calculated from a linear regression of the control sample result against time. Consensus values for the laboratory standards have been obtained from 6-year internal laboratory calibration records against primary reference materials, VSMOW, GISP and SLAP, external 6-member interlaboratory comparison exercise and by back-calculation from the ~170 members IAEA interlaboratory comparison exercise, WICO2012. The Isotrace NZ Ltd laboratories, Dunedin, New Zealand standards and their consensus values were as follows: ICE (18OVSMOW = -32.097 ± 0.075‰), TAP (18OVSMOW = -11.432 ± 0.038‰), SEA (18OVSMOW = -0.029 ± 0.036‰), and as reported also by Stokes et al. (2020).

The roots mass sampling was done on 20 April 2021 (day 33). The bottom meshes of the pots were removed; thus, the soil substrate contained in each pot could be entirely extracted and remain intact. Afterwards, they were split in the middle (0-20 cm and 20-40 cm depth) and manually washed off until the roots were completely clean. The roots were dried in a forced ventilation oven at 60^{o}C for 72 h and weighed as dry matter. Total biomass accumulation was the sum of above- and below-ground dry matter.

3.5.3. Above-ground measurements

The leaf water potential was assessed with a Scholander chamber and pressure bomb (Soil Moisture Equipment Crop, Santa Barbara, California, USA) on 14 April 2021 (day 27) from 10 AM to 03 PM. The newest fully expanded leaf from two tillers per pot was selected, and 10 cm from the tip towards the ligule of each leaf was cut and collected to be used separately in the Scholander chamber. Slowly increasing pressure flux was applied to each sample, and measurement was recorded once the sap was forced out and became visible through a magnifying lens (Turner 1981).

The leaf malondialdehyde concentration (MDA), a product of peroxidation of unsaturated acids and a great indicator to quantify the levels of drought stress on plant tissues, was determined according to Abid et al. (2018) and García-Favre et al. (2021b). On 19 April 2021 (day 32), the newest fully expanded leaf of two random tillers per pot was collected and instantly frozen in liquid nitrogen. The samples were stored in a freezer at -18°C. In the laboratory, each sample separately of the leaf material was weighed and added to 2 mL of 5% trichloroacetic acid (TCA), grounded and centrifuged at 3000 rpm for 10 min. Subsequently, 1 mL of the supernatant obtained was added to 1 mL of 0.67% thiobarbituric acid (TBA), incubated in a boiling water bath for 30 min and centrifuged. The supernatant was displaced in a spectrometer to obtain the absorbance levels at 440, 532 and 660 nm wavelengths (WL). The absorbance levels at the three wavelengths of each sample were corrected according to their initial leaf material weight. The MDA concentration value was calculated by equation 1:

$$[MDA]\ (\mu mol\ L^{-1}) = 6.45 * (WL_{532} - WL_{600}) - 0.56 * WL_{440} \qquad \text{Equation 1}$$

The plant material sampling and storing for plant osmotic potential were done simultaneously with the MDA, following the same sampling procedures. The lamina samples were taken to a laboratory to measure the plant osmotic potential with a Wescor HR-33T microvoltmeter and six C-52 sample chambers (WESCOR Inc, Logan, Utah, USA). The lamina was cut into a 25 mm^2 square to fit in the chamber holder cavity. The sample was placed flattened in the holder, sealed and allowed to equilibrate for 15 minutes until reaching pressure stability, and then the measurement was taken. Each chamber was calibrated against the water potential of five NaCl solutions (0.2, 0.4, 0.6, 0.8, 1.0 molal) at 15ºC (same room temperature as at the plant material measurements). The initial plant osmotic potential values obtained from the plant material were corrected due to the variations of each chamber in which they were specifically measured (Lang 1967).

The number of tillers per pot was assessed weekly (days 0, 8, 15, 22 and 29) from 19 March 2021 to 18 April 2021. To assess the lamina elongation rate, two individual tillers were marked per pot at the beginning of the study period (day 1) and continuously measured every three days (days 1, 4, 7, 10, 13, 16, 19, 22, 25, 28 and 31). A lamina was considered fully expanded when its ligule was visible and measured from the tip to the ligule. A new lamina was assessed when its tip was visible within the previous leaf sheath and measured from its tip to the previous leaf ligule (Poff et al. 2011). The lamina was considered senescing when its extremities began to decolour and dry, and only its remaining green part was measured. The daily lamina elongation rate was calculated as the difference between the accumulated lamina length per tiller of the current date (n) and the accumulated lamina length of the previous measurement date (n-3) divided by the date interval (three days).

The components of the yield, lamina area, and herbage mass were assessed on the final harvest (days 32 and 33). Five tillers per pot (the two marked tillers utilised for previous morphological measurements, plus three with similar leaf regrowth stage and size condition) were collected and fractioned into pseudo-stem, lamina, and dead material. The laminas were measured in an electronic integrator of leaf area (LI-3100C - LI-COR Biosciences, Lincoln, Nebraska, USA). All fractions were separately weighed as fresh, dried in a forced ventilation oven at 60ºC for 72 h and weighed again as the dry matter of each component. The remaining herbage masses in the pots were cut at soil surface level, and fractioned into pseudo-stem, lamina and dead material, separately weighed as fresh, dried in a forced ventilation oven at 60°C for 72 h and weighed again as dry matter of each component. This process was repeated to define the percentage correlation of the tillers sample and the total herbage mass sample and, therefore, define the leaf area index, calculated as the lamina area (cm^2) over the pot surface area (cm^2). The plant morphological responses were measured and depicted as above-ground biomass accumulation of three components of the yield categories (lamina, sheath and dead material) per tiller and pot.

3.5.4. Statistical Analysis

All statistical analyses were performed using SAS v 9.4 (SAS Institute Inc, Cary, NC, USA). The data were examined for normal distribution using the Shapiro-Wilk test, finding that all dependent variables followed a normal distribution.

Analyses of variance for the dependent variables that were recorded only once were performed using the GLIMMIX procedure with the following mixed model:

$$\mathrm{Y}_{ijk} = \mu + \mathrm{Spp}_i + \mathrm{WR}_j + (\mathrm{Spp} * \mathrm{WL})_{ij} + \mathrm{B}_k + \mathrm{e}_{ijk}$$

Where Spp_i is the fixed effect of i$^{\mathrm{th}}$ grass species (Lp, Bv and Dg), WR_j is the fixed effect of the j$^{\mathrm{th}}$ level of water restrictions (HR and LR), $\mathrm{Spp*WR}_{\mathrm{ij}}$ is the fixed effect of the interaction between the i$^{\mathrm{th}}$ grass species and the j$^{\mathrm{th}}$ level of water restriction, B_k is the random effect of the k$^{\mathrm{th}}$ block and $\mathrm{e}_{\mathrm{ijk}}$ is the random residual assumed with mean zero and variance σ_e^2.

Analysis of variance for dependent variables that were repeatedly recorded over time on the same pot was performed using the GLIMMIX procedure with the following mixed model for repeated measures:

$$\mathrm{Y}_{ijk} = \mu + \mathrm{Spp}_i + \mathrm{WL}_j + (\mathrm{Spp} * \mathrm{WR})_{ij} + \mathrm{B}_k + \mathrm{T}_m + (\mathrm{Spp} * \mathrm{T})_{im} + (\mathrm{WR} * \mathrm{T})_{jm} + (\mathrm{SPP} * \mathrm{WR} * \mathrm{T})_{ijm} + \mathrm{e}_{ijkm}$$

Where all terms are as defined above, and $\mathrm{T_m}$ is the fixed effect of the $\mathrm{m^{th}}$ time. To account for repeated measures on the same pot through time, the best structure to model the variances and covariances of the random residuals was determined using the corrected Akaike Information Criteria Value (Wang and Goonewardene 2004). It was found that the best error structure was the Huynh–Feldt model.

F-values for the testing significant effects of fixed factors were calculated using the Satterthwaite approximation as suggested for split-plot design (Satterthwaite 1946). Least square means and standard errors for the fixed effects and combinations between the fixed effects were obtained and used for multiple mean comparisons using Fisher's least significant difference. Significant differences between the least squares means were declared at $p \leq 0.05$.

The relationships between the measured variables were evaluated using multivariate statistical analyses with the CANDISC procedure applied to two different datasets. The first analysis explored the relationships among physiological responses, biomass accumulation and distribution, and the hydraulic lift resulting from soil water stress. The second analysis explored the relationships among general morpho-physiological variables as a result of species types (competitors and stress-tolerators) by Grime

(1974). The canonical variate analysis (Jobson 1992; López et al. 2006) was performed to determine the extent to which variables explained most of the differences between species and soil water restriction levels.

3.6. RESULTS

3.6.1. Biomass accumulation: above- and below-ground dry matter accumulation driven by species and levels of water restriction

There were no significant interactions between the three species and the two levels of water restriction (Spp x WR) for any of the biomass fractions ($p > 0.05$). The total biomass accumulation was significantly different for the Spp and WR; Dg accumulated 25.1% more total biomass than Lp and Bv ($p \leq 0.05$) and LR pots accumulated 25.7% more total biomass than HR ($p \leq 0.01$). Accordingly, the LR pots' above-ground biomass was 41.2% higher than the HR ($p \leq 0.01$). The three species accumulated similar above-ground biomass ($p > 0.05$) (Table 3.1). The total below-ground biomass accumulation ($p \leq 0.001$) and its distribution within the soil depth, as in the 0-20 cm soil layer ($p \leq 0.01$) and the 20-40 cm soil layer ($p \leq 0.05$) were significantly different between the Spp. Overall, Dg had the highest total below-ground biomass - 64.8% higher than Bv and Lp - and the highest root biomass in the 0-20 cm soil layer - 66.7% higher than Bv and Lp (Table 3.1). At the 20-40 cm soil layer, Bv had a similar root biomass as Dg.

Table 3.1. Effect of three plant species (Spp = Bv, Dg and Lp), two water level restrictions (WR = LR and HR) and the interaction between treatments on total biomass (sum of above- and below-ground dry matter [DM]), above-ground biomass (total herbage DM) and below ground-biomass (total roots DM, top-layer DM and bottom-layer DM) at day 32.

	Total Biomass	Above-ground biomass	Below-ground biomass		
	Above- and below-ground DM	Total herbage DM	Total root DM (0-40cm)	Root DM (0-20cm)	Root DM (20-40cm)
	(g)	*(g)*	*(g)*	*(g)*	*(g)*
Spp					
Bromus valdivianus (Bv)	11.4^{b} (±1.0)	5.8 (±0.7)	5.6^{b} (±0.5)	4.5^{b} (±0.4)	1.1^{ab} (±0.1)
Dactylis glomerata (Dg)	14.7^{a} (±1.3)	5.7 (±0.8)	8.9^{a} (±0.7)	7.5^{a} (±0.7)	1.5^{a} (±0.2)
Lolium perenne (Lp)	12.1^{b} (±0.5)	6.9 (±0.4)	5.2^{b} (±0.5)	4.5^{b} (±0.5)	0.7^{b} (±0.2)
Significance	*	NS	***	**	*
WR					
Low Restriction (LR)	14.2^{a} (±0.8)	7.2^{a} (±0.4)	7.0 (±0.9)	5.9 (±0.7)	1.1 (±0.2)
High Restriction (HR)	11.3^{b} (±0.7)	5.1^{b} (±0.4)	6.2 (±0.6)	5.0 (±0.6)	1.1 (±0.1)
Significance	**	**	NS	NS	NS
Interaction Spp x WR					
BvLR	13.3 (±0.6)	7.3 (±0.4)	6.0 (±0.7)	4.8 (±0.7)	1.3 (±0.1)
BvHR	9.4 (±0.8)	4.3 (±0.2)	5.2 (±0.6)	4.1 (±0.4)	1.0 (±0.2)
DgLR	16.8 (±1.4)	6.8 (±1.2)	10.0 (±0.6)	8.4 (±0.5)	1.6 (±0.2)
DgHR	12.5 (±1.5)	4.7 (±0.6)	7.8 (±0.8)	6.5 (±1.2)	1.3 (±0.4)
LpLR	12.3 (±0.2)	7.4 (±0.5)	4.9 (±0.7)	4.5 (±0.7)	0.4 (±0.2)
LpHR	11.8 (±1.0)	6.3 (±0.4)	5.5 (±0.9)	4.5 (±0.8)	1.1 (±0.1)
Significance	NS	NS	NS	NS	NS

Superscripted letters that differ within the same columns and lines for the same fixed effect and/or interaction of fixed effects indicate statistically significant values at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p = 0.0001$ and NS = non-significant. Following each least square mean is the (±) standard error of the mean.

3.6.2. Soil water dynamics and hydraulic lift: phenomena validation and characterisation

On 19 April at 07:45 AM, just before the irrigation of the bottom layers with ^{18}O labelled water, the SWC% of the pots' top and bottom layers were assessed to validate the assessment of further soil-related results. The triple interaction between plant Spp, WR and soil level profile (3x2x2) was significant for the soil volumetric water content ($p \leq 0.001$). There was no significant difference among the top layers' SWC% of all pots just before the moment of the labelled irrigation, with all the measured values under PWP (≤ 0.0935 SWC%). Therefore, ^{18}O labelled water applied in the bottom layers was redistributed via hydraulic lift into homogenous recipients (top layers).

The hydraulic lift was observed and captured from SWC% fluctuations provided by the soil moisture sensors within the pots' top layers (indirect measurements), often occurring the day after the treatments' irrigation was applied. The hydraulic lift via SWC% was identified and measured via SWC% on five occasions (days 9, 16, 19, 22, 27) during the experimental period and was indirectly quantified as the differences between the highest and the lowest SWC% of each day (Table 3.2 and Figure 3.5). The lowest SWC% values were registered around 7 to 8 AM, while SWC% peaks occurred consistently from 12 PM to 3 PM. The hydraulic lift registered as SWC% was significantly related to the Spp on the five occurrence dates ($p \leq 0.05$) and was exhibited to a greater degree by Bv and Dg than Lp (Figure 3.5).

In addition to the SWC% indirect measurements, the hydraulic lift was directly quantified using ^{18}O labelled water (day 33). The hydraulic lift registered using ^{18}O

labelled water significantly differed between the Spp ($p \leq 0.05$). The Bv exhibited the phenomena to a greater degree, approximately double the $\delta^{18}O$ values found in the soil top layer of Dg and Lp pots (Table 3.2). For both indirect and direct methodologies of hydraulic lift assessment, no significant differences due to different WR and no interaction between the fixed effect (Spp x WR) were found ($p > 0.05$). The three pots without the barrier presented a $\delta^{18}O$ of 0.26‰, -0.50‰, 2.99‰ (μ = 0.92‰), while the three pots with the barrier presented a $\delta^{18}O$ of 1.10‰, 1.69‰, -0.73‰ (μ = 1.17‰).

Table 3.2. Effect of three plant species (Spp=Bv, Dg, Lp), two water level restrictions (WR=LR and HR) and interactions between treatments on hydraulic lift indirectly captured as the increase in soil volumetric water content presented by the top layer sensors at day 9, 16, 19, 22 and 27. Effect of three plant species (Spp=Bv, Dg, Lp), two water level restrictions (WR=LR and HR) and the interaction between treatments on hydraulic lift directly captured as $\delta^{18}O$ values by the isotopic analysis from the soil top layer sampled on day 33.

	Hydraulic Lift					
	Soil volumetric water content (top layer)					$\delta^{18}O$
	Day 9	Day 16	Day 19	Day 22	Day 27	Day 33
			(m^3 m^{-3})			*(‰)*
Spp						
Bromus valdivianus (Bv)	0.0102a (±0.0008)	0.0058a (±0.0007)	0.0046a (±0.0006)	0.0055a (±0.0005)	0.0052a (±0.0005)	25.05a (±4.36)
Dactylis glomerata (Dg)	0.0110a (±0.0018)	0.0058a (±0.0007)	0.0037a (±0.0004)	0.0050a (±0.0004)	0.0046a (±0.0011)	13.70b (±3.43)
Lolium perenne (Lp)	0.0050b (±0.0023)	0.0031b (±0.0014)	0.0019b (±0.0009)	0.0027b (±0.0012)	0.0025b (±0.0004)	10.30b (±3.82)
Significance	**	*	**	***	**	*
WR						
Low Restriction (LR)	0.0077 (±0.0020)	0.0039 (±0.0011)	0.0030 (±0.0009)	0.0039 (±0.0060)	0.0035 (±0.0009)	15.29 (±4.69)
High Restriction (HR)	0.0097 (±0.0007)	0.0059 (±0.0004)	0.0039 (±0.0003)	0.0049 (±0.0017)	0.0046 (±0.0004)	17.42 (±2.58)
Significance	NS	NS	NS	NS	NS	NS
Interaction Spp x WR						
BvLR	0.0108 (±0.0012)	0.0059 (±0.0014)	0.0058 (±0.0007)	0.0065 (±0.0003)	0.0057 (±0.0003)	30.23 (±5.86)
BvHR	0.0095 (±0.0010)	0.0057 (±0.0003)	0.0036 (±0.0005)	0.0046 (±0.0008)	0.0046 (±0.0007)	19.87 (±5.80)
DgLR	0.0124 (±0.0009)	0.0057 (±0.0009)	0.0034 (±0.0007)	0.0052 (±0.0007)	0.0049 (±0.0005)	12.22 (±5.66)
DgHR	0.0095 (±0.0020)	0.0059 (±0.0013)	0.0041 (±0.0005)	0.0049 (±0.0007)	0.0044 (±0.0009)	15.20 (±4.95)
LpLR	0.0000 (±0.0000)	0.0000 (±0.0000)	0.0000 (±0.0000)	0.0000 (±0.0000)	0.0000 (±0.0000)	3.40 (±3.17)
LpHR	0.0101 (±0.0008)	0.0062 (±0.0001)	0.0039 (±0.0004)	0.0052 (±0.0005)	0.0049 (±0.0003)	17.96 (±3.93)
Significance	NS	NS	NS	NS	NS	NS

Superscripted letters that differ within the same columns and lines for the same fixed effect and/or interaction of fixed effects indicate statistically significant values at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p = 0.0001$ and NS = non-significant. Following each least square mean is the (±) standard error of the mean.

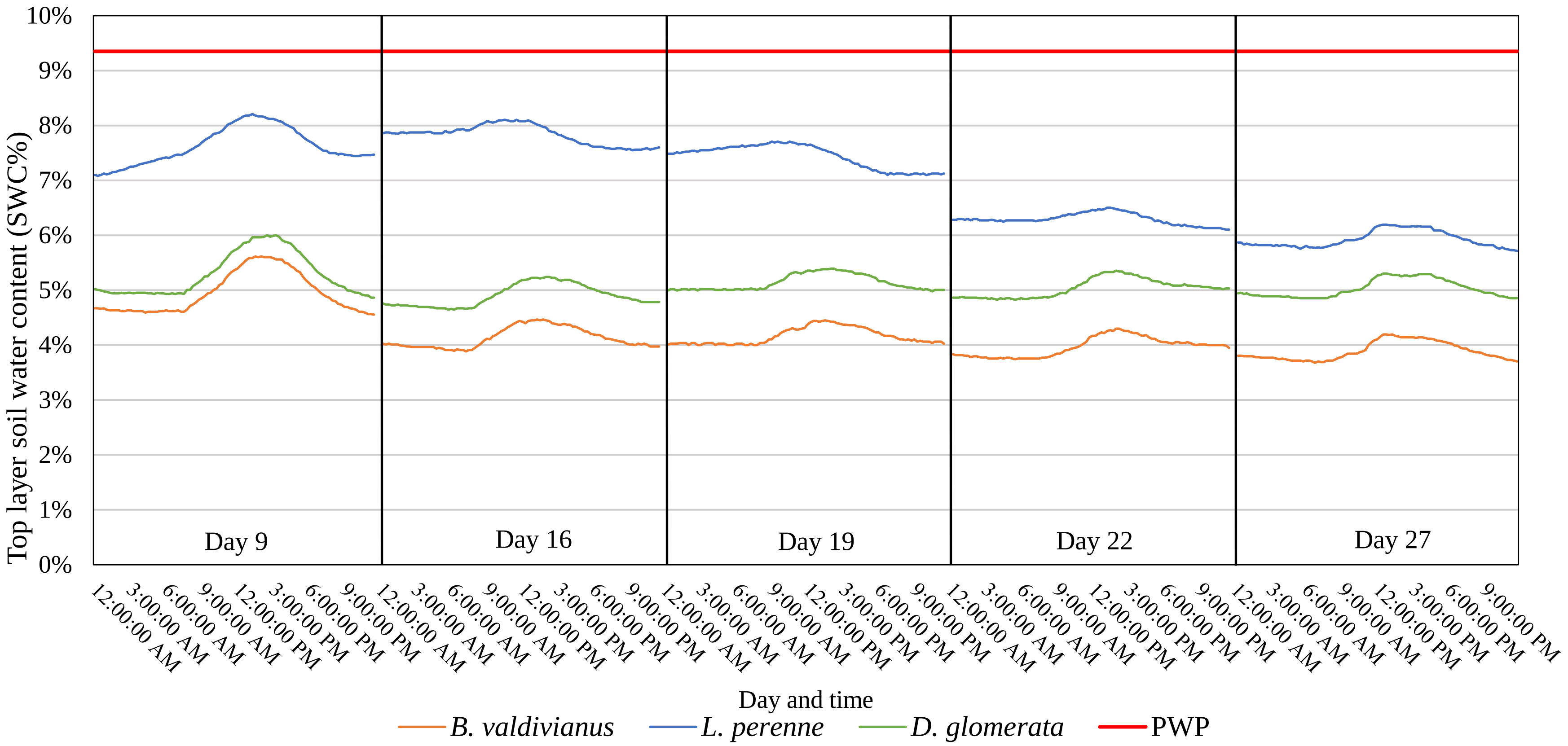

Figure 3-5. Average daily fluctuation readings of volumetric water content captured by SWC% sensors located on the top-layer soil on the five days the hydraulic lift was captured. The SWC% values presented refer to the data by Spp (significantly different as per Table 3.2; *B. valdivianus* in orange, *L. perenne* in blue and *D. glomerata* in green) on day 9, day 16, day 19, day 22 and day 27. The line in red depicts the soil substrate's permanent wilting point (PWP).

3.6.3. Physiological responses: species' metabolic responses regulated by soil water stress.

There were no significant differences in the plant physiological responses evaluated (leaf water potential, plant osmotic potential and MDA) among the three Spp (p> 0.05). However, these three variables were significantly affected by the WR (p≤0.001, p≤0.01, and p≤0.05, respectively). The plants submitted to HR treatment presented more negative values of leaf water potential and plant osmotic potential and higher values of MDA than the plants under LR. There were no significant interactions between the fixed effects (Spp x WR) for leaf water potential and MDA (p>0.05). Significant interactions were found for plant osmotic potential (p≤0.05), in which the LpHR presented the most negative value (Table 3.3).

Table 3.3. Effect of three plant species (Spp = Bv, Dg, Lp), two water level restrictions (WR = LR and HR) and the interaction between treatments on leaf water potential at day 27, plant osmotic potential and malondialdehyde leaf concentration (MDA) at day 32.

	Leaf water potential	Plant osmotic potential	MDA
	Day 27	Day 32	Day 32
	(MPa)	*(MPa)*	*(µmol.L^{-1})*
Spp			
Bromus valdivianus (Bv)	- 1.9 (±0.10)	- 2.0 (±0.05)	1.1 (±0.16)
Dactylis glomerata (Dg)	- 1.9 (±0.09)	- 1.8 (±0.14)	0.7 (±0.12)
Lolium perenne (Lp)	- 1.7 (±-0.11)	- 2.2 (±0.27)	0.9 (±0.06)
Significance	NS	NS	NS
WR			
Low Restriction (LR)	- 1.7^{a} (±0.07)	- 1.7^{a} (±0.08)	0.7^{b} (±0.10)
High Restriction (HR)	- 2.2^{b} (±0.04)	- 2.3^{b} (±0.16)	1.0^{a} (±0.09)
Significance	***	**	*
Interaction Spp x WR			
BvLR	- 1.7 (±0.08)	- 1.9^{a} (±0.04)	0.8 (±0.260)
BvHR	- 2.1 (±0.00)	- 2.1^{a} (±0.06)	1.3 (±0.003)
DgLR	- 1.7 (±0.08)	- 1.7^{a} (±0.17)	0.5 (±0.038)
DgHR	- 2.1 (±0.00)	- 1.9^{a} (±0.21)	1.0 (±0.131)
LpLR	- 1.6 (±0.12)	- 1.7^{a} (±0.12)	0.9 (±0.050)
LpHR	- 1.9 (±0.02)	- 2.8^{b} (±0.20)	0.8 (±0.122)
Significance	NS	*	NS

Superscripted letters that differ within the same columns and lines for the same fixed effect and/or interaction of fixed effects indicate statistically significant values at * p≤0.05, ** p≤0.01, *** p≤0.001, **** p=0.0001 and NS = non-significant. Following each least square mean is the (±) standard error of the mean.

3.6.4. Morphological responses: structural changes in species' strategies determined by soil water

There were no significant interactions (Spp x WR) for lamina, sheath and dead material biomass accumulation and the proportional distribution of lamina weight in relation to the total weight of above-ground biomass (lamina:total biomass proportion) per pot and per tiller ($p > 0.05$). The different WR significantly affected the biomass accumulation of lamina and lamina:total biomass proportion per pot and tiller ($p = 0.0001$ and $p \leq 0.05$, respectively). The plants subjected to the LR treatment accumulated 64.5% and 50.9% more lamina biomass per pot and tiller, respectively, than in HR. Significant differences were found among Spp's dead material biomass accumulation per pot and tiller (both at $p \leq 0.05$), where Bv accumulated dead material was higher than that of Lp and Dg at pot and tiller levels. Meanwhile, significant differences were also found among Spp's sheath accumulation per pot and tiller ($p \leq 0.001$ and $p = 0.0001$, respectively), in which Dg had the greatest sheath mass and Bv the lowest sheath mass for both sampling scales. For the lamina biomass accumulation, significant differences among Spp were found only in the tiller's sampling scale ($p \leq 0.05$), in which the Lp presented the lowest accumulated lamina biomass per tiller. At a pot sampling scale, the three species accumulated similar lamina biomass (Table 3.4).

Table 3.4. Effect of three plant species (Spp = Bv, Dg, Lp), two water level restrictions (WR = LR and HR) and interactions between treatments on the lamina, sheath and dead material accumulation and its proportional distribution in relation to the total weight o of above-ground biomass (lamina:total biomass) per pot and per tiller on Day 32.

	Lamina		Sheath		Dead Material		Lamina:total biomass	
	Pot	Tiller	Pot	Tiller	Pot	Tiller	Pot	Tiller
	(g)	*(mg)*	*(g)*	*(mg)*	*(g)*	*(mg)*	%	
Spp								
Bromus valdivianus (Bv)	3.6 (±0.5)	92.2^{a} (±14.5)	0.8^{b} (±0.2)	20.2^{b} (±2.2)	1.5^{a} (±0.20)	32.9^{a} (±3.5)	60.3 (±3.7)	61.3 (±4.2)
Dactylis glomerata (Dg)	3.5 (±0.5)	104.9^{a} (±14.8)	1.6^{a} (±0.2)	66.6^{a} (±6.2)	0.6^{b} (±0.19)	21.2^{ab} (±5.8)	62.0 (±3.4)	54.5 (±4.7)
Lolium perenne (Lp)	3.7 (±0.3)	44.9^{b} (±6.4)	1.9^{a} (±0.1)	24.5^{b} (±3.1)	1.3^{a} (±0.07)	11.8^{b} (±1.7)	54.0 (±1.8)	55.2 (±1.9)
Significance	NS	**	***	****	*	*	NS	NS
WR								
Low Restriction (LR)	4.4^{a} (±0.2)	97.0^{a} (±14.2)	1.6 (±0.2)	40.4 (±8.8)	1.1 (±0.2)	18.1 (±3.2)	63.0^{a} (±2.1)	62.3^{a} (±1.9)
High Restriction (HR)	2.7^{b} (±0.2)	64.3^{b} (±9.7)	1.2 (±0.2)	33.8 (±7.2)	1.1 (±0.2)	25.7 (±5.0)	54.6^{b} (±2.5)	51.7^{b} (±3.1)
Significance	****	*	NS	NS	NS	NS	*	*
Interaction Spp x WR								
BvLR	4.7 (±0.3)	109.7 (±12.4)	1.1 (±0.14)	24.8 (±0.3)	1.5 (±0.31)	27.3 (±4.9)	64.7 (±3.8)	67.7 (±0.9)
BvHR	2.4 (±0.2)	74.7 (±24.3)	0.4 (±0.04)	15.5 (±1.8)	1.5 (±0.33)	38.4 (±2.1)	56.0 (±6.1)	55.0 (±6.8)
DgLR	4.5 (±0.5)	132.0 (±16.4)	1.8 (±0.48)	73.1 (±9.8)	0.5 (±0.25)	16.2 (±4.9)	66.7 (±4.1)	60.7 (±4.4)
DgHR	2.6 (±0.2)	77.8 (±9.3)	1.4 (±0.22)	60.2 (±7.6)	0.7 (±0.34)	26.2 (±11.0)	57.3 (±4.4)	48.3 (±7.3)
LpLR	4.3 (±0.2)	49.3 (±12.7)	1.9 (±0.26)	23.3 (±5.3)	1.3 (±0.04)	10.7 (±1.7)	57.7 (±0.9)	58.7 (±1.2)
LpHR	3.2 (±0.2)	40.4 (±5.1)	1.9 (±0.08)	25.7 (±4.3)	1.2 (±0.16)	12.9 (±3.2)	50.3 (±1.5)	51.7 (±2.0)
Significance	NS	NS	NS	NS	NS	NS	NS	NS

Superscripted letters that differ within the same columns and lines for the same fixed effect and/or interaction of fixed effects indicate statistically significant values at * *p*≤0.05, ** *p*≤0.01, *** *p*≤0.001, **** *p*=0.0001 and NS = non-significant. Following each least square mean is the (±) standard error of the mean.

The lamina surface area per tiller was significantly different between the Spp ($p \leq 0.001$) and the WR ($p \leq 0.001$) and had a significant interaction (Spp x WR) ($p \leq 0.05$) (Table 3.5). The DgLR had the greatest area of lamina area, with an average increment of 215.0% over the lowest areas presented by LpLR, LpHR and BvHR. There was no significant interaction (Spp x WR) and no significant differences between WR for specific lamina area (lamina area/lamina mass) ($p > 0.05$). Significant differences were only shown by the Spp ($p \leq 0.05$), where Dg had an average SLA 31.0% higher than the other species.

The leaf regrowth stage (LS) significantly differed due to the WR and presented a significant interaction (Spp x WR). The three lowest LS values were found on the plants subjected to a higher WR during the experimental period; DgHR and LpHR presented the lowest values, followed by the BvHR (Table 3.5). In the five different dates of assessment, the number of tillers did not present significant interactions between treatments (Spp x WR) and was not significantly affected by WR ($p > 0.05$). Significant differences were found only for the Spp at all five dates of assessment ($p \leq 0.001$ for all). The same pattern of vegetative growth was observed for all species, with an overall increment in the number of tillers from day 0 (first date of assessment) up to day 29 (final date). Among species, the Lp consistently had the highest number of tillers per pot, generally double the number of tillers that Bv and Dg presented. The Bv and Dg had a similar number of tillers per pot (Table 3.5).

Table 3.5. Effect of three plant species (Spp = Bv, Dg, Lp), two water level restrictions (WR = LR and HR) and interactions between treatments on lamina surface area, specific lamina area, leaf regrowth stage per tiller on Day 32, and the number of tillers on day0, day 8, day 15, day 22, and day 29.

	Lamina surface area	Specific lamina area	Leaf regrowth stage	Number of Tillers				
	D32	D32	D32	D0	D8	D15	D22	D29
	(cm^2)	*(cm^2g^{-1})*						
Spp								
Bromus valdivianus (Bv)	16.9^{b} (±6.2)	47.9^{b} (±9.3)	2.7 (±0.5)	72.8^{b} (±19.9)	75.5^{b} (±22.3)	74.2^{b} (±18.6)	74.3^{b} (±19.4)	76.7^{b} (±22.5)
Dactylis glomerata (Dg)	24.0^{a} (±11.4)	62.3^{a} (±6.3)	2.6 (±0.9)	59.7^{b} (±22.6)	60.0^{b} (±24.2)	60.8^{b} (±23.5)	64.5^{b} (±23.3)	66.2^{b} (±24.0)
Lolium perenne (Lp)	9.5^{c} (±4.2)	47.2^{b} (±3.2)	2.5 (±0.4)	129.3^{a} (±21.1)	132.5^{a} (±21.6)	134.5^{a} (±23.8)	133.0^{a} (±23.6)	138.5^{a} (±25.1)
Significance	***	*	NS	***	***	***	***	***
WR								
Low Restriction (LR)	21.9^{a} (±10.8)	53.9 (±10.5)	3.0^{a} (±0.4)	90.1 (±38.9)	90.9 (±37.6)	90.7 (±37.3)	93.9 (±35.4)	100.6 (±39.9)
High Restriction (HR)	11.7^{b} (±4.6)	51.0 (±8.9)	2.1^{b} (±0.3)	84.4 (±37.0)	87.8 (±41.6)	89.0 (±42.8)	87.3 (±41.3)	87.0 (±40.8)
Significance	***	NS	****	NS	NS	NS	NS	NS
Interaction Spp x WR								
BvLR	21.3^{b} (±2.8)	49.3 (±9.7)	3.0^{ab} (±0.1)	81.3 (±22.9)	83.3 (±25.8)	81.7 (±20.8)	84.0 (±20.7)	87.7 (±25.9)
BvHR	12.6^{c} (±5.5)	46.5 (±10.7)	2.3^{bc} (±0.4)	64.3 (±15.9)	67.7 (±19.9)	66.7 (±16.2)	64.7 (±15.3)	65.7 (±15.4)
DgLR	33.4^{a} (±6.3)	65.9 (±2.0)	3.4^{a} (±0.3)	59.3 (±32.8)	62.3 (±35.9)	63.3 (±34.4)	69.7 (±33.7)	73.3 (±32.6)
DgHR	14.6^{bc} (±4.3)	58.6 (±7.5)	1.8^{d} (±0.2)	60.0 (±14.4)	57.7 (±12.5)	58.3 (±13.1)	59.3 (±12.1)	59.0 (±14.5)
LpLR	10.9^{c} (±5.9)	46.5 (±3.3)	2.8^{b} (±0.4)	129.7 (±23.7)	127.0 (±21.0)	127.0 (±27.1)	128.0 (±26.2)	140.7 (±29.3)
LpHR	8.1^{c} (±1.4)	47.8 (±3.7)	2.2^{cd} (±0.1)	129.0 (±23.4)	138.0 (±25.1)	142.0 (±22.6)	138.0 (±25.1)	136.3 (±26.6)
Significance	*	NS	*	NS	NS	NS	NS	NS

Superscripted letters that differ within the same columns and lines for the same fixed effect and/or interaction of fixed effects indicate statistically significant values at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p = 0.0001$ and NS = non-significant. Following each least square mean is the (±) standard error of the mean.

The daily lamina elongation rate (LER) presented significant triple interactions (Spp x WR x Time) on days 4, 16, 19 and 22 ($p \leq 0.05$, $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.05$, respectively) and also presented a trend of difference on day 13 and 26 ($p \leq 0.1$). On day 4, BvLR and BvHR presented the highest LER, accumulating ~40 mm day^{-1} in total lamina length per tiller. At the same date, LpLR and LpHR elongated slower, accumulating 50% less than BvLR and BvHR, with an average lamina elongation rate of 20 mm day^{-1}. The following significant dates (days 16, 19 and 22), two to three weeks after the first assessment date (day 4), depicted the effect of water restriction throughout the experimental period as it presented a trend of diminishment on the LER for all species. On days 16, 19 and 22, DgHR accumulated less lamina length per tiller at a rate lower than 5 mm day^{-1}.

It is possible to observe the negative impact of water stress on the lamina development per tiller by the end of the experimental period, as DgHR accumulated 65% less lamina length than the DgLR (Figure 3.6). The Bv and Lp plants subject to HR were less affected than the DgHR, with a smaller accumulation decrease of 37% and 25% than their LR pair, respectively. Within the three species submitted to HR treatment, Bv and Dg had a similar response pattern to the water stress, drastically decelerating the lamina elongation rate per tiller within the first ten days. Meanwhile, the Lp had a less accentuated drop in its elongation for the same period and presented a more noticeable deceleration towards the end of the experimental period.

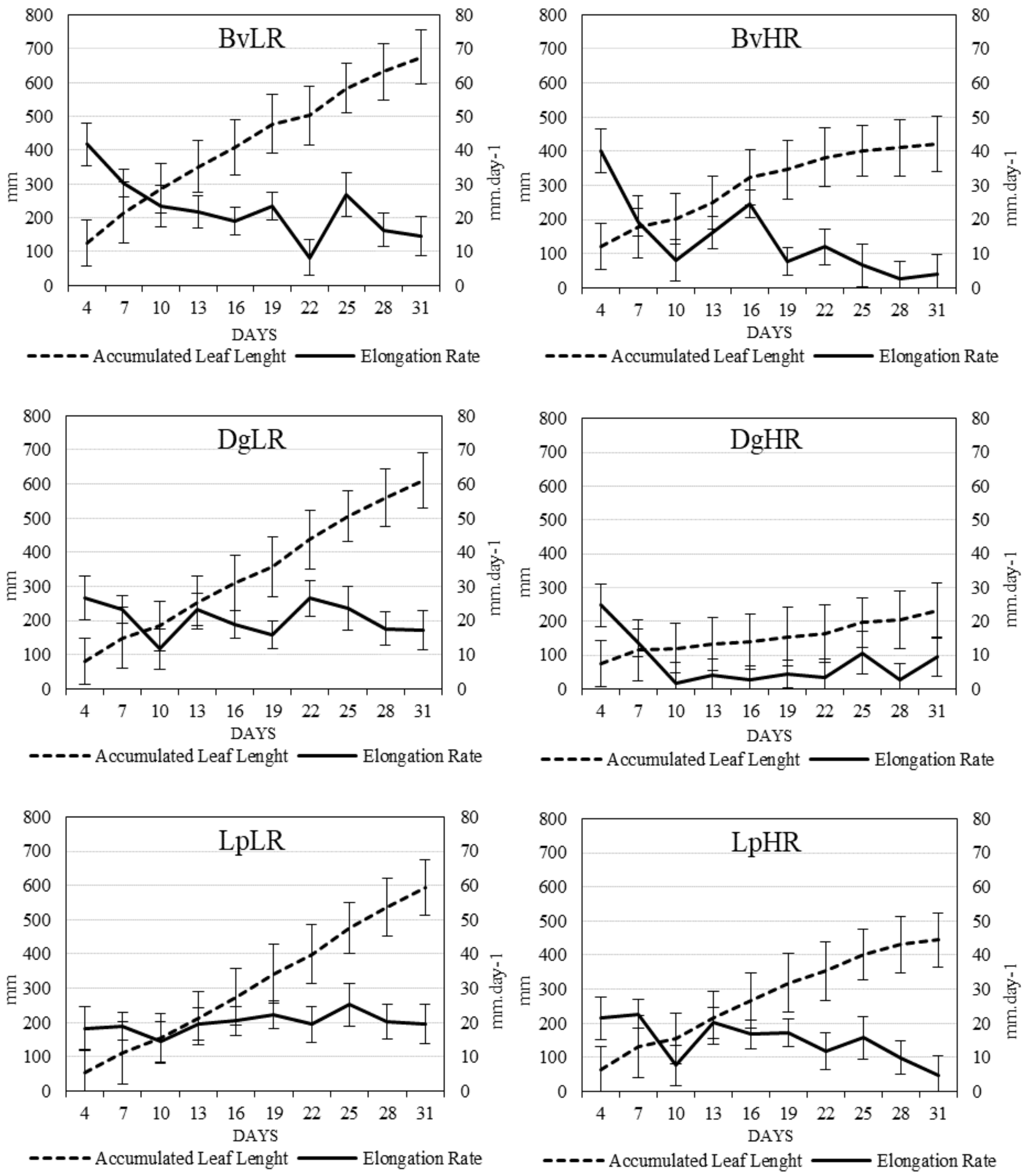

Figure 3-6. The accumulated lamina length (mm - in dashed black) and daily lamina elongation rate (mm.day^{-1} - in solid black) per tiller of the interaction between species, levels of water restriction and time (Spp x WR x Time). Standard errors of the mean are presented as vertical bars and are significantly different across time between species and levels of water restriction for the same response variable.

3.6.5. Canonical variate analysis – identifying the differences among groups of individuals

The canonical variate analysis was significant (Wilk’s Lambda = 0.0001), showing that the variables included in this study explained 95.6% of the results (CAN 1 = 88.4%, $p = 0.0001$, CAN 2 = 7.2%, $p = 0.0031$; Figure 3.7). Within the CAN 1 axe, the number of tillers (NT) was located in its negative direction, showing a contrast with below-ground bottom-layer biomass (BG_bott), hydraulic lift via SWC% sensor readings (HL_swc), and $\delta^{18}O$ values (HL_O18), dead material of the above-ground biomass (AG_demat) and MDA concentration (MDA), which were located in the positive direction of CAN1. In the positive direction of CAN 2, there were located the lamina area (LA), lamina and sheath of the above-ground biomass (AG_lam and AG_shth), below-ground top-layer (BG_top), BG_bott, HL_swc, and in the negative CAN 2 direction there were NT and MDA (Figure 3.8).

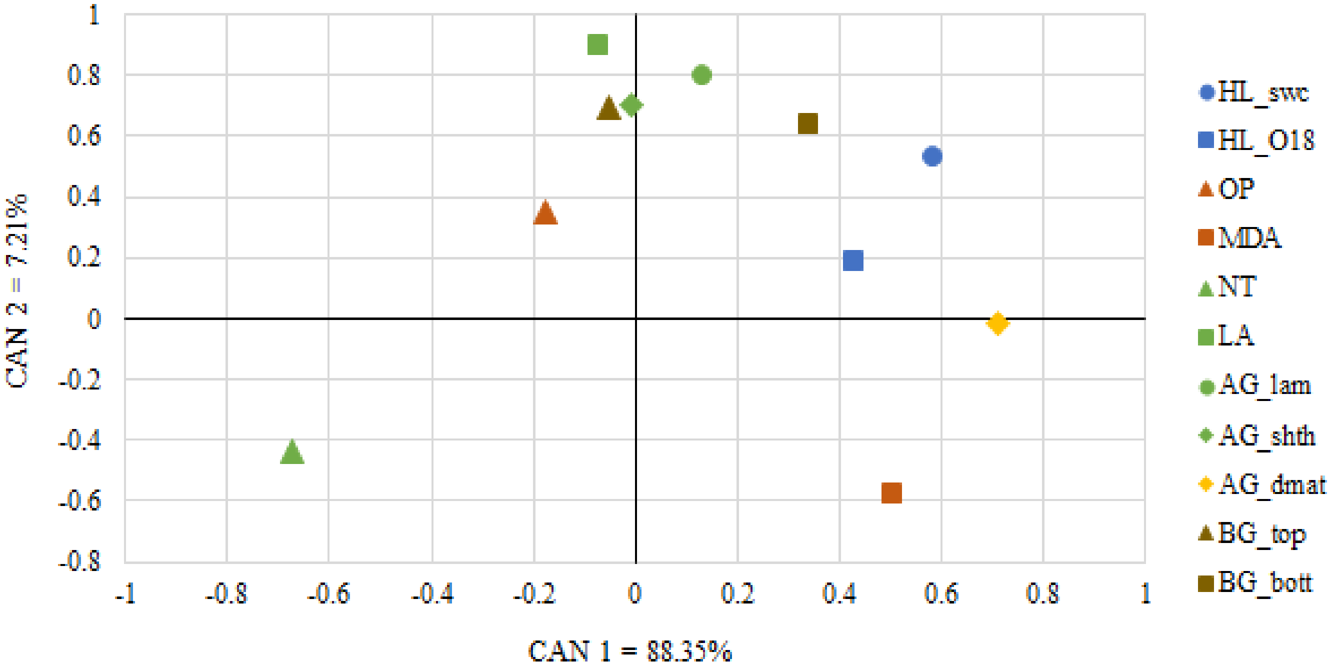

Figure 3-7. Canonical variates of the original variables and their relationships for *Bromus valdivianus*, *Dactylis glomerata* and *Lolium perenne* due to high and low levels of water restriction: hydraulic lift via SWC% sensor readings (HL_swc), hydraulic lift via δ^{18}O values (HL_18O), plant osmotic potential (OP), MDA concentration (MDA), number of tillers (NT), lamina area (LA), lamina of the above-ground biomass (AG_lam), sheath of the above-ground biomass (AG_shth), dead material of the above-ground biomass (AG_demat), below-ground top-layer biomass (BG_top) and below-ground bottom-layer biomass (BG_bott).

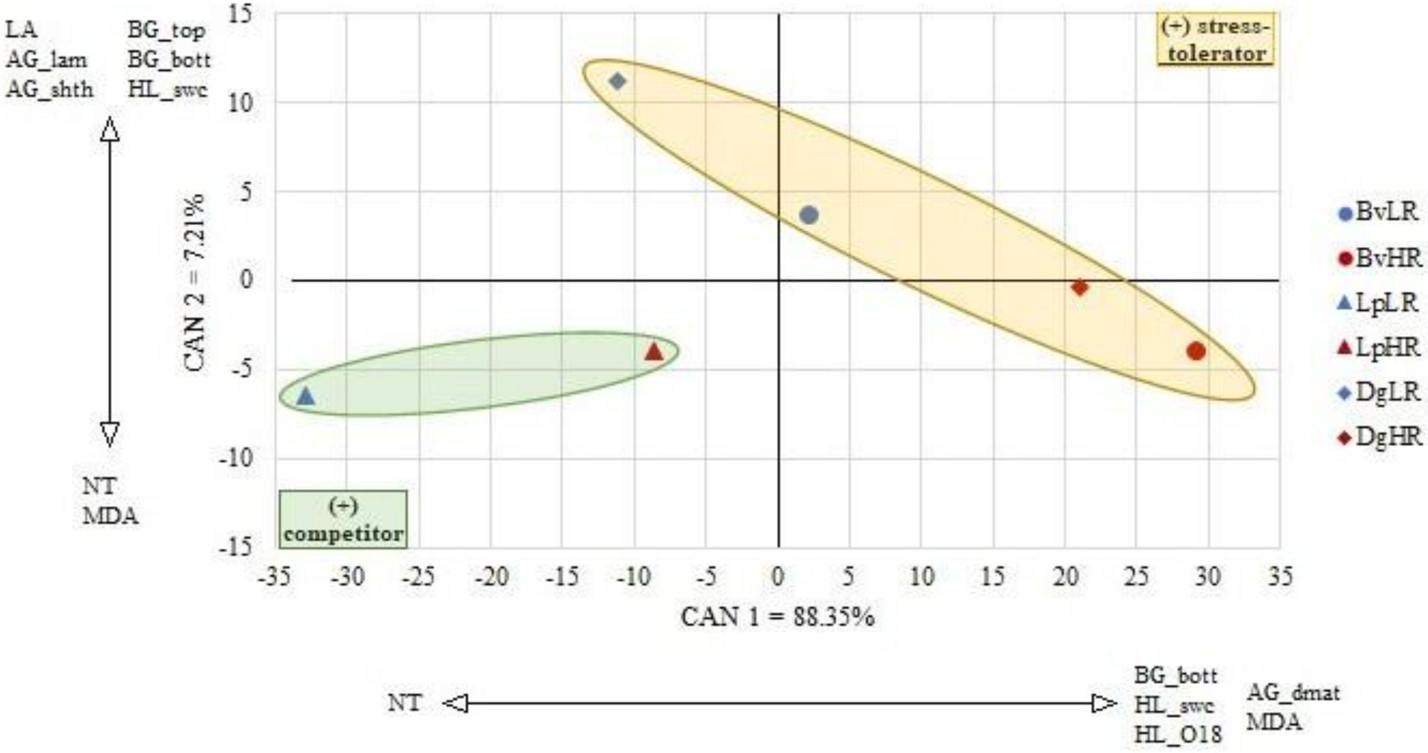

Figure 3-8. Canonical scores for the interaction of fixed effects: species (Spp = *Bromus valdivianus* - Bv, *Dactylis glomerata* – Dg and *Lolium perenne* - Lp) and levels of water restriction (WR = high restriction – HR and low restriction - LR).

3.7. DISCUSSION

The three species displayed some degree of hydraulic lift assessed using both direct (isotopic labelling) and indirect (SWC% sensors) techniques, validating that they can exhibit the phenomena at their own capacity. Results from the current study showed that *B. valdivianus* (to a higher degree), *D. glomerata* (to a medium degree) and *L. perenne* (to a lower degree) redistributed water from the deep to shallower soil layer via the hydraulic lift phenomenon. Complementary information on morpho-physiological responses of each species subjected to different levels of water restriction allowed us to (a) infer how they perform under soil water restriction, (b) correlate their functional strategies with the hydraulic lift phenomenon, (c) hypothesise on future practical applications of the species in a pastoral ecosystem, and (d) suggest new methodologies that can be helpful to the development of this research topic. The role of the capillary barrier utilised in the experimental pots contributed to the robustness of the study data generated.

3.7.1. Can the hydraulic lift be associated with grasses under water stress conditions? If so, what are the drivers that trigger the phenomenon?

Meunier et al. (2017) carried out a study to model the hydraulic lift of *L. multiflorum* in rhizo-boxes, and a day of the application of ^{18}O labelled water found an enrichment of 2.0 and 1.3‰ in comparison to control at the shallower layers (20-25 cm depth). In the present study, the δ^{18}O values found in the top layers evince that the three species exhibited the hydraulic lift at their capacity during daytime and peaking around midday. *Bromus valdivianus* can redistribute water to a greater degree than *D. glomerata* and *L. perenne* under water stress, showing approximately double the δ^{18}O values in the soil top layer. (Table 3.2).

Recent studies have shown that the type of root plays an essential role in the plant's ability to uptake water under drought or water restriction conditions, such that species with shorter root hairs (i.e., *O. sativa* and *Z. mays*) have no contribution to the water uptake (Carminati et al. 2016; Ahmed et al. 2018; Cai and Ahmed 2022). Meanwhile, species with longer root hairs (i.e., *Hordeum vulgare* L.) had an explicit influence on the water uptake levels (Burak et al. 2021; Cai and Ahmed 2022). The root morphology and allocation in depth may partially explain the hydraulic lift occurrence. *Bromus valdivianus* and *D. glomerata* were the species that presented greater root mass in the bottom layer (deep-rooted if compared to *L. perenne*) and are known to present root growth in depth as a functional trait of both species (García-Favre et al. 2022; Crush et al. 2005). Meanwhile, *L. perenne* allocates fewer roots in depth (shallow-rooted) (Wedderburn et al. 2010; García-Favre et al. 2022), a functional trait likely to constrain the species' hydraulic lift occurrence. In the present study, a more even root system distribution was found in *B. valdivianus*, which had a dry matter ratio of approximately 4:1 (0-20cm:20-40cm), while *D. glomerata* and *L. perenne* had approximately 6:1 and 5:1, respectively. In the present study, *L. perenne* under low water restriction did not exhibit hydraulic lift, as evidenced by δ^{18}O value [3.40‰ (±3.17)]. This leads us to conclude that probably shallow-rooted plants are less capable of exhibiting hydraulic lift under low levels of soil water restriction because of this morphological restrictive attribute.

The fluctuations of SWC% displayed on five different days, with an increase in the soil water availability during the hours of highest temperature (towards midday), demonstrated that *B. valdivianus* under low and high restriction, *D. glomerata* under low and high restriction, and *L. perenne* under high restriction can exhibit hydraulic lift depending on the levels of soil water stress (Figure 3.5). On day 9, the highest fluctuations in SWC% were registered, interpreted as the day in which the highest hydraulic lift was exhibited. On this day, the vapour pressure deficit rose to 41.34 hPa, leading to a linear transpiration rate increase (Rawson et al. 1977). In addition, on these five dates, similar environmental conditions were observed, in which the daily light integral was at or above 80 mol m^{-2} s^{-2}, or double the value in relation to the previous day. The energy required to evaporate water from leaves and plants comes mostly from direct solar radiation, which also leads to an increment in the transpiration rate and, consequently, an increased movement of water and solutes by mass flow through the xylem to transpiring surfaces of shoots (Kramer and Boyer 1995). The peaking values of SWC% observed around midday, interpreted as the hydraulic lift, can be associated with two complementary factors: (a) increased transpiration rates that result in a higher sap rising flow within the xylem to subsidise water for leaves and shoots and (b) within a higher rate of water movement, some water was released (or lost) to the soil, driven by the extremely low top layer SWC% (below PWP). The most negative plant osmotic potential was found in the *L. perenne* under high water restriction, which can be related to a higher physiological stress level due to the high level of water restriction applied (Table 3.3). In the present study, even though the water potentials were not specifically measured in all soil and plant compartments, it is possible to infer through the soil water retention curve and plant water potential measurements (plant osmotic potential and leaf water potential) that the hydraulic lift was driven by the water potential soil gradient. When the top layer was permanently under PWP (-15,000 hPa), and the plants had a plant osmotic potential and leaf water potential of ~2.0 MPa, part of the water flowing more intensively during the transpiration peak is transferred passively to the soil due to differences in water potential.

Beyond that, differences in the root membrane permeability of each species could be hypothesised as an additional morpho-physiological factor that can facilitate water transit and, therefore, redistribution inter mediums. However, there is not enough evidence to support this argument in the present study. Specific functional traits of roots (i.e., maximum rooting depth and proportional distribution, water acquisition and utilisation, and association with mycorrhizal fungi) focusing on the potential hydraulic lift occurrence that a species can exhibit remain gaps to be fulfilled in this research topic (Prieto et al. 2012; Freschet et al. 2021). A major barrier to overcome in such studies includes methodologies to infer, non-destructively and accurately, the hydraulic lift's occurrence, magnitude and potential agricultural applicability (i.e., bioirrigation). Hence, the necessity to apply interdisciplinary approaches, such as the combination of high-frequency laser spectrophotometry to analyse the eddy covariance of isotopes during soil-plant-atmosphere processes (isotopic flux partitioning) (Good et al. 2014) or highly sensitive and real-time and non-invasive imaging techniques (e.g., δ^2H neutron imaging) (Cai et al. 2022).

3.7.2. Is the hydraulic lift a characteristic of competitors and/or stress-tolerators? How does it relate to morpho-physiological responses on grasses under soil water restriction?

The phenotypic plasticity of the three species under soil water restriction was evinced by the morphological responses of the plant components in contrast to the above-ground biomass accumulation (leaf growth x tillering). A compensatory effect in response to the environment alters resource utilisation, often seen as changes in the development rate among organs due to abiotic stress (Huber et al. 1999). In the present study, *L. perenne* presented a higher number of tillers per pot and a lower lamina area and lamina dry matter accumulation per tiller; *B. valdivianus* and *D. glomerata* presented an opposite pattern, with a lower number of tillers but a higher lamina surface and lamina dry matter accumulation per tiller (Tables 3.4 and 3.5). However, the final lamina dry matter mass accumulated and the ratio of 'lamina mass:total biomass' were similar between the three species. This is an important compensatory effect, previously assessed for grasses (López et al. 2013), in which the species present different growth strategies whilst accumulating similar lamina mass and total above-ground mass. These growth strategies can be taken to a field scale when trying to optimise the species selection for the establishment of diverse pastures (Nelson 2000) and can also be used to optimise the species' performance for sites of variable environmental conditions (i.e., dry season, topography, flood periods, etc.).

Regarding water restriction levels, the MDA concentration showed that when submitted to HR treatments, the three species were more stressed (1.04 µmol L^{-1}), negatively impacting total biomass accumulation (Tables 3.1 and 3.3). The LR pots' above-ground biomass was 41.2% higher than the HR pots for all three species, with increment over time driven mostly by the lamina accumulation, as this was the only component with significant differences in above-ground biomass accumulation due to water stress (Table 3.1 and 3.4). Changes in soil water availability initially affect the lamina elongation rate rather than the tiller density (Bahmani et al. 2003; García-Favre et al. 2021a). During the three initial dates of lamina elongation assessment, the BvLR, BvHR, DgLR and DgHR treatments had a clear downward trend in their LER, and that can be related to the levels of water stress that the plants were subjected to. After the third assessment date, DgHR had an LER close to zero, corroborating previous studies on *D. glomerata* under water stress (Volaire et al. 1998).

Meanwhile, *L. perenne* under both water restriction regimes were not substantially affected by the top-layer drought imposed and the two respective levels of water restriction on the bottom layers (Figure 3.6). It is well established that soil water restriction results in an initial plateau in the herbage accumulation, followed by overall diminishment and, after a prolonged time, negatively affects plant persistence and survival (Thomas and James 1999; Arredondo and Schnyder 2003; Skinner et al. 2006; Nie and Norton 2009; Turner et al. 2012; Rogers et al. 2019). López et al. (2013) conducted a study showing that *B. valdivianus,* under water stress, tends to accentuate its below-ground accumulation rather than above-ground. In a drought resistance study with *L. perenne* and *D. glomerata* under full irrigation and prolonged summer drought, the higher survival rate after the drought was closely related to the species' ability to maintain roots at deeper depths and readjust their water potential on the surviving leaves (Volaire et al. 1998). Conversely, in another study with *L. perenne* and *B. valdivianus* as mixtures and monocultures, the deeper-rooted species (*B. valdivianus*) was responsible for a 15% increment in herbage mass production during the low water availability season, in comparison to the monoculture of the shallower-rooted species (*L. perenne*) (García-Favre et al. 2022).

The different responses of grasses to abiotic stress can determine pasture succession in the long term. Hence, it is possible to divide the three species studied here according to their short-term morpho-physiological responses to the drought conditions imposed, whereby *L. perenne* could be classified as a drought-resistant species that continues growing under moderate drought conditions (e.g., up to a month in the present study). In comparison, *B. valdivianus* and *D. glomerata* could be classified as drought survivors that almost or entirely cease their growth and can, therefore, survive periods of more prolonged drought (Volaire 2018). Conversely, the Competitors (C), Stress-tolerators (S), and Ruderals (R) theoretical model proposed by Grime (1974) categorises species due to their functional roles in the ecosystem, intra- and inter-specific competition ability under abiotic and biotic stress and succession mechanisms. According to the C-S-R model, competitors can monopolise resource capture faster by the spatially dynamic foraging of roots and shoots, stress-tolerators have long-lived tissues and mechanisms to cope with environmental stress where growth is severely restricted, and ruderals are short-lived with the tendency to invest in resources caption to produce offsprings rapidly. The present study analyses the three grass species according to the C-S-R model and, under current experimental conditions, *B. valdivianus* and *D. glomerata* fit in the stress-tolerators class and *L. perenne* as a competitor. The CVA analysis highlighted the differences in morpho-physiological strategies adopted by the three species (*B. valdivianus*, *D. glomerata*, and *L. perenne*) under levels of water restriction (high and low water restriction), enforcing both Grime's and Volaire's propositions around abiotic stress effects on individuals (Figures 3.7 and 3.8). It is important to reinforce that in this current study, the baseline for all species was a drought condition with the top layer continuously under PWP, and the corresponding amounts of water were irrigated only into the bottom layer. In that respect, the responsiveness of *L. perenne* under different levels of drought was very subtle. At the same time, *B. valdivianus* and *D. glomerata* exhibited varied morpho-physiological responses due to the imposed moderate (low restriction treatments) or severe (high restriction treatment) drought conditions.

The positive positioning of variables in CAN 1 and CAN 2 was closely related to the species' ability as a stress-tolerator, whereas the negative positioning was mostly explained by the species' ability as a competitor (Grime 1974). The *L. perenne* under both water restriction regimes was closely located in the left bottom zone of the CVA, towards the negative direction of CAN 1 and CAN 2 (Figures 3.7 and 3.8). The close positioning and small variation within the CAN 2 axis showed that *L. perenne* strategies did not change under different levels of water stress, whereas the graph depicts a considerable differentiation of Bv and Dg strategies under the two water stress conditions. The *B. valdivianus* and *D. glomerata* under low water restriction were located towards the positive direction of CAN 2, exhibiting a high LA, lamina and sheath weight per tiller. Meanwhile, the *B. valdivianus* and *D. glomerata*, under high water restriction, were located around the central area of the CAN 2 axis, which shows a low mobilisation of resources to grow. This short-term strategic response is probably associated with the higher level of water stress applied. In addition, the CAN 1 location of *B. valdivianus* and *D. glomerata* under high water restriction in the far positive direction indicated how they were affected by the drought imposed, with clear responsiveness in the form of MDA and dead material per tiller. Although the *B. valdivianus* and *D. glomerata* plants had different responses in herbage growth due to water stress, they had root allocation as a common characteristic. Along the CAN 2 axis, *B. valdivianus* and *D. glomerata*, under low water restriction, were placed in the positive direction, following an increase in the top and bottom layer root accumulation. Concomitantly, along the CAN 1 axis, the *B. valdivianus* and *D. glomerata*, under high water restriction, were placed in the far positive direction, where the hydraulic lift and bottom-layer root allocation increased. Hence, *B. valdivianus* and *D. glomerata* plants can be characterised as drought survivors and stress-tolerators due to the root allocation and hydraulic lift (functional traits) but can also present competitor characteristics when under more favourable conditions of soil water availability, as seen by the greater herbage accumulation of the *B. valdivianus* and *D. glomerata* under low water restriction.

Grime's theory and Volaire's propositions facilitate the selection process of suitable species to compose a pastoral ecosystem given the chemical and physical attributes of the soil, rainfall conditions, latitude, and altitude. Moreover, potential species with the desirable characteristics to comprise a diverse pasture can be selected because of their complementary functionality, with different roles that fulfil agroecological spaces in a pastoral ecosystem. Such a combination of species can be a key process to strategically overcome pasture persistence threats related to more regular extreme climatic events.

3.8. CONCLUSION

The isotopic labelling methodology and soil water content monitoring methodologies adopted in the study enabled the validation and assessment of the hydraulic lift in grass species. These methodologies indicated that all three species exhibited hydraulic lift, *B. valdivianus* to a greater, *Dactylis glomerata* to a medium and *L. perenne* to a lesser degree. The phenomenon occurred between 10 AM and 3 PM daily, with the top layer SWC% peaking when the temperatures were the highest in the day, at around 12 PM. The root morphology and allocation at depth may explain the phenomenon occurrence, as a more even root system distribution was found in Bv, which had a dry matter ratio of approximately 4:1. In contrast, *L. perenne* and *D. glomerata* had approximately 6:1 and 5:1, respectively.

The negative impact of water stress affected the three species differently, with *B. valdivianus* and *D. glomerata*, drastically decelerating the lamina elongation rate per tiller within the first ten days, while *L. perenne* did not completely slow its growth, presenting a postponed response with a more noticeable deceleration towards the end of the experiment period. In addition to that, the multivariate analysis highlighted the three grass species' morpho-physiological strategies to cope with soil water stress according to the C-S-R model. *Lolium perenne* strategies did not change under different levels of water content, whereas the graph depicts a considerable differentiation of both *B. valdivianus* and *D. glomerata* strategies. Therefore, the study indicates that *B. valdivianus* and *D. glomerata* presented attributes of stress-tolerators, and under lower levels of water restriction can also present attributes of competitors, while *L. perenne* only presented attributes of competitors, highlighted by its lack of adaptative responsiveness under water stress.

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CHAPTER 4

Productive and nutritional parameters in diverse pastures composed of complementary species (*Lolium perenne* L., *Bromus valdivianus* Phil., *Dactylis glomerata* L., and *Trifolium repens* L.) under the leaf regrowth stage defoliation criterion

European Journal of Agronomy, Research Article, Under review.

Oliveira B, Lopez I, Cranston L, Kemp P, Donaghy D, Lopez-Villalobos N (2025) Productive and nutritional parameters in diverse pastures composed of complementary species (*Lolium perenne* L., *Bromus valdivianus* Phil., *Dactylis glomerata* L., and *Trifolium repens* L.) under the leaf regrowth stage defoliation criterion. [Under review] European Journal of Agronomy.

Journal of New Zealand Grassland Association, Research Article, Published.

Oliveira B, Lopez I, Cranston L, Kemp P, Donaghy D (2023) Using leaf regrowth stage to define defoliation interval for diverse pastures of complementary species (*Lolium perenne* L., *Bromus valdivianus* Phil., *Dactylis glomerata* L. and *Trifolium repens* L.). Journal of New Zealand Grasslands:309-320. doi:10.33584/jnzg.2023.85.3642

International Grassland Congress, Conference Proceeding, Published

Oliveira B, Lopez I, Cranston L, Kemp P, Donaghy D (2023) Agricultural Performance of Diverse Pastures of Complementary Species and Monoculture Pastures Defoliated According to the Leaf Regrowth Stage Window of Opportunity Criterion. XXV International Grassland Congress (IGC 2023). 1283-1286. https://doi.org/10.52202/071171-0312

4.1. ABSTRACT

The diversification of plant species within intensively managed pastures has been proposed as an alternative to conventional pure sward systems in an effort to address climatic-driven issues of pasture-based livestock production. In a diverse pasture composed of complementary species (DPCS), individual species fulfil different agroecological niches and complement each other. It is expected that DPCS have a greater capability to tolerate and perform under increasingly extreme climate events; however, appropriate grazing management strategies for diverse pastures still need to be fully elucidated. In the present study, DPCS comprised *Lolium perenne* L., *Bromus valdivianus* Phil., *Dactylis glomerata* L., and *Trifolium repens* L. The leaf regrowth stage [LS; *L. perenne* (2.5-3.0LS), *B. valdivianus* (3.5-4.0LS) and *D. glomerata* (3.5-4.0LS)] is proposed to be an applicable method of defoliation management for DPCS due to a potential overlapping of their optimal defoliation intervals. The productive, morpho-physiological, and nutritional parameters of DPCS and their respective single-grass and *T. repens* pastures were assessed for two years under the LS criterion for defoliation. Within the DPCS, the three grass species presented a temporal overlapping of their respective LS intervals. Significant differences were found in the dry matter seasonal accumulation; DPCS exhibited seasonal asynchronous growth among species and presented an enhanced ability to withstand the ingress of volunteer species. In all pastures, the nutritional parameters were mostly influenced by season and LS criterion. *Lolium perenne*, *B. valdivianus*, and *D. glomerata* were shown to be complementary grass species, presenting growth asynchrony and an overlapping defoliation interval that optimised production. In this study, grazing management that focuses on the morpho-physiological development

of grasses within a diverse pasture was successfully applied, with *B. valdivianus* LS optimal interval for defoliation being the most appropriate criterion for determining a grazing regime for DPCS.

4.2. GRAPHIC ABSTRACT

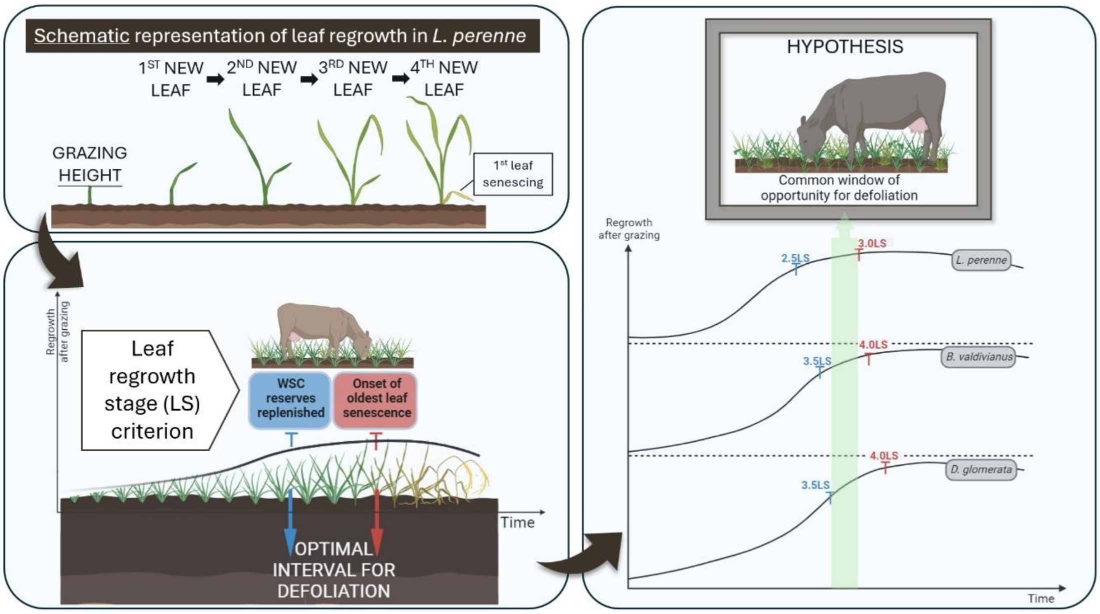

Figure 4-1. Graphic abstract

4.3. KEYWORDS

Grazing management; pasture ecology; phyllocron; growth asynchrony; development synchrony

4.4. INTRODUCTION

Increased extreme climatic events caused by climate change (i.e., higher average temperatures, precipitation variability with concentrated rainfall followed by long periods of rainfed water restriction, and elevated carbon dioxide concentrations) are changing the production dynamics of temperate grasslands (Soussana et al., 2013; Tubiello et al., 2007). New Zealand's livestock production systems are predominantly pasture-based, with 13.3 million hectares (ha) of New Zealand’s land area covered in productive pastures, representing 49.6% of the country's land area (Manaaki Whenua, 2023). The sown pastures in these areas are mostly composed of *Lolium perenne* L. and *Trifolium repens* L. and are intensively managed to achieve high levels of animal productivity (Caradus et al., 2021). Future New Zealand’s pastoral systems are expected to display a detrimental greater degree of inter-annual production variability and seasonal feed surpluses and shortages (Keller et al., 2021; Lee et al., 2013). In addition to the increased climate-driven seasonality of production, in these pasture-based systems, unadjusted rotational grazing interval and over-grazing are a problem causing pugging damage, soil erosion, bare soil areas (Brock and Hay, 2001; Brougham, 2011; Greenwood and McKenzie, 2001) and depletion of plant reserves that support regrowth after grazing (Thornton et al., 2000). In the long term, these imbalances can lead to a decrease in the population of sown species, increasing the susceptibility to weed invasion, and reducing productivity and quality (Briske and Richards, 1995).

Species diversity has been proposed as an alternative to conventional and less complex (pure stand or binary) pasture systems that are intensively managed in an effort to address climate-driven issues facing productive grassland systems [see meta-analysis by Isbell et al. (2015)]. Species diversity can contribute to stabilising production due to growth asynchrony and potential facilitation processes between species (Lüscher et al., 2022). For instance, in naturalised and semi-naturalised extensive pastures, the presence of several species is common, and in these ecosystems, each species fulfils specific functional roles (Descalzi et al., 2019; Kemp and López, 2016). However, on a high-productive pasture-based livestock farm, many challenges emerge with establishing and managing a multi-species system. These challenges originate from the inherited characteristics of forage species when coping with disturbance, competition, and stress (Grime, 1974). For instance, not all species present in extensively managed grasslands can cope with practices of intensive systems, such as fertilisation (Hautier et al., 2009) and grazing frequency and intensity (Barreta et al., 2023; Fischer et al., 2019) - these practices contribute to the competitive exclusion of some less adapted species (Isselstein et al., 2007). Consequentially, intensive management practices may favour or prejudice a given species, resulting in the loss of sown species' population density and increased opportunity for volunteer species to overtake (Tracy and Sanderson, 2004).

The addition per se of different species in a pasture can lead to a natural ‘drift’ in the population of some species caused by inter-species niche competition (Sanderson et al., 2007). Therefore, the choice of species (Komainda et al., 2020; Küchenmeister et al., 2012) and the complementarity of functional groups and specific traits among these chosen species are fundamental for the success of a diverse pasture [see definition of ‘complementarity’ by Barry et al. (2019)]. Diversification of species based on their complementarity may be appropriate for dealing with climatic-driven problems. Yet, research is needed to determine the progression or degradation of these diverse pastures under intensive grazing management practices.

In a diverse pasture composed of complementary species, individual species fulfil different agroecological niches (Oliveira et al., 2023) and complement each other to enhance ecological diversity through spatial and temporal resource partitioning and abiotic facilitation. The different functional roles (i.e., root exploitation or the season of activity) and the trait-mediate plant responses to disturbance, competition, and stress will dictate species' succession and productivity. In the present study, a diverse pasture is composed of *L. perenne* [shallow-rooted, winter-active, waterlogging tolerator, competitor (Crush et al., 2010; Grime et al., 1988; Hoekstra et al., 2014; Stewart, 2006)], *Bromus valdivianus* Phil. [deep rooted, summer-active, drought tolerator, facilitator via hydraulic lift phenomena (García-Favre et al., 2021; López et al., 1997; Meier et al., 2024; Stewart, 1996)] *Dactylis glomerata* L. [drought resistant, year-round-active, competitor and facilitator via hydraulic lift phenomena (Grime et al., 1988; Gross et al., 2007; Volaire, 1995)] and *T. repens* [shallow-rooted, high nutritive value, biological N-fixator (Hoekstra et al., 2014; Widdup and Barrett, 2011)].

The challenge of diversification is enhanced by the fact that animal intake requirements often dictate the conventional defoliation management in pasture-based systems (Roche et al., 2017), where pasture is allocated according to availability, which, in turn, is dictated by climatic conditions for growth. Currently, the morpho-physiological development stage of the pasture species is not often taken into consideration when determining the defoliation regime (Donaghy et al., 2021). In this sense, a grazing criterion based on the morpho-physiological maturity of the plants was developed, relating growth stages to the lamina appearance of individual tillers (Fulkerson and Donaghy, 2001).

The leaf regrowth stage (LS) criterion for defoliation is an interval that ranges from a minimum LS value associated with the replenishment of water-soluble carbohydrate reserves in the stubble and a maximum LS value associated with the onset of leaf senescence (see ‘Material and Methods’ section for detailed information) (Chapman et al., 2011; Fulkerson and Donaghy, 2001; Fulkerson and Slack, 1994; Gatti et al., 2016; Ordóñez et al., 2021; Turner et al., 2006). Previous studies in Australia, Chile, and New Zealand have confirmed the relation between the water-soluble carbohydrate reserves and the optimal LS targets for *L. perenne* [2.0 to 3.0 LS (Fulkerson and Donaghy, 2001; Lee et al., 2008)], *B. valdivianus* [3.5 to 4.0 LS, (García-Favre et al., 2022; Ordóñez et al., 2021)] and *D. glomerata* [around 4.0 LS (Gatti et al., 2016; Turner et al., 2006)]. The LS criterion is endorsed by New Zealand, Chile and Australia’s research and advisory institutions (Dairy Australia, n.d.; DairyNZ, n.d.; Proyecto FIA, 2007) and utilised by some farmers as an attempt to mitigate the negative effects of defoliation rotation practices that are purely animal- or timeframe-focused (Clarke et al., 2021; Donaghy et al., 2021).

This investigation advances plant-focused defoliation management as a key factor in the performance of intensively managed diverse pastures and introduces LS as a potentially suitable management criterion. If the individual species present a similar chrono-physiological development regrowth pattern, resulting in a common window of opportunity among species for optimal defoliation in the following grazing cycles, positive outcomes in terms of resilience and short-term persistence are expected to be seen, as defoliation disturbance will not favour or prejudice any species. The three grass species are hypothesised to present an overlap of their individual leaf regrowth stage interval for defoliation, resulting in a common window of opportunity for optimal defoliation of all grass species in diverse pastures comprising *L. perenne*, *B. valdivianus* and *D. glomerata* (see ‘graphic abstract’). In contrast to the single-grass pastures, it is expected that the proposed diverse pasture presents complementarity and, consequentially, less seasonality, higher short-term persistence, and similar nutritive value. The current study evaluated the LS of different species present in diverse and single-grass pastures, their seasonal and annual herbage mass accumulation, growth patterns, botanical and morphological composition, and nutritive value parameters compared to single-grass pastures.

4.5. MATERIAL AND METHODS

4.5.1. Site and experimental setup

The study was undertaken at Massey University’s Dairy 1 (Palmerston North, Manawatu, New Zealand) from 15 June 2021 to 14 June 2023, with the approval of the Massey University Animal Ethics Committee (Approval number 21/24). The soil type is Manawatu silt loam over sand (Landcare Research National Soil Data Base, Lab. No SB10036). Soil samples were collected at three depths for chemical fertility analysis on 26 February 2021 (Table 1). The area received annual maintenance fertilisation on 1 March 2021 and 17 March 2022, at 500 kilograms (kg) ha^{-1} and 400 kg ha^{-1} of superphosphate [0% nitrogen (N), 22.5% phosphorus (P), 0% potassium (K), 27.5% sulphur (S) and 50% calcium (Ca)]. Post-grazing N fertilisations were applied as urea (46% N) in July 2021, November 2021, January 2022, August 2022, November 2022, and February 2023, each at 30 kg N ha^{-1}.

Table 4.1. Soil chemical characteristics at three depths (0-15, 15-30, 50-65 cm) of the experimental area on 26 February 2021.

Soil Layer	pH	OM	Olsen P	K	Ca	Mg	CEC	Tot. Base Sat.	Ext Org S	C/N
cm	*$CaCl_2$*	*%*	*mg L^{-1}*	*----------ME 100 g^{-1}------------*				*%*	*mg kg^{-1}*	
0-15	5.60	3.33	29.0	0.21	6.80	1.35	13.00	64.00	3.00	10.0
15-30	5.53	2.53	2.30	0.20	6.33	1.30	12.70	63.30	3.00	9.57
50-65	5.67	1.30	5.33	0.16	6.10	1.15	10.70	71.00	2.30	9.70

Organic matter (OM), Olsen phosphorus (Olsen P), potassium (K), calcium (Ca), magnesium (Mg), cation exchange capacity (CEC), total base saturation (Tot Base Sat), extractable organic sulphur (Ext Org S), carbon:nitrogen ratio (C/N).

The climate is classified as Marine Climate - Cfb [Köppen-Geiger's climate classification (Beck et al., 2018)]. Monthly rainfall, ground minimum temperature, and air minimum and maximum temperature are presented in Figure 1 [NIWA/AgResearch Weather Station, ~800 metres (m) from the field site].

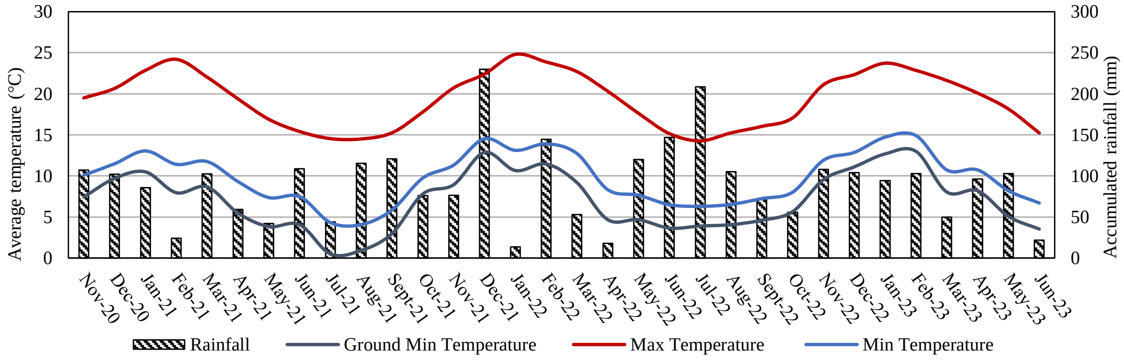

Figure 4-2. Monthly accumulated rainfall (mm), monthly averaged ground minimum (°C), and monthly averaged air maximum and minimum temperatures (°C) during the establishment and experimental period (November 2020 to June 2023). Bars indicate accumulated rainfall, lines indicate averaged temperatures.

On 24 November 2020, the area was sprayed out with N-(phosphonomethyl) glycine (glyphosate WeedMaster® G360) at 6 litres (L) ha^{-1} and Thifensulfuron-methyl (Harmony® 50 SG) at 30 grams (g) ha^{-1} to control the weed seed population. From 10 to 18 December 2020, the area was ploughed, power-harrowed and levelled. On 18 December 2020, mixtures (Mix) of *L. perenne* (Lp), *B. valdivianus* (Bv), and *D.*

glomerata (Dg) and their respective single grass pastures (Mono) were established. *Trifolium repens* (Tr) was sown at the same rate in all pastures (Table 2). In December 2020, the area was irrigated for one day with a mobile gun sprinkler to ensure a successful establishment. Following this, the pastures were rainfed.

The study was arranged in a randomised complete block design, with three blocks (n=3), each with the six experimental treatments in 20 m by 20 m plots. From June 2021 onwards, the seasonal interval for defoliation was determined according to the LS criterion of each species and applied to the single-grass pastures and mixtures (hereafter referred to as diverse pastures of complementary species – DPCS; Table 2). These resulted in six experimental treatments: MonoLp (defoliated at *L. perenne* LS), MonoBv (defoliated at *B. valdivianus* LS), MonoDg (defoliated at *D. glomerata* LS), MixLp (defoliated at *L. perenne* LS), MixBv (defoliated at *B. valdivianus* LS), MixDg (defoliated at *D. glomerata* LS). In the MixLp, for instance, the *L. perenne* plants are the targeted species (α) and thus justified defoliation based on the *L. perenne* LS interval, whereas the *B. valdivianus, D. glomerata,* and *T. repens* neighbouring plants are the companion species.

Massey University’s Dairy 1 herd is mostly comprised of crossbred cows (Jersey with Holstein-Friesian) ranging from 2 to 8 years old (herd average = 4.2 years old), with the whole herd calving season concentrated over late July and August. The farm runs as a year-round pasture-based system, with maize silage being supplied as a complement when pasture growth rates are below herd dry matter (DM) requirements. Within this study's experimental area, dairy cows grazed the plots during the day from around 07 AM to 05 PM, on a one-day grazing event basis (10-

15 animals per plot depending on herbage allowance; animal average = 13 kg DM animal^{-1}) until the pasture reached a stubble height of 5 to 8 centimetres (cm) from ground level (Hodgson, 1990). Pre- and post-grazing data were collected over eight agricultural seasons (winter 2021, spring 2021, summer 2021-22, autumn 2022, winter 2022, spring 2022, summer 2022-23, and autumn 2023), with the first four seasons comprising year 1 and the last four seasons comprising year 2.

Table 4.2. Establishment description of the four pastures species composition and sowing rate, cultivars, resulting experimental treatments, and seasonal leaf regrowth stage (LS) optimal interval for defoliation.

Pasture composition (sowing rates, *kg ha*$^{-1}$)	Cultivars	Experimental treatment	Species role *	Winter Summer Autumn (LS interval)	Spring † (LS interval)
Lp + Tr (20 + 6)	*L. perenne* cv. Maxsyn + *T. repens* cv. Weka	MonoLp	Lp *α*	2.5 – 3.0	2.25 – 2.75
Bv + Tr (50 + 6)	*B. valdivianus* cv. Bareno + *T. repens* cv. Weka	MonoBv	Bv *α*	3.5 – 4.0	3.0 – 3.5
Dg + Tr (15 + 6)	*D. glomerata* cv. Greenly II + *T. repens* cv. Weka	MonoDg	Dg *α*	3.5 – 4.0	3.0 – 3.5
Lp + Bv + Dg + Tr (10 + 15 + 12+ 6)	*L. perenne* cv. Maxsyn + *B. valdivianus* cv. Bareno + *D. glomerata* cv. Greenly II + *T. repens* cv. Weka	MixLp	**Lp *α*** Bv$_{\text{CSpp}}$ Dg$_{\text{CSpp}}$	**2.5 – 3.0**	**2.25 – 2.75**
		MixBv	Lp$_{\text{CSpp}}$ **Bv *α*** Dg$_{\text{CSpp}}$	**3.5 – 4.0**	**3.0 – 3.5**
		MixDg	Lp$_{\text{CSpp}}$ Bv$_{\text{CSpp}}$ **Dg *α***	**3.5 – 4.0**	**3.0 – 3.5**

Lp: *L. perenne*, Bv: *B. valdivianus*, Dg: *D. glomerata*, Tr: *T. repens*; α: targeted species that determined each defoliation event; CSpp: companion species; LS: leaf regrowth stage; *All pastures have *T. repens* in their composition. † LS interval was slightly anticipated to alleviate detriments in pasture quality due to flowering stems.

4.5.2. Defoliation criteria

The leaf regrowth stage is a practical criterion that requires the assessment of individual tillers in the field, and it is measured as the sum of representative quarters of live leaves in a tiller (Figure 2). To assess the LS of *L. perenne* tillers, for example, it is necessary to know that this species grows one leaf at a time. Often, the tiller will present one residual leaf (oldest leaf) from the prior defoliation that may be accounted for if representing at least 0.50 LS. Subsequently, any new fully expanded leaves (visible ligule) are accounted individually as 1.00 LS. Lastly, any expanding leaf is accounted as per the relative size it represents in comparison to the newest fully expanded leaf (closest leaf) and recorded as either 0.25, 0.50 or 0.75 LS. The LS values of each leaf are then added, and the resulting sum is the LS for that given *L. perenne* tiller (Figure 2). In the present study, the LS targets for *L. perenne* are 2.5-3.0 LS in summer, autumn and winter, and 2.25-2.75 LS in spring, and for *B. valdivianus* and *D. glomerata* are 3.5-4.0 LS in summer, autumn and winter, and 3.0-3.5 LS in spring.

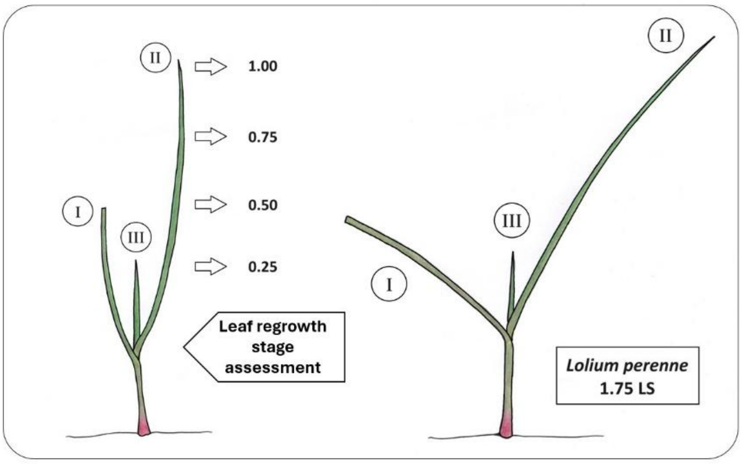

Figure 4-3. Conceptual image of the leaf regrowth stage (LS) assessment in the same tiller of Lolium perenne. The tiller presents a residue leaf from previous grazing (I), and two new leaves, one fully expanded (II) and one expanding (III). Utilising the concept of quarters to define the stages (indicated by arrows), this tiller is at 1.75 LS, resulting from the sum of leaves I, II and III's LS values.

4.5.3. Leaf regrowth stage, apparent dry matter accumulation, botanical and morphological composition

The LS of the target species (α) and companion species was assessed on nine randomly chosen mature tillers of each grass species per plot on the day before each grazing event (García-Favre et al., 2022). The herbage mass was measured pre- and post-grazing by cutting to ground level three randomly placed 0.1 m2 quadrats in each plot. Three samples were collected per plot, with quadrats randomly placed in areas representing the average sward status prior to and following defoliation (Frame 1993). Post-grazing samples were washed when necessary to eliminate residual detritus and soil. The samples were dried for at least 72 hours in a forced-air oven at 60 ℃ and weighed to determine their DM. The apparent accumulated DM (season or year) was the sum of apparent herbage growth of different grazing events over a given period, calculated as the difference between the pre-grazing DM at the current grazing event 'n' and the post-grazing DM of the previous grazing event 'n-1'

(Campbell, 2009). The daily growth rate of the pasture was calculated by dividing the apparent accumulated DM by the number of days between consecutive grazing events. The average daily growth rate per season was calculated as a ‘weighted mean’ balanced by the number of days of each daily growth rate from different grazing events (‘n; n+1; n+2; etc.’) within a given season.

The botanical and morphological composition were assessed at every second grazing event. Two sub-samples of approximately 12.5% from two out of three herbage mass samples were randomly taken to be fractionated into individual components of *L. perenne*, *B. valdivianus*, *D. glomerata*, *T. repens*, dead material and volunteer species (every other species that was not the original sown species of each treatment) for the botanical composition; and lamina, sheath (leaf sheath + stem), and flower (above flag leaf) of *L. perenne*, *B. valdivianus*, *D. glomerata* for the morphological composition (Oliveira et al., 2023). The fractioned materials were oven-dried at 60 Celsius degrees (°C) for at least 72 hours and weighed to determine the percentage of each botanical and morphological component on a DM basis.

4.5.4. Nutritive value

Throughout the study, the experimental treatments were defoliated on different dates because of the applied defoliation criteria, which were related to each respective targeted species LS. Once a season and within the same grazing rotation time frame of the different treatments, the nutritional parameters of the apparent harvestable herbage were assessed. To reflect the defoliation management that sampling purports to simulate, at least ten sub-samples were cut down to 5 cm residual height in the early morning of the grazing event day (Frame, 1993), avoiding the warmer hours of the day. These sub-samples generated one composite sample of approximately 150 g per plot in each block. The sample was instantly sealed in a zip-lock bag, packed into a thermal bag, refrigerated at 4 °C (Atkin and Tjoelker, 2003) and sent on the same day for analysis at Hill Laboratories Ltd., Hamilton, New Zealand. Hill Laboratories participates in both national (International Accreditation New Zealand - IANZ) and international (Association of American Feed Control Officials - AAFCO) inter-laboratory comparison programmes (ILCP) for the feed test reference methods.

The total nitrogen content was estimated by near-infrared spectroscopy (NIRS), calibration based on Dumas combustion (Chang and Zhang, 2017) and corrected to a fully dry basis assuming 5% residual moisture. The crude protein content (CP) was obtained by multiplying N by 6.25. The organic matter digestibility in-vivo (OMD) was determined using AFIA (Australian Fodder Industry Association) in-vitro Pepsin-Cellulase procedure and derived as in-vivo using a linear regression based on calibration samples from Lincoln University, Lincoln, New Zealand. The metabolisable energy content (ME) was calculated from OMD from AFIA Method 7R (modified), using AFRC (Agriculture & Food Research Council, UK) and Lincoln University’s standard formulae. The neutral detergent fibre content (NDF) was estimated by NIRS, calibration based on NDF by NFTA method. The lignin content (LGN) was Estimated by NIRS, calibration based on acid detergent extraction followed by treatment with 72% sulphuric acid in the Ankom Daisy Incubator. The non-structural carbohydrates content (NSC) was calculated as the difference between 100% and the sum of CP, ash, crude fat, and NDF percentages.

The results by NIRS are obtained using samples dried at 62 °C and grounded to 1 millimetre-sized particles. Measurement results are calculated on a dry matter basis and calibrated using a multipurpose analyser (MPA II NIR - Bruker Corporation, Billerica, Massachusetts, USA). The algorithm used to construct the calibration from NIRS data (NIR spectra) firstly pre-processes the NIR spectra using a first derivative, the Savitzky-Golay smoothing algorithm (Savitzky and Golay, 1964), vector normalisation and wavelet transformation. Then, the NIR spectra originating from the multipurpose analyser instrument are transformed into a single stream using calibrated transformation matrixes. Following, the transformed NIR spectra associated with each sample pass through a bootstrap re-sampling model, which uses a partial least square data reduction filter with 20 components, a local weighted Euclidean distance (500 nearest neighbours) and a support vector machine model. The bootstrap re-samplings created 20 different random calibrations from the dataset for each on-the-fly prediction. Lastly, the mean of the 20 different predictions is reported as the measurement results.

4.5.5. Statistical analyses

Statistical analyses were performed using SAS v 9.4 (SAS Institute Inc, Cary, NC, US) and Statistica™ v 14.0.0.15 (TIBCO Software Inc, Hamburg, DE). The data were examined for normal distribution using the Shapiro-Wilk test, finding that all dependent variables followed a normal distribution.

Datasets of annual and seasonal apparent DM accumulation, average daily growth rate, seasonal percentage of individual components in the botanical composition, and seasonal LS per species were analysed with the following mixed model using the GLIMMIX procedure:

$$\mathrm{Y}_{ij} = \mu + \mathrm{P}_i + \mathrm{B}_j + \mathrm{e}_{ij} \qquad \text{Equation 1}$$

Where Y_{ij} is the dependent variable, Pi is the fixed effect of ith experimental treatment (MonoLp, MonoBv, MonoDg, MixLp, MixBv, MixDg), Bj is the random effect of the jth block, and eij is the random residual assumed with mean zero and variance σ_e^2.

Least square means and standard errors for the fixed effect were obtained and used for multiple mean comparisons using Fisher's least significant difference test. Significant differences between the least squares means were declared at $p \leq 0.05$. Datasets composed of percentage values were transformed into arcsine. Results related to the transformed variables were subsequently back-transformed to the original scale and presented in the results section.

The nutritive value of each pasture treatment was modelled across successive seasons for two years using polynomial regression models of the nutritive value on the day of the year. The choice of polynomial order was guided based on the goodness of fit between the regression curve and the data points, with considerations of the R-squared (R2) and the relative prediction error (RPE; equation 2). The RPE was used to evaluate the fitness of the predicted nutritive value and defined as the positive square root of the mean square prediction error (MSPE; equation 3) and expressed as a percentage of the mean of the actual values (O'Neill et al., 2012):

$$RPE = \frac{\sqrt{MSPE}}{\mu_{A_i}} \times 100 \qquad \text{Equation 2}$$

$$MSPE = \frac{1}{n}\sum_{i=1}^{n}(A_i - P_i)^2 \qquad \text{Equation 3}$$

Where A represents the actual observed values for nutritive value, and P represents the predicted values. An RPE value lower than 10% indicates satisfactory prediction, whereas an RPE between 10% to 20% indicates a relatively acceptable prediction, and an RPE greater than 20% indicates poor prediction (Fuentes-Pila et al., 1996).

4.6. RESULTS

4.6.1. Leaf regrowth stage

In the first year, the defoliation criteria applied resulted in 11 grazing events for MonoLp and MixLp, 10 for MonoBv and MixBv, and 9 for MonoDg and MixDg, and in the second year, the same defoliation criteria resulted in 10 grazing events for all six treatments. During these two years, the targeted seasonal LS intervals for defoliation according to each α species were achieved in the six treatments. In the MixLp, the companion *B. valdivianus* was defoliated earlier than recommended in winter 2021 and summer 2021-22 (< 3.5 LS in Table 3; see Table 2 and ‘defoliation criteria’ section for recommended LS intervals), while the companion *D. glomerata* was mostly defoliated earlier than recommended during the four seasons of the first year, and in the second-year winter (winter 2022) (< 3.5 LS). During both years, the MixBv had most companion species defoliated within their respective LS intervals, except for autumn and winter 2022, when the companion *D. glomerata* was defoliated slightly earlier than recommended. In the MixDg, the companion *L. perenne* was defoliated later than its recommended LS in both winters (> 3.0 LS), while the companion *B. valdivianus* was defoliated later than recommended during the first-year winter (winter 2021) (> 4.0 LS) and just at its LS interval maximum point limit (4.0 LS) in the second-year winter (winter 2022).

Significant differences in the LS of the same species within each different mixture (*L. perenne* in MixLp, MixBv, and MixDg, *B. valdivianus* within MixLp, MixBv, and MixDg and *D. glomerata* within MixLp, MixBv, and MixDg) were found in both winters and summers (Table 3; $p<0.05$). Overall, when differences were found, higher LS values of all species were present in MixDg. In winter 2021 and 2022, the *L. perenne* LS was higher in MixDg than in MixLp and MixBv, and the *B. valdivianus* LS was higher in MixDg than in MixLp and MixBv. In winter 2021, the *D. glomerata* LS was higher in MixDg and MixBv than in MixLp, while in winter 2022, it was higher in MixDg than MixLp and MixBv. In summer 2021-22, *L. perenne* LS was higher in MixDg than in MixLp and MixBv, while in summer 2022-23, MixDg and MixLp were higher than MixBv. In summer 2021-22, *B. valdivianus* LS was higher in MixDg and MixBv than MixLp. In summer 2021-22, the *D. glomerata* LS was higher in MixDg than in MixLp and MixBv, while in summer 2022-23, it was higher in MixBv than MixLp and MixDg.

Table 4.3. Seasonal average leaf regrowth stage (LS) of the same species within each of the three mixtures prior to defoliation (*L. perenne* in MixLp, MixBv and MixDg; *B. valdivianus* in MixLp, MixBv and MixDg; *D. glomerata* in MixLp, MixBv and MixDg) in winter 2021, spring 2021, summer 2021-22, autumn 2022, winter 2022, spring 2022 and summer 20221-22.

Species within each mixture	Winter 2021	Spring 2021	Summer 2021-22	Autumn 2022	Winter 2022	Spring 2022	Summer 2022-23	Autumn 2023
Lolium perenne								
MixLp	2.6 c (±0.006)	2.5 (±0.041)	2.6 b (±0.034)	2.7 (±0.021)	2.6 c (±0.048)	2.5 (±0.075)	2.8 a (±0.029)	2.7 (±0.042)
MixBv	3.0 b (±0.017)	2.5 (±0.032)	2.6 b (±0.019)	2.6 (±0.045)	2.8 b (±0.023)	2.6 (±0.035)	2.7 b (±0.042)	2.9 (±0.039)
MixDg	3.3 a (±0.035)	2.7 (±0.057)	2.9 a (±0.068)	2.7 (±0.061)	3.1 a (±0.048)	2.5 (±0.039)	2.9 a (±0.021)	2.7 (±0.060)
Significance	***	NS	*	NS	**	NS	*	NS
Bromus valdivianus								
MixLp	3.3 c (±0.051)	3.1 (±0.021)	3.2 b (±0.052)	3.6 (±0.024)	3.5 c (±0.056)	3.3 (±0.041)	3.7 (±0.074)	3.6 (±0.102)
MixBv	4.0 b (±0.008)	3.0 (±0.037)	3.6 a (±0.029)	3.6 (±0.027)	3.8 b (±0.016)	3.4 (±0.042)	3.7 (±0.000)	3.7 (±0.012)
MixDg	4.2 a (±0.028)	3.2 (±0.077)	3.6 a (±0.141)	3.7 (±0.038)	4.0 a (±0.037)	3.4 (±0.008)	3.9 (±0.107)	3.6 (±0.127)
Significance	****	NS	*	NS	**	NS	NS	NS
Dactylis glomerata								
MixLp	3.2 b (±0.003)	2.9 (±0.044)	3.2 c (±0.038)	3.4 (±0.075)	3.0 c (±0.034)	3.2 (±0.065)	3.5 b (±0.055)	3.5 (±0.049)
MixBv	3.7 a (±0.158)	3.1 (±0.070)	3.5 b (±0.021)	3.4 (±0.025)	3.3 b (±0.084)	3.4 (±0.028)	3.7 a (±0.003)	3.5 (±0.075)
MixDg	3.7 a (±0.029)	3.2 (±0.088)	3.6 a (±0.044)	3.5 (±0.052)	3.6 a (±0.046)	3.1 (±0.033)	3.5 b (±0.016)	3.5 (±0.013)
Significance	*	NS	**	NS	**	NS	*	NS

Letters that differ within columns for the same species indicate values that are significantly different; NS=non-significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; following each species LS least square mean is the (±) standard error of the mean.

4.6.2. Annual and seasonal apparent dry matter accumulation and seasonal growth rates

In the first year, the total accumulated pasture herbage mass did not differ ($p>0.05$) between all treatments, with an average annual accumulation of 20.26 (±1.17) tonnes (t) DM ha^{-1}. Nonetheless, in the first year, significant differences were found in the seasonal accumulated DM (Table 4). In the second year, significant differences in the total herbage mass accumulation were found ($p<0.01$) - MixDg was greater than MonoBv, MixLp, and MonoLp but similar to MonoDg and MixBv; MonoLp accumulated 15% less than MixDg. Significant seasonal differences were found in all first-year seasons and the second year, in winter 2022 and spring 2022 (Table 4 and Figure 3). The MixLp had greater growth rates than MonoLp in spring 2021 and spring 2022 (41.5% and 22.8%, respectively; $p<0.05$), summer 2021-22 (13.9%; $p<0.05$), and autumn 2022 (37.7%; $p<0.05$) (Figure 3). The MixBv had greater growth rates than MonoBv in winter 2021 (69.6%; $p<0.05$), winter 2022 (41.2%; $p<0.001$), and autumn 2022 (43.8%; $p<0.05$). MixDg presented a greater growth rate in autumn 2022 (78.7%; $p<0.05$) and winter 2022 (71.8%; $p<0.05$) and lower growth in winter 2021 than MonoDg (23.6%; $p<0.001$).

Table 4.4. Effect of defoliation criteria on experimental treatments (MonoLp, MonoBv, MonoDg, MixLp, MixBv, MixDg) on seasonal (winter 2021, spring 2021, summer 2021-22, autumn 2022, winter 2022, spring 2022, summer 2022-23 and autumn 2023) and annual (1st and 2nd year) dry matter accumulation (t DM ha^{-1}).

	Apparent DM accumulation *(t DM ha^{-1})*									
Treatment	Winter 2021	Spring 2021	Summer 2021-22	Autumn 2022	1st Year	Winter 2022	Spring 2022	Summer 2022-23	Autumn 2023	2nd Year
MonoLp	3.33 a	5.45 bc	6.90 c	2.98 ab	18.67	2.56 abc	6.30 c	6.52	4.25	19.62 c
	(±0.32)	(±0.37)	(±0.36)	(±0.49)	(±1.08)	(±0.16)	(±0.26)	(±0.47)	(±0.39)	(±0.21)
MonoBv	1.86 c	4.75 c	9.23 ab	1.99 b	17.82	2.23 bc	6.57 c	7.98	4.30	21.12 b
	(±0.19)	(±0.54)	(±0.34)	(±0.19)	(±0.79)	(±0.21)	(±0.34)	(±0.81)	(±0.56)	(±0.30)
MonoDg	3.50 a	6.54 ab	7.40 c	2.24 b	19.68	1.69 c	8.07 a	6.61	5.86	22.22 ab
	(±0.24)	(±1.16)	(±0.69)	(±0.18)	(±2.15)	(±0.25)	(±0.29)	(±0.35)	(±0.68)	(±1.10)
MixLp	3.67 a	7.71 a	7.86 abc	4.11 a	23.35	2.81 ab	7.81 ab	6.72	3.66	21.00 bc
	(±0.18)	(±0.49)	(±0.06)	(±0.35)	(±0.47)	(±0.16)	(±0.12)	(±0.73)	(±0.24)	(±0.72)
MixBv	3.15 ab	5.68 bc	9.44 a	2.86 ab	21.13	3.21 a	6.67 bc	6.73	5.45	22.07 ab
	(±0.14)	(±0.74)	(±0.90)	(±0.34)	(±1.80)	(±0.11)	(±0.44)	(±0.18)	(±0.13)	(±0.29)
MixDg	2.68 b	6.72 ab	7.55 bc	4.00 a	20.94	2.90 ab	7.96 a	7.20	4.88	22.94 a
	(±0.07)	(±0.38)	(±0.27)	(±0.81)	(±0.70)	(±0.55)	(±0.48)	(±0.29)	(±0.68)	(±0.39)
Signific.	***	*	*	*	NS	*	*	NS	NS	**
SEM season	0.20	0.50	0.55	0.42	1.17	0.29	0.36	0.31	0.53	0.45

Letters that differ within columns indicate values that are significantly different; NS=non-significant, * p<0.05, ** p<0.01, *** p<0.001; following each experimental treatment, the least square mean is the (±) standard error of the mean; SEM season: standard error of the mean per season.

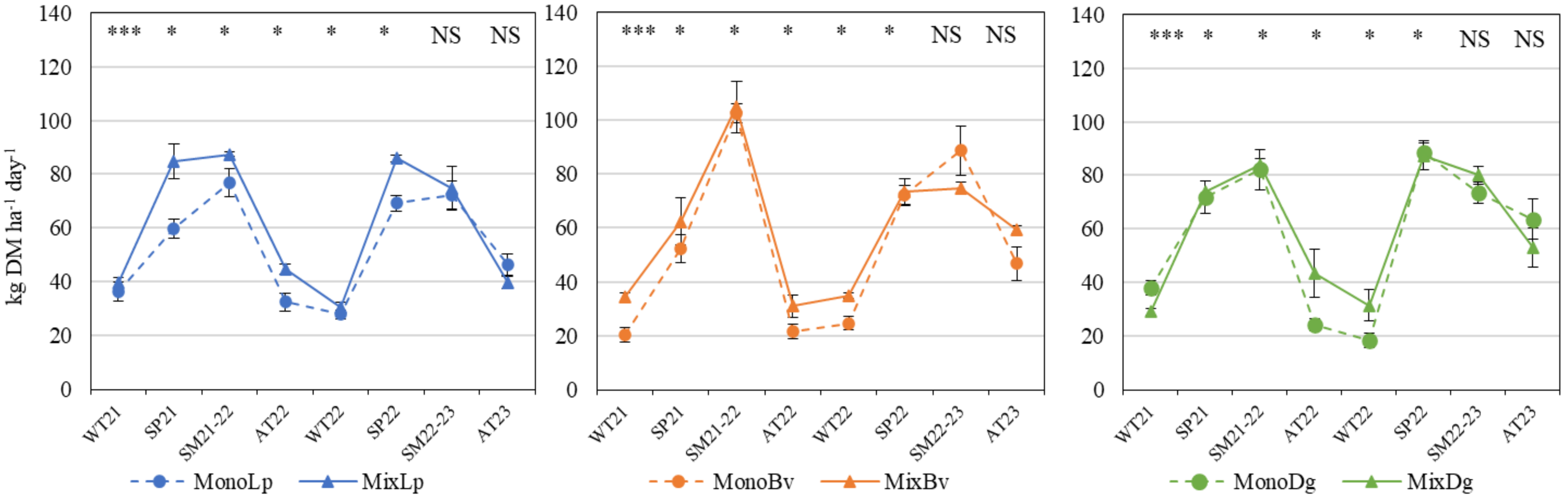

Figure 4-4. Average daily growth rate (kg DM ha-1 day-1) of the treatments (MonoLp - dashed blue line and blue circles, MixLp - solid blue line and blue triangles, MonoBv - dashed orange line and orange circles, MixBv - solid orange line and orange triangles, MonoDg - dashed green line and green circles, and MixDg - solid green line and green triangles) in winter 2021 (WT21), spring 2021 (SP21), summer 2021-22 (SM21-22), autumn 2022 (AT22), winter 2022 (WT22), spring 2022 (SP22), summer 2022-23 (SM22-23) and autumn 2023 (AT23). The figure was split according to the αLS of the same species to facilitate the visualisation of growth rate lines. Levels of significance are indicated in the graphs per season as NS=non-significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. The vertical bars indicate (±) S.E.M. within the same season for the six treatments.

4.6.3. Botanical and morphological composition

In both years, the *L. perenne* percentage within the MonoLp and the *B. valdivianus* percentage within the MonoBv treatment presented a consistent and marked decrease from winter to summer (Figure 4). In MonoLp, from winter to summer, the *L. perenne* percentage dropped from 77% to 22% in year 1 and from 73% to 44% in year 2. In MonoBv, *B. valdivianus* percentage dropped from 61% to 18% in year 1 and from 65% to 23% in year 2. Conversely, in summer 2021-22, MonoLp had a significant peak at 33% of volunteer plant species' proportion in the sward, followed by MonoBv peak of 24% ($p<0.001$). MonoBv also had significant peaks of volunteer plant species in the sward ($p<0.05$) in spring 2022 (19%) and summer 2022-23 (30%). In MonoDg, the *D. glomerata* percentage presented variations with no apparent seasonal consistency.

In MixLp, the *L. perenne* percentage was significantly higher than in the other two mixtures in both springs, when it dominated approximately 46% of the sward, compared to approximately 30% in MixBv and MixDg ($p<0.001$). Throughout seasons, the proportion of *L. perenne* in the mixtures remained overall around 30%. The *B. valdivianus* proportion in the mixtures markedly decreased from 30% in winter 2021 to 5% in summer 2022-23, remaining between 5% and 10% for the rest of the studied period. In contrast, the *D. glomerata* proportion consistently increased from winter 2021 until autumn 2023. In MixDg throughout the study, the average *D. glomerata* percentage in the sward became eight times greater, MixBv six times, and MixLp five times.

Across seasons, MixLp, MixBv, and MixDg had at most 4% of the sward dominated by volunteer species. In summer 2021-22, MonoLp and MonoBv presented the highest proportion of volunteer species in the sward (average of 28%; $p<0.001$). In winter 2022, MonoDg presented the highest proportion of volunteer species in the sward (20%; $p<0.001$). In spring 2022, summer 2022-23, and autumn 2023, MonoBv presented the highest proportion of volunteer species in the sward (19%, 30%, and 14%, respectively; $p<0.05$). In both winters, MonoDg had the highest *T. repens* presence in the sward (average of 20%; $p<0.01$), and in summer 2021-22, MonoBv had the highest *T. repens* presence in the sward (36%; $p<0.001$).

The morphological composition also varied with season. In MonoLp, the lamina:sheath ratio remained stable throughout the eight seasons, with the *L. perenne* plants comprising approximately 70% of laminas. In MonoBv, the morphological composition significantly changed from winter to spring in both years, when the lamina proportion dropped from 73% to 26% in the first year and from 75% to 47% in the second year, and the sheath and flower proportion increased from 27% to 74% in the first year and from 25% to 53% in the second year. In MonoDg, a similar fluctuation in the morphological composition was also observed towards spring, with an increased presence of sheath and flowers (Figure 5). Within the mixtures, a more significant presence of sheath and flowers during spring 2021 was observed in MixDg (71%), followed by MixBv (52%) and then MixLp (39%).

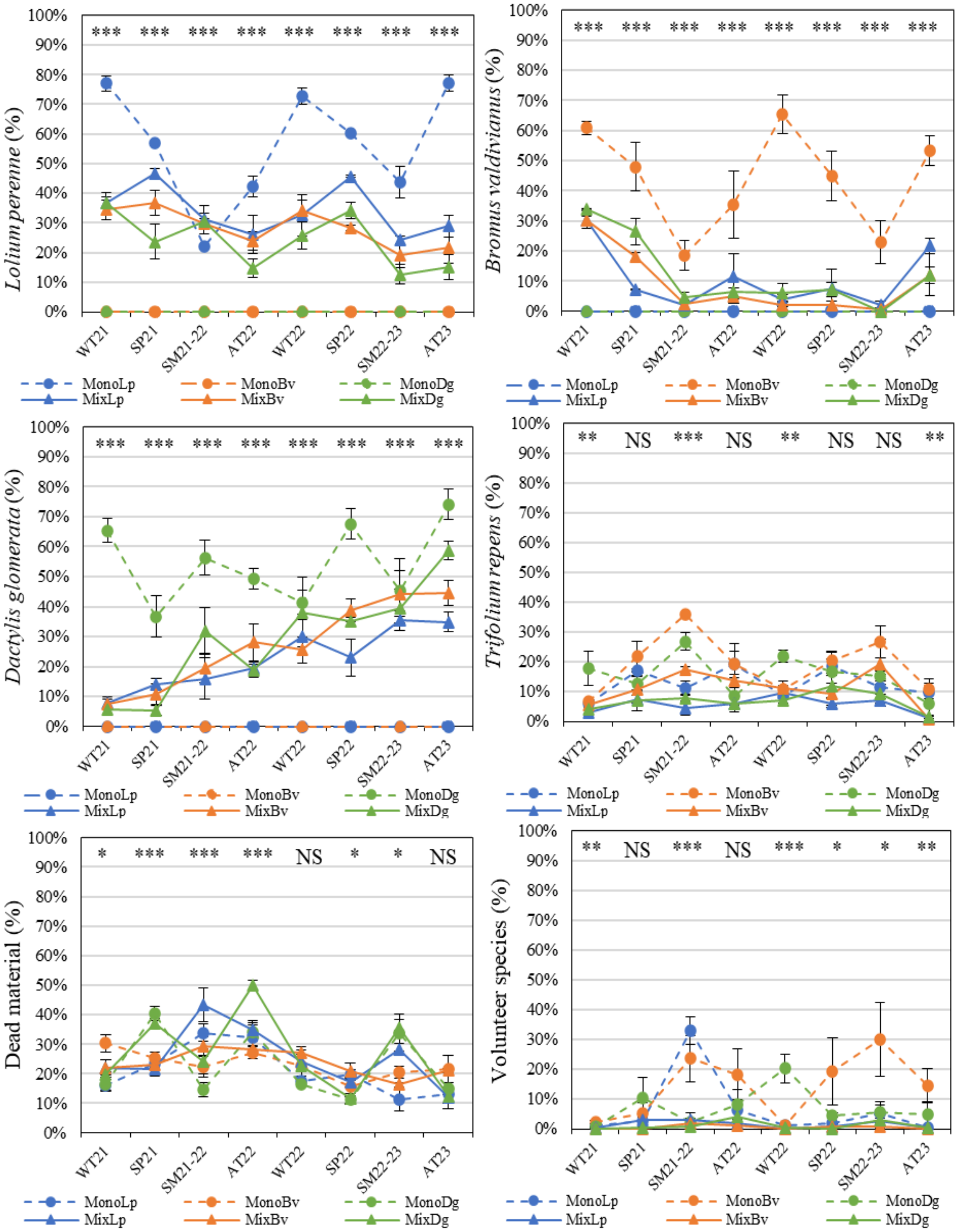

Figure 4-5. The average percentage of individual components in the botanical composition of MonoLp (dashed blue line and blue circles), MonoBv (dashed orange line and orange circles), MonoDg (dashed green line and green circles), MixLp (solid blue line and blue triangles), MixBv (solid orange line and orange triangles), and MixDg (solid green line and green triangles) during winter 2021 (WT21), spring 2021 (SP21), summer 2021-2022 (SM21-22), autumn 2022 (AT22), spring 2022 (SP22), winter 2022 (WT22), spring 2022 (SP22), summer 2022-23 (SM22-23) and autumn 2023 (AT23). Levels of significance are indicated in the graphs per season as NS = non-significant, * p<0.05, ** p<0.01, *** p<0.001. The vertical bars indicate (±) S.E.M. within the same season for the six treatments.

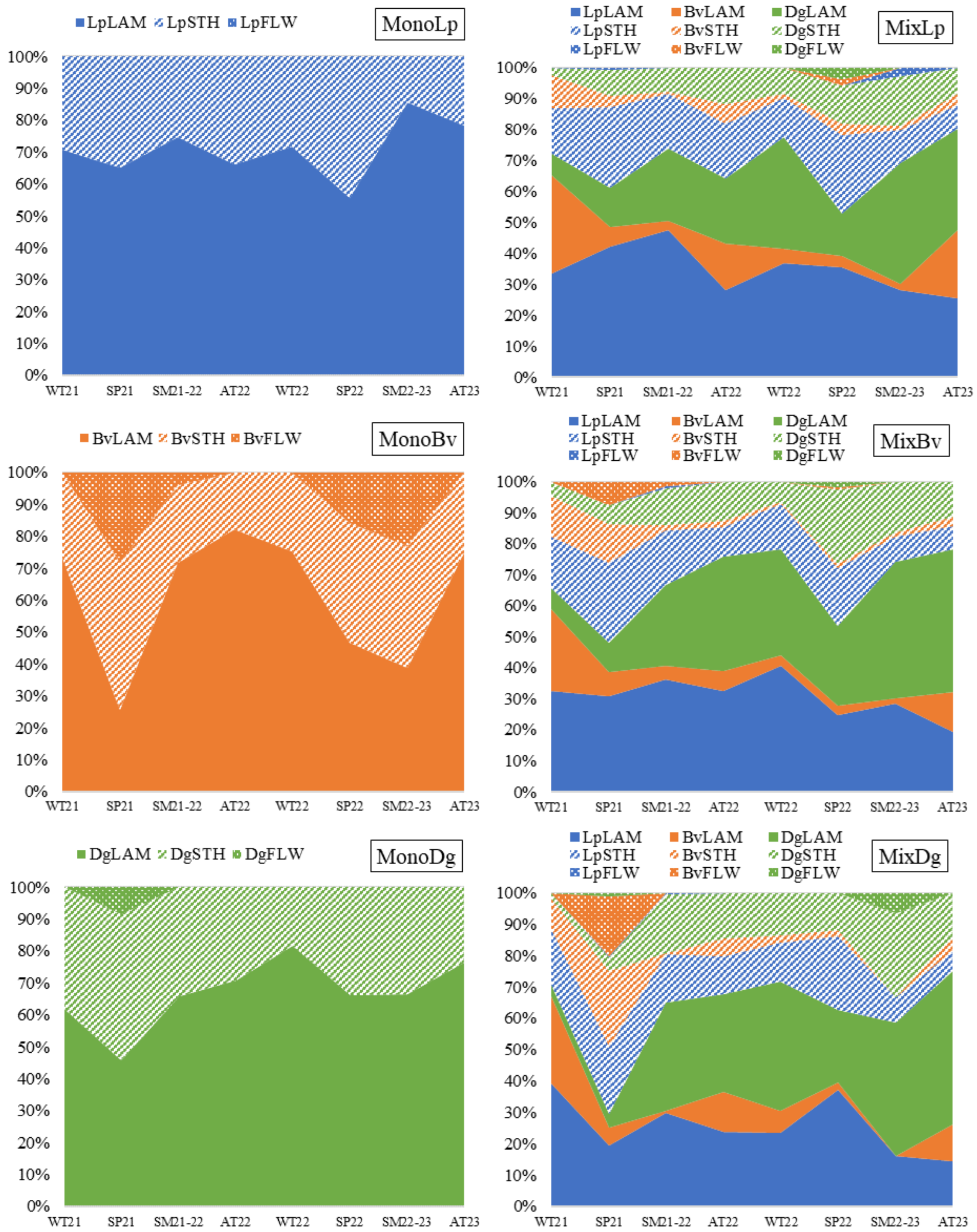

Figure 4-6. Seasonal fluctuations of the average percentage of individual components in the morphological composition [lamina (LAM - solid area), sheath (STH - dashed area) or flower (FLW - pointed area)] of each species (Lp = *Lolium perenne* - in blue, Bv = *Bromus valdivianus* - in orange and Dg = *Dactylis glomerata* - in green) per treatment (MonoLp, MonoBv, MonoDg, MixLp, MixBv, MixDg) winter 2021 (WT21), spring 2021 (SP21), summer 2021-2022 (SM21-22), autumn 2022 (AT22), spring 2022 (SP22), winter 2022 (WT22), spring 2022 (SP22), summer 2022-23 (SM22-23) and autumn 2023 (AT23).

4.6.4. Nutritive quality

In all treatments, the polynomial regression models for ME and OMD provided a good fit to the data (R2-values > 0.7) and a satisfactory prediction of the model (RPE values < 10%). In MonoLp, MonoBv, MixLp, and MixBv, ME peaked at an average of 12 MJ kg^{-1} DM in winter and dropped to approximately 10 MJ kg^{-1} DM in the transition from spring to summer. In MonoDg and MixDg, ME values were lower than 11 MJ kg^{-1} DM in summer, spring, and most of autumn. For OMD, values lower than 80% were observed in the transition from spring to summer in MonoLp, MonoBv, MixLp, and MixBv. In MonoDg, the OMD values were overall lower than 80% during the four seasons and in MixDg during spring, summer, and autumn (Figure 6).

The polynomial regression models for CP in MonoLp, MonoBv, MixLp, and MixBv provided a good fit to the data (R2-values > 0.7) and satisfactory prediction of the model (RPE values < 10%). For MonoDg and MixDg, the polynomial regression models did not present a desirable fit to the CP data (R2-values < 0.7) and only provided a relatively acceptable prediction of the model (RPE values between 10 and 20%). Overall, the numerically lowest values of CP were found during spring, with the lowest values in MonoLp. The highest predicted values of CP were found on the transition from autumn to winter when MonoLp, MonoBv, MixLp, and MixBv presented CP values higher than 25%. The polynomial regression models for NDF of MonoLp, MonoDg, MixLp and MixDg provided a good fit to the data (R2-values > 0.7) and satisfactory prediction of the model (RPE values < 10%). For MonoBv and MixBv, although the RPE values provided a relatively satisfactory prediction, the polynomial regression model could not explain the variation in the data (R2-

values < 0.7). In MonoDg, an NDF above 50% was observed most of the year, while in MixDg and MixLp, this level was only recorded during spring and summer.

The polynomial regression models for LGN did not present a desirable fit to the data (R2-values < 0.7), although presenting a relatively acceptable prediction of the model (RPE values between 10% to 20%); therefore, the models only explained relative trends of the LGN content dataset. Overall, MonoBv, MonoDg, and MixDg presented higher LGN contents than the other treatments. The polynomial regressions for the NSC in MonoLp, MixLp, MixBv and MixDg provided a good fit to the data (R2-values > 0.7) and a relatively acceptable prediction of the model (RPE values between 10% to 20%). However, for the NSC in MonoBv and MonoDg, the polynomial regression did not provide a good fit to the dataset nor a satisfactory prediction of the models. In MonoLp, MonoBv, MixLp, and MixLp, a peak of NSC was observed at the end of winter, remaining high during spring and summer and dropping towards autumn and early winter; meanwhile, MonoDg and MixDg presented different NSC patterns, with a high NSC value in late autumn and early winter, then dropping during spring and in the case of MonoDg, presenting a secondary peak in early summer (Figure 7).

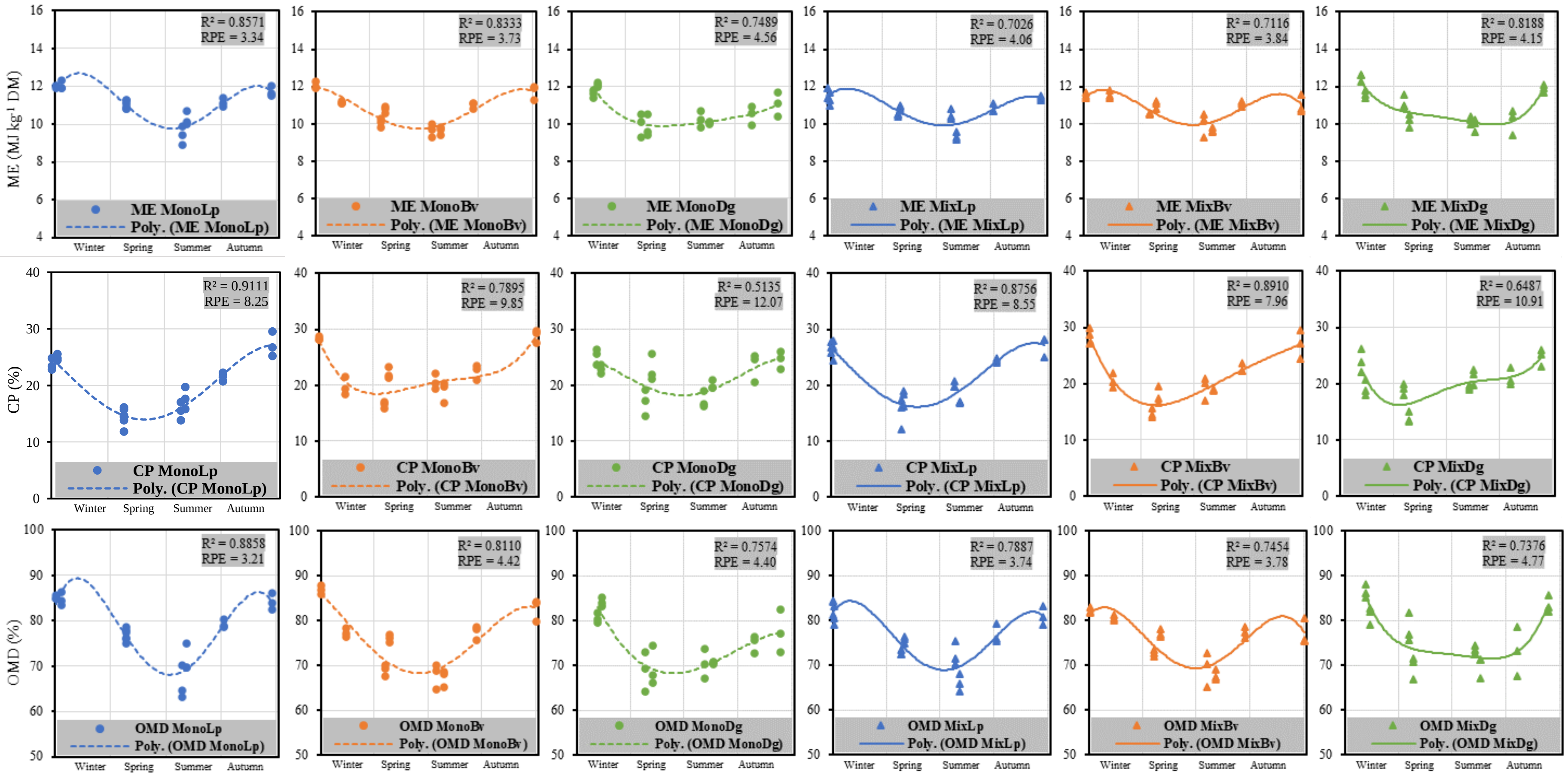

Figure 4-7. Metabolisable energy content (first row - ME, MJ kg DM^{-1}), crude protein percentage (second row - CP, %) and organic matter digestibility in-vivo percentage (third row - OMD, %) in the dry matter of treatments (MonoLp - blue points; MonoBv - orange point; MonoDg - green points; MixLp - blue triangles, MixBv - orange triangles, MixDg - green triangles) per season (winter, spring, summer, and autumn). Polynomial regressions (Poly.) of annual predicted values are estimated based on sampled data points (MonoLp - blue dashed line; MonoBv - orange dashed line; MonoDg - green dashed line; MixLp - blue solid line, MixBv - orange solid line, MixDg - green solid line). R^2=r-squared value. RPE=relative prediction error (%).

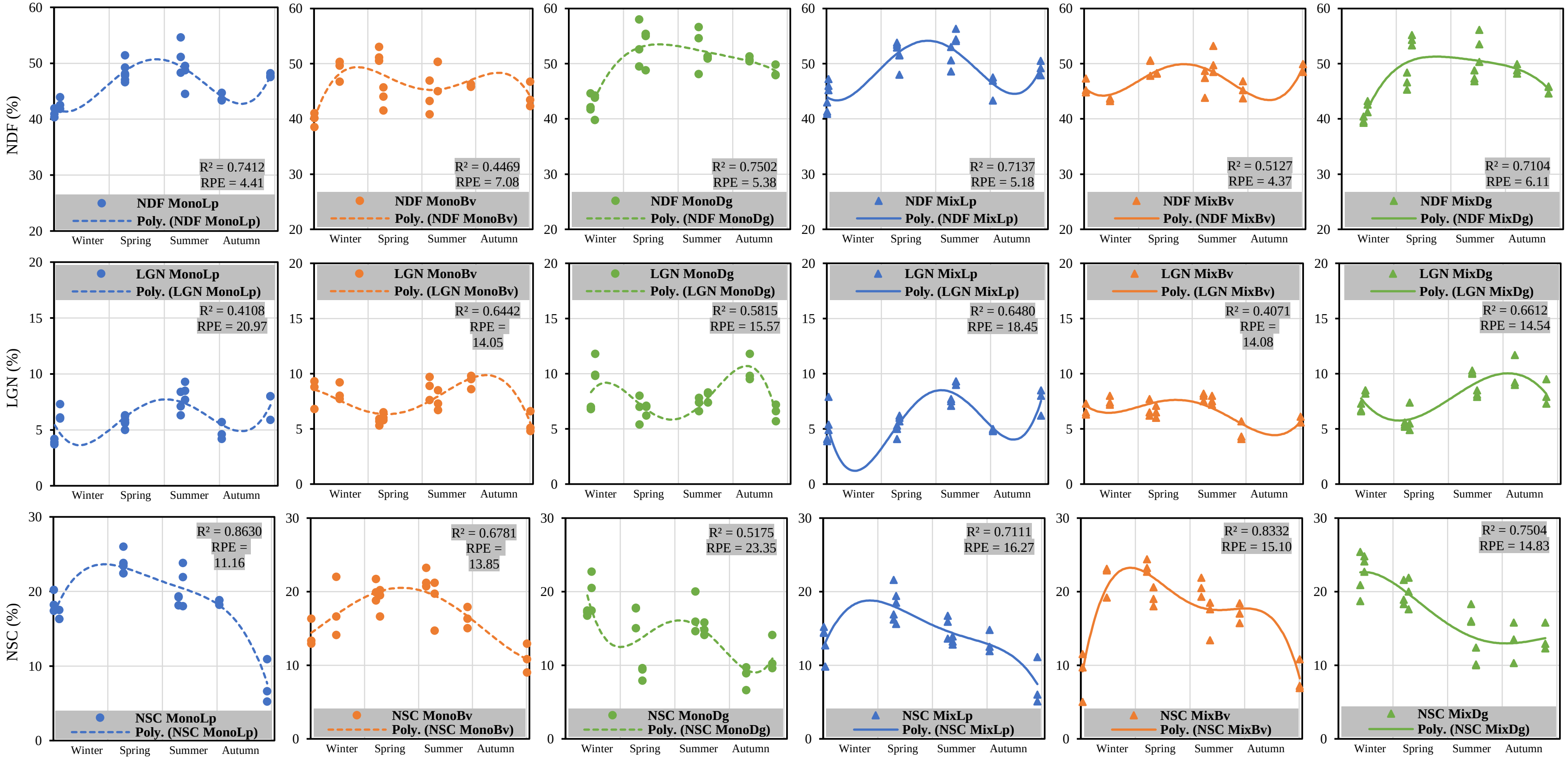

Figure 4-8. Neutral detergent fibre percentage (first row - NDF, %) and lignin percentage (second row - LGN, %) non-structural carbohydrates percentage (third row - NSC, %) in the dry matter of treatments (MonoLp - blue points; MonoBv - orange point; MonoDg - green points; MixLp - blue triangles, MixBv - orange triangles, MixDg - green triangles) per season (winter, spring, summer, and autumn). Polynomial regressions of annual predicted values are estimated based on sampled data points (MonoLp - blue dashed line; MonoBv - orange dashed line; MonoDg - green dashed line; MixLp - blue solid line, MixBv - orange solid line, MixDg - green solid line). R^2=r-squared value. RPE=relative prediction error.

4.7. DISCUSSION

The challenge associated with selecting species or cultivars that comprise a diverse pasture is 'situation-specific' and depends on the climate, soil type, and pasture utilisation (Pembleton et al., 2015). Moreover, it requires a strategic approach for appropriate defoliation management 'rules' that prevent the pasture from being dominated by one or two species (Donaghy et al., 2021). These aspects underpin the successful performance and persistence of a diverse pasture under intensive pastoral systems, enabling diversity to become a viable alternative to adapt to climatic changes.

In the present study, the information acquired at a pasture level (annual and seasonal DM accumulation, average daily growth rates per season, and nutritive value parameters) and at an individual species level (botanical and morphological composition) allowed us to: (i) infer the suitability of the optimal LS interval as a criterion to defoliate DPCS and define the temporal overlapping among different species that result in a common window of opportunity for defoliation; (ii) characterise the seasonal patterns of yield, fluctuations in botanical and morphological composition and susceptibility to volunteer species ingression in DPCS and single-grass pastures; (iii) identify complementary effects (e.g., herbage growth asynchrony, LS intervals synchronicity) underlying the combination of *L. perenne*, *B. valdivianus* and *D. glomerata*; and (iv) understand the seasonal fluctuations of nutritive parameters in DPCS and single-grass pastures. Further research is needed to test DPCS in years with longer drought periods.

4.7.1. Diverse pastures of complementary species and the use of leaf regrowth stage

According to the present study, a diverse pasture comprising *L. perenne*, *B. valdivianus*, *D. glomerata*, and *T. repens* is a viable alternative to the conventional ryegrass-based pastures in temperate humid climates. All treatments recorded a high yield in the first two years, yielding on average 20.27 t DM ha^{-1} (±1.17) in Year 1 and 21.50 t DM ha^{-1} (±0.45) in Year 2. This overall performance can be associated with the intensified management practices implemented, including maintenance fertilisation and regular application of N (Whitehead, 2000), and the use of optimal post-grazing residuals [e.g. stubble heights of 50-80 mm, and post-grazing targets of 1200-1700 kg DM ha^{-1} (Chapman et al., 2011; Lee et al., 2008)]. Further, the climatic conditions during the experimental period were favourable to herbage growth due to a prevailing La Niña pattern [warmer summers and milder winters with precipitation around 900-1300 mm year^{-1}, reliable and distributed throughout the year in the Manawatu region (Mosley, 2000)]. In New Zealand, pastoral growth is often constrained during the summer, which is a dry and warm season (Matthew et al., 2012; McCahon et al., 2021) and is sometimes subjected to periods of low soil moisture and heat that can cause temperate pastures to cease growth completely (Norton et al., 2008). However, under La Niña's influence (summer 2021-22 and 2022-23), the present study registered pastures growing all year round, with summer growth rates at around 80 kg DM ha^{-1} day^{-1}. Other commercial dairy farms in the Lower North Island (same region as the current experiment) also reported high pasture growth rates during both summers, from around 40 kg DM ha^{-1} day^{-1} up to approximately 70 kg DM ha^{-1} day^{-1} (DairyNZ, 2024).

Additionally, the LS criteria for defoliation also contributed to the high yielding found in all treatments. The LS optimal interval for defoliation, which is based on the leaves' lifespan, allows the replenishment of plant energy reserves, resumption of root growth, increased tillering, and consequently, maximum growth rates are achieved (Donaghy et al., 2021; Lemaire et al., 2009). Extension studies with farmers have shown increased pasture accumulation when the grazing rotation is realigned to fit the LS interval of *L. perenne* in ryegrass-based pastures (Clarke et al., 2021). In the present study, the seasonal optimal LS intervals of targeted (α) species were achieved in all single-grass and diverse pastures. Meanwhile, the companion species were mostly defoliated within, and sporadically defoliated out of their optimal LS intervals. In MixBv (see Table 2 for the LS target in α species), most grazing events had companion species defoliated within their optimal LS interval, except *D. glomerata*, which was defoliated slightly earlier than recommended in autumn and winter 2022 (<3.5 LS; Table 3). Overall, this indicates that when *B. valdivianus* LS is the grazing criteria, the three grass species present a recurrent overlapping of their optimum defoliation intervals, showing that they present a common window of opportunity for defoliation. In winter, summer, and autumn, these overlapping intervals were found to be 2.6-3.0 LS for *L. perenne*, 3.6-4.0 LS for *B. valdivianus* and 3.5-3.7 LS for *D. glomerata*, and in spring they were 2.5-2.6 LS for L. perenne, 3.0-3.4 LS for *B. valdivianus* and 3.1-3.4 LS for *D. glomerata*. These findings corroborate those of García-Favre et al. (2022), who found that mixed pastures of *L. perenne* and *B. valdivianus* have overlapping optimal LS intervals.

However, in the other two DPCS (MixLp and MixDg), the defoliation interval based on the α species (*L. perenne* and *D. glomerata*, respectively) did not always match the optimal LS interval of the other two companion species. In the MixLp (*L. perenne* as α), *B. valdivianus* was defoliated earlier than recommended in winter 2021 and summer 2021-22 (<3.5 LS; Table 3), and *D. glomerata* in winter 2021, spring 2021, summer 2021-22, autumn 2022 and winter 2022 (<3.5 LS in summer, autumn, and winter; <3.0 LS in spring; Table 3). Overall, in MixLp, the three species' LS were closer to the lower limit within their LS interval (*L. perenne* closer to 2.5 LS, and *B. valdivianus* and *D. glomerata* closer to 3.5 LS). Conversely, in MixDg (*D. glomerata* as α), *L. perenne* was defoliated later than recommended in winter 2021 and winter 2022 (>3.0 LS) and *B. valdivianus* in winter 2021 (>4.0 LS; Table 3). Overall, in the MixDg, the three species' LS were closer to the upper limit within their LS interval (L. perenne closer to 3.0 LS, and *B. valdivianus* and *D. glomerata* closer to 4.0 LS; Table 3). From these findings, it is possible to conclude that *D. glomerata* takes slightly longer to reach its optimal LS interval for defoliation compared to *L. perenne* and *B. valdivianus*. In a mini-sward study with mixtures of *D. glomerata* and *Bromus wildenowii* Kunth., it was demonstrated that *D. glomerata* took 19 winter days longer than *B. wildenowii* to get to 4 LS (Gatti et al., 2016).

Considering the theoretical background of the LS criteria, for the grazing events in which the companion species were defoliated slightly earlier or later than the recommended LS intervals (MixLp or MixDg), it is possible to infer that there were trade-offs at a species level between water-soluble carbohydrate reserves for regrowth and optimising feed productivity and quality. In other words, a farmer may choose to utilise a faster rotation with pasture being defoliated just before the companion species' minimum interval limit to maximise feed quality or, alternatively, a longer rotation with pastures defoliated just after the maximum interval limit to maximise herbage yield and full replenishment of WSC reserves. In the present study, the species in the DPCS were mostly grazed within their optimal LS interval for defoliation, which may also encourage pasture short-term persistence. More research and extension promoting the use of LS as a defoliation criterion is needed to evaluate on-farm long-term effects on diverse pasture persistence and resilience.

4.7.2. The effects of complementarity in diverse pastures: growth asynchrony and overyielding

By combining summer- and winter-active species, the DPCS showed that growth asynchrony can result in seasonal overyielding, exhibited as greater seasonal growth rates of DPCS compared to their respective (same α LS species) single-grass pastures. For example, MixLp consistently presented significantly higher daily growth rates in the warmer seasons (both spring periods and summer 21-22) than MonoLp (Figure 3). Similarly, MixBv consistently presented significantly higher daily growth rates than MonoBv in the cooler seasons (Figure 3). The single-grass pastures presented significant season-to-season variation, highlighted by production peaks in one season followed by a trough in the next (Table 4). For instance, MonoLp presented relatively high accumulated DM masses in winter 2021 and 2022 and in autumn 2021, followed by relatively low accumulated DM masses in spring 2021 and 2022 and in summer 2021-22. MonoBv was among the treatments that presented the lowest accumulated masses in winter 2021 and 2022, spring 2021 and 2022, and autumn 2022, and a peak of production in summer 2021-22. Lastly, MonoDg had variations in accumulated herbage masses, however, with no apparent seasonal consistency.

Previous studies under different conditions (glasshouse, plot, farm, up to continental scale) have registered growth asynchrony among species in diverse pastures (Descalzi et al., 2019; Finn et al., 2013; García-Favre et al., 2022; Komainda et al., 2020; Küchenmeister et al., 2012). However, these same studies have not always found seasonal or annual overyielding of diverse pastures in relation to less complex pastures (pure stand or binary). For example, in the main production year of two different glasshouse experiments utilising pure stands, three-species diverse pastures, and five-species diverse pastures (*L. perenne*, *D. glomerata*, *T. repens*, *Plantago lanceolata* L., and *Taraxacum officinale* Agg.), it was found that *T. repens* pure stands presented the highest accumulated DM in relation to all other possible combinations of species (Komainda et al., 2020; Küchenmeister et al., 2012). The diverse treatments containing the legume presented medium to high accumulated biomass, and the forb and grass pure stands were among the lowest accumulated DM values. Both studies suggested that these findings were primarily driven by the legume N supply, and secondarily driven by forbs or grass species alternatively experiencing optimal temperatures for growth across seasons.

A field study by Descalzi et al. (2019) demonstrated the growth asynchrony in fertilised naturalised pastures composed mostly of *Agrostis capillaris* L., *Holcus lanatus* L., *L. perenne*, *Vulpia dertonensis* All., *Anthoxanthum odoratum* L., and a diverse pasture of *L. perenne*, *B. valdivianus*, *H. lanatus*, *D. glomerata* and *T. repens*, albeit not presenting overyielding in relation to the control *L. perenne* and *T. repens*. On the other hand, field studies by Nyfeler et al. (2009) observed overyielding in diverse pastures compared with pure stands, up to the point of 'transgressive' overyielding, where diverse pastures produced more than the best pure stand treatment. In a plot-scale experiment with sheep grazing pure stands and mixed swards of *B. valdivianus* and *L. perenne*, García-Favre et al. (2022) found growth asynchrony in the mixtures that, in this case, resulted in 15% higher accumulated DM (overyielding) when compared to the pure stands. Moreover, in an intercontinental scale study with the same experimental design across 31 different locations, Finn et al. (2013) found that four species mixtures containing two grass and two legume components presented 'transgressive' overyielding in relation to pure stands in 97% of the comparisons over multiple years.

Although not all studies have reported the overyielding of diverse pastures in relation to less complex pastures, one common factor was consistently reported: diverse pastures presented a clear effect of annual or seasonal yield stability as a buffering ability to respond to varied abiotic and biotic disturbances. Finally, due consideration to species identity and functional roles must be given when selecting species to comprise a diverse pasture (Sanderson et al., 2007). The succession and persistence of a diverse pasture depend not upon including more species but on the causal relationship of combining all these species.

4.7.3. The effects of complementarity in diverse pastures: volunteer species suppression and botanical composition dynamics

In intensive dairy pastoral systems such as New Zealand's, susceptibility to weed invasion is associated with a decline in pasture resilience caused by various issues, such as overgrazing (Hernández-Garay et al., 1997), pugging damage (Drewry et al., 2008) and lower persistence traits of perennial-ryegrass cultivars (Chapman et al., 2015), amongst others (McCahon et al., 2021). The reduced presence of volunteer species in diverse pastures is an ecological side effect, resulting in enhanced niche competitiveness and consequent higher resource exploitation and utilisation (Knops et al., 1999; Naeem et al., 2000), termed by Tracy and Sanderson (2004) as "resource use complementarity". A probable secondary explanation for this reduction is the "sampling effect" which describes the higher probability that at least one of the pasture species is at the peak of its competitiveness and actively growing, which in turn reduces the opportunities for non-desired species ingression (Aarssen, 1997; Huston, 1997). Tracy and Sanderson (2004) suggest that both processes occur concomitantly in diverse pastures, which may also be the case in this study.

The present study found that DPCS have an enhanced ability to withstand the ingress of volunteer species in the sward. Seasonal peaks of volunteer plant species presence (~20%) were often registered in the single-grass pastures (summer 2021-22, winter 2022, spring 2022, summer 2022-23, and autumn 2023), while the DPCS had at most 4% presence of volunteer plant species. These significant differences in volunteer species proportion were, anecdotally, associated with a significant decrease in the sown species proportion (MonoLp and MonoBv in summer 2021-22, MonoDg in winter 2022, and MonoBv in spring 2022), up to an extreme in which MonoLp had significantly less *L. perenne* percentage (22%) than in MixLp, MixBv and MixDg (average of 30% among DPCS). These events were driven by the aggressive invasion of volunteer spring- and summer-active species (Rumex obtusifolius L., Paspalum dilatatum Poir.) and volunteer winter-active species (Poa annua L.). These findings agree with other studies comparing pure stands and different mixed pastures in North America [*Phleum pratense* L., *Poa pratense* L., *Trifolium pratense* L. and *T. repens* (Sturludóttir et al., 2013)] and in Europe [*L. perenne*, *D. glomerata*, *T. repens* and *T.*

pratense L., or *L. perenne, P. pratense* L., *T. repens* and *Trifolium ambiguum* M. Bieb. (Connolly et al., 2009)]. Furthermore, the seasonal fluctuations in botanical composition found in this study are probably related to competition (for *T. repens*), environmental constraints (for *B. valdivianus*), opportunistic overtake (for *D. glomerata*), and defoliation criteria (for *L. perenne*).

Many factors can compromise the persistence of legumes in pastures, including establishment issues, livestock grazing selection, treading, and resource competition for light, water, and nutrients (Brock and Hay, 2001). The higher *T. repens* proportion in single-grass pastures than in DPCS may have resulted from severe interspecific plant competition (Annicchiarico et al., 2014) by the three grass species that present characteristics of competitors (Grime et al., 1988). In both summers, the higher rainfall events caused by La Niña [especially December 2021, with 229.8 mm recorded, compared with the long-term (1981-2010) average of 87.5 mm (NIWA, n.d.) resulted in long-term saturated soil conditions, and the *B. valdivianus* proportion drastically dropped in all treatments. Bromus spp are recommended for well-drained soils as they do not tolerate low oxygen levels in soils (López et al., 1997; Stewart, 1996). Such sensitivity is probably due to small-sized aerenchyma, contrasting with *L. perenne*, which presents well-developed aerenchyma (Zhang et al., 2023).

As the *B. valdivianus* proportion in the mixtures significantly decreased, the *D. glomerata* proportion concomitantly increased (Figure 4), showing an opportunistic overtake of one species by another. *Dactylis glomerata* plants have larger tillers and longer leaves than both *L. perenne* and *B. valdivianus*, which may result in a competitive advantage, especially at longer grazing rotations. The ‘umbrella effect’ is a phenomenon that occurs in diverse pastures when species that are taller than their companion species shade out the neighbouring tillers, giving them an advantage in capturing light (Bryant et al., 2015). However, such phenomena can be manipulated in diverse pastures by introducing faster grazing rotations. Carlen et al. (2002) assessed the competitive ability of *D. glomerata* in mixtures with Festuca pratensis and reported *D. glomerata*'s overtake by the second growing season under infrequent defoliation treatment. In the present study, MixLp and MixBv, the treatments in which the LS targets resulted in faster grazing rotations, showed a more even proportion between the morphological components of *L. perenne* and *D. glomerata* (Figure 5). In MixDg, the longer grazing rotations resulted in *D. glomerata* components representing a greater proportion of the sward, favouring its spatial dominance over other species.

4.7.4. Grazing management and diversity effects on the pastures' nutritive parameters

In New Zealand's dairy pastoral systems, the recommended CP content for lactating cows is in the order of 16% to 18% during early and mid-lactation (spring and summer), 14% during late lactation (autumn), and 12% during the dry period (winter) (DairyNZ, 2023). In the present study, although all experimental treatments experienced a trough in CP content during spring and early summer, pastures still presented adequate CP for early and mid-lactation cows during that period (except for MonoLp with CP ~14% in spring). The trough in CP content may be associated with an increased pasture growth rate during spring that led to a dilution effect on a percentage basis. Conversely, all experimental treatments presented excessively high CP content during autumn and winter (above 25% DM), which can result in environmental issues and, potentially, be toxic to animals (Pacheco and Waghorn, 2008). In pastures containing CP content higher than dairy cattle requirements, the ingested N surplus (~70%) is excreted predominantly as a waste product (Marshall et al., 2021), which can ultimately result in nitrate leaching into the water catchments and reservoirs (Selbie et al., 2015). In the present study, the high CP values in winter and autumn were associated with the presence of legumes in the pastures (ranging from 5 to 20%), and also probably the result of years of breeding programs focusing on higher nutritive value (Wilkins, 1991).

In pasture-based dairy systems, ME is usually the first limiting factor of production (van Vuuren and Meijs, 1987). Normally, the minimum threshold for lactating dairy cows to maintain milk yield is around 11 MJ kg^{-1} DM (AFRC, 1993). All pastures presented slightly low ME values (~10 MJ ME^{-1} DM) in late spring and summer, a well-known problem in pasture-based systems caused by the onset of the reproductive phase in grasses, where higher lamina:stem ratios result in higher fibre levels (Delagarde et al., 2000). In a broad study with C3 and C4 grasses, Fulkerson et al. (2007) concluded that, overall, the nutritional parameters are higher during cool seasons (winter and autumn) and are often followed by a decline in quality over warmer seasons, a fact associated with reproductive development. This clear pattern was also found in New Zealand by Roche et al. (2009a, 2009b), suggesting that, at that time of the year, feed supplementation may be necessary to maintain milk production.

In the current study, MonoDg and partially MixDg did not record the post-summer recovery in ME content, as observed in MonoLp, MonoBv, MixLp, and MixBv. As previously discussed, the *D. glomerata* LS treatments were defoliated fewer times throughout the study period. In some seasons (spring 2021, autumn 2022, and summer 2022-23), MonoDg and MixDg recorded a higher proportion of dead material than the other treatments (over 35%), along with a higher proportion of sheaths and flowers (35-40%), up to an extreme in spring 2021 when flowers and sheaths comprised 70% of MixDg and 55% of MonoDg. This higher proportion of dead material, along with sheaths and flowers, resulted in higher NDF and LGN contents because fibre levels are high in sheaths and flowers (Griggs et al., 2007). MonoBv also contained high levels of LGN and NDF throughout spring and summer (30% and 5% in year 1, and 15% and 20% in year 2, respectively), which can be associated with a relatively longer reproductive phase than the other two grass species (Balocchi et al., 2001).

Despite significantly increased biomass, Suter et al. (2021) found that forage quality was generally similar between mixtures and pure stands. According to their study, this results in greater units of CP, ME, or OMD per area (i.e., kg CP ha^{-1}) in mixtures. Therefore, it is possible that mixtures can maintain a higher stocking capacity than pure stands, which is an area requiring further research. In this study, the differences in nutritive parameters were mostly driven by the defoliation criteria and, to a lesser extent, by the diversity of the pasture. Therefore, the *B. valdivianus* LS criteria can be proposed as the most suitable defoliation criteria for DPCS because it (i) covers the optimal LS interval for the defoliation of all three grass species more often, (ii) results in adequate nutritive quality, and (iii) it also resulted in overyielding and reduced susceptibility to volunteer species compared to the single-grass pastures. In addition, on some occasions during spring and summer when herbage growth was faster, the *L. perenne* LS criteria can be a suitable defoliation criterion since it (i) resulted in a more balanced species proportion, (ii) adequate nutritive quality, and (iii) it also resulted in overyielding and reduced susceptibility to volunteer species compared to the single-grass pastures. The LS can be used as a flexible management tool to optimise production, quality, and utilisation of diverse pastures.

4.8.CONCLUSIONS

The LS criterion was a suitable management tool for DPCS and can be considered successful due to an overall high annual yield, seasonal overyielding in relation to single-grass pastures, low susceptibility to ingression of volunteer species, and satisfactory levels of crude protein and metabolisable energy. It is possible to conclude that DPCS have growth asynchrony among different species DM accumulation and development synchrony among species LS. Within DPCS, a common window opportunity for optimal defoliation of *L. perenne, B. valdivianus* and *D. glomerata* was achieved mostly when utilising the *B. valdivianus* LS target. The *L. perenne* LS could be a target alternative when aiming to buffer the competition by *D. glomerata*. The *D. glomerata* LS is not the most appropriate grazing criterion since the resulting longer grazing rotation causes an increase in dead material, sheath and flower proportions, overall lower nutritive quality and promotes the overtake of *D. glomerata* over the other species.

Finally, the hypothetical bases of the study (DPCS defoliated according to the LS criterion) can become an important alternative for temperate grazing and have the potential to be applied to diversified pastoral ecosystems of other climates and soil types; nonetheless, the combined species must present complementary traits. Further studies are necessary to assess the production and quality parameters of DPCS defoliated according to the LS criterion in years with longer or more extreme drought periods.

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Chapter 5

Animal behaviour and dietary preference of dairy cows grazing binary and diverse pastures under the leaf regrowth stage defoliation criterion

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5.1. ABSTRACT

In New Zealand, intensively managed pasture-based dairy systems rely on binary pastures mostly comprised of *Lolium perenne* L. and *Trifolium repens* L.. More frequent and extreme climatic events have been negatively affecting the persistency and production of these pastures, which now present increased seasonality, with marked peaks and troughs of production throughout the year. Diversification of plant species offers a solution to deal with increased seasonality. However, little is known about animal behaviour and dietary preferences of dairy cows grazing diverse pastures. The present study aimed to assess the grazing preferences of dairy cows when unrestrictedly offered binary (*L. perenne* and *T. repens*; Bi) and diverse pastures (*L. perenne*, *Bromus valdivianus* Phil., *Dactylis glomerata* L. and *T. repens*; Mix) subjected to three different leaf regrowth stage (LS) defoliation criteria. Secondarily, the study aimed to determine the main plant-related drivers for any potential animal preference. The treatments were MixLp (defoliated at *L. perenne* LS), BiLp (defoliated every time MixLp was defoliated), MixBv (defoliated at *B. valdivianus* LS) and BiBv (defoliated every time MixBv was defoliated), MixDg (defoliated at *D. glomerata* LS) and BiDg (defoliated every time MixDg was defoliated). Dairy cattle were evaluated over five agricultural seasons. The response variables were grazing time and location, bite rate, animal behavioural activity, pre-grazing herbage mass, undisturbed sward height, lamina:stem ratio, crude protein, metabolisable energy, organic matter digestibility, non-structural carbohydrates, neutral detergent fibre and lignin. Where significant differences were found, binary pastures presented lower sward height and higher non-structural carbohydrate content in comparison to the diverse pastures under the same LS defoliation criteria.

However, no significant differences were found in the percentage of time that cows spent grazing both pastures. Season was the greatest contributor to the proportion variation found in all response variables, with values ranging from 47.55% up to 88.77%. In winter and spring, cows modulated their grazing behaviour (proportional time spent grazing, ruminating, or idling), investing more time actively grazing pastures under *L. perenne* LS interval of defoliation (2.5-3.0 LS), the criteria which resulted in shorter grazing rotations. This study allowed us to understand the suitability of diverse pastures from an animal perspective, and highlighted that independent of the pasture type, the positive productive and nutritional effects of defoliation management based on the LS may also extend themselves to positive outcomes in animal preference, interpreted as the percentage of time dairy cows spend grazing rather than ruminating or idling across and within seasons.

5.2. KEYWORDS

Grazing behaviour, dairy cows, perennial ryegrass, pasture brome, cocksfoot, white clover

5.3. INTRODUCTION

The strategic advantage of low-cost and efficient dairy farming in New Zealand has been largely explained by its reliance on pasture-based systems (Caradus et al., 2023; Macdonald and Roche, 2023; Roche et al., 2017), specifically, pastures comprising *Lolium perenne* L. and *Trifolium repens* L. (Caradus et al., 2021). These two species have been a recurrent subject of study in pastoral research for almost a century (Blackman and Templeman, 1940; Brougham, 1959, 1960; Da Silva et al., 2004; Hernández-Garay et al., 2010a; Hernández-Garay et al., 2010b; Macdonald and Roche, 2023). The interest in these two species is mostly due to their adaptability to intensively managed dairy systems since both species present high levels of productivity, great nutritive value and dietary acceptability by dairy cows (Brock and Hay, 2001; Cosgrove, 2011; Hunt and Easton, 1989). However, these two species are highly sensitive to soil water stress and high temperatures (Cosgrove, 2011), and under the current climatic scenario, the persistence and continued high productivity of *L. perenne* and *T. repens* binary pastures are threatened by more frequent and intense extreme weather events (Keller et al., 2021; Lee et al., 2013).

One of the main advantages of pasture-based dairy systems utilising *L. perenne* and *T. repens* binary pasture is that, for most of the year, the pasture annual growth curve matches dairy cows' feed requirements. Firstly, by aligning the peak of herbage production and animal calving period in spring and, secondly, by the lower herbage production when cows dry off during late autumn and early winter (Garcia and Holmes, 2010; Holmes and Roche, 2007). However, due to a more marked seasonality of production as an unfolding situation triggered by climate change (Kalaugher et al., 2017; Keller et al., 2021), herbage production of these binary pastures is no longer sufficient to meet cows' feed requirements throughout the year, and farmers are increasingly using external feed sources to maintain milk production (Clark, 2011; Holmes et al., 2002). In New Zealand, a review on pasture resilience showed that the amount of non-pasture feeds (harvested or imported supplements) utilised in dairy farms increased from an average of 4.0% to 18.8% per cow from 1990-91 to 2017-18 (Rys et al., 2021). Although the inclusion of external source feed may result in the production of extra milk solids, it does not necessarily result in increased profitability as it consequentially increases the cost of production (NZD $0.30 to $0.40 per extra kg of dry matter) (Silva Villacorta et al., 2005).

A large body of research has been done on the inclusion of new forage species within New Zealand pasture-based dairy farms as an attempt to buffer the increasing seasonality of production in *L. perenne* and *T. repens* binary pastures. The inclusion of more species within a pasture-based system conceives the idea behind diverse pastures and could occur as the addition of either herbs or legumes such as *Cichorium intybus* L. (Cranston et al., 2015; Li and Kemp, 2005), *Plantago lanceolata* L. (Bryant et al., 2019; Kemp et al., 2013; Nobilly et al., 2013), *Trifolium pratense* L. (Brock et al., 2003), *Medicago sativa* L. (Moot et al., 2016), or other grasses, such as *Dactylis glomerata* L. and *Bromus valdivianus* Phil. (García-Favre et al., 2022; Oliveira et al., 2023). In many of these studies, positive outcomes related to plant production (e.g. overyielding and growth asynchrony), environmental solutions (e.g. reduced nitrate leaching), and improved nutritive value (e.g. nutritive contribution over summer) were found. Yet, little is known about these diverse pastures' dietary acceptability over common binary pastures of *L. perenne* and *T. repens* and, moreover, their effects on dairy cows' grazing behaviour.

The complex animal-plant dietary relationship in experimental conditions is often conceived as a two-step process composed of animal grazing ‘preference’ and dietary ‘selection’ (Gregorini et al., 2015; Hodgson, 1979; Parsons et al., 1994). Preference has been defined as relative feed intake or relatable measures when access to different feeds is unrestricted under grazing conditions (Allen et al., 2011; Hodgson, 1979). Meanwhile, dietary selection is a behavioural expression of preference when access to feed is affected by the environment or opportunity for selection, resulting in the removal of some sward components (plant patches or plant parts) (Allen et al., 2011; Hodgson, 1979). When animals are unrestrictedly offered a diverse array of forage types and have the opportunity to exhibit their preference fully, different layers of processes or features occurring at a plant level will affect the animal's preferences (Poli, 1998). For example, seasonal or diurnal variability (i.e. plant phenological state), morphological and structural characteristics of the sward (i.e. sward height or lamina:stem ratio), and lastly, nutritive parameters of the forages (i.e. energy and carbohydrates content or fibrous composition) (Poli, 1998). Several grass species, such as *L. perenne*, *B. valdivianus*, *Bromus willdenowii* Kunth, *D. glomerata*, *Festuca arundinacea* Schreb., have been accessed as monocultures in preferential grazing trials (Garcia-Favre et al., 2023; Horadagoda et al., 2009; Villalba et al., 2015). In general, for grasses within these aforementioned studies, Bromus spp. were the most preferred species, followed by *L. perenne* and secondarily by *D. glomerata*, while *F. arundinacea* was the least preferred species. To our knowledge, although a wide range of theoretical models have been proposed explaining the reason behind the dietary preference of animals for mixing different sources of nutrition in the diet (Rutter, 2006), very little research has been done on animal preference when grazing diverse pasture mixes (Soder et al., 2007).

Considering the adoption of plant species diversification in farming systems (Cranston et al., 2015; Isbell et al., 2015; Sanderson et al., 2007) and given that grazing preference of dairy cows is commonly driven by nutritive value, sward structure and plant morphology, the present study hypothesised that dairy cows prefer binary pastures (*L. perenne* and *T. repens*) due to their potentially higher nutritive value and greater morphological structure of the sward in comparison to diverse pastures (*L. perenne*, *B. valdivianus*, *D. glomerata* and *T. repens*). Therefore, this study aimed to assess the grazing preference of dairy cows when they were unrestrictedly offered binary (*L. perenne* and *T. repens*) and diverse pastures (*L. perenne*, *B. valdivianus*, *D. glomerata* and *T. repens*) subjected to three different leaf regrowth stage (LS) defoliation criteria. The LS is a plant-focused defoliation criterion which consists of an optimal interval for defoliation based on a minimum point set by the replenishment of water-soluble carbohydrate (WSC) reserves in the tillers and a maximum point by the onset of senescence in the older parts of the tiller (see material and methods section for further details) (Fulkerson and Slack, 1994). Secondarily, the study aimed to determine what were the main plant-related attributes that may lead to preferential grazing.

5.4. MATERIAL AND METHODS

5.4.1. Experimental site

The present study was conducted over five agricultural seasons between February 2022 and March 2023 within a wider pastoral study undertaken at Massey University’s Dairy 1 (Palmerston North, Manawatu, New Zealand; 40°22'36.1"S 175°36'40.2"E), with the approval of the Massey University Animal Ethics Committee (Approval number 21/24).

The soil type is Manawatu silt loam over sand (Landcare Research National Soil Data Base, Lab. No SB10036). The results of soil chemical analysis (0-15 cm soil depth) collected on 26 February 2021 indicated 5.60 pH [CaCl2 0.01 M (1:2.5)], 3.33% organic matter content, 29.0 Olsen P (mg/L), 0.21 exchangeable K (me/100g), 6.80 exchangeable Ca (me/100g), 1.35 exchangeable Mg (me/100g), 13.0 cation exchange capacity (me/100g), 64.0% total base saturation content, 3 extractable organic sulphur (mg/kg) and 10.03 carbon:nitrogen ratio. The area received annual maintenance fertilisation in the form of superphosphate on 1 March 2021 at 112.5 kg P/ha, 137.5 kg S/ha and 250 kg Ca/ha, and on 17 March 2022 at 90 kg P/ha, 110 kg S/ha and 200 kg Ca/ha. Post-grazing nitrogen fertilisations were applied at a rate of 30 kg N/ha in the form of urea (46% N) in July 2021, November 2021, January 2022, August 2022, November 2022, and February 2023. The climate is classified as Marine Climate - Cfb (Köppen-Geiger's climate classification) (Beck et al., 2018). Monthly rainfall, ground minimum temperature, and air minimum and maximum temperature are presented in Figure 1 (NIWA/AgResearch Weather Station, ~800 m from the field site).

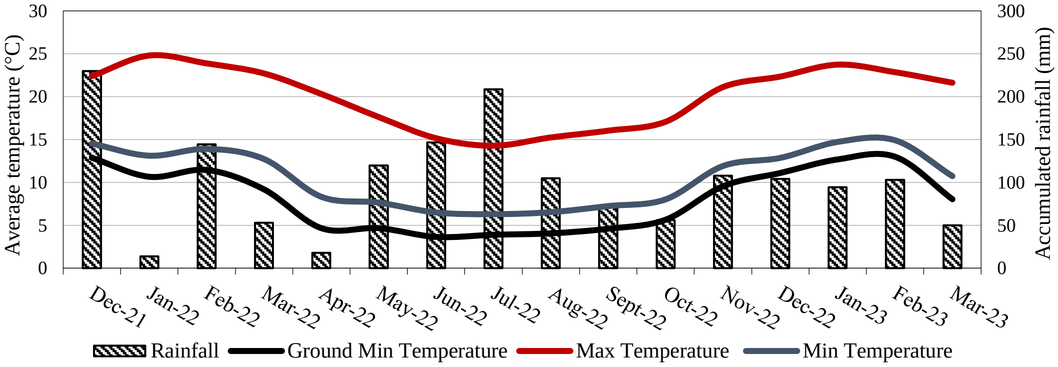

Figure 5-1. Monthly accumulated rainfall, monthly averaged ground minimum, and monthly averaged air maximum and minimum temperatures during the experimental period (five agricultural seasons, from December 2021 to March 2023). Bars indicate accumulated rainfall; lines indicate average temperatures.

On 24 November 2020, the area was sprayed out with N-(phosphonomethyl) glycine (glyphosate WeedMaster® G360) at 6 L/ha and thifensulfuron-methyl (Harmony® 50 SG) at 30 g/ha to control weed seed bed and population. From 10 to 18 December 2020, the area was ploughed, power harrowed and levelled. On 18 December 2020, the two types of pastures, diverse (Mix) and binary pastures (Bi) (see pasture composition and sowing rates in Table 1), were established side-by-side (see Figure

3 for plot details) with a roller drill, and the area was lightly irrigated for one day with a mobile gun sprinkler to ensure a successful establishment. Following this, the pastures were rainfed.

5.4.2. Experimental design and treatments

The binary and diverse pastures were regularly grazed for two production years according to the methodology utilised in a previous wider pastoral study (Oliveira et al., 2023). The wider study had its grazing protocol started in June 2021 with dairy cows grazing on a one-day event basis until the pasture reached an undisturbed post-grazing height of 5 to 8 cm from ground level. The defoliation criteria to define different treatments were based on the LS interval of each grass species, *L. perenne* (2.5-3.0 LS), *B. valdivianus* (3.5-4.0 LS) and *D. glomerata* (3.5-4.0 LS) (Figure 2). Detailed information about the LS defoliation criteria for diverse pastures utilised in the present study can be found in Oliveira et al. (2023).

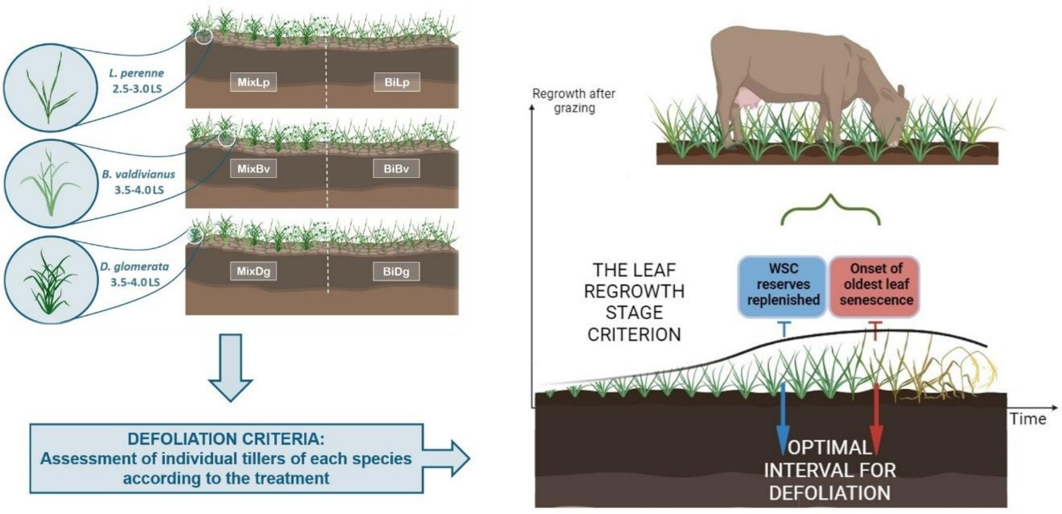

Figure 5.2. Conceptual diagram of the defoliation criteria methodology, which displays the leaf regrowth stage (LS) assessment of individual tillers of each species within diverse pastures for defining the experimental treatments based on the optimal interval for defoliation for *L. perenne* (2.5-3.0 LS), *B. valdivianus* (3.5-4.0 LS) and *D. glomerata* (3.5-4.0 LS). This image was partially created in BioRender.com

Table 5.1. Establishment description of the two pastures species composition and sowing rates, cultivars, resulting experimental treatments, and defoliation parameter.

Pasture composition (acronym)	Cultivars and sowing rates (kg ha^{-1})	Experimental treatment	Defoliation parameter
Lp + Bv + Dg + Tr (Mix)	*L. perenne* cv. Maxsyn (10) + *B. valdivianus* cv. Bareno (15) + *D. glomerata* cv. Greenly II (12) + *T. repens* cv. Weka (6)	MixLp	*L. perenne* LS
		MixBv	*B. valdivianus* LS
		MixDg	*D. glomerata* LS
Lp + Tr (Bi)	*L. perenne* cv. Maxsyn (20) + *T. repens* cv. Weka (6)	BiLp	Following MixLp
		BiBv	Following MixBv
		BiDg	Following MixDg

Lp: *L. perenne*, Bv: *B. valdivianus*, Dg: *D. glomerata*, Tr: *T. repens*; Mix: diverse pasture, Bi: binary pasture; LS: leaf regrowth stage.

All response variables were assessed once a season, for five seasons [summer 2022 (summer 1), autumn 2022 (autumn), winter 2022 (winter), spring 2022 (spring), and summer 2023 (summer 2)]. The animal-related dependent variables were grazing time (GT; %) and location, bite rate (BR; bites per minute), and animal behavioural activity (percentage of time spent grazing, ruminating, and idling). The plant-morphology dependent variables were pre-grazing dry matter herbage mass (HM; ton DM/ha), pre-grazing undisturbed sward height (HT; cm), and lamina:stem ratio (LSR). On a dry matter basis, the plant nutritive parameters were crude protein (CP; g/kg), metabolisable energy (ME; MJ/kg DM), organic matter digestibility in-vivo (OMD; %), non-structural carbohydrates (NSC; g/kg), neutral detergent fibre (NDF; g/kg) and lignin (LGN; g/kg).

For summer 1, the grazing events and experimental assessments occurred on 16 February 2022 (MixLp versus BiLp), 10 February 2022 (MixBv versus BiBv) and 02 March 2022 (MixDg versus BiDg); for autumn on 21 April 2022 (MixLp versus BiLp), 14 April 2022 (MixBv versus BiBv) and 11 May 2022 (MixDg versus BiDg); for winter on 13 July 2022 (MixLp versus BiLp), 11 July 2022 (MixBv versus BiBv) and 16 August 2022 (MixDg versus BiDg); for spring 2022 in 08 November 2022 (MixLp versus BiLp), 07 November 2022 (MixBv versus BiBv) and 22 November 2022 (MixDg versus BiDg); and for summer 2 on 08 February 2023 (MixLp versus BiLp), 09 February 2023 (MixBv versus BiBv), and 22 February 2023 (MixDg versus BiDg).

5.4.3. Animal measurements

Twenty dairy cows from Massey University's Dairy 1 herd were selected according to their attributes, aiming for a homogeneous and representative average group of New Zealand dairy farm cows. The farm has adopted once-a-day milking for the entire season since 2013. Out of the twenty dairy animals selected, fifteen were continuously utilised in the experiment, and five were in the replacement group, utilised in case one of the original animals was unavailable for one specific grazing event. The dairy cows' attributes utilised to select the experimental group of fifteen animals were breed [Holstein Friesian × Jersey (F × J), namely, kiwi-cross], breed proportion ($0.375 \leq F \leq 0.625$ and $0.375 \leq J \leq 0.625$), age (3 to 5 years) and average daily milk production (15 to 23 L/day). The dairy cows' attributes utilised to select the experimental group of five replacement animals were most of the same as the original animals, except for one cow being seven years old and the other two cows with a daily milk production of approximately 10.5 L/day. The cows were predominantly pasture-fed but were supplemented with maize silage (2 kg of dry matter per cow) in autumn and winter.

Following a 6 AM milking (spring, summer, autumn) or at 7 AM in winter (dry-off season), the fifteen cows were randomly subdivided into groups of five animals, accounting for one group per block (Figure 3). All cows had previously grazed all the tested pastures so there was no novel feed (Horadagoda et al., 2009). Even though, aiming to reinforce the experiment's robustness, before every grazing event, each group was allocated for 20 minutes into an external adaptation area containing the diverse pasture treatment of the given day. Following the adaptation period, the cows were allocated to the treatment areas containing, side-by-side, 140 m2 of a diverse pasture and 140 m2 of a binary pasture, offering both pastures simultaneously (Figure 3). The animals were then evaluated for two hours during their first post-milking grazing (Figure 3), the moment in which the grazing is generally more active (Gibb et al., 1998).

Three cameras (GoPro HERO8 Black – GoPro Inc, San Mateo, California, USA) were placed at a strategic point close to each experimental plot in such a way that each camera covered the whole evaluated area within each block, thus the five cows' behavioural activity could be posteriorly assessed, and the two different pasture treatments could be thoroughly distinguishable (Figure 3). As soon as the cows were transferred from the adaptation area to the experimental plot, the recording started and proceeded for the next two hours. Afterwards, a group of five trained people analysed the video footages captured during the five seasons of the study. Briefly, each person would follow one of the five animals within a plot and detect which type of activity the animal was performing every 30 seconds timeframe - grazing (actively grazing or selecting forage with its head down), ruminating (while standing up or lying down) or idle (every other behaviour that not grazing or ruminating) (Penning et al., 1984; Sheahan et al., 2013). For the grazing activity, the location in which each animal was grazing (mix or binary) was also recorded. When an animal was grazing within 0.5 m from the plot's edges, it would be accounted as a lost data point. In this study, over 50,000 data points were collected to assess the behavioural activity of the cows. The resulting data of this evaluation is presented as the seasonal GT in each pasture type and seasonal percentage fluctuation of the three behavioural activities.

The counting of bites was performed in the field for optimal accuracy. A group of three trained observers were distributed within the experimental area and were responsible for assessing five animals, each within the same block. The measurement was recorded every 10 minutes, following the same randomly defined order of cows, one by one assessed as the time it took to perform 30 bites (Forbes and Hodgson, 1985) and in which pasture type it occurred (Figure 3). If the animal changed the pasture type in which it was grazing, or if the time between two consecutive bites in the same pasture type exceeded 15 seconds, the measurement would be reset, and the counting started again from the new particular position (Balocchi et al., 2002). The animals were assessed for two hours, within the same time frame as the recording for animal behavioural activity, resulting in a total of twelve rounds of bite counting measurement per animal. The resulting data from this evaluation is presented as seasonal BR in each pasture type.

5.4.4. Plant measurements

The HM was measured by cutting to ground level the herbage within three quadrats (0.1 m2) randomly placed in each plot. The samples were dried for at least 72 hours in a forced-air oven at 60 oC and weighed to determine their dry matter content. The HT was obtained through the average of 40 zig-zagged random points per plot taken with a sward stick the day before each grazing event (Barthram, 1986).

The nutritive parameters of the apparent harvestable herbage were assessed by randomly harvesting down to 5 cm residual height at least ten sub-samples per plot (Frame, 1993) in the early morning of the previous day to the grazing event, avoiding the warmer hours of the day. These sub-samples generated one composed sample of approximately 150 g per block. The sample was instantly sealed in a zip-lock bag, packed into a thermal bag, refrigerated at 4 oC (Atkin and Tjoelker, 2003) and sent on the same day for analysis at Hill Laboratories Ltd., Hamilton, New Zealand. Hill Laboratories participates in both national (International Accreditation New Zealand - IANZ) and international (Association of American Feed Control Officials - AAFCO) inter-laboratory comparison programmes (ILCP) for the feed test reference methods.

The total nitrogen content was estimated by near-infrared spectroscopy (NIRS), calibration based on Dumas combustion (Chang and Zhang, 2017), and corrected to a fully dry basis assuming 5% residual moisture. The CP was obtained by multiplying N by 6.25. The OMD in-vivo was determined using AFIA (Australian Fodder Industry Association) in-vitro Pepsin-Cellulase procedure and derived as in-vivo using a linear regression based on calibration samples from Lincoln University, Lincoln, New Zealand. The ME content was calculated from OMD from AFIA Method 7R (modified), using AFRC (Agriculture & Food Research Council, UK) and Lincoln University's standard formulae. The NDF was estimated by NIRS, calibration based on NDF by NFTA method. The LGN was Estimated by NIRS, calibration based on acid detergent extraction followed by treatment with 72% sulphuric acid in the Ankom Daisy Incubator. The NSC was calculated as the difference between 100% and the sum of CP, ash, crude fat, and NDF percentages.

The results by NIRS are obtained using samples dried at 62 oC and grounded to 1 mm-sized particles. Measurement results are calculated on a dry matter basis and calibrated using a multipurpose analyser (MPA II NIR - Bruker Corporation, Billerica, Massachusetts, USA). The algorithm used to construct the calibration from NIRS data (NIR spectra), firstly pre-processes the NIR spectra using a first derivative, Savitzky-Golay smoothing algorithm (Savitzky and Golay, 1964), vector normalisation and wavelet transformation. Then, the NIR spectra originating from the multipurpose analyser instrument are transformed into a single stream using calibrated transformation matrixes. Following, the transformed NIR spectra associated with each sample pass through a bootstrap re-sampling model, which uses a partial least square data reduction filter with 20 components, a local weighted Euclidean distance (500 nearest neighbours) and a support vector machine model. The bootstrap re-samplings created 20 different random calibrations from the dataset for each on-the-fly prediction. Lastly, the mean of the 20 different predictions is reported as the measurement results.

5.4.5. Statistical analysis

All statistical analyses were performed using SAS v 9.4 (SAS Institute Inc, Cary, NC, USA). The data were examined for normal distribution using the Shapiro-Wilk test, finding that all dependent variables followed a normal distribution using the UNIVARIATE procedure. The homogeneity of variance between pasture treatments was examined with Bartlett’s test using the GLM procedure.

Analyses of variance, within seasons of the year, for the dependent variables (GT, BR, HM, HT, LSR, CP, ME, OMD, NDF, LFN and NSC were performed using the GLIMMIX procedure with the following mixed model:

$$\mathrm{Y}_{ijk} = \mu + \mathrm{P}_i + \mathrm{B}_j + \mathrm{e}_{ij} \qquad \textit{equation 1}$$

Where P_i is the fixed effect of ith pasture type (MixLp, BiLp, MixBv, BiBv, MixDg, BiDg), B_k is the random effect of the jth block, and e_{ij} is the random residual assumed with mean zero and variance σ_e^2.

Least square means and standard errors for the pasture treatments were obtained and used to perform pair-wise comparisons (MixLp and BiLp; MixBv and BiBv; MixDg and BiDg) using Fisher's least significant difference (LSD post hoc test). Significant differences between the least squares means were declared at $P \leq 0.05$.

Out of the eleven dependent variables analysed via analysis of variance and the post-hoc test, nine were selected to undergo a multivariate statistical analysis, allowing the characterisation of relationships between them. Two dependent variables were excluded: OMD due to its direct relationship with ME values and LGN due to its direct relationship with NDF. The dataset of the nine selected dependent variables was standardised (Gittins, 1985). The chosen multivariate analysis was the canonical variate analysis (CVA), performed to determine the extent to which variables explained most of the differences between pasture types and seasons of the year, and at the same time, to explore the relationships between the measured variables in the pastures and grazing cows through the seasons (Garcia-Favre et al., 2023; Jobson, 1992). The CVA was performed using the CANDISC procedure with the following mixed model:

$$\mathrm{Y}_{ijk} = \mu + \mathrm{P}_i + \mathrm{S}_j + (\mathrm{P} * \mathrm{S})_{ij} + \mathrm{B}_k + \mathrm{e}_{ijk} \qquad \text{equation 2}$$

Where Pi is the fixed effect of ith pasture type (MixLp, BiLp, MixBv, BiBv, MixDg, BiDg), Sj is the fixed effect of the jth season (Summer 1, autumn, winter, spring and summer 2), 〖P*S〗_ij is the fixed effect of the interaction between the ith pasture type and the jth season, Bk is the random effect of the kth block and eijk is the random residual assumed with mean zero and variance σ_e^2.

Posteriorly, utilising the same model as for the CVA, the fixed factors of pasture type and season, their interaction and the random effect of the block were analysed with the GLM procedure to obtain the proportion of contribution (V) in the variation of each response variable within the experiment, utilising the following equation:

$$V_e = \frac{\mathrm{F}_e}{\sum F} \qquad \textit{equation 3}$$

Where V is the variation proportion explained by one of the effects (random = block; fixed = pasture, season and interaction between pasture and season), and Fe is the F ratio value of the same one of the effects (F-value).

5.5. RESULTS

5.5.1. Pair-wise comparisons of dependent variables

The percentage of time spent grazing did not differ between pairs of treatments within seasons ($P > 0.05$; Table 2). The bite rate differed between MixLp and BiLp in summer 1 and autumn ($P \leq 0.05$), with BiLp presenting the highest rates in both seasons. The bite rate also differed between MixBv and BiBv in summer 2 ($P = 0.04$), with BiBv presenting the highest rate (Table 5.2).

Table 5.1. Effect of pasture type in the bite rate and percentage of the time allocated grazing in each treatment during summer 1, autumn, winter, spring, and summer 2 as a pair-wise comparison (MixLp and BiLp; MixBv and BiBv; MixDg and BiDg) followed by respective *p*-values.

	Summer 1	Autumn	Winter	Spring	Summer 2
			Grazing time (%) *		
MixLp	39.6 (±5.5)	39.8 (±6.7)	42.0 (±3.82)	35.3 (±0.78)	39.5 (±4.8)
BiLp	54.4 (±7.9)	39.7 (±2.0)	41.3 (±2.33)	40.0 (±7.29)	53.3 (±5.9)
p-value	0.38	0.99	0.72	0.62	0.31
MixBv	49.6 (±7.8)	51.1 (±2.2)	36.1 (±5.3)	21.0 (±5.9)	43.6 (±8.1)
BiBv	48.6 (±7.0)	46.4 (±3.7)	48.9 (±0.5)	33.8 (±0.6)	54.0 (±6.1)
p-value	0.96	0.63	0.32	0.30	0.66
MixDg	45.1 (±2.7)	45.7 (±2.7)	31.8 (±6.7)	26.5 (±4.1)	45.6 (±3.8)
BiDg	49.5 (±1.2)	52.1 (±4.2)	27.9 (±4.7)	34.0 (±3.0)	52.8 (±3.8)
p-value	0.26	0.45	0.51	0.19	0.44
			Bite rate (*bites min^{-1}*)		
MixLp	57.38^{b} (±0.41)	57.90^{b} (±1.30)	56.20 (±2.92)	47.56 (±0.81)	49.04 (±3.44)
BiLp	65.45^{a} (±1.89)	63.35^{a} (±1.38)	56.20 (±4.25)	56.76 (±3.37)	54.35 (±2.68)
p-value	0.04†	0.03†	0.97	0.08	0.17
MixBv	58.20 (±3.32)	58.75 (±2.46)	55.18 (±1.10)	52.51 (±1.12)	52.72^{b} (±4.54)
BiBv	60.78 (±2.03)	60.66 (±3.26)	56.59 (±0.19)	51.39 (±4.54)	59.92^{a} (±3.32)
p-value	0.43	0.38	0.35	0.78	0.04†
MixDg	50.61 (±3.38)	61.98 (±2.35)	63.10 (±2.36)	51.27 (±1.87)	57.80 (±2.12)
BiDg	55.75 (±4.63)	64.38 (±2.68)	62.27 (±2.35)	54.38 (±1.50)	60.42 (±2.04)
p-value	0.08	0.45	0.37	0.15	0.31

Following each least square mean value is the (±) standard error of the mean. P-values refer to the pair-wise comparison between Mix and Bi pasture types under the same LS criteria within the same seasons. *The grazing time does not add to 100% due to time spent idling or ruminating. † Significant P-values.

Overall, the HM did not differ between pairs of treatments within seasons ($P > 0.05$), with the exception of MixBv and BiBv in autumn ($P = 0.02$), when MixBv accumulated 0.6 t DM/ha more than BiBv (Table 5.3). The HT significantly differed between MixLp and BiLp in summer 1, autumn and summer 2 ($P \leq 0.02$), between MixBv and BiBv in summer 1, autumn and summer 2 ($P \leq 0.02$), and between MixDg and BiDg in summer 1 and summer 2 ($P \leq 0.01$), with Mix treatments consistently presenting greater heights when compared to their respective Bi treatments (Table 5.3). In winter and spring, the HT did not differ between any treatment pairs ($P > 0.05$). Overall, the LSR did not differ between pairs of treatments within seasons (P

> 0.05), with the exception of MixDg and BiDg in spring (P = 0.03), with MixDg presenting a ratio 47% higher than BiDg (Table 5.3).

Table 5.2. Effect of pasture type in the pre-grazing herbage mass, pre-grazing undisturbed sward height and lamina:stem ratio in each treatment during summer 1, autumn, winter, spring, and summer 2 as a pair-wise comparison (MixLp and BiLp; MixBv and BiBv; MixDg and BiDg) followed by respective *p*-values.

	Summer 1	Autumn	Winter	Spring	Summer 2
			Pre-grazing herbage mass (*ton DM* ha^{-1})		
MixLp	3.50 (±0.14)	3.65 (±0.19)	2.30 (±0.12)	3.92 (±0.07)	4.18 (±0.41)
BiLp	3.14 (±0.06)	3.26 (±0.26)	1.98 (±0.31)	3.44 (±0.47)	3.73 (±0.08)
p-value	0.13	0.45	0.45	0.42	0.44
MixBv	4.40 (±0.35)	2.94^{a} (±0.14)	2.24 (±0.13)	3.52 (±0.22)	3.61 (±0.19)
BiBv	3.56 (±0.06)	2.34^{b} (±0.11)	2.26 (±0.24)	3.30 (±0.35)	3.02 (±0.07)
p-value	0.18	0.02†	0.95	0.62	0.15
MixDg	5.37 (±0.20)	3.46 (±0.46)	2.21 (±0.27)	3.70 (±0.18)	3.38 (±0.20)
BiDg	4.90 (±0.70)	2.34 (±0.22)	2.32 (±0.05)	3.32 (±0.32)	3.02 (±0.58)
p-value	0.55	0.08	0.76	0.21	0.54
			Pre-grazing undisturbed sward height (*cm*)		
MixLp	21.5^{a} (±0.3)	23.1^{a} (±1.5)	19.4 (±0.7)	31.6 (±2.3)	34.5^{a} (±1.3)
BiLp	15.7^{b} (±1.0)	17.6^{b} (±1.8)	19.5 (±2.3)	30.0 (±1.1)	22.6^{b} (±1.2)
p-value	0.02†	0.02†	0.93	0.35	0.01†
MixBv	19.6^{a} (±0.5)	25.6^{a} (±0.4)	21.6 (±2.3)	34.0 (±1.3)	36.1^{a} (±1.6)
BiBv	13.0^{b} (±0.7)	20.1^{b} (±0.8)	21.1 (±0.5)	29.1 (±0.9)	21.9^{b} (±0.6)
p-value	0.01†	0.01†	0.86	0.10	0.02†
MixDg	31.7^{a} (±0.7)	17.2 (±0.9)	13.2 (±1.1)	36.9 (±2.5)	33.7^{a} (±1.3)
BiDg	19.6^{b} (±0.7)	16.0 (±0.4)	14.2 (±0.9)	33.3 (±1.2)	22.5^{b} (±1.4)
p-value	0.01†	0.16	0.28	0.27	0.002†
			Lamina:stem ratio		
MixLp	2.46 (±0.46)	1.87 (±0.13)	3.72 (±0.17)	1.31 (±0.04)	2.10 (±0.30)
BiLp	3.38 (±0.32)	2.02 (±0.11)	2.49 (±0.41)	1.31 (±0.19)	3.10 (±0.16)
p-value	0.30	0.55	0.16	0.96	0.09
MixBv	2.15 (±0.33)	3.07 (±0.09)	4.47 (±0.35)	1.36 (±0.08)	2.96 (±0.47)
BiBv	2.04 (±0.29)	3.49 (±0.39)	2.73 (±0.21)	1.37 (±0.09)	3.54 (±0.77)
p-value	0.83	0.38	0.08	0.97	0.57
MixDg	1.97 (±0.15)	2.30 (±0.31)	2.59 (±0.12)	0.57^{b} (±0.08)	1.76 (±0.10)
BiDg	1.54 (±0.26)	1.98 (±0.12)	2.43 (±0.01)	0.84^{a} (±0.04)	2.58 (±0.22)
p-value	0.11	0.21	0.29	0.03†	0.06

Letters that differ between the pair of treatments indicate significantly different values; following each least square mean value is the (±) standard error of the mean. † Significant *p*-values.

The The CP content differed between MixLp and BiLp in summer 2 (P = 0.04), with BiLp presenting 22 g/kg DM more CP than MixLp (Table 4). The CP content also differed between MixDg and BiDg in autumn and winter ($P \leq 0.01$), with BiDg presenting the highest content in autumn and MixDg the highest content in winter. The ME content differed between MixLp and BiLp in summer 1 ($P = 0.03$), when MixLp presented 0.3 MJ/kg DM more than BiLp (Table 5.4). Also, the ME content differed between MixBv and BiBv in winter ($P = 0.04$), when BiBv presented 0.5 MJ/kg DM more than MixBv. Lastly, the ME content differed between MixDg and BiDg in summer 2 ($P = 0.04$), when BiDg presented 0.8 MJ/kg DM more than MixDg. The OMD percentage differed between MixLp and BiLp in spring ($P = 0.01$), with BiLp presenting the highest percentage. Also, the OMD percentage differed between MixDg and BiDg in summer 1 ($P = 0.04$), with BiLp presenting 6.1% more OMD than MixDg (Table 5.4).

Table 5.3. Effect of pasture type in the crude protein, metabolisable energy and organic matter digestibility content in each treatment during summer 1, autumn, winter, spring, and summer 2 as a pair-wise comparison (MixLp and BiLp; MixBv and BiBv; MixDg and BiDg) followed by respective *p*-values.

	Summer 1	Autumn	Winter	Spring	Summer 2
			Crude protein (%)		
MixLp	19.7 (±0.72)	24.5 (±0.23)	26.4 (±1.01)	17.9 (±0.85)	17.0^{b} (±0.05)
BiLp	18.7 (±0.20)	20.6 (±3.07)	25.9 (±1.85)	15.3 (±0.30)	19.2^{a} (±0.20)
p-value	0.20	0.64	0.78	0.10	0.04†
MixBv	18.4 (±0.29)	22.8 (±0.42)	28.6 (±0.81)	17.9 (±0.83)	18.9 (±0.09)
BiBv	16.9 (±0.52)	21.1 (±1.14)	25.4 (±1.63)	15.1 (±0.29)	19.7 (±0.28)
p-value	0.19	0.36	0.13	0.09	0.17
MixDg	19.1 (±0.45)	21.1^{b} (±0.87)	24.0^{a} (±1.19)	19.1 (±0.55)	21.4 (±0.84)
BiDg	17.5 (±0.08)	23.0^{a} (±0.86)	18.7^{b} (±0.92)	18.2 (±1.36)	23.3 (±1.06)
p-value	0.07	0.002†	0.01†	0.43	0.42
			Metabolisable energy (*MJ kg DM*$^{-1}$)		
MixLp	10.3 (±0.08)	10.8 (±0.13)	11.3 (±0.20)	10.9 (±0.06)	9.3 (±0.13)
BiLp	10.0 (±0.08)	11.2 (±0.31)	11.6 (±0.13)	11.1 (±0.06)	9.8 (±0.22)
p-value	0.03†	0.49	0.42	0.08	0.21
MixBv	10.1 (±0.24)	11.1 (±0.09)	11.5^{b} (±0.09)	11.0 (±0.12)	9.7 (±0.10)
BiBv	10.1 (±0.26)	11.7 (±0.20)	12.0^{a} (±0.03)	11.2 (±0.15)	9.9 (±0.08)
p-value	0.96	0.35	0.04†	0.55	0.26
MixDg	10.3 (±0.09)	10.2 (±0.39)	12.5 (±0.10)	11.2 (±0.22)	9.9^{b} (±0.18)
BiDg	9.9 (±0.15)	11.2 (±0.19)	12.8 (±0.03)	11.8 (±0.12)	10.7^{a} (±0.01)
p-value	0.18	0.12	0.13	0.07	0.04†
			Organic matter digestibility (%)		
MixLp	71.5^{a} (±0.72)	76.9 (±1.19)	80.9 (±1.22)	75.7^{b} (±0.44)	66.0 (±1.10)
BiLp	70.2^{b} (±0.44)	78.9 (±2.74)	83.4 (±0.30)	78.5^{a} (±0.28)	70.0 (±1.23)
p-value	0.05	0.65	0.21	0.01†	0.16
MixBv	70.0 (±1.74)	77.4 (±0.72)	82.2^{b} (±0.34)	77.2 (±0.50)	67.8 (±0.72)
BiBv	70.5 (±1.23)	81.2 (±1.11)	84.9^{a} (±0.38)	79.4 (±0.79)	70.3 (±0.77)
p-value	0.83	0.18	0.06	0.22	0.05
MixDg	73.7 (±1.21)	73.2 (±3.15)	86.6 (±0.88)	78.1 (±1.86)	70.0^{b} (±1.38)
BiDg	72.0 (±0.86)	79.5 (±0.63)	88.7 (±0.47)	82.5 (±0.79)	76.1^{a} (±0.33)
p-value	0.30	0.20	0.19	0.09	0.04†

Following each least square mean value is the (±) standard error of the mean. P-values refer to the pair-wise comparison between Mix and Bi pasture types under the same LS criteria within the same seasons. † Significant P-values.

The NDF content differed between MixLp and BiLp and MixBv and BiBv in summer 2 ($P \leq 0.05$) and between MixDg and BiDg in autumn ($P = 0.01$), with all Mix treatments presenting higher values than Bi treatments (Table 5.5). The LGN content did not differ between pairs of treatments within seasons ($P > 0.05$; Table 5.5). The NSC content differed between MixLp and BiLp in autumn and spring ($P \leq 0.04$), between MixBv and BiBv also in autumn and spring ($P \leq 0.03$), and between MixDg and BiDg in autumn, winter and spring ($P \leq 0.02$; Table 5.5). In all events where differences were found, the Mix treatments presented greater NSC content than the Bi treatments.

Table 5.4. Effect of pasture type on the neutral detergent fibre, lignin and non-structural carbohydrates content in each treatment during summer 1, autumn, winter, spring, and summer 2 as a pair-wise comparison (MixLp and BiLp; MixBv and BiBv; MixDg and BiDg) followed by respective P-values.

	Summer 1	Autumn	Winter	Spring	Summer 2
Neutral Detergent Fibre (%)					
MixLp	51.7 (±0.31)	45.9 (±1.31)	46.1 (±0.58)	50.4 (±1.18)	54.9^{a} (±0.68)
BiLp	52.0 (±0.70)	43.5 (±2.04)	44.9 (±1.27)	48.8 (±0.58)	48.7^{b} (±0.80)
p-value	0.68	0.55	0.55	0.83	0.05†
MixBv	47.1 (±1.67)	45.2 (±0.90)	45.8^{a} (±0.78)	48.2 (±0.03)	54.7^{a} (±0.92)
BiBv	47.7 (±0.69)	42.2 (±0.83)	42.0^{b} (±1.33)	47.7 (±1.59)	48.8^{b} (±0.49)
p-value	0.80	0.20	0.04	0.78	0.01†
MixDg	48.0 (±1.04)	49.0^{a} (±0.50)	39.8 (±0.33)	46.8 (±0.90)	53.3 (±1.69)
BiDg	48.1 (±0.92)	43.9^{b} (±0.38)	38.3 (±0.47)	43.3 (±0.58)	46.7 (±0.58)
p-value	0.96	0.01†	0.21	0.14	0.09
Lignin (%)					
MixLp	8.4 (±0.31)	4.9 (±0.06)	6.1 (±0.93)	6.0 (±0.15)	9.2 (±0.09)
BiLp	8.6 (±0.58)	6.1 (±0.45)	6.9 (±0.81)	5.2 (±0.25)	8.4 (±0.63)
p-value	0.86	0.09	0.49	0.12	0.34
MixBv	7.8 (±0.32)	4.7 (±0.50)	6.7 (±0.31)	6.5 (±0.32)	8.5 (±0.40)
BiBv	8.9 (±0.61)	5.3 (±0.49)	6.7 (±0.65)	5.2 (±0.09)	7.9 (±0.28)
p-value	0.09	0.51	0.90	0.06	0.34
MixDg	8.8 (±0.66)	9.9 (±0.87)	6.9 (±0.22)	5.4 (±0.12)	8.2 (±0.17)
BiDg	9.3 (±1.22)	6.7 (±0.63)	6.0 (±0.34)	6.0 (±0.32)	7.3 (±0.19)
p-value	0.59	0.06	0.10	0.10	0.12
Non-structural carbohydrates (%)					
MixLp	15.1 (±0.99)	13.1^{b} (±0.88)	10.8 (±0.97)	17.8^{b} (±1.15)	13.3 (±0.32)
BiLp	14.7 (±1.01)	20.8^{a} (±2.21)	11.8 (±3.53)	21.0^{a} (±1.43)	16.2 (±0.94)
p-value	0.83	0.02†	0.91	0.04†	0.11
MixBv	20.9 (±1.22)	17.0^{b} (±0.78)	18.7 (±1.94)	19.2^{b} (±0.76)	12.3 (±0.65)
BiBv	21.4 (±0.28)	22.7^{a} (±0.75)	16.3 (±3.10)	22.1^{a} (±2.26)	16.4 (±0.21)
p-value	0.64	0.02†	0.46	0.03†	0.41
MixDg	17.0 (±0.54)	13.2^{b} (±1.59)	21.7^{b} (±1.97)	19.6 (±1.01)	10.8^{b} (±0.80)
BiDg	16.8 (±2.60)	17.6^{a} (±1.18)	29.7^{a} (±1.21)	24.3 (±0.79)	14.7^{a} (±0.43)
p-value	0.92	0.02†	0.01†	0.52	0.01†

Letters that differ between the pair of treatments indicate significantly different values; following each least square mean value is the (±) standard error of the mean. † Significant *p*-values.

5.5.2. Canonical variate analysis and proportions of variation

Out of the eleven dependent variables of this study analysed via the univariate procedure, nine were selected to undergo a CVA (animal-related: GT and BR; plant morphology: HM, HT and LSR; plant nutritive parameters: CP, ME, NDF and NSC). These variables had a significant Wilk's Lambda value ($p < 0.0001$) and explained 71.1% (CAN 1 = 37.9%; CAN 2 = 33.2%; Figure 4) of the differences between treatments when the interaction of pasture treatments and seasons were analysed (MixLp, BiLp, MixBv, BiBv, MixDg and BiDg in summer 1, autumn, winter, spring and summer 2; Figure 5). Along CAN 1 and CAN 2, the error is declared at ±0.3, in such a way that CAN 1 has four contributing response variables in the positive direction (GT, BR, CP, LSR) and two in the negative direction (HM, HT), while CAN 2 has four contributing variables in the positive direction (GT, HM HT, NDF), and three in the negative direction (BR, ME, NSC).

Within the pasture treatments, groupings due to the seasonal effect were found for spring and autumn, with winter highlighted by a circle in blue (Figure 5). The pasture treatment grouping formed in spring was mostly explained by CAN 1, closely associated with HT and HM, and oppositely associated with GT, BR, CP and LSR. The pasture treatment grouping formed in autumn and winter was explained by CAN 1 and CAN 2, oppositely associated with HT and HM (CAN 1) and NDF (CAN 2), and neutrally to closely associated with GT, BR, CP and LSR (CAN 1) and with NSC and ME (CAN 2). For both summers, no grouping containing all treatments was found; however, along CAN 1, it was possible to observe that Mix treatments were always more closely associated with HT and HM (towards the left side) than their respective Bi treatment, which were more oppositely associated to HT and HM (towards the right side) (Figure 5).

The same nine variables in the CVA were used in an F-value test to obtain the proportion of contribution in a variation of each response variable within the experiment (Table 6). The season was the only fixed effect that was significant (Pr<0.0001) for all tested variables, consistently presenting the highest proportion of contribution in variation (highest V values). The season factor contributed to 69% of variation occurring in GT, 48% in BR, 72% in HM, 77% in HT, 74% in LSR, 87% in CP content, 89% in ME, 72% in NDF and 42% in NSC. Therefore, season was the main source of variation for all tested response variables in the present study. In addition to that, following the season effect of variation, pasture type presented a relatively high percentage of contribution in the variation occurring for BR (18%), NDF (22%) and NSC (38%).

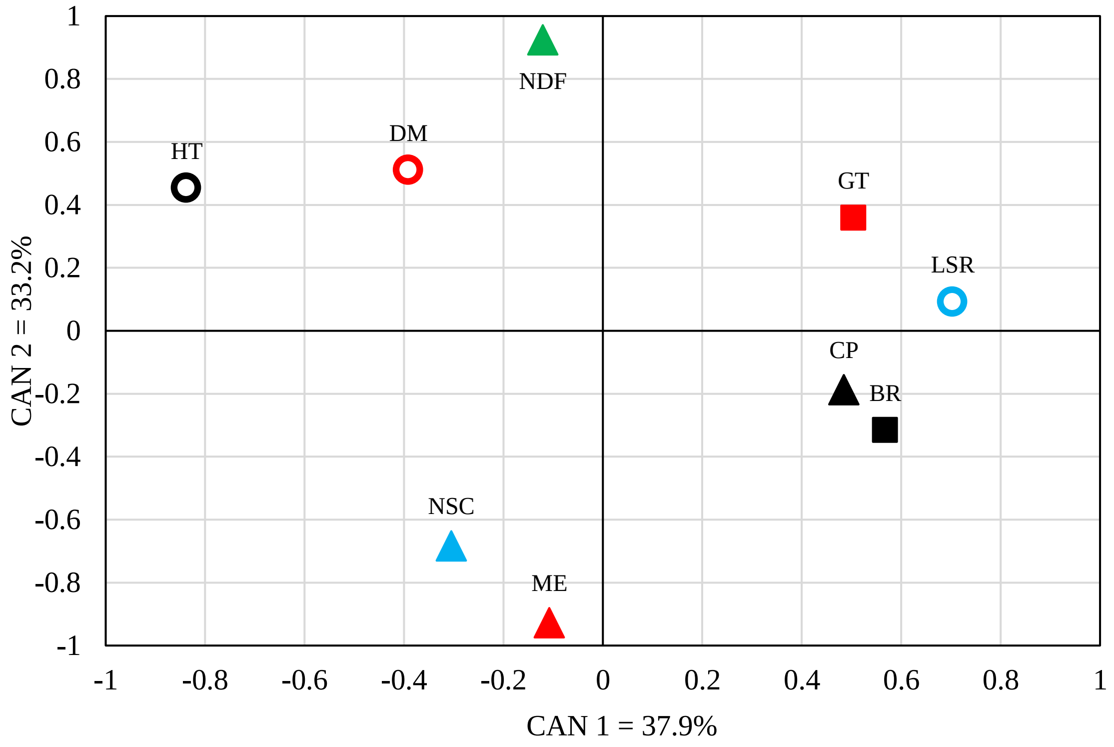

Figure 5-2. Distribution of the original response variables according to their weight on the first two canonical variates (CAN 1 and CAN 2). Animal-related variables: Grazing time (GT; black triangle), bite rate (BR); Plant morphology variables: pre-grazing herbage mass (HM), pre-grazing undisturbed sward height (HT), lamina:stem ratio (LSR); Plant nutritive parameters: crude protein (CP), metabolisable energy (ME), non-structural carbohydrates (NSC) and neutral detergent fibre (NDF).

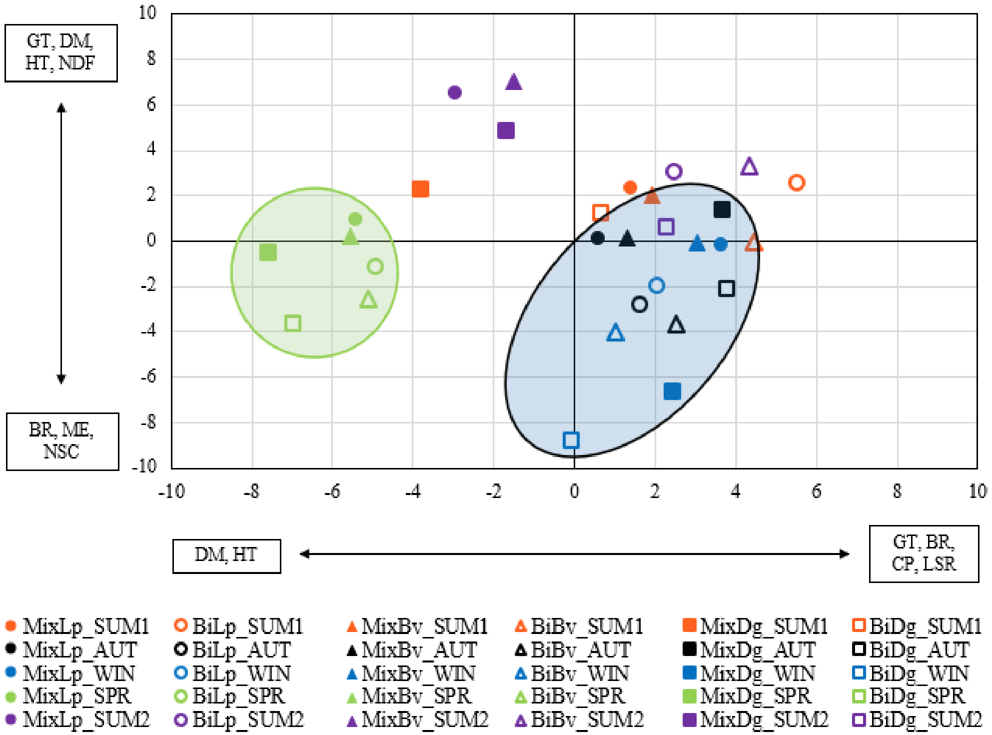

Figure 5-3. Canonical scores for the interaction of fixed effect pasture (MixLp, BiLp, MixBv, BiBv, MixDg, BiDg) and season (SUM1: summer 1, AUT: autumn, WIN: winter, SPR: spring, SUM2: summer 2) averaged by the random effect of the block. Arrows transpose the significant (>0.3 or <-0.3) response variables for CAN 1 (x-axis) and CAN 2 (y-axis). Response variables abbreviations: Grazing time (GT), bite rate (BR), pre-grazing herbage mass (HM), pre-grazing undisturbed sward height (HT), lamina:stem ratio (LSR), crude protein (CP), metabolisable energy (ME), non-structural carbohydrates (NSC) and neutral detergent fibre (NDF).

Table 5.5. F ratio values (F Value), statistical significance (Pr > F), and variation proportion (V) explained by the effects (random = block; fixed = pasture type, season and interaction pasture type x season) utilised in the model for canonical multivariate analysis per each response variable (grazing time, bite rate, pre-grazing herbage mass, pre-grazing undisturbed sward height, lamina:stem ratio, crude protein, metabolisable energy, neutral detergent fibre and non-structural carbohydrates).

	Grazing time			Bite rate			Pre-grazing herbage mass		
	F Value	Pr > F	V	F Value	Pr > F	V	F Value	Pr > F	V
Block	1.61	0.2103	9.44%	5.42	0.0069	25.49%	8.68	0.0005	13.27%
Pasture type	2.22	0.0671	13.02%	3.9	0.0041	18.34%	5.52	0.0003	8.44%
Season	11.82	<.0001	69.33%	10.11	<.0001	47.55%	47.34	<.0001	72.40%
Pasture*Season	1.4	0.1713	8.21%	1.83	0.038	8.61%	3.85	<.0001	5.89%

	Pre-grazing undisturbed sward height			Lamina:stem ratio			Crude protein		
	F Value	Pr > F	V	F Value	Pr > F	V	F Value	Pr > F	V
Block	2.61	0.0818	1.32%	0.62	0.543	1.14%	0.7	0.502	1.08%
Pasture type	30.32	<.0001	15.31%	9.51	<.0001	17.46%	2.45	0.0444	3.80%
Season	152.85	<.0001	77.20%	40.26	<.0001	73.93%	56.39	<.0001	87.40%
Pasture*Season	12.22	<.0001	6.17%	4.07	<.0001	7.47%	4.98	<.0001	7.72%

	Metabolisable energy			Neutral detergent fibre			Non-structural carbohydrates		
	F Value	Pr > F	V	F Value	Pr > F	V	F Value	Pr > F	V
Block	1.15	0.3253	0.65%	0.06	0.9423	0.07%	0.45	0.6399	1.21%
Pasture type	13.14	<.0001	7.37%	20.19	<.0001	22.18%	14	<.0001	37.61%
Season	158.19	<.0001	88.77%	65.9	<.0001	72.40%	15.52	<.0001	41.70%
Pasture*Season	5.73	<.0001	3.22%	4.87	<.0001	5.35%	7.25	<.0001	19.48%

5.5.3. Animal behaviour activity

Because the fixed effect ‘season’ was the greatest contributor to the variation found in the response variables, a graph with the seasonal fluctuations in animal behaviour (grazing, rumination or idleness) was plotted to depict changes across seasons according to each of the three different defoliation criteria (*L. perenne* LS, *B. valdivianus* LS and *D. glomerata* LS) utilising an average between both binary and diverse pastures values (Figure 6). Animals grazing the pasture treatments defoliated according to the *L. perenne* LS interval presented little fluctuation in GT time across seasons, spending over 70% of their time grazing in autumn, winter and spring and over 90% in both summers. Still, within these treatments, the time spent by the animal in rumination and idleness was often around 10% and did not present major fluctuations within seasons. On the other hand, animals grazing pastures defoliated according to the *B. valdivianus* LS and *D. glomerata* LS presented much more seasonal fluctuation in their behavioural activity. For *B. valdivianus* LS, the most striking feature in animal behaviour changes is a major shift starting in winter and peaking in spring, in which, at the expense of grazing time, animals begin to spend more time idling and secondarily ruminating. Similarly, but more markedly, the shift in the behavioural activity of the animals grazing pastures, according to *D. glomerata* LS, also began in winter and remained during spring, both seasons in which animals spent around 60% of their time grazing, and from 10 to 30% of their time idling or ruminating.

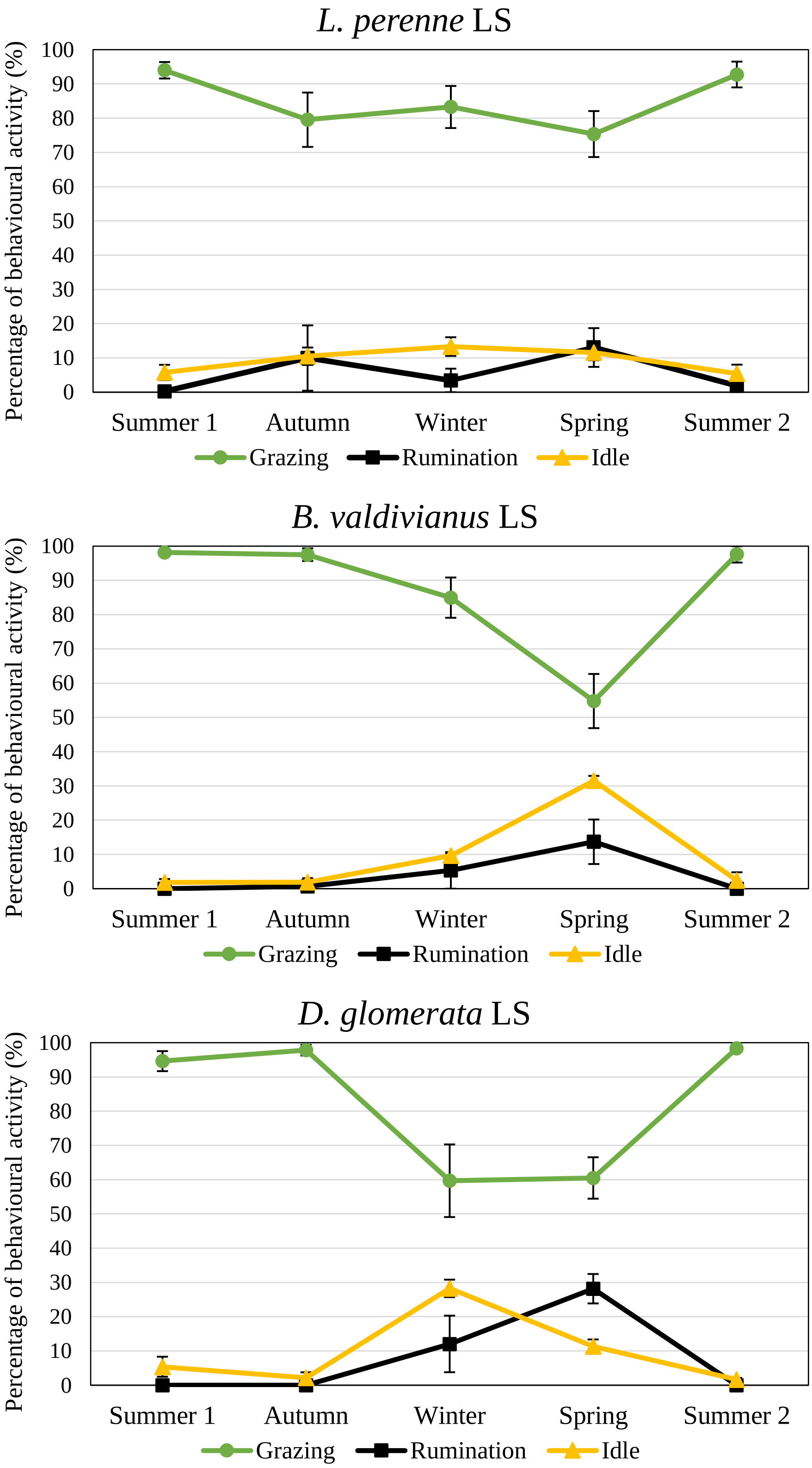

Figure 5-4. Seasonal percentage fluctuation (summer 1, autumn, winter, spring and summer 2) of the behavioural activity (grazing, ruminating or idle) exerted by dairy animals during their first two hours in a pasture sward after the morning milking period. Graphs were done using an average between binary and diverse pasture behaviour values according to each LS interval criterion. The vertical bars indicate the (±) standard error of the mean per activity within each season.

5.6. DISCUSSION

The univariate analysis showed recurrent significant differences in HT and NSC within the pair-wise comparison of binary and diverse pastures, which could have driven cows to exhibit preference. However, no significant differences were found in the percentage of time that cows spent grazing binary or diverse pastures under the same LS criteria for defoliation within the same season. Further multivariate and univariate analysis highlighted that season was the greatest contributor to the proportion of variation found in all response variables. Within the same season, LS defoliation criteria were responsible for modulating cows' grazing behaviour (proportional time spent grazing, ruminating, or idling).

5.6.1. The role of pasture productivity and morphology on the dietary preference of dairy cows

The utilisation of plant-focused criteria (LS) to define the most appropriate time for defoliation is an effective alternative to enhance pasture productivity and persistence (Donaghy et al., 2021) (Figure 2). However, different grass species will present different intervals for optimal defoliation (Fulkerson and Donaghy, 2001; Ordóñez et al., 2021; Turner et al., 2006). Consequently, in a rotational grazing system containing multispecies mixes, not always all species will be coincidently and simultaneously 'prepared' for defoliation. For the case of this study, this temporal mismatching between species is noteworthy in such a way that pasture treatments following *L. perenne* LS were under a more frequent defoliation basis, followed by *B. valdivianus* LS and, ultimately, *D. glomerata* LS (less frequent defoliation events).

Grazing frequencies directly affect plant growth accumulation, morphological and structural characteristics, and nutritive parameters (Gordon, 2000; Hodgson, 1990). Thus, clarifying the consequences of grazing frequencies on animal dietary preference is essential to understanding the role and potential suitability of the LS defoliation interval as a criterion for diverse pastures.

In the present study, the herbage mass offered did not differ between the pairs of treatments (Bi versus Mix), with only one exception out of fifteen grazing events (MixBv versus BiBv in autumn; Table 2). Several studies have found that the herbage on offer strongly influences dietary preference (Demment et al., 1993; Illius et al., 1992; Kenney and Black, 1986), process related to potential dry matter intake and plant density within an area and the consequent energetic efficiency for the animal once it invests its time grazing the given area (Kacelnik and Bernstein, 1988). Since no significant differences in HM were found in the pair-wise comparisons, it is possible to affirm that, for the present study, herbage allowance was not a factor of influence. Such results enhanced the robustness of this study because animals had the chance to choose and show preference, if any, without external interferences caused by herbage on offer and potential intake.

Unlike HM, the HT presented several significant differences between Bi and Mix treatments over time (Table 2). In the literature, the influence of sward height on animal dietary preference is contradictory. Forbes and Hodgson (1985) found significant differences in the time spent grazing (625 ± 15 minutes/day against 580 ± 16 minutes/day) two different *L. perenne* treatments with statistically similar sward heights (~ 18.3 cm). Anecdotally, Smit et al. (2006) found that there were no significant relationships between cattle preference and pre-grazing surface sward heights when testing six cultivars of *L. perenne* in three different experimental arrangements (experiment 1: ~ 18.6 cm; experiment 2: ~ 14.0 cm; experiment 3: ~ 25.4 cm). Other studies, however, suggest that cattle tend to prefer higher swards (Bailey, 1995; Gibb et al., 1997; Griffiths et al., 2003a, b). Griffiths et al. (1997) carried out a study in which *L. perenne* pasture treatments presented progressively increasing targets for pre-grazing sward height (8.9 to 19.6 cm). They found that cows regularly sampled from all but the shortest swards (8.9 cm). These contradictions may be explained by potential minimum thresholds limiting animal preference so that, at pre-grazing conditions, only the extremely short swards [i.e. 8.9 cm in Griffiths et al. (1997)] represent a non-preferred area for cattle. In the present study, although differences in HT between binary and diverse pastures were found, they did not affect the time that cows spent grazing (Tables 1 and 2), likely because all HT were relatively high and above a potential minimum threshold.

5.6.2. The role of pasture nutritive value on the dietary preference of dairy cows

Seasonal Beyond the structural characteristics of the sward, plant nutritive parameters can also affect animal preference for species (Horadagoda et al., 2009) or cultivars (Smit et al., 2006). Plant nutritive parameters are highly affected by biotic factors, such as the choice and proportion of each species in a mix (Rutter et al., 2004), the defoliation intensity and the frequency of grazing (Waghorn and Clark, 2004). Because nutritive parameters can drive animals' preference and selection (Provenza et al., 2007; Waghorn and Clark, 2004), the statistical approach of this study adopted two analyses. Firstly, the univariate analysis between a pair of treatments focused on the comparison between treatments under the same LS defoliation criterion. Secondly, the multivariate analyses (see next section) embraced broader differences among treatments occurring due to seasonal fluctuations and different LS defoliation criteria.

Among nutritive parameters, WSC is a factor of great influence on grazing preference and selectivity. In a cafeteria study carried out over eight seasons, Horadagoda et al. (2009) found that cows spent more time grazing *B. willdenowii*, *Pennisetum clandestinum* Chiov., *T. repens*, *M. sativa*, and less time grazing *F. arundinacea*, *Paspalum dilatatum* Poir., *C. intybus* and *P. lanceolata*. Horadagoda et al. (2009) concluded that animal preference can be reasonably well predicted in pastures comprised of plants with higher WSC content and lower nitrate-nitrogen content. Similarly, Smit et al. (2006), in a study with six cultivars of *L. perenne*, also found that cows preferred the pastures containing higher concentrations of WSC.

In the present study, significant differences in NSC (for temperate pastures, analogous to WSC) content were found between Mix and Bi treatments (Table 5). However, they did not appear to greatly impact cows' preferences, as seen in Table 6, where the pasture type is non-significant (Pr = 0.0671) for the proportion of variation (V = 13.02%) in grazing time. This may be associated with the fact that WSC values were still relatively high, with an average of 16.11% for Mix and 21.22% for Bi in grazing events where the treatments differed, as opposed to the rejected species (*F. arundinacea*, *Paspalum dilatatum* Poir., *C. intybus* and *P. lanceolata*) in Horadagoda et al. (2009) presenting a WSC mostly below 10%, and to the rejected cultivars (*L. perenne* cv. Agri, cv. Barezane, cv. Herbie, cv. Respect) in Smit et al. (2006) presenting WSC ranging from 10.46% to 8.17%.

The fibre content in grasses, although necessary to maintain rumen function (AFRC, 1993; SCA, 1990), has often been associated with a negative impact on dairy cows' selection and preference (Horadagoda et al., 2009; Jacobs et al., 1999; Smit et al., 2006). In particular, the lignin fraction of fibre content in grasses has a direct impact on tensile strength (Baumont et al., 2000), which makes difficult feed prehension and utilisation (Inoué et al., 1994). In the present study, no differences between LGN content were found between Mix and Bi treatments within the same season, which corroborates the cows not exhibiting preference.

5.6.3. Seasonal fluctuations and defoliation criteria influencing dairy cows' behavioural activity

Within seasons, the pair-wise comparisons (Bi versus Mix) did not show significant differences in the time cows spent grazing. In this study, cows' preference was mostly driven by seasonal effects rather than pasture type. Changes in the patterns of grazing behaviour (GT and BR) according to the seasons were found in the CVA (Figures 4 and 5) and F-value analysis (Table 5). For all the response variables shown in Table 5, 'season' presents the greatest percentage contributing to variation.

Seasonal fluctuations in the nutritive value occur due to changes in plant physiology, phenology and growth stages (O'Reagain and Schwartz, 1995). As an active response to these seasonal fluctuations in pasture quality, cows modulate their grazing behaviour. In a three-year farm-level study conducted by Iqbal et al. (2022) with cows continuously monitored by automated collar devices, the season was the second most influential source of variance (after individual cow choice) and explained 5 to 12 % of the variation in time spent grazing. In a study by Cullen et al. (2017), the nutritive value of vegetative and reproductive tillers of four grass pure stands (*L. perenne*, *D. glomerata*, *B. wildenowii* and *F. arundinacea*) were assessed in winter (only vegetative) and spring. For all four species, the nutritional drop in quality is explained by the growth of reproductive tillers, when, in spring, CP and ME significantly decreased while fibre content significantly increased. These studies corroborate the CVA 'green' grouping in Figure 5, when the morphological and nutritional characteristics of the plants 'per se' became less preferable to the animals. That grouping depicts a close association of spring with HM and HT and an opposite association with GT, BR, CP and LSR.

Fluctuations in dietary behaviour can also be attributed to nutritive quality and morphological structure as affected by grazing management criteria. In the present study, grazing based on the *D. glomerata* LS resulted in less frequent grazing events [see (Oliveira et al., 2023)]. In spring, Mix and Bi pastures under *D. glomerata* LS presented extremely low LSR, respectively 0.57 and 0.84 (Table 2). These are similar to the results found in a study by Griffiths et al. (1997), showing that animals prefer swards with a high LSR (2.53 ± 0.0004), deliberately avoiding the swards with a low LSR (0.74 ± 0.0004). In that respect, it is possible to conclude that changes in behavioural activities within spring were partially driven by changes in plant morphological characteristics caused by different LS criteria grazing intervals.

Furthermore, these changes in behavioural activities as affected by grazing management criteria can be well exemplified by two results of this present study. Firstly, by the marked shift in the behavioural activity when grazing pastures defoliated according to *D. glomerata* LS, in which cows spent 60% of their time grazing and 40% idling or ruminating during winter and spring (Figure 6). Secondly, by the ‘green’ grouping in spring (Figures 4 and 5), where Mix and Bi pastures defoliated according to *D. glomerata* LS are located the furthest from GT and BR.

According to the results presented in this study and corroborating previous results in the same experimental area by Oliveira et al. (2023), grazing management is critical to maintaining high-quality nutritive parameters in pastures as a continuous attempt to buffer inherent and detrimental seasonal effects. In addition, this study shows that the positive effects of defoliation management based on LS may also extend itself to positive outcomes in animal preference, interpreted as the percentage of time dairy cows spend grazing rather than ruminating or idling.

Finally, the initial study hypothesis is rejected since, within the same seasons, dairy cows did not exhibit preference - as in time spent grazing - when unrestrictedly offered binary (*L. perenne* and *T. repens*; Bi) or diverse pastures (*L. perenne, B. valdivianus*, *D. glomerata*, and *T. repens*; Mix) that had been subjected to the same LS defoliation criteria.

5.7. CONCLUSION

Most of the differences detected by pair-wise comparisons showed that HT and WSC often differ between binary (*L. perenne* and *T. repens*) and diverse pastures (*L. perenne*, *B. valdivianus*, *D. glomerata* and *T. repens*) under the same LS interval of defoliation within the same season. However, these differences did not influence the actual time that animals spent grazing either binary or diverse pastures. Therefore, the present study provided evidence that dairy cows did not exhibit any grazing preference for pastures subjected to the same grazing management.

In this study, pastures that were less frequently defoliated (*D. glomerata* LS) had animals spending less time actively grazing during winter and spring, increasing the time spent idling and ruminating. Furthermore, seasonal effects contributed the greatest to the variation occurring in the response variables. These findings show that grazing management and seasonal effects are more relevant than plant species diversity for modulation of grazing behaviour. Diverse pastures comprising *L. perenne*, *B. valdivianus*, *D. glomerata* and *T. repens* do not affect dairy cows' grazing preference and can, therefore, be considered a reliable option for dairy farmers that wish to diversify their pasture-based systems.

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Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

6.1. OVERALL DISCUSSION

In a fundamentalistic conception, a successful perennial pasture (monoculture, binary or diverse) relies on four main attributes: persistence, productivity, nutritional quality and animal acceptance (Hodgson 1990; Langer 1990; McCall and Sheath 1993; Fahey Jr et al. 1994; Hodgson and Illius 1996; Holmes and Roche 2007). The general hypothesis of this thesis was that *L. perenne*, *B. valdivianus*, *D. glomerata*, and *T. repens* have complementary functional traits that enable these species to coexist and function as a diverse pasture and, therefore, present high herbage productivity, enhanced short-term persistence, and high-quality nutritional feed resources for pasture-based dairy systems. In this thesis, the proposed species to comprise a diverse pasture were thoroughly assessed in regard to their individual functional traits and plant community features that would provide enhanced persistence (Chapter 3), high productivity (Chapters 3 and 4), adequate nutritional quality for animal requirements (Chapter 4 and 5), and preference by dairy cows (Chapter 5). The diverse pasture comprising *L. perenne*, *B. valdivianus*, *D. glomerata* and *T. repens* proved to be functionally complementary and, thus, it is referred to as a diverse pasture composed of complementary species (DPCS).

In addition, rotational grazing management [defined by Hodgson (1979) as "the practice of imposing a regular sequence of grazing and rest from grazing upon a series of grazing areas"] dictates the unfolding outcomes of a given pasture by allowing those aforementioned four components of 'the successful perennial pasture' to be accordingly achieved. In that sense, this thesis also explored the outcomes of utilising the leaf regrowth stage interval as a grazing criterion. Results from Chapters 4 and 5 demonstrated that, for DPCS, the leaf regrowth stage interval for defoliation is a suitable grazing management practice that enables diverse pastures to achieve high productivity, adequate nutritional quality for animal requirements and acceptance by dairy cows.

6.1.1. Root morpho-physiological and functional traits: Can hydraulic lift become a 'free irrigation' for pasture-based production systems?

Roots are the principal means through which plants reach and extract water and nutrient resources from the soil (Siddique et al. 2016). The importance of these resources is relatable and attested by the investment in fertilisers and irrigation by commercial farms. Thus, the greater the understanding of the roots' role in production systems, the more it will be possible to save in costs of out-sourced resources and buffer prejudicial drought-related environmental effects (Atkinson 2000). However, research on underlying drivers of root traits, growth, development, turnover, architecture and functionality - and whether these are genetically or environmentally driven - has proven to be difficult to conduct and extremely disturbing to the soil-root-plant continuum. In addition to that, less disturbing techniques, such as computer-assisted tomography, magnetic resonance imaging (Asseng et al. 2000), root imaging with minirhizotrons (Richner et al. 2000), and isotope techniques (Bingham et al. 2000) are rather expensive due to the need for specialised devices and labour.

In grasslands, however difficult, the study of root growth (below-ground net primary production), turnover, and shoot:root ratio (analogue to C:N net allocation) is considered extensive comparatively to other biomes (Gill and Jackson 2008). In studies with *L. perenne*, for instance, it was found that the root turnover is dependent on the phytomer development (Robin et al. 2010), and its below-ground biomass presents seasonal patterns of growth, with larger and longer roots growing in-depth during spring, potentially as a preventive strategy for dry periods in summer, season in which root growth ceases (Matthew et al. 2016; Robin et al. 2021). Part of the results in Chapter 3 contributed to the body of information regarding below-ground biomass distribution in *L. perenne, B. valdivianus* and *D. glomerata*, providing insights on root growth and allocation according to different grass species functional roles. However, the link between growth, architecture and functional traits of roots, and the consequential outcomes in intensively managed grassland ecosystems are yet unclear. As suggested in an extensive and comprehensive analysis of root traits to understand the effects of plant diversity on grassland productivity by Bakker (2018), the next step would be to (i) assess the role of functional traits for complementarity on the soil resources utilisation and exploitation, (ii) how agricultural management practices, such as defoliation, alter root growth and, (iii) further elucidate the mechanisms underlying positive biodiversity effects driven by root functional traits.

Grime et al. (1988) suggested that plant evolutionary strategies could be divided into three functional trait groups: competitors, stress tolerators and ruderals. Despite most of the species utilised in perennial pastures presenting characteristics of two or three functional trait groups, one will invariably prevail in a given environmental condition. Results from Chapter 3, under extreme drought conditions, demonstrated that *L. perenne* presented functional traits of a competitor, which could mean a potential dependence upon the ability to sustain high rates of resource capture (Atkinson 2000). Hence, for competitors, it may be necessary to constantly reinvest high rates of captured resources in new thin adventitious roots, which in turn shows a dependence on being continuously exposed to unrestricted edaphic resources within the top centimetres of the soil profile. On the other hand, *B. valdivianus* and *D. glomerata* presented characteristics of stress tolerators, being dependent upon their capacity to both capture and retain scarce resources and utilise adaptive growth and functional strategies. This tends to mean that the roots of stress-tolerators will be long-lived with plasticity expressed by morpho-physiological changes (Atkinson 2000). Moreover, regarding water uptake, stress-tolerators will express their ability to exploit vast soil layers via root architecture and distribution and access water due to the hydraulic conductivities among root types and along the root length (Siddique et al. 2016). Beyond below-ground biomass distribution and functional roles, Chapter 3 also provided initial insight into the link between these two aspects (growth and function) and validated a new physiological phenomenon in C_3 grass species: the hydraulic lift. Thus, the hydraulic lift can be one of the many different functional traits that grass roots can present.

On an ecosystem scale, Bogie et al. (2018) and Singh et al. (2020) presented the concept of ‘bioirrigation’ as a potential positive outcome resulting from hydraulic lift. The bioirrigation concept is based on the deliberate inclusion of deep-rooted species into monocultures, such that the redistributed water by the added deep-rooted species benefits the neighbouring shallow-rooted plant species, enhancing their chance of survival or growth during drought periods. Globally, frequent freshwater shortages, extreme rainfall events, and desertification dictate water resource use efficiency. In that sense, a growing body of information and evidence shows hydraulic lift to be of substantial systematic benefit to agroforestry systems in arid, semi-arid and mesic ecosystems (Horton and Hart 1998; Burgess 2010).

With increasing temperatures and more frequent extreme climatic events, agricultural production in mid-latitude regions needs to readjust their production systems to cope with the drought threat (Robinson 2021; Yu et al. 2022; Zhao et al. 2023). Under these scenarios, hydraulic lift can become an innovative, sustainable, and low-cost strategy to mitigate scarce water resources by utilising a larger soil profile volume (Alagele et al. 2021). Thus, hydraulic lift brings a new perspective on how the combination of functional species (e.g., different root system architecture and root functional traits) benefits the ecosystem, whereby commensalism allows one drought-exposed species to cope with periods of water restriction that can negatively affect the ecosystem's stability.

In a pastoral ecosystem, however, the process of choosing specific combinations of deep-rooted grasses or forages that have the potential to exert the hydraulic lift and, in addition, function as a DPCS is not yet clear and is an area with a huge scope for further research. This may increase the understanding of the links between root functional traits and subsequent positive biodiversity effects. Nevertheless, it is evident that hydraulic lift as a source of bioirrigation has the potential to become a key factor in the persistence and survival of diverse pastures, or in other words, a type of 'free irrigation' for future diverse pasture-based production systems.

6.1.2. Causes of complementarity in a diverse pasture composed of *L. perenne*, *B. valdivianus*, *D. glomerata*, and *T. repens*

One of the main factors affecting the performance of diverse pasture is the targeted species and their designed combination to comprise a mixed sward, which should aim to achieve functional complementarity in regard to relevant traits that will enhance resource acquisition and resource use efficiency in a spatial and temporal scale (Gross et al. 2007; Tilman et al. 2014; Lüscher et al. 2022). One of the general objectives of this thesis was to determine whether *L. perenne*, *B. valdivianus*, *D. glomerata*, and *T. repens* grown as a diverse pasture present enhanced ecosystem functionality due to their complementary functional traits. Throughout this thesis, resource partitioning, abiotic facilitation, and biotic feedback, which are the drivers behind a functional ecosystem (Figure 6.1), were discussed and explored on different occasions.

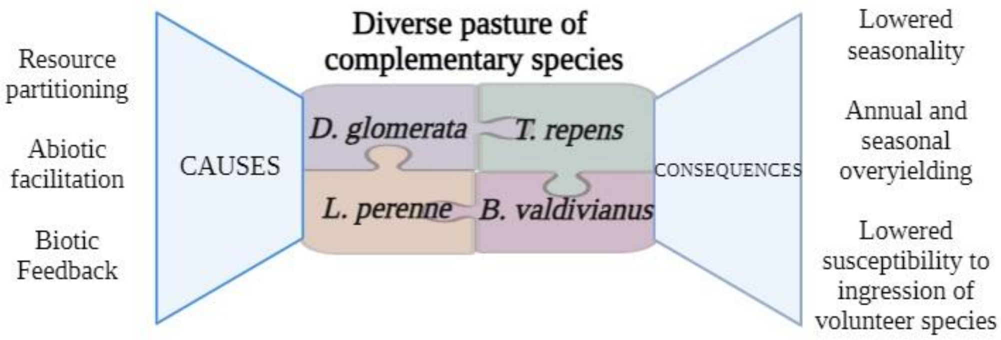

Figure 6-1. Conceptual framework of potential complementarity causes and observed complementary consequence of a diverse pasture comprising *L. perenne*, *B. valdivianus*, *D. glomerata* and *T. repens*. Adapted from: Barry et al. (2019).

Firstly, Chapter 3 discussed the hydraulic lift phenomenon in C_3 grass species, which has the potential to become a process of abiotic facilitation in field conditions and can result in enhanced functionality of these species when comprising a diverse pasture. Secondly, Chapter 4 discussed the growth asynchrony (e.g. winter- or summer-active species), which is a type of temporal resource partitioning. Thirdly, Chapter 4 also discussed the association of different root functional traits (e.g. deep- and shallow-rooted species), which is a type of spatial resource partitioning, being represented by a vertical niche differentiation and implying that species were able to acquire water and nutrients from different layers within the soil profile. Lastly, Chapter 4 also presented another type of abiotic facilitation, given by the presence of a legume component (*T. repens*) in the pastures, providing biological N supply to the grass's components. These aspects of complementarity have resulted in a pasture ecosystem with enhanced functionality, and within an agricultural perspective, were proven in the field study (Chapter 4) by three important consequences: lowered seasonality of production, annual and seasonal overyielding, and lowered susceptibility to ingression of volunteer species (Figure 6.1).

From an ecological perspective, ecosystems will present the population of some species declining while the population of other species increases, and the averaging of the fluctuations in species proportion will stabilise the collective productivity of the aggregate community and ecosystem (Tilman et al. 1998; Hector et al. 2010). These 'unmatching' plant responses, namely growth asynchrony, can be interpreted as a form of temporal niche differentiation (Loreau 2000). In pastoral science, the growth asynchrony is analogous to the season of active growth of a given species within a diverse pasture (e.g., summer- and winter-active species or warm- and cool-season species). Differences in seasonal peaks of productivity may lead to stability of production throughout the year (Isbell et al. 2009) or at least a less variable pasture-based feed supply. Growth asynchrony has been reported by other authors in different diverse pasture systems, for instance, Cranston et al. (2015) with *C. intybus*, *P. lanceolata*, *T. repens* and *T. pratense*, García-Favre et al. (2022) with *L. perenne* and *B. valdivianus*, Husse et al. (2016) with *L. perenne*, *C. intybus*, *T. repens* and *T.*

pratense, Descalzi et al. (2019) with *L. perenne*, *B. valdivianus, H. lanatus, D. glomerata* and *T. repens*.

Finally, it is important to highlight that within diverse pasture ecosystems, inter- and intra-specific plant relationships such as competition (Tilman et al. 1998; Lehman and Tilman 2000) and relationships between different trophic levels such as grazing disturbance (Grime et al. 1988; Thébault and Loreau 2005) could also affect the degree of population growth asynchrony. These interactions are strongly driven by grazing management and require further research in future experiments involving DPCS.

6.1.3. In a future scenario of climatic change, what would be the benefits of utilising diverse pastures composed of complementary species in pasture-based dairy systems?

A chapter about pasture-based systems from late 90s research (Snaydon 1987) already suggested the theoretical benefits of utilising species with different patterns of seasonal production (growth asynchrony) to achieve a more even production distribution throughout the year. Currently, in the context of future climate change scenarios, enhanced functionality by complementarity in diverse pastures has a fundamental role in maintaining year-round productivity and long-term persistence in pasture-based systems (Lüscher et al. 2022). This is of major importance, particularly for New Zealand dairy systems, which are systematically organised in a way to synchronise (i) daily, seasonal, and annual pasture feed supply and (ii) the animal feed requirements with appropriate calving and drying off dates (Holmes and Roche 2007). In typical New Zealand rainfall years, dairy farmers calving their herd in spring face two periods of pasture feed deficit (Matthews et al. 2004; Garcia and Holmes 2005; Holmes and Roche 2007). Firstly, from July to September, when dairy cows are calving, their feed requirements exponentially increase, while temperature has not yet risen enough to support higher growth rates in the conventional *L. perenne* and *T. repens* pastures. Secondly, from January to March, when dairy cows are in mid-lactation and, therefore, still in need of appropriate feed supply to maintain production and body score, while the conventional *L. perenne* and *T. repens* growth rates are already limited due to soil water restriction or drought.

These periods of pasture shortage can be better visualised with feed profile planning charts, which involve setting out patterns of expected pasture production and animal feed requirements. Figure 6.2 depicts a feed profile developed utilising pasture production data from this thesis and a simulation of animal requirements by adapting data from Matthews et al. (2004) and Garcia and Holmes (2005). The four pasture production curves presented in Figure 6.2 were calculated as the average of Year 1 and Year 2 for (a) MonoLp, (b) MonoBv and (c) MonoDg. The calculated average for DPCS contains data from Year 1 and Year 2 of MixLp and MixBv treatments, which were a posteriori found to be the treatments with the more recurrent overlapping optimal LS intervals of different species. Moreover, these are the two mixtures that presented greater nutritional parameters models (Chapter 4). The animal feed requirements curve is a simulation for a seasonal dairy farm calving in early August (early spring), stocked at 3.5 cows per hectare, producing 300-350 kg MS cow per year [adapted from Matthews et al. (2004) and Garcia and Holmes (2005)].

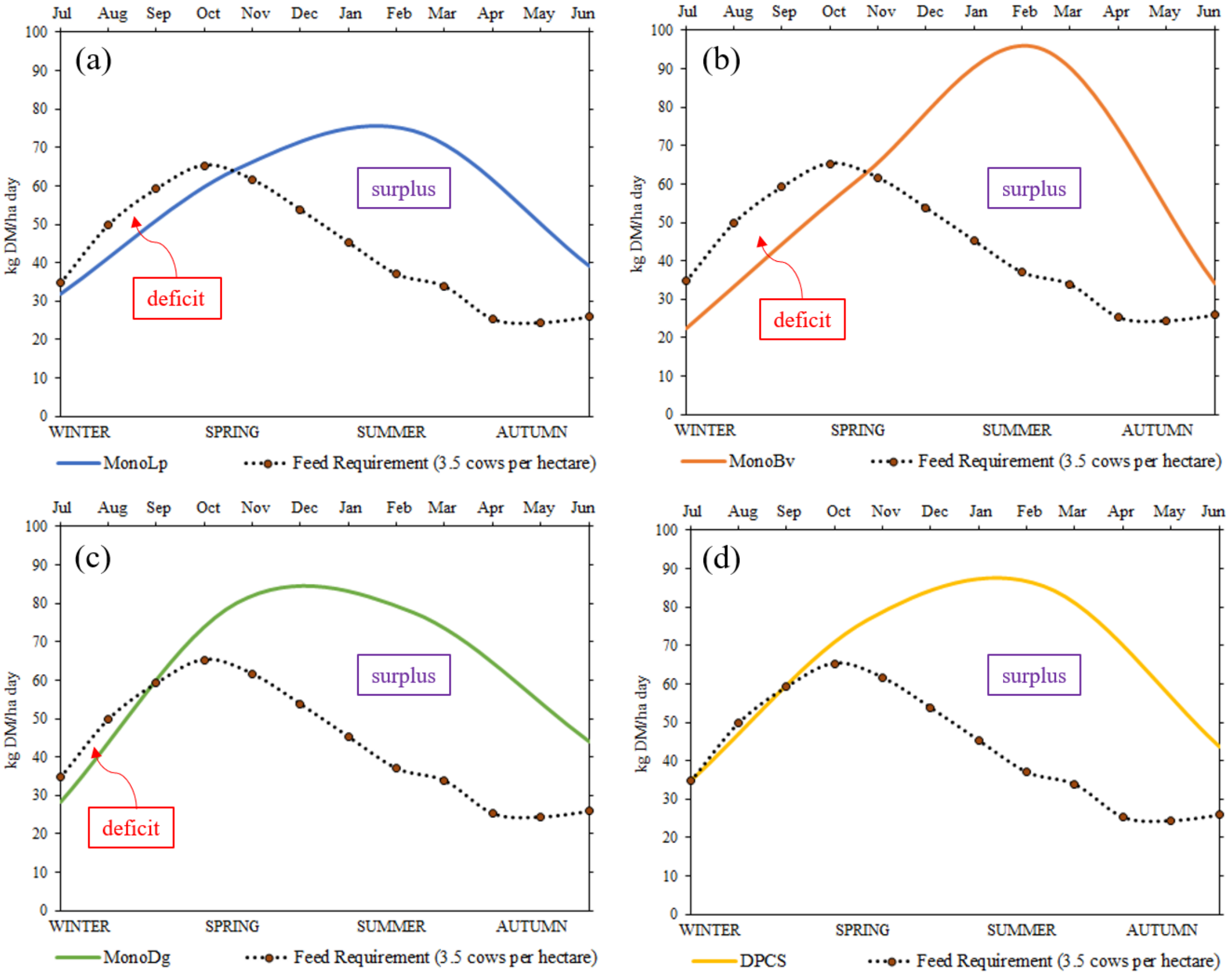

Figure 6-2. Adaptive illustration of feed profile for a seasonal dairy farm calving in early August (early spring), stocked at 3.5 cows per hectare, producing 300-350 kg MS cow per year, and indicating seasonal variations (surplus and deficit) in the balance between feed supply and feed requirements. The pasture growth daily accumulation curves are based on (a) MonoLp in blue, (b) MonoBv, (c) MonoDg and (d) diverse pasture of complementary species (DPCS) data presented in this thesis. The animal feed requirements curve is a simulation with data adapted from Matthews et al. (2004) and Garcia and Holmes (2005).

As shown in Chapters 4 and 5, over the two years that the field study was carried out, the Manawatu region was under the strong influence of La Niña, which means atypical rainfall conditions, mild winters and extreme rainfall events occurring over summer (e.g. December 2021 with monthly accumulated rainfall of 216 mm). Therefore, all four types of pastures presented a large pasture feed surplus during the summer. Under such ideal conditions for pasture growth (high water supply and temperatures), it was not possible to capture a growth advantage of DPCS during water restriction periods. However, as previously discussed in Chapter 2, Chapter 4 and Chapter 6, it would be expected that DPCS perform better under periods of water restriction.

On the other hand, Figure 6.2 also shows that DPCS had a productive advantage over all other single-grass species pastures during the early calving season, being the only pasture able to supply enough feed to maintain approximately 3.5 cows per hectare. Among single-grass pastures, MonoDg presented the lower pasture feed deficit in early calving; however, it is important to highlight the less appropriate nutritional parameters found in MonoDg, posteriorly associated with its leaf regrowth stage (LS) interval based on *D. glomerata* plants, resulting in a slower rotation over the experimental years. In the case of single-grass pastures, a deficit in feed supply in the early stages of calving means that either (i) the animal's daily intake will be below optimal, and the farmer may see a drop in milk production, or (ii) it will be necessary for the farmer to import out-sourced feed to complement the animal dietary requirement in a way to main production and body score condition. In both scenarios, the farmer will be under a financial constraint, by either needing to spend with supplements or producing less milk. By theoretical and numerical means, it is evident that the growth asynchrony and consequent overyielding experienced in DPCS are a great advantage for pasture-based production systems.

As previously discussed, New Zealand dairy farms are predominantly running under a grass-fed production system, in which peak of lactation and peak of forage production match in a way to optimise forage utilisation and milk production (Holmes and Roche 2007). Although similar in systems, farms spread across the country are also running under a large array of soil profiles that present different physical, chemical and biological properties. In that sense, it is important to highlight some reservations regarding the utilisation of DPCS in some soils found in New Zealand. The main emphasis goes towards what is known by the farmers as the “heavy soils”. These soils (e.g., Pallic soils, covering 12% of New Zealand) present a high potential for slaking or dispersion and slow permeability (opposite of ‘free-drainage’) (Manaaki Whenua, n.a), which becomes a problematic factor for the persistence of *B. valdivianus*. In this scenario, the substitution of *B. valdivianus* for another species that has a better performance when in soils that are saturated for most of the year (e.g., *Holcus lanatus*) could be advantageous (López et al. 1997; López et al. 2009).

6.1.4. Growth asynchrony and development synchrony, the counterbalanced arrangement: What are the implications of the leaf regrowth stage as a criterion for defoliating diverse pastures composed of complementary species?

Another general objective of this thesis was to determine whether there is a temporal overlapping of the different species' optimal intervals for defoliation and, if so, whether productivity, seasonality and persistence were affected. The concept behind temporal overlapping is based on utilising the LS interval, which in turn has its theoretical basis in the plant phyllochron. Wilhelm and McMaster (1995) debate the overall concept of phyllochron in grass species and detail the recurrent misconception that plant growth and plant development represent the same aspect of biomass increase and, therefore, are analogous. Plant growth and development are distinct but related processes (Wilhelm and McMaster 1995), and the distinction between the two processes is critical, mostly when intending to apply the concept of LS in pastoral practices.

Plant growth is defined as an irreversible and permanent increase in plant volume (Salisbury and Ross 1969; Wetmore and Steeves 1971). For grasses, it can be interpreted as lengthening in leaf blade tissue, increase in leaf area, and increase in herbage or roots dry matter (DM) weight over time. Plant development is, however, more complex to be defined. In functional terms, it refers to processes by which cells, organs, tissues and plants pass through various identifiable stages during their life cycle (Wilhelm and McMaster 1995) and includes processes of organ initiation (morphogenesis) but extends to organ differentiation and ultimately must include the process of senescence (Salisbury and Ross 1969; Greulach 1973). Both plant growth and development may occur simultaneously, but in specific conditions or over some periods, they will occur at different rates (sigmoidal, quadratic or linear), or one may even occur without the other (i.e. under environmental stress) (Haun 1973; Wilhelm and McMaster 1995).

A practical example of this differentiation is observed when analysing results from Chapters 4 and 5 (studies that utilise the LS as a criterion for defoliation) and comparing them to the recommended targets based on herbage mass accumulation (t DM ha^{-1}) utilised by New Zealand dairy farmers when budgeting their seasonal and annual pasture production. Matthews et al. (2004) advised seasonal pre-grazing pasture targets for seasonal supply in dairy farms at around 2.50 to 2.70 t DM ha^{-1}. More recently, slightly higher values are found on the DairyNZ website in the section entitled ‘Effective grazing management’, which advises that to achieve high grazing intakes, pre-grazing pasture targets must be at between 2.80 to 3.20 t DM ha^{-1} (DairyNZ 2023). Generally, these are the targets that New Zealand dairy farmers aim for when monitoring and budgeting their systems.

In Chapters 4 and 5 of the present thesis, which followed pre-established optimal LS intervals for defoliation according to *L. perenne*, *B. valdivianus* and *D. glomerata*, great variation was found in the pre-grazing herbage mass values. Within the most ideal and a posteriori recommended LS interval to be followed (*B. valdivianus* and *L. perenne* LS), raw data values of pre-grazing DM herbage mass cut at ground level were in average 3.05 t DM ha^{-1} in winter 2021, 4.45 t DM ha^{-1} in spring 2021, 4.50 t DM ha^{-1} in summer 2021-22, 3.50 t DM ha^{-1} in autumn 2022, 2.35 t DM ha^{-1} in winter 2022, 4.00 t DM ha^{-1} in spring 2022, 3.80 t DM ha^{-1} in summer 2022-23 and 3.45 t DM ha^{-1} in autumn 2023. Along these lines, the results presented in this thesis, in contrast with current practical management pre-grazing targets, may lead us to consider whether pastures in New Zealand are under constant pressure caused by over-grazing (in frequency scale), often defoliated before being physiologically ready. Moreover, the often-mentioned lack of pasture persistence may be the inevitable corollary of such an over-grazing situation.

Studies utilising the LS optimal defoliation interval in a paddock (García-Favre et al. 2022) and on a farm scale (Clarke et al. 2021) have found high productivity annual yields. In Chapter 4, one of the reasons explaining high productivity results, in both single-grass and diverse pastures, was the utilisation of optimal LS intervals as the grazing management parameter. Pastures which are defoliated appropriately have the chance to regrow at their full plant capacity and, therefore, perform better, presenting enhanced productivity and persistence (Donaghy et al. 2021). Beyond growth-related results, another interesting feature observed within the DPCS was the overlapping optimal LS interval of *L. perenne*, *B. valdivianus* and *D. glomerata*. These three species' optimal LS intervals, although numerically different (*L. perenne* from 2.5 to 3.0 LS, *B. valdivianus* and *D. glomerata* from 3.5 to 4.0 LS), were temporarily overlapping, which shows a ‘development synchrony’ among these three grass species (Chapter 4). This development synchrony among species is crucial when comprising an intensively managed diverse pasture. It will allow them to function as an ecosystem that presents an ecological succession of species and maintains stability (Tilman 1993; Hoekstra et al. 2014; Suter et al. 2021).

In summary, it is possible to relate the development synchrony and the growth asynchrony (discussed in the previous section 6.1.2.) as the ecological and morpho-physiological features that allowed the optimally defoliated DPCS to present the seasonal overyielding (Chapter 4) and the great nutritional quality maintained over all seasons (Chapters 4 and 5). This shows that DPCS presented a counterbalanced arrangement between growth asynchrony and development synchrony.

6.1.5. What are the next levels of grazing understanding to be acquired in DPCS?

As the last part of the fundamentalistic conception of successful perennial pastures (monoculture, binary or diverse), the animal acceptance was also tested, and results were presented in Chapter 5. The last general objective of this thesis was to assess and determine the diet preference of dairy animals when offered ryegrass-based and diverse pastures simultaneously. Altogether, Chapter 5 showed that dairy cows accept and graze DPCS, which validates initial insights about the plant-animal relationship between dairy cows and diverse pastures. However, these plant-animal relationships and their mechanisms reach beyond grazing behaviour and foraging preferences, and therefore, it is necessary to investigate them further.

Firstly, forage intake, which is a product of grazing time, bite rate, and bite mass (Thompson and Poppi 1990), will allow a better understanding of herbage consumption on a DM basis. Secondly, diet selection, which is the behavioural expression of preference when access to feed is affected by the environment or opportunity for selection, results in the removal of some sward components (plants or plant parts (Hodgson 1979) and will allow a better understanding on the consumption rates of digestible nutrients, for example, on a relative feed value or relative forage quality index basis (Allen et al. 2011). Lastly, feed utilisation, which is the conversion of herbage mass and feed nutrition into milk, for instance, will allow further understanding of the financial perspectives of utilising DPCS.

The multipolar relationship between different trophic levels is difficult to assess and measure. They require a holistic approach so that hypotheses are straightforward while still being comprehensive enough to cover the whole soil-plant-animal continuum. This is the challenge associated with research that proposes to elucidate the dynamic interconnection between grazing disturbance (Grime et al. 1988; Thébault and Loreau 2005) and intra- and inter-specific competition (Tilman et al. 1998; Lehman and Tilman 2000). These interactions should be considered in future research involving diverse pastures.

Lastly, to expand this research line ‘agricultural overview’, there is a great scientific gap that deserves further development: metabolomic studies for the species present in DPCS. Metabolomic studies are responsible for extracting and characterising primary and secondary compounds that can exert both anti-nutritional and nutritionally beneficial effects upon forage feeding value (Barry et al., 2021). Primarily as in effects on the animal, seeing as food intake, digestive processes (Xu et al., 2006), animal production (including body and wool growth, lactation and reproduction) and animal health (bloat and parasite control). Secondarily, as in potential effects on human health (Van Vliet et al., 2021).

6.2.CONCLUSIONS

6.2.1. Further work

Some specific topics that are of merit for further investigation have been discussed throughout this thesis. However, a few other points of relevance are important to be raised. Firstly, the observation that the optimal LS interval for DPCS was often within the minimal point of *the D. glomerata* optimal interval for defoliation raises a question in regard to the long-term effects of shortened rotation in the *D. glomerata* plant population. Secondly, data suggests that DPCS are expected to perform better than monocultures or binary pastures under periods of water restriction. Therefore, their continued evaluation under a typical rainfall year in New Zealand (El Niño) would be extremely valuable in terms of production and understanding the underlying morpho-physiological adaptive mechanisms conferring DPCS a more robust response to water and heat stress.

6.2.2. Practical implications for local dairy farmers

Among the general objectives presented in Chapter 1, the practical aim of this thesis was to provide answers for farmers who wish to diversify their pasture systems. Several strategies and practices for adopting such systems were identified and are listed as follows:

i. Diverse pastures of complementary species comprising *L. perenne*, *B. valdivianus*, *D. glomerata* and *T. repens* should be grazed according to *B. valdivianus* LS: 3.5 to 4.0 LS in winter, summer and autumn; 3.0 to 3.5 LS in spring.
ii. Occasionally, it is possible to utilise *L. perenne* LS intervals (2.5-3.0 LS) to buffer the competition created by *D. glomerata* so that pastures have a more uniform sward structure and do not become ‘clumpy’.
iii. Post-grazing targets must range between 5 to 8cm residual height and should also help farmers achieve and maintain a uniform sward structure.

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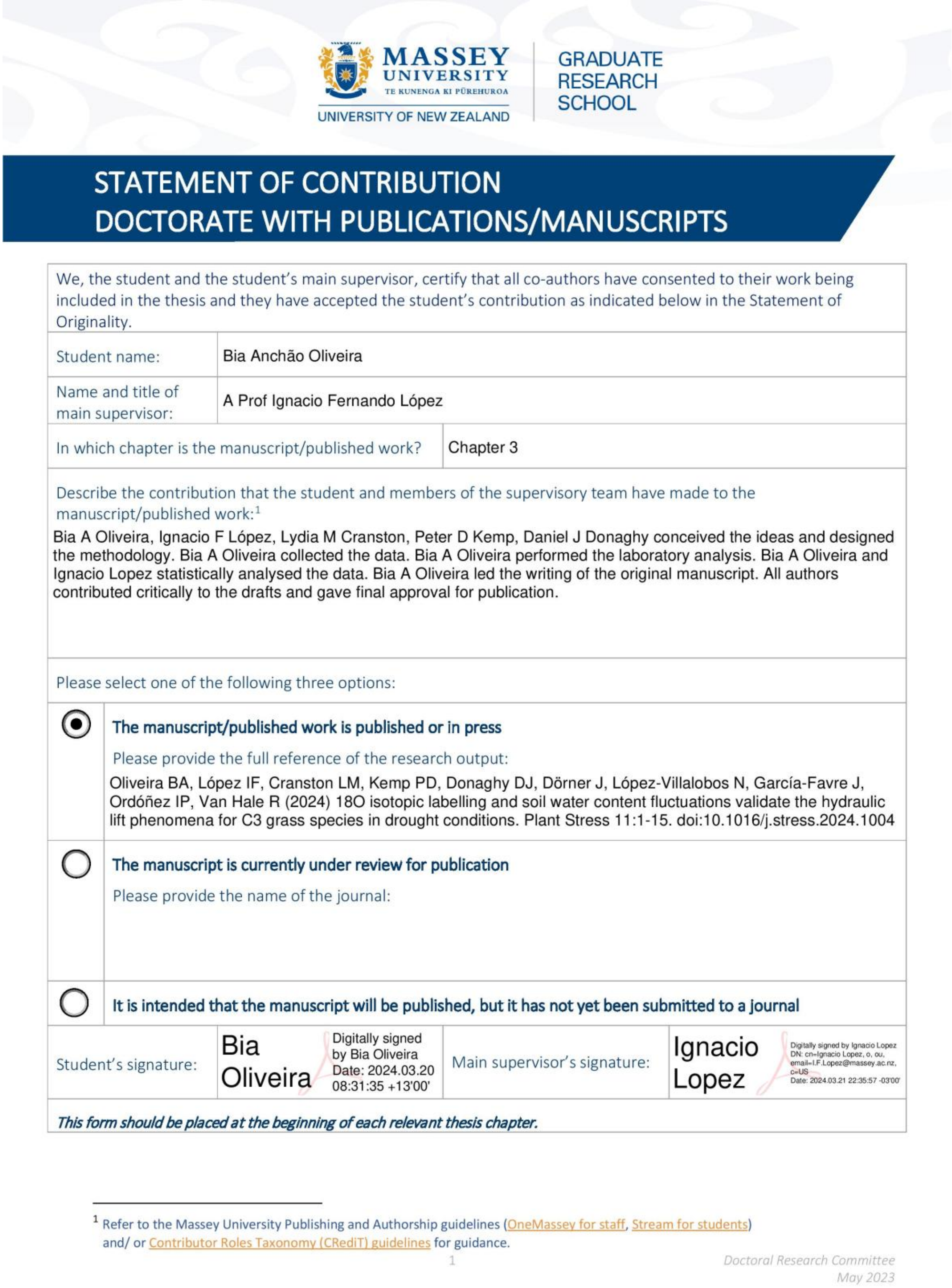
Appendix 1



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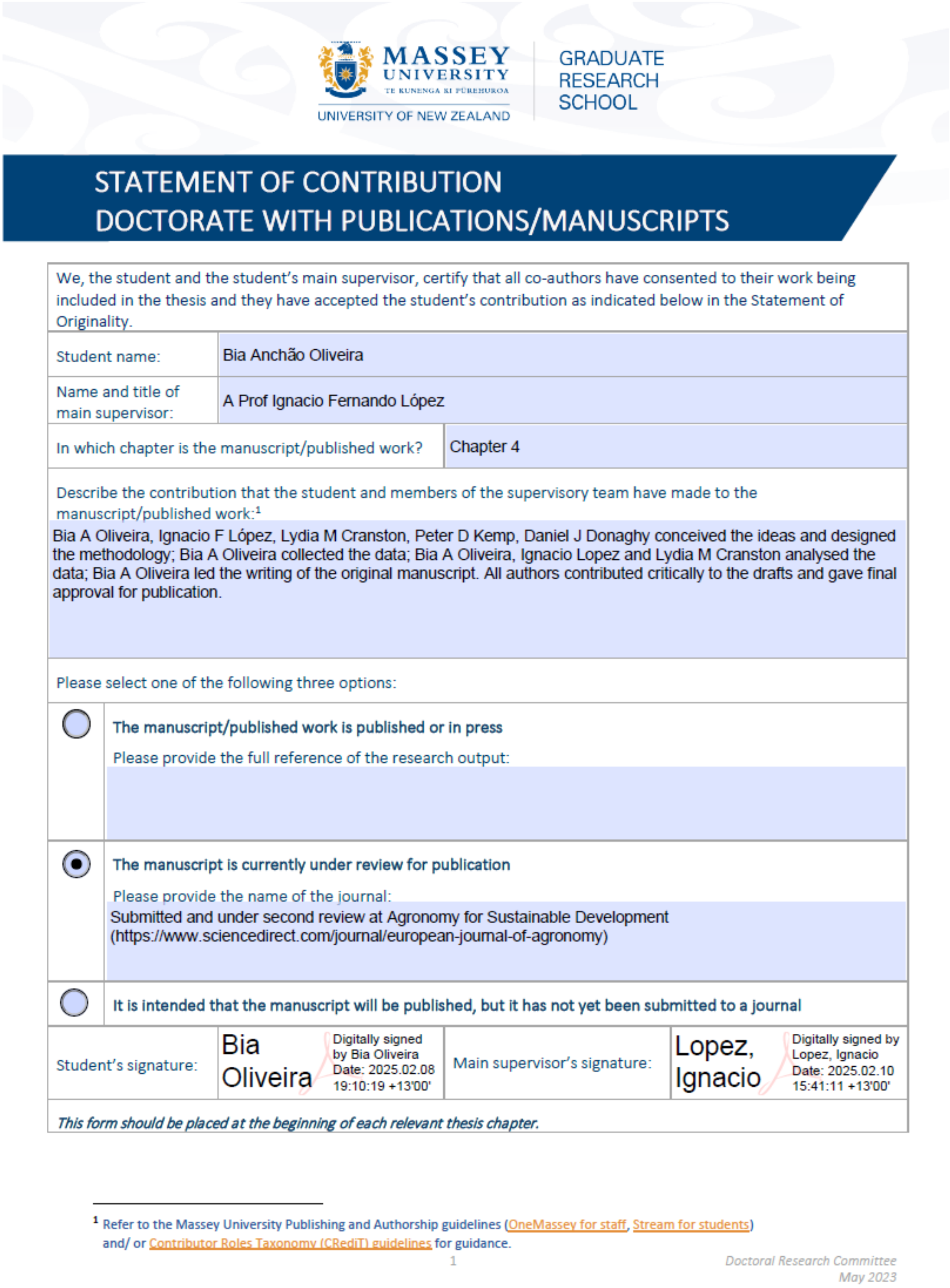

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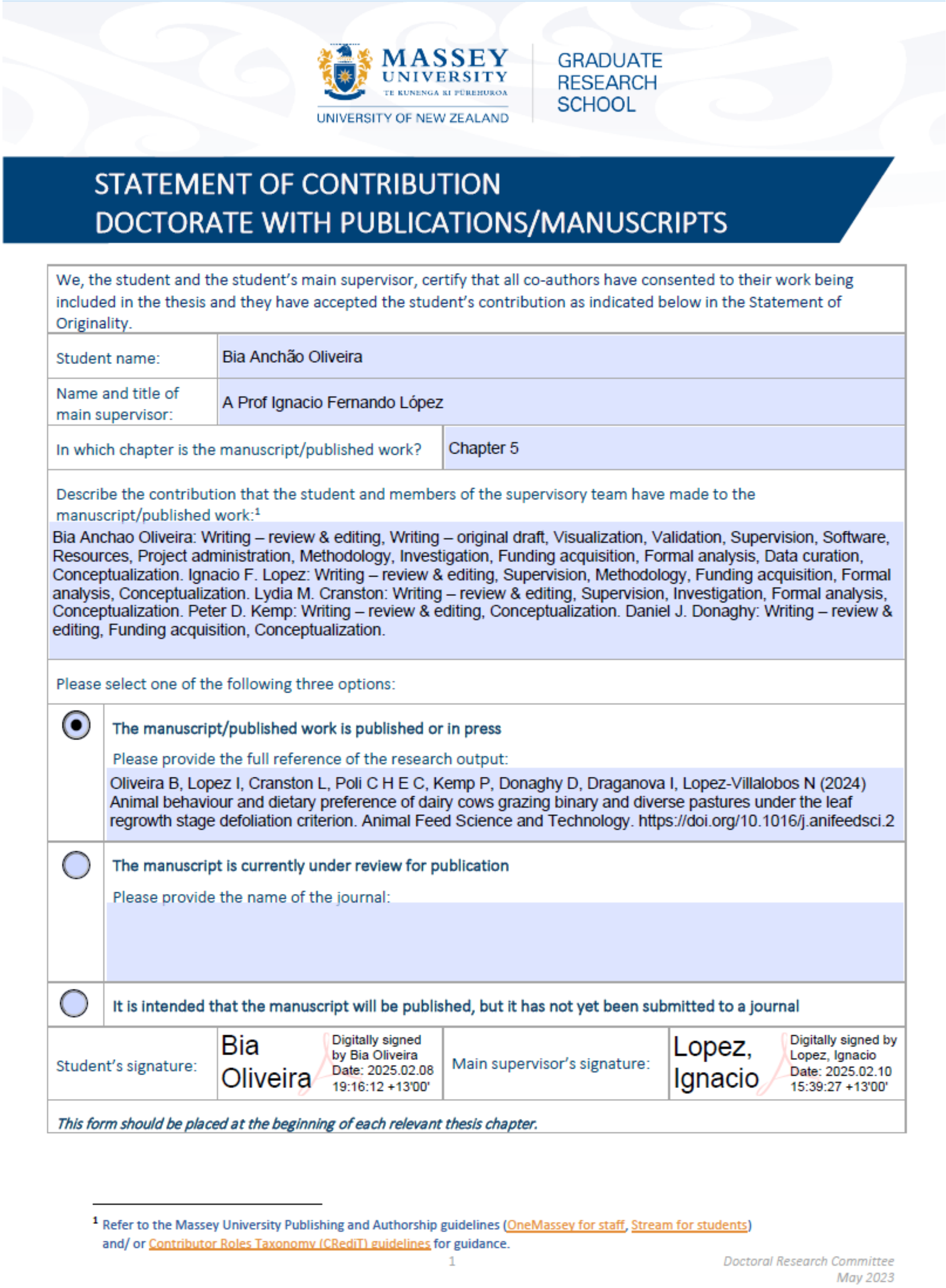

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Bia Anchao Oliveira: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. Ignacio F. Lopez: Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. Lydia M. Cranston: Writing – review & editing, Supervision, Investigation, Formal analysis, Conceptualization. Peter D. Kemp: Writing – review & editing, Conceptualization. Daniel J. Donaghy: Writing – review & editing, Funding acquisition, Conceptualization.

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