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**Growth and nutritive value of pastures under climate
extremes**

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This thesis is dedicated to

God, my Lord, my Savior, my guide, my inspiration and my strength.

*My husband, Tiago, and parents, Henrique and Clea, from whom I found love, support,
inspiration and encouragement, for which I am eternally grateful.*

In loving memory of my grandmother, Antônia.

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

A black rectangular box redacting the signature of the author.

Karen Luanna Marinho Catunda Rodrigues

Declarations

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Thesis Abstract

Grasslands are a major part of the global ecosystem that significantly contribute to food security by providing part of the feed requirements of ruminant livestock. Improved grasslands, such as pastures, are central to emerging challenges for food production in the face of climate change. Predicted increases in the frequency, intensity and duration of climate extremes, including heatwaves and droughts, are likely to have large consequences for pasture systems, and these consequences generate concern for the sustainability of livestock production around the globe. Despite the magnitude of potential impacts, the effects of extreme climate conditions on forage production and nutritive value in different pasture species are understudied. This is especially true for future scenarios with concurrent changes to temperature and water availability.

The main aim of this thesis was to investigate the impacts of future climate extreme scenarios, including increased temperatures and severe droughts, on the nutritive value of key pasture grassland species. My research involved the evaluation of changes in forage nutritional composition and digestibility under contrasting climate regimes. In addition, I looked at possible explanations for the observed changes in forage nutritive value associated with plant growth and morphological traits, including productivity and structural allocation (e.g. dead material and leaf:stem biomass ratios).

In order to improve the understanding of climate change impacts on forage nutritive value of future pasture systems, I conducted a series of experiments (short- and long-term) at the Pastures and Climate Extremes experimental field facility and a glasshouse facility at the Hawkesbury Campus of Western Sydney University, Richmond, New South Wales, Australia. The pasture species comprised a range of functional diversity (C_3/C_4 grasses, legumes, annuals and perennials) and species' origins (native grasses, tropical and temperate pastures) that were grown under ambient and experimentally-imposed severe drought conditions; a sub-set of

species were also exposed to warming in factorial combination with drought. Experimental droughts consisted of a 60% reduction in soil water content from maximum soil water holding capacity (glasshouse experiment) or winter and spring periods of 60% rainfall reduction (field experiments). Warming treatments included +4 °C above ambient temperature (glasshouse experiment) or a continuous +3 °C above-ambient canopy temperature (field experiment). These climate treatments represent the upper end of climate predictions for average surface air temperatures and winter/spring droughts for the study region. In view of the large number of samples collected during this study, I used near-infrared reflectance spectroscopy to develop calibration models to estimate the nutritional composition of the studied pasture species.

I predicted that both forage production and nutritive value would be adversely impacted by extreme scenarios of warming and drought. I further predicted that the combination of these scenarios would have a more pronounced negative impact on forage than the single climate treatments. I found that the impacts on pasture production and forage nutritive value were greater under severe drought than under warming, and their combination resulted in effects that were not always greater than those associated with warming or drought treatments on their own. In addition, I observed that climate extremes strongly impact pasture productivity, resulting in low forage production and increased dead biomass, with the magnitude varying by species. For instance, in the two-year experiment, warming, drought and warming + drought treatments reduced total annual productivity, respectively, by 42%, 48% and 64% in *Festuca arundinacea*, and by 21%, 32% and 34% in *Medicago sativa*. The effects of climate treatments on forage nutritive value (including nutritional composition and digestibility) were mixed, including both marginal increases and declines and null effects. For example, in the 6-month drought experiment involving nine pasture species, the species most adversely impacted by drought in terms of nutritive value were *Chloris gayana* (presenting a percent increase of up to 103% in acid detergent lignin) and *Lolium perenne* (an increase of up to 9% in neutral detergent

fibre). The least affected species were *Biserrula pelecinus* (an increase of up to 12% in crude protein) and *Themeda triandra* (an increase of up to 31% in non-structural carbohydrates), which both increased their nutritive value in the drought. *Medicago sativa* and *Phalaris aquatica* were the only species with no significant change in nutritive value under drought. This variation in nutritional responses, besides depending on pasture species, also depended on the nature of the climate stress and timing when the stress was imposed in relation to the developmental stage of the plants. The long duration of my study (from 2018 to 2020) and its incorporation of multiple harvests allowed me to capture the range of variation, across different pasture species, in productivity and nutritive value under extreme climate scenarios associated with differences in phenology.

The outcomes of this thesis show the importance of exploring species-specific nature in response to seasonal droughts and elevated temperatures to better understand climate change impacts on pasture systems and inform future planting decisions and breeding programmes. Further, this thesis provides insights for grazing industries in Australia and abroad about the potential impacts of extreme events on pasture management and animal production.

CHAPTER 1: General Introduction

1.1 Forage nutritive value

Forage is a widespread term that typically refers to plant materials, mostly leaves and stems, that are consumed by grazing livestock (e.g. cattle, sheep and goats). It encompasses pasture species (usually grasses or legumes), crop residues, hay and silage (Collins and Newman, 2017). The nutritional quality of forage can be defined as the extent to which forage consumption translates into animal performance (e.g. rates of daily weight gain, milk production and reproduction; Ball et al., 2001; Coleman and Moore, 2003). The translation of forage consumption into animal performance and production depends on sward structure and nutrient content, which in turn determines digestibility and forage intake (a measure of the amount of food consumed in a given time, for example, per day), both of which are used as indicators of potential animal production (Coleman and Moore, 2003; Dumont et al., 2015; Heaney, 1970). Therefore, determining the nutritional composition of forage is crucial for determining its suitability for meeting production goals, as well as guiding livestock managers and farmers to determine how much forage and ration supplementation is required to optimise food use efficiency for a specific animal and production goal (Ball et al., 2001). Historically, the nutritional quality of forage can be evaluated by assessment with animal trials, including *in vivo* and *in sacco* digestibility with fistulated animals, however these methods have limitations due to expense, labour, time investment and amount of feed required in the trials. Instead, proximate analysis of forage chemical composition (nutrients composition; **Section 1.1.1**), bioassays (e.g. *in vitro* digestibility; **Section 1.1.2**) and near-infrared reflectance spectroscopy (**Section 1.1.3**) have been used successfully to evaluate the nutritive value of forage (Coleman and Moore, 2003). According to the international terminology for grazing lands and grazing animals published by the International Forage and Grazing Terminology Committee (Allen et

al., 2011), the definition of forage nutritive value is "*the predicted animal response based on chemical composition, digestibility and nature of digested products, as estimated by in vitro or in vivo chemical analyses*". Therefore, this term was selected to refer to the nutritional composition and digestibility of forage in this thesis.

1.1.1 Chemical composition

There is a long history of assessment of forage nutritive value, beginning in the middle of the 1800s, through proximate analysis of food (also known as centesimal composition). According to the Weende method (Henneberg and Stohmann, 1864), modified by Van Soest (1967) for the fibre fraction, typical laboratory analyses of forages aim to obtain information on the following food components:

- **Dry Matter (DM)** – This is the portion of the feed remaining after all the water has been removed by drying the feed sample in an oven until the sample reaches a stable weight. The nutrients in feeds are part of the DM portion of the feed, this includes protein, fibre, fat, minerals, etc (Silva and Queiroz, 2002). There is considerable variation in the water content of forages, thus excluding the water or expressing the nutrient levels on a dry matter basis provides the essential common basis for direct comparison of the nutrient contents across different forages and easier formulation of diets (Saha et al., 2017).
- **Ash** – This is the residue after the total combustion of organic matter, which indicates the total mineral concentration (inorganic compounds) in a forage. Measuring ash helps to understand what proportion of the forage is potentially digestible organic material (Silva and Queiroz, 2002).
- **Fibre** – The most abundant component of the dry matter, which represents plant cell wall material. Fibre is an essential and unavoidable constituent for ruminants since the volatile fatty acids produced during the microbial fermentation of fibre are the main source of energy for the

animal. In addition, fibre is responsible for the effectiveness and fibrosity that stimulates rumination and saliva production, influencing the digestibility of food and consequently animal consumption (Van Soest, 1994). The fibre can be broken down into three crude fractions: cellulose and hemicellulose that are called structural carbohydrates (polysaccharides), and lignin. Cellulose and hemicellulose are largely digestible in the rumen, however, lignin is totally indigestible and when present in high concentrations it can act as a physical barrier to the microbial enzymes that break down cellulose and hemicellulose. These constituents are most commonly analysed using the proximate detergent method developed by Van Soest (1967; 1994):

- **Neutral Detergent Fibre (NDF):** Through the use of a neutral detergent solution (sodium dodecyl sulfate + ethylenediaminetetraacetic disodium salt + sodium borate + sodium phosphate dibasic + triethylene glycol) it is possible to separate the cell contents plus pectins (the part of the forage soluble in water and in neutral detergent that corresponds to the fraction of forage that is easily digested by ruminants) from the cell wall (the part insoluble in neutral detergent that corresponds to the content difficult to digest by ruminants). The cell contents comprise mainly proteins, fats and soluble carbohydrates. Neutral detergent fibre includes cell walls comprising cellulose, hemicelluloses, lignins, ash, fibre-bound nitrogen and tannin complexes. The NDF concentration of a forage can predict dry matter intake because a high NDF concentration means the animal feels full longer because the animal takes longer to digest the components.

- **Acid Detergent Fibre (ADF):** Acid detergent solution (cetyl trimethylammonium bromide + sulfuric acid) dissolves the hemicellulose component of NDF, and the residue is called ADF. The determination of ADF can be performed separately or sequentially using the residual material from NDF. This is the least digestible cell wall

component of forage and comprises cellulose, lignin, ash and traces of other substances resistant to this acid. The ADF concentration is inversely related to digestibility.

- **Acid Detergent Lignin (ADL)**: After digestion in acid detergent, the residual material (ADF) is sequentially digested in sulfuric acid, which dissolves the cellulose, allowing the concentration of residual lignin to be determined. Lignin is one of the main anti-nutritional factors in ruminant feed as it acts as a barrier to fibre degradation by rumen microbes, making energy from fibre unavailable to the ruminants, thus decreasing forage digestibility (Buxton et al., 1995; Jung et al., 1997).

- **Crude Protein (CP)** – This component is estimated from the plant nitrogen concentration multiplied by a conversion factor (6.25) that transforms the result into crude protein (AOAC, 1990). Protein is an essential macronutrient for all animals, as it supplies fixed nitrogen for rumen microorganisms and amino acids to the small intestine for absorption and use by the animal for growth and reproduction. Crude protein is generally positively related to digestibility, and as crude protein increases, so does livestock performance (e.g. weight gain and milk production; Coleman and Moore, 2003).

- **Crude Fat or Ether Extract (EE)** – This is a portion of dry matter extracted with an ether-based solvent (e.g. petroleum ether). This component is a measure of all fat substances, including fatty acids, sterols, oils, plant pigments, and waxes. Fats are important for ruminants to provide energy, absorb some vitamins, provide insulation, protection and for neural functions (Silva and Queiroz, 2002).

- **Soluble carbohydrates or non-structural carbohydrates (NSC)** – The proximate analysis of NSC provides an estimate of readily available energy for ruminal microorganisms, primarily from sugars and starches (Ball et al., 2001). It is often calculated by difference from directly measured constituents, using the formula $NSC = 100 - [CP + EE + Ash + NDF]$ (Sniffen et al., 1992).

1.1.2 Digestibility

Digestibility (absorption efficiency) is important for interpreting the quality of ingested nutrients. In practice, it refers to the proportion of dry matter that can be broken down by the animal and its rumen microbiota, and absorbed in the animal's body (Saha et al., 2017). For example, if forage has 80% apparent digestibility (a widely used measurement of digestibility), this would mean that 80% of the forage eaten by the animal is used for body maintenance, growth, reproduction (pregnancy + lactation) and activity, with the remaining 20% by weight excreted as faeces. As a general guide, 70-80% digestibility is required for high livestock production; 60-70% is required for moderate production; 55-60% is required to maintain dry stock; and below 55% digestibility, the dry stock will lose weight (DPI, 2020). Digestibility of forage and nutrients can be assessed in the laboratory using *in vitro* simulated digestions that use ruminal fluid (obtained from slaughtered or ruminally-fistulated animals; Tilley and Terry, 1963) or purified enzymes (e.g. Aufrere and Michaletdoreau, 1988). Alternatively, digestibility can also be predicted from ADF and CP concentrations (Linn and Martin, 1989; NRC and NASEM, 2016; Oddy et al., 1983). This is due to a strong and well-studied relationship between *in vitro* dry matter digestibility (the proportion of plant dry matter which is digestible) and chemical composition, wherein digestibility is positively correlated with CP, and negatively with ADF (Augustine et al., 2018).

1.1.3 Near-infrared reflectance spectroscopy

In recent decades, near-infrared reflectance spectroscopy (NIRS), a non-destructive sample analysis technique, has been used as an alternative to conventional methods to determine forage nutritive value. This technique uses the absorption and reflectance of near-infrared light from a sample to predict the chemical composition of forages (Foley et al., 1998, Reddersen et al., 2013, Stuth et al., 2003). The reflectance spectrum of near-infrared light from a sample is

influenced by the nature of chemical bonds between hydrogen and carbon, nitrogen, and oxygen in that sample, and consequently by the nature and quantity of complex carbon-and nitrogen-containing compounds (e.g. CP, NDF, ADF, and others; Foley et al., 1998). Statistical procedures, particularly partial least squares regression, are used to develop predictive models (“calibrations”) between the reflectance data spectra collected from samples and reference values that are obtained using traditional wet chemistry methods, like those described above (Stuth et al., 2003). Accurate NIRS predictions depend upon a calibration set (i.e. a large database) that is representative of the variation in chemical composition seen in the target population (Reddersen et al., 2013). After the development of reliable calibrations, new samples can be scanned in NIRS and bypass the need for wet chemistry (Foley et al., 1998; Puigdomènech et al., 1997; Shenk and Westerhaus, 1991). After start-up costs, this method is advantageous because it is inexpensive in the long term, time-efficient, produces no chemical waste, is non-destructive, and requires small sample size and minimal preparation of samples (Foley et al., 1998).

1.1.4 Factors that influence forage nutritive value

The nutritive value of forage is affected by interrelationships among many factors, including the stage of development/phenology of plants, plant morphology traits (mainly tissue arrangement or structure such as leaf:stem ratio), plant species, ecological strategy and functional type (e.g. legumes/grasses and cool/warm season) and environmental growing conditions (Ball et al., 2001; Buxton, 1996; Collins, 2017). Particular attention should be paid to one of the most important factors that influences forage nutritive value: environmental growing conditions. These can have acute (short-term and immediate) and chronic (long-term, cumulative) effects. Factors that have the greatest effect include temperature, soil water deficit and soil nutrients (Waghorn and Clark, 2004). Understanding this is vital because

anthropogenic climate change is altering plant exposure to these drivers. In this context, an important research gap to be addressed is how heat and water stress influence the nutritive value of forage species – knowledge that is crucial for optimising and planning the future of livestock industry across the world. In addition, this knowledge is needed to allow better predictions of when forage should be harvested or grazed for optimal nutritive value, what plant species or cultivars will perform best, where grazing and pasture production will remain viable and to predict animal health, nutrition and production under future climate conditions (AbdElgawad et al., 2014; Buxton, 1996; Dumont et al., 2015).

1.2 A changing climate

Since the mid-nineteenth century, a constant increase in the levels of anthropogenic greenhouse gases in the Earth's atmosphere has been observed. These gases affect the radiative balance of the Earth, causing it to warm, leading to numerous changes in the global climate (IPCC, 2019). Global surface temperatures are now about 1.1 °C higher than in 1850-1900 (IPCC, 2021). These increases in average temperature are indicative of a systematic change in climate that manifests uniquely in different areas of the globe. In particular, a changing climate leads to changes in the frequency, intensity, duration, and timing of weather and extreme climate events worldwide (IPCC, 2019). Climate extremes (extreme weather or climate events) are generally defined as *"as the upper or lower statistical tails of the observed range of values of climate variables or climate indicators (e.g. temperature/rainfall or drought/aridity indices respectively)"* (IPCC, 2019).

Predicted changes in temperature and rainfall patterns are likely to include more frequent and severe extreme events such as heatwaves in most parts of the world and droughts in some regions that are already drought-prone (CSIRO and BOM, 2015; IPCC, 2021). Heatwaves are periods of excessively hot weather and can be defined operationally as periods

of at least three days where the combined effect of high temperatures and excess heat is unusual within the local climate (Australian Bureau of Meteorology, 2012; Nairn and Fawcett 2013), although other field and application specific definitions also exist (IPCC, 2019). Disagreement can also be found around drought, with formal definitions often varying by discipline and the types of measurements taken into consideration (e.g. precipitation, soil moisture), but in essence, it refers to an acute water shortage. The Bureau of Meteorology in Australia has defined drought as “*a prolonged, abnormally dry period when the amount of available water is insufficient to meet our normal use*”. Droughts can be measured in many ways, for example, hydrologists assess droughts as periods of precipitation shortfalls on surface or subsurface water supply (e.g. reservoir and lake levels, and groundwater; Wilhite and Glantz, 1985), and meteorologists assess the extent and severity of droughts in terms of rainfall deficits (Bureau of Meteorology-Australian Government). A rainfall deficit occurs when the total rainfall of an area over a period is less than the long-term average for that period. The term severe rainfall deficiency is defined as when rainfall falls within the fifth percentile of observations for the period in question (Bureau of Meteorology - Australian Government).

In Australia, extreme climate events are projected to increase in both number and magnitude (IPCC, 2021). A report about climate change in Australia shows that rising greenhouse gases have contributed to an increase in Australian average surface air temperatures of 0.9 °C since 1910 (CSIRO and BOM, 2015). Since 2000, there has been an increase in extremely warm months and a reduction in cold ones, as well as an increase in heatwaves in terms of duration, frequency, and intensity in most regions of the country (Australian Bureau of Meteorology, 2012). Temperature projections across Australia predict warming of 1.5 °C under low-emission scenarios and 5.5 °C under high-emission scenarios by the end of the century (IPCC, 2021). While there is considerable variation and uncertainty in projections of rainfall patterns in Australia due to strongly contrasting results from global climate models,

rainfall in the cool season (winter and spring) is projected to decline in southern Australia, increasing the duration of drought conditions. Associated with these predicted patterns of precipitation, the nature of the drought is projected to change, with a greater frequency of extreme droughts, especially in the southern regions, and less frequent moderate to severe droughts projected for all regions of Australia (CSIRO and BOM, 2015; IPCC, 2021).

1.3 Overview of climate change impacts on pasture-based systems

Climate variables relevant to the Earth's ecosystems are predominantly temperature and precipitation-related. Therefore, climate extremes, including individual, short/long-term, or their combination, will significantly impact ecosystems, including natural and managed grasslands, across a number of spatial and temporal scales (IPCC, 2019). Grasslands make up the largest proportion of terrestrial landcover and provide important ecosystem services such as supporting a diversity of animals (e.g. wild and domestic herbivores) and plant species that make them suitable for natural and managed grazing systems (ABARES, 2016; Gibson, 2009). Grazing lands (pastures and rangelands) account for 37% of the total global land use, with permanent pastures accounting for most of this area, including intensive and extensive pastures (IPCC, 2019). In Australia, livestock grazing is widespread and occupies 54% of land use (i.e. grazing natural vegetation 44.87%, grazing modified pastures 9.24% and irrigated pastures 0.08%; ABARES, 2016). The total land area used to raise livestock reflects the high demand for supporting food production globally. Consequently, the potential impacts of climate extremes on grassland ecosystems pose major future challenges for global food security (IPCC, 2019).

Elevated temperatures and severe drought have been identified as key future concerns for pasture-based systems in many parts of the world, as they are likely to have consequences for future forage production and nutritive value (Chang-Fung-Martel et al., 2017; Howden et

al., 2008; IPCC, 2012; McKeon et al., 2009). Furthermore, there are likely to be many direct and indirect impacts of climate change on livestock. The most significant direct impacts come from animal thermal stress, which will result in a decrease in food intake, milk/meat production and quality, reproductive efficiency, animal health, and in extreme cases cause mortality. The indirect impacts are related to the reduced availability of food for animals and water resources, for example, a reduction in the quantity of pasture and water demand for irrigation (Sejian, 2016). Aside from changes in quantity, there are also key potential changes in the nutrient composition of forage species that are likely to alter tissue quality, digestibility and subsequent animal performance under future climate change scenarios, and these impacts are comparatively less well understood (AbdElgawad et al., 2014; Dumont et al., 2015).

Pasture is the most economical source of nutrients in the ruminant diet, however, supplementary nutrition (e.g. grains, protein meals, mineral and vitamin complexes) for grazing ruminants is often required to meet nutritional needs, and that comprises the most expensive foods in livestock systems. Therefore, if available pasture forage does not provide the necessary nutrients to meet the nutritional needs of the animals, the proportion of supplemental feed increases, a critical factor that may increase input costs in livestock production systems under future climate change scenarios (Dairy Australia, 2018; Howden et al., 2008; Rojas-Downing et al., 2017). Thus, understanding the consequences of elevated temperatures and droughts on the forage nutritive value of pasture species is of great importance for the future in order to adapt or implement compensation strategies against possible negative effects (AbdElgawad et al., 2014; Dumont et al., 2015; Grant et al., 2014).

1.3.1 Pasture responses to elevated temperatures and drought

Pasture nutritive value responses to high temperatures and drought will reflect the severity of heat, and deficiencies in soil moisture and nutrient availability, as well as plant-specific phenological and morphological responses.

Elevated temperature, as an isolated factor, has the potential to change the length of the growing season and can increase soil mineralization rate, consequently increasing plant growth; although these positive effects are often reported in relatively colder environments (Bloor et al., 2010; Dieleman et al., 2012; Rustad et al., 2001). Countering this, extremely high temperatures have been shown to increase evapotranspiration, resulting in a decline in soil moisture and nutrient availability, consequently reducing plant growth (Rustad et al., 2001). Furthermore, reductions in tiller emergence and leaf:stem ratio (Mitchell, 1956; Wilson et al., 1991), accelerated senescence, and significant plant tissue damage, which can cause mortality, due to heat stress were reported under high-temperature conditions (Wahid et al., 2012).

In terms of forage nutritive value responses, studies have reported varying responses of nutritional composition that might be associated with the severity of warming imposed, differences in plant species and phenology, and baseline climatic differences between studies (Lee et al., 2017). Results range from no change to increases in fibre (Dumont et al., 2015; Waghorn and Clark, 2004), no change/increases/decreases in CP (Dumont et al., 2015; Dieleman et al., 2012; Habermann et al., 2019) and no change/decreases in NSC (Dumont et al., 2015; Habermann et al., 2019). Furthermore, there is evidence that high temperatures may affect the digestibility of C₃/C₄ and legume/grass species differently. For example, a study investigating species from different functional groups under high temperatures found that the digestibility of C₄ grasses (*Cynodon dactylon*, *Panicum maximum* var. *trichoglume*, *Panicum laxum*) decreased more than did a C₃ legume (*Medicago sativa*; Wilson et al., 1991). The existing literature shows that additional research is needed specifically to investigate plant

chemical composition and digestibility responses of different functional groups of pasture species under high temperatures, including extreme temperatures (Dumont et al., 2015). Studies of this nature are indispensable in order to identify heat-tolerant species and traits associated with species able to produce under these conditions.

Drought, as an isolated factor, has been shown to affect pasture in different ways depending on the severity and timing of water restrictions. For example, moderate drought stress can delay plant maturation and growth, and cause moderate senescence (Buxton, 1996). In addition, it has been shown to have inconsistent effects on nutritive value, including no change or a reduction in fibre, and no change or slight improvement in CP concentrations and digestibility (Deleglise et al., 2015; Dumont et al., 2015; Kuchenmeister et al., 2013). In contrast, severe drought stress has been shown to inhibit plant growth and speed maturation, leading to severe senescence and decreases in leaf:stem ratio (Bruinenberg et al., 2002; Ren et al., 2016). This increase in senescent material results in a significant decrease in the forage nutritive value (e.g. digestibility; Deleglise et al., 2015; Dumont et al., 2015; Ren et al., 2016). Belowground, severe water limitation reduces soil moisture, diffusion of nutrients and nutrient uptake (Durand et al., 2010; Evans and Burke, 2013; Gonzalez-Dugo et al., 2005). In general, studies have reported reduced forage nutritive value under severe drought conditions through an increase in the fibrous fraction and a decrease in CP, NSC and digestibility (Deleglise et al., 2015, Ren et al., 2016, Durand et al., 2010, Buxton, 1996, Schonbach et al., 2009). Furthermore, a particular concern for pasture systems is the species-specific responses to severe drought, since they may respond differently in terms of nutritive value (McGranahan and Yurkonis, 2018, Grant et al., 2014). For example, there is evidence that C₄ grasses are generally more resistant to drought than C₃ grasses, as a result of their higher water use efficiency (Evans et al., 2011; McGranahan and Yurkonis, 2018). In the context of increasing frequency and severity of drought, a valuable goal would be to identify key species and traits with improved

drought resistance that maintain nutritive value at advanced stages of maturity (Buxton, 1996; Grant et al., 2014).

While it is important to understand the isolated effects of elevated temperatures and drought on pasture systems, it is also essential to identify the complex interactions of these factors, as they inevitably occur concurrently and are not independent of each other – higher temperatures increase plant demand for water and the rate of water loss from pastures (Bloor et al., 2010; Dumont et al., 2015; Tubiello et al., 2007). Several studies and meta-analysis have emphasized the need for research with combinations of future scenarios such as climate extremes in pasture systems (AbdElgawad et al., 2014; Cullen et al., 2009; Dellar et al., 2018; Dumont et al., 2015), particularly in terms of nutritive value across different species (Howden et al., 2008; McGranahan and Yurkonis, 2018). Understanding and dealing with instability in pasture nutritive value due to unpredictable climatic change is an ongoing challenge for scientists, farmers, and livestock industries, so studies in this area can lead to more efficient use of resources, better economic outcomes, and consequently, an improvement in the future of sustainable livestock production.

1.4 Research objectives

Based on the identified knowledge gaps, the overall aim of this PhD project was to investigate the impacts of climate extremes, such as warming and/or drought, on the forage nutritive value of a diverse range of globally important pasture species. This involved relating nutritional composition to digestibility and plant traits, including biomass productivity and morphology.

To achieve this aim, the experiments in this thesis were conducted in a novel project that combines a world-first Pastures and Climate Extremes (PACE) field facility investigating pasture responses to future climate scenarios under common growing conditions (background climate, soils), with glasshouse experiments aimed at understanding some of the mechanisms

driving field responses in a more closely controlled environment. The PACE project is located at the Hawkesbury Campus of Western Sydney University, in southeastern Australia, and is co-funded by Western Sydney University, Meat and Livestock Australia and Dairy Australia. The PACE facility comprises rainout shelters that are divided into plots containing pasture species from a range of functional groups (C_3 legumes, C_3/C_4 grasses, annuals, and perennials) and origins (tropical and temperate introduced, and native) that were exposed to warming, including heatwaves, and/or seasonal droughts since 2018. In line with future climate predictions for southeastern Australia by the end of the century, the experimental treatments included continuous elevated temperature (+3 °C) achieved using infra-red heating lamps, and extreme drought, comprising a 60% reduction in winter and spring rainfall. Detailed descriptions of facilities and experimental design are located in an industry report (Power et al., 2020) and in associated scientific publications (Churchill et al., 2021; Zhang et al., 2021).

The specific objectives and approaches of each research chapter of this thesis were as follows:

- **Chapter 2** evaluated the performance of near-infrared reflectance spectroscopy calibrations in predicting standard nutritional composition of a large set of common pasture species covering a range of functional diversity, origins and environmental conditions (warming and drought). The calibration models were developed using samples collected in Chapters 3, 4 and 5. I was able to develop accurate calibrations that achieved excellent predictions for key nutritional parameters of more than 2600 samples collected in this thesis. Therefore, this chapter was essential for generating data for all the experimental chapters.
- **Chapter 3** investigated the effects of elevated temperature (+4 °C warming) and short-term drought (60% reduction in soil water content from maximum soil water holding capacity), alone and in combination, on plant morphological traits and nutritive value

of two important temperate pasture species, *Festuca arundinacea* and *Medicago sativa*. This 4-month study was conducted in glasshouse chambers and I measured plant biomass, percentage of dead material, plant height, number of tillers/stems and leaf:stem ratio. I also analysed nutritional composition and digestibility.

- **Chapter 4** investigated the effects of 2-years of continuous warming (+3 °C) and two consecutive 6-month periods (2018 and 2019) of severe drought in winter/spring (60% rainfall reduction), as isolated factors and in combination, on two common temperate pasture species (*Festuca arundinacea* and *Medicago sativa*). This study was conducted in the field facility and I collected samples during regular harvests to quantify forage productivity, nutritional composition and digestibility.
- **Chapter 5** investigated the effects of a 6-month period of severe winter/spring drought (60% rainfall reduction) on plant structural allocation and nutritional composition in nine common pasture species from a range of functional diversity and origins. The species included *Biserrula pelecinus*, *Chloris gayana*, *Digitaria eriantha*, *Festuca arundinacea*, *Lolium perenne*, *Medicago sativa*, *Phalaris aquatica*, *Rytidosperma caespitosum* and *Themeda triandra*. This study was conducted in the field facility and I collected samples at regular harvests to quantify biomass productivity, percentage of dead material, leaf:stem biomass allocation, and nutritional composition (whole-plant and within the leaf and stem tissues).

1.5 Thesis outline and structure

This thesis contains original work conducted by myself, in which, in addition to writing, I collected, analysed and interpreted the data from all chapters, with guidance from my supervisory panel and co-authors. The thesis is divided into six chapters with four original research chapters (Chapters 2-5): Chapter 2 is a methods chapter and Chapters 3-5 are

experimental chapters. Repetition across chapters was unavoidable when recurrent methodologies were used. All four research chapters were written as separate manuscripts, that have been published, in review, or are intended to be published in peer-reviewed journals, as detailed below. In addition, as each chapter is written as it appears, or will appear in publications, I used the collective pronoun in the research chapters.

Chapters	Title
Chapter 1	General introduction
Chapter 2 (Methods)	Near-infrared spectroscopy calibration strategies to predict multiple nutritional parameters of pasture species from different functional groups <i>(under review in Journal of Near Infrared Spectroscopy)</i>
Chapter 3 (Experiment 1)	Short-term drought is a stronger driver of plant morphology and nutritional composition than warming in two common pasture species <i>(published in Journal of Agronomy and Crop Science)</i>
Chapter 4 (Experiment 2)	Productivity of temperate pasture species is impacted more than nutritive value by two years of simulated climate extremes <i>(in preparation for submission to Global Change Biology)</i>
Chapter 5 (Experiment 3)	Plant structural and nutritional responses to drought differ among common pasture species <i>(under review in Crop & Pasture Science)</i>
Chapter 6	General discussion

1.6 Relevant additional information

- **Data accessibility:** Raw data collected in the research chapters were uploaded to the data management system of the Hawkesbury Institute for the Environment at Western Sydney University (HIEv) and archived. Plant samples collected were properly labelled and stored on the Hawkesbury campus for potential future analyses.
- **Candidate further contributions:** In addition to leading, as first author, the manuscripts from each chapter of this thesis, the PACE project gave me the opportunity to actively collaborate on other important research projects during my PhD, to which I devoted a significant amount of time, although they were not included in this thesis. For example, pasture systems research that investigated the effects of 1) climate extremes (drought and warming) on different combinations of C₃ and C₄ grass and legume species growing in mixtures; 2) inoculation of arbuscular mycorrhizal fungi on pasture species under drought and elevated temperature conditions; and 3) elevated carbon dioxide on tropical pasture species (legumes and grasses) growing in monocultures and mixtures. These collaborations are particularly noteworthy as they led to co-authored peer-reviewed publications, industry reports and conference presentations that used data I helped to collect and analyse, and further manuscripts that are currently in preparation for publication, as presented below:

Peer-review publications:

-Zhang, H., Powell, J. R., Power, S. A., Churchill, A. C., Plett, J. M., Macdonald, C. A, Jacob, V., Kim, G. W., Pendall, E., Tissue, D., **CATUNDA, K. M.**, Igwenagu, C., Carrillo, Y., Moore, B. D., & Anderson, I. C. (2021). Arbuscular mycorrhizal fungal-mediated reductions in N₂O

emissions were not impacted by experimental warming for two common pasture species.

Pedobiologia Journal, 87-88, 150744. <https://doi.org/10.1016/j.pedobi.2021.150744>

-Zhang, H., Powell, J. R., Plett, J. M., Churchill, A. C., Power, S. A., Macdonald, C. A., Jacob, V., Kim, G. W., Pendall, E., Tissue, D., **CATUNDA, K. M.**, Igwenagu, C., Carrillo, Y., Moore, B. D., & Anderson, I. C. (2021). Climate warming negates arbuscular mycorrhizal fungal reductions in soil phosphorus leaching with tall fescue but not lucerne. *Soil Biology and Biochemistry*, 152, 108075. <https://doi.org/10.1016/j.soilbio.2020.108075>

-Churchill, A. C., Zhang, H., Fuller, K. J., Amiji, B., Anderson, I. C., Barton, C. V. M., Carrillo, Y., **CATUNDA, K. L. M.**, Chandregowda, M. H., Igwenagu, C., Jacob, V., Kim, G. W., Macdonald, C. A., Medlyn, B. E., Moore, B. D., Pendall, E., Plett, J. M., Post, A. K., Powell, J. R., Tissue, D. T., Tjoelker, M. G., & Power, S. A. (2021). Pastures and Climate Extremes: Impacts of cool season warming and drought on the productivity of key pasture species in a field experiment. *Accepted for publication in Frontiers in Plant Science*. (preprint): <https://doi.org/10.1101/2020.12.21.423155>

-Kim, G. W., Zhang, H., Powell, J. R., Plett, J. M., Churchill, A. C., Power, S. A., Macdonald, C. A., Jacob, V., Tissue, D. T., **CATUNDA, K. L. M.**, Igwenagu, C., Carrillo, Y., Anderson, I.C., & Pendall, E. (2021). Drought and warming interact to stimulate N₂O emissions in pasture crops. *Under review in the Journal of Environmental Management*.

-Churchill, A. C., Zhang, H., **CATUNDA, K. L. M.**, Kim, G. W., Anderson, I. C., Moore, B. D., Pendall, E., Powell, J. R., & Power, S. A. (2021). Nutrient resource partitioning and

consequences for productivity in tropical legume-grass mixed swards under elevated CO₂. *In preparation for submission to Global Change Biology.*

Industry report:

-Power, S. A., Churchill, A. C., Zhang, H., **CATUNDA, K. L. M.**, Jacob, V., Chandregowda, M. H., Kim, G. W., Igwenagu, C., Tissue, D. T., Moore, B. D., Powell, J. R., Plett, J. M., Macdonald, C. A., Pendall, E., Carrillo, Y., Tjoelker, M. G., Medlyn, B. E., & Anderson, I. C. (2020). Sustainable pasture systems under climate extremes. (Industry Report). Published by Meat and Livestock Australia Limited, North Sydney, New South Wales, Australia. <https://www.mla.com.au/research-and-development/reports/2020/sustainable-pasture-systems-under-climate-extremes/#>

Conference presentations:

-**CATUNDA, K. L. M.**, Churchill, A. C., Power, S. A., & Moore, B. D. Forage production and nutritional quality of tall fescue and alfalfa under long term warming and severe drought. In: American Forage & Grassland Council Annual Conference, 2022, Kansas, USA.

-**CATUNDA, K. L. M.**, Churchill, A. C., Zhang, H., Power, S. A., & Moore, B. D. Effects of warming and drought on nutritional quality of two temperate pasture species. In: Recent Advances in Animal Nutrition – Australia Conference, 2021, Gold Coast, Australia.

-Churchill, A. C., Zhang, H., **CATUNDA, K. M.**, & Power, S. A. Pasture plant-plant competition and nutrient facilitation under extreme drought and warming. In: Ecological Society of America Annual Meeting, 2020, Virtual. <https://eco.confex.com/eco/2020/meetingapp.cgi/Paper/83572>

-Churchill, A. C., Zhang, H., **CATUNDA, K. L. M.**, Kim, G. W., Pendall, E., Moore, B. D., Anderson, I. C., & Power, S. A. Nutrient facilitation between tropical legumes and grasses under elevated CO₂. In: Ecological Society of Australia Conference, 2019, Launceston, Tasmania.

-Churchill, A. C., Zhang, H., Fuller, K., **CATUNDA, K. L. M.**, Chandregowda, M., Power, S. A., & PACE Scientists. Consequences of extreme climate conditions on plant productivity and recovery among diverse pastures. In: Australian Rangelands Society Conference, 2019, Canberra, ACT.

-Churchill, A. C., Barton, C., Fuller, K., **CATUNDA, K. M.**, Power, S. A. Detecting shifts in pasture ecosystem health and function under extreme climate conditions using canopy greenness. In: American Geophysical Union, Fall Meeting, 2019, San Francisco, USA.
<https://ui.adsabs.harvard.edu/abs/2019AGUFM.B33K2638C/abstract>

-Churchill, A., Barton, C., Fuller, K., **CATUNDA, K. M.**, Power, S. Canopy greenness highlights phenological shifts in pasture resistance and resilience under extreme climate conditions. In: The Ecological Society of America Annual Meeting, 2019, Louisville, USA.
<https://eco.confex.com/eco/2019/meetingapp.cgi/Paper/78155>

CHAPTER 2: Near-infrared spectroscopy calibration strategies to predict multiple nutritional parameters of pasture species from different functional groups

2.0 ABSTRACT

Near-infrared reflectance spectroscopy (NIRS) has been used by the agricultural industry as a high-precision technique to quantify nutritional chemistry in plants both rapidly and inexpensively. This study aimed to evaluate the performance of NIRS calibrations in predicting the nutritional composition of ten pasture species that underpin livestock industries in many countries. These species comprised a range of functional diversity (C_3 legumes; C_3/C_4 grasses; annuals/perennials) and origins (tropical/temperate; introduced/native) that grew under varied environmental conditions (control and experimentally induced warming and drought) over a period of more than 2 years ($n = 2,622$). A maximal calibration set including 391 samples was used to develop and evaluate calibrations for all ten pasture species (global calibrations), as well as for subsets comprised of the plant functional groups. We found that the global calibrations were appropriate to predict the six key nutritional parameters studied for our pasture species, with the highest accuracy found for ash (ASH), crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF), and the lowest for ether extract (EE) and acid detergent lignin (ADL) parameters. The plant functional group calibrations for C_3 grasses performed better than the global calibrations for ASH, CP, ADF and EE parameters, whereas for C_3 legumes and C_4 grasses the functional group calibrations performed less well than the global calibrations for all nutritional parameters of these groups. Additionally, our calibrations were able to capture the range of variation in forage nutritive value caused by future climate scenarios of warming and severe drought.

2.1 INTRODUCTION

Forage nutritive value depends on plant nutritional composition, which influences digestibility and forage intake, and which are typically used as indicators of potential animal production (Ball et al., 2001; Coleman and Moore, 2003; Dumont et al., 2015). In view of this, evaluation of nutrient composition is essential for determining whether forage nutritive value is adequate for animal production and in guiding livestock managers/farmers in determining how much forage and supplementation is needed to optimize food use efficiency for a particular animal and production goal (Ball et al., 2001). Traditionally, forage was evaluated by animal trials (e.g. *in vivo* and *in sacco* digestibility with fistulated animals), however, this method has a number of constraints, including high costs, labour, time investment and amount of feed required in the trials. Consequently, this method is not suitable for examining large numbers or types of forage samples. Alternatives that have been used successfully to evaluate forage nutritive value include proximate analysis of forage nutritional composition (nutrients and anti-nutrients), *in vitro* digestibility assays and near-infrared reflectance spectroscopy (NIRS; Coleman and Moore, 2003).

Near-infrared reflectance spectroscopy has been embraced by the agricultural industry as a high-precision technique for measuring nutritional chemistry that is rapid, time-efficient, inexpensive, produces no chemical waste, and requires a small sample size and minimal preparation of samples (Abrams et al., 1987; Foley et al., 1998; Murray, 1993; Roberts et al., 2004). This is a non-destructive technique that uses the absorption and reflectance of near-infrared light, and often visible wavelengths as well, from a sample to predict the chemical composition and other traits (e.g. digestibility, palatability, etc.; Foley et al., 1998; Moore et al., 2010; Reddersen et al., 2013; Stuth et al., 2003). The reflectance spectrum of near-infrared light is influenced primarily by the nature of chemical bonds between hydrogen and carbon, hydrogen and nitrogen, and hydrogen and oxygen in each sample, and consequently by the

nature and quantity of complex carbon and nitrogen-containing compounds, such as crude protein, fibre, and other plant constituents (Foley et al., 1998; Parrini et al., 2018; Smith et al., 2019). Based on these, concentrations can often be accurately predicted from the near-infrared reflectance spectra by developing standardized calibrations with samples of known nutritional composition (Foley et al., 1998). The process involves well-established statistical procedures used to develop, assess and improve predictive calibration equations for reflectance spectra based on reference values obtained by a variety of standard wet chemistry or other analytical techniques (Stuth et al., 2003). Importantly, accurate NIRS predictions of unknown samples depend on a calibration set (i.e. a large database) that is representative of the chemical and spectral variation encountered in the target population (Foley et al., 1998; Puigdomènech et al., 1997; Reddersen et al., 2013; Shenk and Westerhaus, 1991). After the development of reliable calibrations, NIR spectra of new samples can then be acquired and used for immediate quantification of multiple parameters, bypassing the need for wet chemistry analyses, and the associated expense of the latter.

Near-infrared reflectance spectroscopy has been shown to accurately predict forage nutritive value, such as in studies involving mixed bulk samples of central European grasslands (Berauer et al., 2020), fresh samples from natural pastures in Italy (Parrini et al., 2019), warm-season legumes in the United States (Baath et al., 2020), and native and temporary grasses in the United Kingdom (Bell et al., 2018). However, few studies report on the establishment of NIRS calibration models for predicting multiple nutritional constituents, such as ash, crude protein, ether extract, neutral detergent fibre, acid detergent fibre and acid detergent lignin, and fewer still include calibrations using large sample sets comprised of multiple species (Parrini et al., 2018; Parrini et al., 2019; cf. Norman et al., 2015; Norman et al., 2020). In addition, many calibration models for predicting nutritional composition do not capture variation across

a range of functional diversity and origins of pasture species, particularly growing under future environmental conditions such as climate change scenarios (Berauer et al., 2020).

In southern Australia, pasture systems are based on a diverse range of grasses and legumes, for which information on nutritional composition under a wide range of environmental (including climatic) conditions is limited (Howden et al., 2008; Lee et al., 2013; Norman et al., 2020; Norman et al., 2021). To address this knowledge gap, we used the Pastures and Climate Extremes (PACE) experimental facility to evaluate the nutritional responses of a wide range of pasture/rangeland species (including tropical/temperate, introduced/native and grasses/legumes) to year-round warming (including intensification of heatwaves) and extreme winter/spring drought events. We specifically aimed to evaluate the performance of NIRS calibrations in predicting nutritional composition of the species under differing climatic conditions. The species used in this study comprised a range of functional diversity (C_3 legumes; C_3/C_4 grasses; annuals and perennials) and origins (tropical and temperate; introduced and native) with a wide variation in concentrations of chemical constituents. Additionally, we tested different calibration strategies using combined datasets from all studied pasture species (global calibrations) and different independent datasets of plant species groups (plant functional group calibrations), in order to contribute to the improvement of NIRS calibrations and wider use of this technology in the research and development of livestock nutrition. We tested the hypothesis that the global calibrations would provide accurate predictions across a range of pasture species for the six key nutritional parameters (ash, crude protein, ether extract, neutral detergent fibre, acid detergent fibre, and acid detergent lignin). We also hypothesised that predictions from the global calibrations will be more accurate than those from calibrations derived from plant functional groups.

2.2 MATERIALS AND METHODS

2.2.1 Pasture sample collection

Representative samples of the pasture species were collected at the Pastures and Climate Extremes (PACE) experimental field facility (see Chapters 4 and 5) and from an associated glasshouse study (see Chapter 3) at the Hawkesbury Campus of Western Sydney University, at Richmond, New South Wales, Australia (S33.610, E150.740, elevation 25 m). The field site has a mean annual precipitation of 800 mm and a mean annual temperature of 17.2 °C, with monthly means peaking in January (22.9 °C) and at their lowest in July (10.2 °C; Australian Government Bureau of Meteorology, Richmond - UWS Hawkesbury Station). At the field facility, the soil was loamy sand with a volumetric water-holding capacity of 15-20%, pH of 5.7, plant available N of 46 mg/kg, plant available (Bray) P of 26 mg/kg and 1% soil organic carbon (more details are reported in Churchill et al., 2021). In field, the plants were planted in subplots (2 m x 2 m), each with a different pasture species, with a total of 10 species: *Biserrula pelecinus*, *Chloris gayana*, *Digitaria eriantha*, *Festuca arundinacea*, *Lolium perenne*, *Medicago sativa*, *Phalaris aquatica*, *Rytidosperma caespitosum*, *Themeda triandra* and *Trifolium subterraneum* (Catunda et al., 2021a; Churchill et al., 2021; see Chapters 4 and 5). In a companion study using a glasshouse facility, two of the species from the field site (*Festuca arundinacea* and *Medicago sativa*) were grown in pots (3.7 L, 150 mm diameter, 240 mm height) using field soil (Catunda et al., 2021b; see Chapter 3). A detailed overview of the experimental facilities descriptions and pasture management are reported in Catunda et al. (2021a; field), Catunda et al. (2021b; glasshouse), Churchill et al. (2021; field), and Zhang et al. (2021; glasshouse). The ten different pasture species used in this study include a range of plant functional groups (C₃ legumes; C₃/C₄ grasses; annuals and perennials) and origins (tropical and temperate; introduced and native) and are commonly used as forage in grasslands in southeastern Australia and, except for the grass *Rytidosperma caespitosum*, internationally

(**Table 2.1**). Plants were grown under a wide range of environmental conditions in the field and glasshouse experiments, including control, warming and/or drought treatments, while other conditions were held constant (soils, fertilization, pests, etc.). Each species had 6 replicates per treatment in the field facility (Catunda et al., 2021a; Churchill et al., 2021; see Chapters 4 and 5) and 8 replicates per treatment in the glasshouse (Catunda et al., 2021b; see Chapter 3). Experimental droughts consisted of a 60% reduction in soil water content from maximum soil water holding capacity (glasshouse experiment) or winter and spring periods of 60% rainfall reduction (field experiment). Warming treatments included +4 °C above ambient temperature (glasshouse experiment) or a continuous +3 °C above-ambient canopy temperature (field experiment). These experimentally manipulated conditions maximised the range of variation in the concentrations of chemical components in the plant samples (Catunda et al., 2021a; Catunda et al., 2021b; see Chapters 3, 4 and 5).

Plant samples from the field were collected throughout a period of 2 years and 4 months (from November 2017 to March 2020) in regular harvests of aboveground biomass based on cut and carry recommendations used by local farmers (Clements et al., 2003); perennial species were harvested 3-5 times per year and annual species 2-3 times (Clark et al., 2016). Samples from the glasshouse study were collected in June and August 2018 following the same harvest protocol adopted in the field. Swards from the field and glasshouse were cut at 5 cm above the soil surface and weighed (fresh weight). Each sample collected in the field facility was composed of 100 g of fresh biomass per subplot (2 m x 2 m) per species, while from the glasshouse, the samples were composed of the total aboveground biomass present in the entire pot (varied from 5 to 27 g/pot). The representative samples were composed of leaves, stems/tillers, and flowers when present, as well as a mixture of both live and dead material as present. Weeds were excluded of the samples. A total of 2,622 samples (2,238 from the field

and 384 from the glasshouse) were collected and used for evaluation as described below (**Table 2.1**).

Table 2.1. Information about pasture species, climate conditions and sample number included in the study.

Type	Photosynthetic Pathway	Common name	Latin name (cultivar)	Origin	Lifecycle	Climate conditions [#]	Sample number
Legumes*	C ₃	Biserrula	<i>Biserrula pelecinus</i> (Casbah)	Temperate, introduced	Annual	Control and drought	139
	C ₃	Lucerne	<i>Medicago sativa</i> (SARDI 7 series 2)	Temperate, introduced	Perennial	Control, drought and warming	542
	C ₃	Sub-clover	<i>Trifolium subterraneum</i> (Campeda)	Temperate, introduced	Annual	Control, drought and warming	29
Grasses	C ₃	Phalaris	<i>Phalaris aquatica</i> (Holdfast GT)	Temperate, introduced	Perennial	Control, drought and warming	332
	C ₃	Ryegrass	<i>Lolium perenne</i> (Kidman)	Temperate, introduced	Annual	Control and drought	92
	C ₃	Tall Fescue	<i>Festuca arundinacea</i> (Quantum II MaxP)	Temperate, introduced	Perennial	Control, drought and warming	429
	C ₃	Wallaby	<i>Rytidosperma caespitosum</i>	Temperate, native	Perennial	Control, drought and warming	185
	C ₄	Digit	<i>Digitaria eriantha</i> (Premier)	Tropical, introduced	Perennial	Control and drought	300
	C ₄	Kangaroo grass	<i>Themeda triandra</i>	Tropical, native	Perennial	Control, drought and warming	417
	C ₄	Rhodes	<i>Chloris gayana</i> (Katambora)	Tropical, introduced	Perennial	Control and drought	157

*Legumes received appropriate rhizobium inoculant during sward establishment: ALOSCA granular inoculant for *Biserrula pelecinus* (Group BS; ALOSCA Technologies, Western Australia, Australia); Easy Rhiz soluble legume inoculant and protecting agent to *Medicago sativa* (Group AL; New Edge Microbials, New South Wales, Australia); and NoduleN for the *Trifolium subterraneum* (Group C; New Edge Microbials).

[#]A detailed overview of the climate conditions is reported in Catunda et al. (2021a; field), Catunda et al. (2021b; glasshouse), Churchill et al. (2021; field), Zhang et al. (2021; glasshouse) and Chapters 3, 4 and 5.

2.2.2 Sample preparation and spectral data collection

Forage samples were either immediately frozen (-18 °C) and later freeze-dried (for the glasshouse facility) or microwaved at 600 W for 90 seconds to deactivate enzymes (Landhäusser et al., 2018), followed by oven-drying at 65 °C for at least 48 hours (for the field facility). To homogenize samples, dried plants were ground through a 1-mm screen in a laboratory mill (Foss Cyclotec Mill, Denmark) and stored in airtight plastic containers in the dark at room temperature prior to the collection of near-infrared reflectance spectra and wet chemical analysis. For the nitrogen analysis, plant samples were ground further, using a ball-mill to produce a fine powder (Retsch® MM200; Hann, Germany).

Near-infrared spectra were collected on a FOSS XDS Rapid Content™ Analyzer with XDS near-infrared technology (FOSS Analytical, Hilleroed, Denmark). All samples were analysed in the range of 400-2500 nm with a spectral resolution of 0.5 nm. Spectra were acquired with ISIscan™ Routine Analysis Software (Foss, Denmark). Samples were presented to the NIR in circular quartz sample cups, non-rotating, with a minimum sample diameter of 21.6 mm and scanned sample area of 233.7 mm². Each spectrum was the average of 32 scans per sample. Samples were repacked and spectra collected in duplicate, and the average of the two spectra was used for subsequent calibrations and predictions. A subset of 391 representative samples was selected for determining nutritional composition by wet chemistry using the ‘select’ function in the software WinISI 4.8.0 (FOSS Analytical A/S, Denmark). The selected samples for wet chemical analyses were representative across sample collection periods, field and glasshouse settings, functional diversity and species’ origin. Furthermore, the subset covered the range of spectral variation in the full scanned population of plant samples, summarized by a principal component analysis to minimize spectral redundancy.

2.2.3 Wet chemical analysis

Samples were subjected to analyses of dry matter (DM) and ash (ASH) according to the official methods and procedures for animal feed outlined by the Association of Official Analytical Chemists (AOAC, 1990). Nitrogen (N) concentration was determined from ~ 100 mg of the sample using an automated combustion method with a Leco TruMac CN analyzer (Leco Corporation, USA). Crude protein (CP) concentration was then calculated by applying a 6.25 conversion factor to the N concentration (AOAC, 1990). Ether extract (EE) was determined according to the American Oil Chemists' Society (AOCS) high-temperature method using petroleum ether (B.P. 40-70 °C) and the Soxhlet method (Buchi 810 Soxhlet Multihead Extract Rack, UK). Fibre fractions were determined with an ANKOM Fibre Analyzer (model 200, ANKOM® Technology, NY, USA) with the use of neutral and acid detergent solutions and correction for dry matter content (Goering and Van Soest, 1970). The samples were analysed for neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) by the sequential method of Van Soest and Robertson (1980). Sodium sulphite and α -amylase were added to the solution for NDF determination. Each sample was analysed in duplicate and nutrient concentrations were expressed on a DM basis (as a percentage).

2.2.4 Calibration development and statistics

NIRS calibration models were established using WinISI software version 4.8.0 (FOSS Analytical A/S, Denmark). We adopted two calibration strategies (**Figure 2.1**): 1) global and 2) plant functional group-based. For the global calibration models development, samples from all pasture species were subjected to wet chemistry analysis ($n = 391$), and randomly assigned to either a calibration set ($n = 313$ samples) or an external validation set ($n = 78$ samples). We also investigated the performance of dedicated calibration models for each of three functional group categories: (1) C_3 legumes, (2) C_3 grasses and (3) C_4 grasses (**Figure 2.1**). For each of

these plant functional group models, 80% of the chemistry/spectra pairs were used for the calibration set and the remainder were kept for external validation.

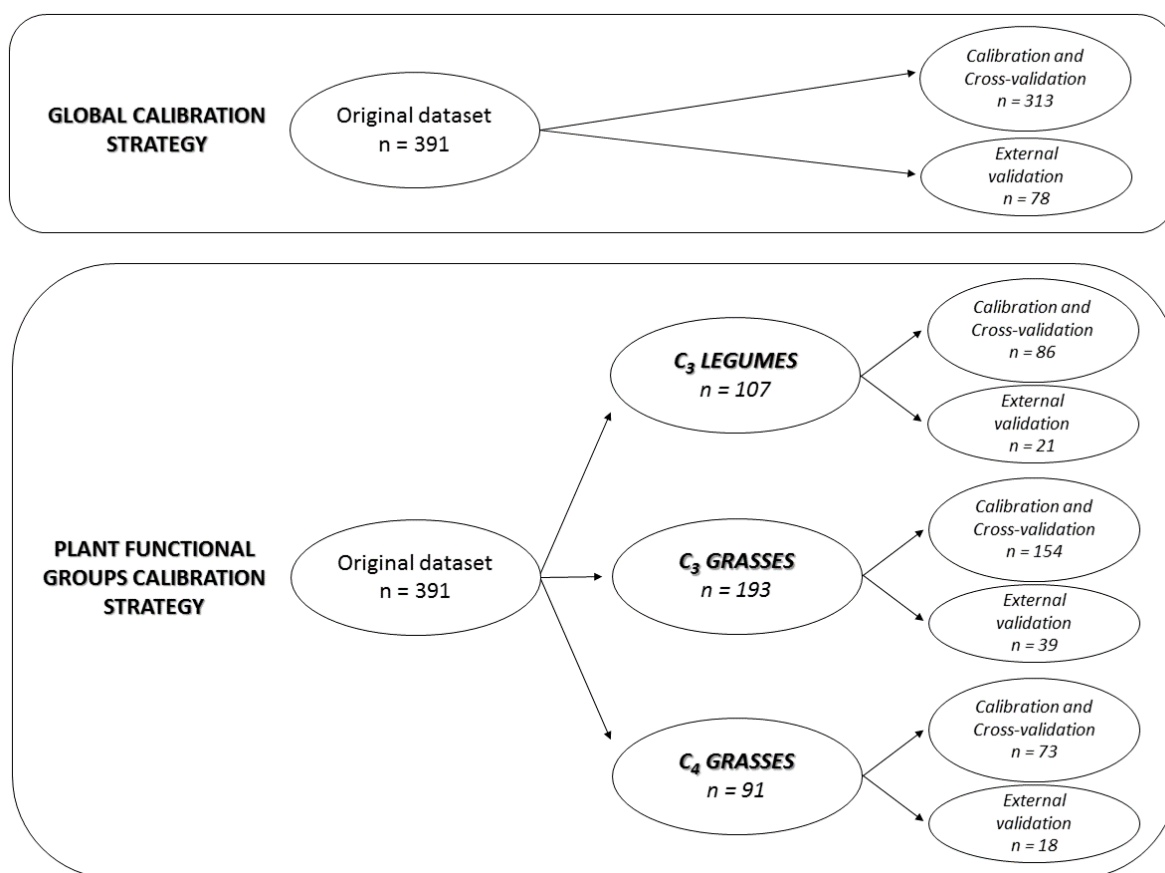


Figure 2.1. Summary of the datasets, calibration and validation processes used in two calibration strategies: a global calibration that included all species and dedicated plant functional group calibrations that modelled C₃ legumes, C₃ grasses and C₄ grasses separately.

Predictive equations based on samples analysed with wet chemistry were then developed using modified partial least-squares regression (MPLS), with cross-validation to prevent the overfitting of models (Shenk and Westerhaus, 1991). The best performing equations were selected by testing a range of scattering pre-treatment options with different derivative gaps and smoothing using the software WinISI 4.8.0. The variety of scattering pre-treatments tested included none, standard normal variate + detrend, standard normal variate only, detrend only, standard multiplicative scatter correction, and weight multiplicative scatters

correction in order to reduce the influence of the sample particle size on NIRS spectra and the path-length variations. The different derivative mathematical pre-treatments tested for each calibration equation to decrease noise effects were coded as follows: “1,4,4,1,” “1,6,4,1,” “1,8,6,1,” “2,4,4,1,” “2,6,4,1,” “2,8,6,1,” “2,10,10,1,” and “3,10,10,1,” where the first digit is the number of the derivative, the second one is the gap over which the derivative is calculated, the third one is the smoothing segment and the last one is the secondary smoothing segment. For the six key nutritional parameters, the best models were selected on the basis of the highest coefficient of determination of calibration (R^2) and the internal cross-validation (one minus the variance ratio, 1-VR), along with the lowest standard error of calibration (SEC) and internal cross-validation (SECV), and the smallest difference between SEC and SECV; this was achieved using the software WinISI 4.8.0 (Andueza et al., 2011; Norman et al., 2020). To compare the predictive ability of calibration equations among different parameters, the coefficient of determination of actual and predicted values (R^2) obtained in the external cross-validation set was evaluated. In addition, to support data interpretation and allow comparison with other studies, we calculated the ratio performance deviation (RPD) by dividing the standard deviation of the reference data by the standard error of prediction (Williams and Sobering, 1996; Williams, 2014). All scatter plots were created using R software, version 4.0.0 (R Core Team, 2020).

2.3 RESULTS AND DISCUSSION

The ranges and means for the six nutritional parameters (ASH, CP, EE, NDF, ADF and ADL) of pasture species used in the calibration models are presented in **Table 2.2**. Our wet chemistry results reveal a broader range of values than those reported in previous studies with forage species (Andueza et al., 2011, Parrini et al., 2019, Parrini et al., 2018), allowing our NIRS calibration models in turn to be applied to samples spanning a broader range of nutritive values.

This may reflect the fact that, as well as comprising a broad range of functional diversity and species origins, our plants were also grown under very varied environmental conditions, including control conditions as well as warming and severe droughts, imposed individually and in combination. However, it is important to note that our NIRS calibration models might not be applicable to other species, in particular tropical legumes, as there were no species from this functional group included in our study.

Table 2.2. Descriptive statistics for the nutritional composition (data expressed as percentage of dry matter) of pasture species analysed with wet chemistry for the global and C₃ grasses calibration models.

Calibrations	Parameter	Range (%)	Mean (%)	Standard Deviation	Standard Error
Global	Ash	3.1 - 19.0	8.1	2.3	0.1
	Crude Protein	1.7 - 32.0	12.0	5.1	0.3
	Ether Extract	0.1 - 7.6	2.9	1.9	0.1
	Neutral Detergent Fibre	22.0 - 84.2	58.2	12.1	0.6
	Acid Detergent Fibre	15.8 - 54.7	31.9	6.0	0.3
	Acid Detergent Lignin	0.3 - 18.3	6.1	3.0	0.2
C ₃ grasses	Ash	3.4 - 18.9	8.7	2.6	0.2
	Crude Protein	2.8 - 27.8	11.5	5.1	0.4
	Ether Extract	0.1 - 7.6	3.3	1.9	0.1
	Acid Detergent Fibre	19.1 - 46.1	30.6	6.1	0.4

Overall, our calibrations accurately predicted the nutritional composition of pasture biomass across a range of species and plant functional groups, which accords with previous studies predicting forage nutritive value by NIRS (Andueza et al., 2011; Smith et al., 2019). Optimal calibrations were obtained using different calibration parameters for each of the key nutritional parameters (**Table 2.3**), including different mathematical spectral pre-treatments for each constituent, although standard normal variate + detrend was the most effective scattering pre-treatment in all cases, as also reported by Barnes et al. (1989).

The samples in our study, across all pasture species, were first value-predicted for each nutritional parameter using equations developed by the global calibrations (**Figure 2.1**), then samples from each plant species group (C₃ legumes, C₃ grasses, and C₄ grasses) were separated and predicted using their respective plant functional group calibrations in order to improve the results that were obtained with the global calibrations. From this step, C₃ legumes and C₄ grasses functional group calibrations did not improve the accuracy of any nutritional parameters compared to results obtained using the global calibrations, however, the C₃ grasses functional group calibration improved predictions, relative to the global calibrations, for some nutritional parameters (ASH, CP, EE and ADF). Norman et al. (2015; 2020) investigated the use of NIRS calibrations to predict the nutritional value of grasses, legumes and forbs, concluding that separating taxonomically similar species into groups did not lead to more accurate predictions than broad, mixed species calibrations. The best-performing calibration models obtained in our study for each nutritional parameter based on optimal wavelengths, mathematical pre-treatments, scattering processing and regression methods are summarized in **Table 2.3** (global and C₃ grasses calibration models).

Table 2.3. Details of treatment of spectra, calibration and validation sets, and performance statistics of the global and C₃ grasses calibration models for nutritional parameters (expressed as percentage of dry matter) of pasture species.

Calibrations	Parameter	Wavelengths (nm)	Mathematical treatment*	Calibration						Validation			
				N	SEC	R ² c	SECV	1-VR	RPD	N	SEP	R ² v	RPD
Global	ASH	400-2492	2,8,6,1	307	0.41	0.96	0.73	0.88	5.0	78	0.91	0.89	3.0
	CP	700-2492	3,10,10,1	309	0.89	0.97	1.13	0.95	5.9	78	1.10	0.95	4.5
	EE	400-2492	2,6,4,1	306	0.54	0.92	0.89	0.79	3.6	78	0.99	0.72	2.0
	NDF	400-2492	2,8,6,1	311	1.78	0.98	2.50	0.96	7.1	78	2.34	0.97	5.9
	ADF	400-2492	2,4,4,1	308	1.41	0.94	1.80	0.89	4.2	78	2.06	0.91	3.3
	ADL	400-2492	3,10,10,1	309	1.14	0.85	1.51	0.73	2.6	78	1.55	0.72	2.0
C ₃ grasses	ASH	400-2492	3,10,10,1	151	0.35	0.98	0.87	0.87	7.1	39	0.73	0.93	3.8
	CP	700-2492	2,4,4,1	153	0.30	0.99	0.73	0.97	10.0	39	0.87	0.97	5.9
	EE	400-2492	2,4,4,1	150	0.43	0.95	0.80	0.83	4.5	39	0.98	0.75	2.0
	ADF	400-2492	2,4,4,1	153	0.83	0.98	1.67	0.92	7.1	39	1.38	0.95	4.5

ASH: ash; CP: crude protein; EE: ether extract; NDF: neutral detergent fibre; ADF: acid detergent fibre; and ADL: acid detergent lignin.

*Mathematical treatment describes the approach used for spectral analysis (stored as $\log(1/\text{reflectance})$). The first two numbers describe the derivative used, the third and fourth numbers indicate the degrees of primary and secondary smoothing performed on the derivative. Thus 2,4,4,1 indicates that the second derivative was calculated with a gap size of 4 nm and that a maximal primary smooth but no secondary smooth was used. N: The number of samples in the calibration or validation set; SEC: standard error of calibration; R²c: coefficient of determination of calibration; SECV: standard error of the internal cross validation; 1-VR: coefficient of determination of the internal cross-validation; RPD: ratio of standard error of performance: standard deviation of calibration or validation; SEP: standard error of prediction; and R²v: coefficient of determination of validation.

Most models used wavelengths from 400-2500 nm, except for CP, which used wavelengths from 700-2500 nm (**Table 2.3**). Among the chemical constituents, different chemical bonds absorb at different wavelengths, thus identifying these regions located in the spectrum contributes to better estimation of the concentration of these nutritional parameters (Foley et al., 1998). In addition, the best calibration statistics in our study were found using the second derivative mathematical treatment for most of the parameters calibration models, whereas the third derivative treatment was best for CP and ADL in the global calibration and ASH for the C₃ grasses calibration models (**Table 2.3**).

The global calibrations had high accuracy and predictive power in both internal (cross) and external validation for ASH, CP, NDF and ADF (**Table 2.3; Figure 2.2**). The accuracy was lower for EE and ADL than for other parameters examined in this study (**Table 2.3; Figure 2.2**), and this may be due to the occurrence of samples with low concentrations of these constituents (**Table 2.2**). The low concentrations found here could cause changes in the spectrum wavelength and absorption, making it difficult to measure using NIRS, compared to the other nutritional parameters, as reported by Roberts et al. (2004). Only a few studies have reported NIRS calibrations for EE and ADL nutritional parameters, making comparison and clarification challenging; this may reflect a general difficulty in achieving satisfactory calibrations. Berauer et al. (2020) in a study with 512 (calibration set) bulk samples of European species-rich montane pastures, reported similar predictions for EE ($R^2c = 0.86$, $R^2v = 0.73$). In contrast, Parrini et al. (2018) presented calibration models using 105 bulk pasture samples in their calibration set collected from Tuscany (Italy) that were able to predict EE and ADL with higher accuracy than in our study (for both parameters $R^2c = 0.99$, $R^2v = 0.98$). In the latter case, the small number of samples used may have contributed to the low variability of values between samples used in the calibration sets and lower errors in the associated predictions.

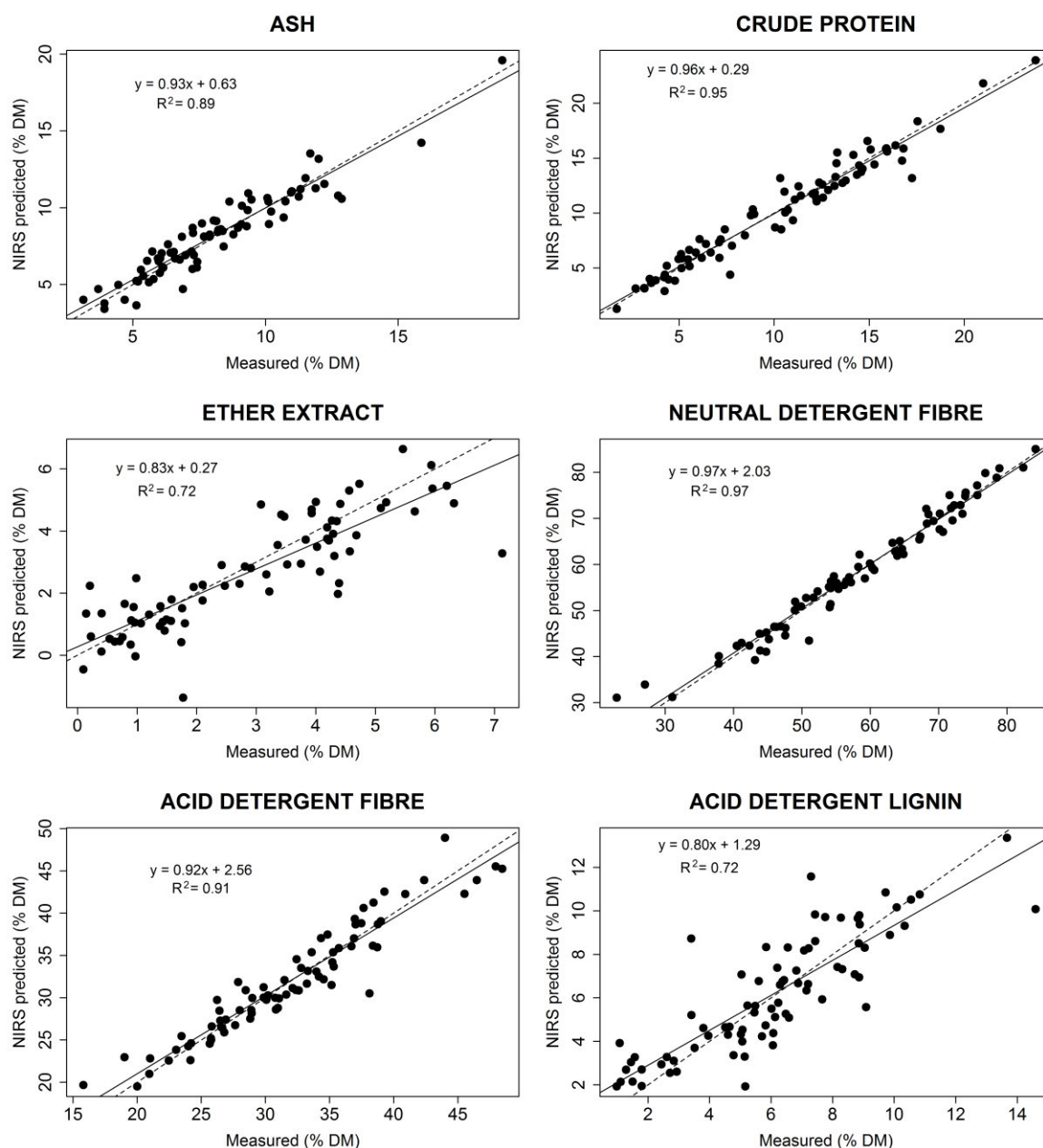


Figure 2.2. Relationships between values measured in laboratory analysis and values predicted by NIRS in the external validation set using the global calibrations for nutritional parameters (ash, crude protein, ether extract, neutral detergent fibre, acid detergent fibre and acid detergent lignin), expressed as a percentage of dry matter (% DM). Solid lines indicate ordinary least-squares linear regressions, and dashed lines show a 1:1 relationship.

Plant functional groups are widely used to describe trait variation within and across plant communities (Thomas et al., 2019). Despite the widespread use of plant functional groups to describe common plant morphological, physiological, biochemical and phenological traits (Pérez-Harguindeguy et al., 2013), in our study, calibration models based on plant functional groups were only superior to the global calibrations for C₃ grasses. The predictions for C₃ grass samples were superior using the C₃ grass functional group calibration models for ASH, CP, EE and ADF (Table 2.3; Figure 2.3).

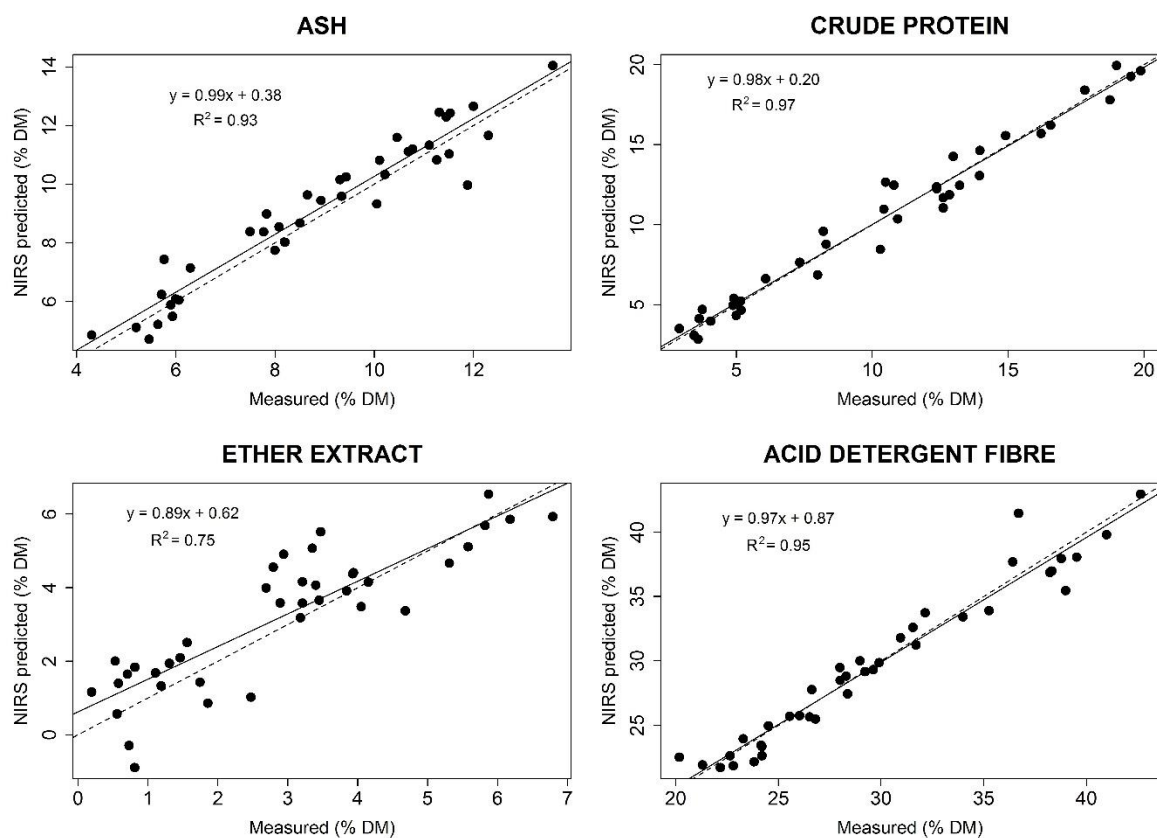


Figure 2.3. Relationships between values measured in laboratory analysis and values predicted by NIRS in the external validation set using the C₃ grass functional group calibrations for nutritional parameters (ash, crude protein, ether extract, acid detergent fibre) of C₃ grasses, expressed as a percentage of dry matter (% DM). Solid lines indicate ordinary least-squares linear regressions and dashed lines show a 1:1 relationship.

In order to evaluate the accuracy of a calibration model and to allow standard comparison with other studies, we calculated the RPD (ratio performance deviation), a non-dimensional statistic for the quick evaluation and classification of NIR spectroscopy calibration models which has been widely used in NIRS studies (Williams and Sobering, 1996; Williams, 2014). In our study, RPD values from the validation set for ASH, CP, NDF and ADF (**Table 2.3**) were acceptable for quality control, ranging from 3 for ASH (global calibrations) to 6 for the NDF (global calibrations) and CP (C_3 grasses calibrations) equations, with respectively “good” to “excellent” classification according to Williams (2014). The high accuracy found in our study for predictions of NDF and CP may be related to the wide range of concentrations in biomass samples included in this study, as also reported by Andrés et al. (2005) in predictions of forage species from Leon (Spain). RPD values (validation set) for EE (global and C_3 grasses calibration models) and ADL (global calibrations) were lower (both RPD = 2) than the other parameters, although these are considered acceptable for screening purposes (Williams, 2014). For Australian forage species, Norman et al. (2020) developed calibration models that included 3 years of data collection of forage species, and found RPD values for validation datasets of CP, NDF and ADF that are comparable to those we report here; however, they did not report EE and ADL parameters, so comparison for these is not possible. Other studies with multiple forage species showed lower NIRS predictive ability than our models for specific nutritional parameters such as ASH (Andueza et al., 2011; Berauer et al., 2020; Norman et al., 2020), CP (Andueza et al., 2011; Berauer et al., 2020), EE (Berauer et al., 2020), NDF and ADF (Smith et al., 2019).

Our NIRS calibrations provided satisfactory accuracy (predictive power) to be able to detect changes in forage nutritive value of the studied pasture species associated with differences in phenology and warming and/or drought scenarios over the 2 years of sample collections. These characteristics qualify our calibrations to assess the effects of seasonality on

forage nutritive value and suggest the feasibility of future development of real-time in-field NIRS monitoring approaches to monitor seasonal and interannual changes in nutrient concentrations of pasture species (Bell et al., 2018; Murphy et al., 2021). These abilities will help farmers/industry to assist with regular feed management decision-making, including in the face of climate change and associated climate extremes, such as are increasingly being experienced across Australia and, indeed, worldwide.

2.4 CONCLUSIONS

This study showed that global NIRS calibrations for a diverse range of pasture species were able to predict multiple key nutritional parameters. Predictions of nutritional metrics for C₃ grass biomass were improved by using a plant functional group-specific calibration for ash, crude protein, ether extract and acid detergent fibre, whereas those for C₃ legumes and C₄ grasses were accurately predicted using the global calibrations. In addition, our calibrations explicitly capture the range of variation in forage nutritive value brought about by warming and drought treatments in this suite of pasture species. High-quality, accurate NIRS calibrations are an essential tool to help rapidly track/monitor forage nutritive value changes in response to management interventions and climate conditions, consequently improving pasture management practice in the future.

CHAPTER 3: Short-term drought is a stronger driver of plant morphology and nutritional composition than warming in two common pasture species

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Author Contributions: KLMC, ACC and HZ collected samples. KLMC processed and analysed the samples, conducted statistical analyses (with input from ACC and HZ) and wrote the manuscript. All authors designed the experiment and contributed to manuscript development.

3.0 ABSTRACT

Under warmer and drier future conditions, global livestock and dairy production are threatened by impacts on the productivity and nutritive value of pastures. However, morphological and nutritional adjustments within plants in response to warming and drought vary among species, and less is known about how these relate to production and forage nutritive value. To investigate this, we grew two common pasture species, tall fescue (*Festuca arundinacea*: grass) and lucerne (*Medicago sativa*: legume), in a climate-controlled facility, under different temperatures (ambient and elevated) and watering regimes (well-watered and droughted). We found that drought had a strong negative impact on biomass production, morphology and nutritive value, while warming only significantly affected both species when response metrics were considered in concert, although to a lesser degree than the drought. Furthermore,

interactions between warming and drought were only seen for lucerne, with a reduction in biomass and an increase in dead material and dry matter. In tall fescue, drought had bigger impacts on nutritional composition than on morphological traits, while in lucerne, drought affected all morphological traits and most nutritional parameters. These findings suggest that in future climate scenarios, drought may be a stronger driver of changes in the morphology and nutritional composition of pasture grasses and legumes, compared to modest levels of warming.

3.1 INTRODUCTION

As the climate changes, more extreme and frequent periods of heat stress and water deficiency have become the most common and critical limiting factors for productivity and nutritive value (i.e. nutritional composition and digestibility) of pastures and grasslands across the globe (Chang-Fung-Martel et al., 2017; Deléglise et al., 2015; IPCC, 2014). Grazing livestock require a reliable and consistent supply of forage of high nutritive value (Herrero et al., 2013; Lee et al., 2017) to achieve high animal performance and maintain profitable production (Dairy Australia, 2018; Laca et al., 2001). However, research suggests that pasture systems will be challenged to meet demands for forage in some regions of the world as predicted climate change will impact annual pasture production by driving shifts in plant phenology and increasing the inter-annual variability of production. Both of these outcomes pose an increased climate risk to the dairy and meat industries (Perera et al., 2020; Rojas-Downing et al., 2017). Along with changes in pasture productivity, there are also potential shifts in the morphological traits and nutritional composition of pasture species that are likely to alter forage digestibility (AbdElgawad et al., 2014; Fernández-Habas et al., 2021; Herrero et al., 2015; Howden et al., 2008). In contrast to shifts in productivity, these changes and their consequences under future climate scenarios, such as warming and drought, are relatively understudied.

Warming can affect plant growth directly, with the nature of the response dependent upon the optimal temperature of a plant species. For example, positive warming effects on growth can be expected for species where production is limited by cold temperatures (Bloor et al., 2010), whereas neutral or negative responses may be more likely in warmer environments (Dukes et al., 2005). Warming above the optimal temperature of the plant may cause temperature stress via direct effects on plant physiology and metabolism and indirectly via increased evapotranspiration and lowered plant water availability (Rustad et al., 2001). These stresses can negatively influence pasture forage nutritive value via affecting morphological traits, such as reduced leaf size, tiller emergence and leaf:stem ratios (Mitchell, 1956; Wilson et al., 1991). In addition, some studies have reported a reduction in the nutritive value of forage under warming (Lee et al., 2017) through increases in concentrations of fibre, and decreases in concentrations of crude protein (Waghorn and Clark, 2004) and non-structural carbohydrates (Habermann et al., 2019; Wilson et al., 1991).

Drought and its associated reduction in soil moisture content result in a wide range of impacts on plant morphology and nutritional composition, with the magnitude of impacts dependent on the developmental stage of plants and the severity and duration of the drought (Gray and Brady, 2016; IPCC, 2014). Severe drought inhibits growth and accelerates maturation of existing plant tissue, death of tillers and leaf senescence that result in decreasing the leaf:stem ratio and an increase in fibre concentration (Bruinenberg et al., 2002; Ren et al., 2016). Additionally, as senescence proceeds, severe water deficits increase nutrient translocation (such as nitrogen and soluble carbohydrates) from leaves to roots, thus reducing the concentrations of nutrients aboveground (Buxton, 1996; Durand et al., 2010). In contrast, moderate drought stress typically induces different morphological responses, including delays in plant maturation and growth, especially for perennial species, which then results in only mild to moderate leaf loss (Buxton, 1996). Studies focused on nutritional responses have reported

seemingly idiosyncratic changes among different nutritional parameters, such as no effect or reductions in fibre concentrations, and no effect or slight improvements in crude protein concentrations and digestibility of forage under moderate water deficit conditions (Bittman, 1988; Buxton, 1996; Deleglise et al., 2015; Dumont et al., 2015; Kuchenmeister et al., 2013; Staniak and Harasim, 2018). In these cases, unchanged or reduced fibre concentrations could be explained by reduced growth and increases in leaf:stem ratio reported under moderate conditions (Bruinenberg et al., 2002; Deleglise et al., 2015). Increased crude protein concentrations could be attributed to delayed maturation and lower biomass production under moderate water limitation, allowing nutrients such as nitrogen to become concentrated in plant tissues (Dumont et al., 2015; Grant et al., 2014).

While studies have been conducted on the responses of species to a single climate change variable, there is a lack of information as to what may happen with concurrent changes in temperature and water availability (Borjas-Ventura et al., 2019; Chang-Fung-Martel et al., 2017; Vicedo et al., 2021). In addition, how morphological and nutritional adjustments can affect each other in different plant species under climate change scenarios, especially how warming and drought interact within pasture species, are not well understood and need to be investigated. To address these research gaps, we conducted a study in a climate-controlled facility to investigate the effects of warming (+4 °C) and short-term drought (60% reduction in soil water content from maximum soil water holding capacity), alone and in combination, on plant morphological traits and nutritional composition of two important temperate pasture species: tall fescue (*Festuca arundinaceae* – a C₃ grass) and lucerne (*Medicago sativa* – a C₃ legume). These species have been widely cultivated due to their value as high-quality perennial forage pastures that are adapted to a wide range of growing conditions. They show reasonable potential to tolerate drought (Bouton, 2012; Gibson and Newman, 2001) and exhibit optimum growth temperatures of 15-30 °C for tall fescue and 15-25 °C for lucerne (Hill et al., 1985;

Mcdonald et al., 2003). We hypothesized that warming and drought, as isolated factors, will negatively affect biomass production, morphological traits and nutritive value, with the magnitude dependent on species-specific responses. We further predict that drought in combination with warming will have a more pronounced negative impact on plant morphological and nutritional traits than as isolated climate factors, as warming can exacerbate the negative effects of water stress on plants.

3.2 MATERIALS AND METHODS

3.2.1. Site and experimental design

The experiment was conducted between mid-April and early-August 2018 in four climate-controlled glasshouse chambers located at the Hawkesbury Campus of Western Sydney University, Richmond, New South Wales, Australia (33°36'40" S, 150°44'43" E). Two agriculturally important temperate pasture species were used in this experiment: the C₃ grass, tall fescue (*Festuca arundinacea* Schreb., Cultivar: Quantum II MaxP, sourced from Elders Australia) and the C₃ legume, lucerne also known as alfalfa (*Medicago sativa*, Cultivar: SARDI 7 Series 2, sourced from Heritage Seeds Australia). These are the same cultivars used in a parallel field experiment manipulating rainfall and temperature, and data associated with the performance of these species in the field manipulation experiment are reported in Churchill et al. (2021). The two-factor experimental design included two temperatures (ambient, aT; elevated, eT) and two watering regimes (well-watered, W; droughted, D), giving four treatment combinations (aT.W, aT.D, eT.W, eT.D), each with eight replicate pots and thus 32 pots per species. Watering regimes were nested within the temperature treatments. Treatment replicates were divided evenly among four chambers, with two chamber replicates maintained at aT and the other two at eT. Pot positions within chambers were re-randomized every two weeks to minimize potential within-chamber effects.

3.2.2. Plant growth conditions and treatments

We collected field soil from the Pastures and Climate Extremes (PACE) field experimental facility, also located on the Western Sydney University Hawkesbury Campus (33°36'S, 150°44'E; Churchill et al., 2021). The soil used in this experiment had a sandy-loam texture (~84% sand) with an average pH of 5.6, plant available N of 55 mg kg⁻¹, plant available (Bray) P of 39 mg kg⁻¹ and 1.1% soil organic carbon (Zhang et al., 2021). The soil was sieved (5 mm), air-dried and mixed with quartz sand (7:3, v/v), then 3.9 kg of soil was placed into each plastic pot (3.7 L, 150 mm diameter, 240 mm height). Seeds of each species were surface-sterilized with 1.25% NaOCl for 10 min, rinsed with deionized water 10 times and germinated in Petri dishes with sterilized water for 1 week. Five germinated seedlings were transplanted into each pot and then thinned to four healthy individuals per pot after 2 weeks. The legume pots were supplied with appropriate rhizobia (Easy Rhiz soluble legume inoculant, Group AL, New Edge Microbials, New South Wales, Australia) necessary for nodulation (Zhang et al., 2021). As a legume, lucerne is dependent upon rhizobia to meet its nitrogen demand. Inoculation is a standard and recommended practice in agricultural/pasture systems (Adam et al., 2016; Gemell and McDonald, 2017).

Temperature treatments were initiated at the same time as transplantation. The temperature treatments included an ambient regime (aT; 26/18 °C day/night) and an elevated temperature regime (warming) with + 4 °C warming (eT; 30/22 °C day/night) using a 15:9 light: dark cycle. Humidity was controlled at 60% and the effectiveness of achieving the temperature conditions within the glasshouse chambers can be seen in **Supplementary Table S3.1**. The ambient regime reflected the average daily maximum temperature for the site over the previous 20 years (Richmond RAAF site; Australian Bureau of Meteorology, 2018), and the elevated regime represented the predicted maximum temperature increase of 4 °C for this region within this century (Pearce et al., 2007).

To maintain consistent water availability, an automated irrigation system was used to ensure pots were well-watered every second day to 100% water holding capacity (WHC, i.e. until drainage just occurred after watering events). All pots were maintained at well-watered conditions until the two watering regimes (well-watered and droughted) were initiated three weeks before the final harvest. Pots in well-watered treatments were maintained in well-watered condition as before. In contrast, pots in drought treatments had watering withheld for 4 days (until the majority of the plants started to wilt). Thereafter, we weighed pots every other day to maintain the drought treatment pots at 40% WHC for one week (adding an appropriate amount of water when necessary). After one week, we re-watered the drought treatment pots to bring the soil water condition back to 100% WHC. This oscillating drought regime (shifting between 100% and 40% WHC) was repeated for the remaining three weeks of the experiment, ending just prior to the final harvest. Within each watering regime, pots under aT and eT were maintained at similar WHC, to be able to test the direct effects of warming and minimize the interactive influence of warming on soil water content. Following typical pasture management practices, aboveground biomass was clipped at 5 cm above the soil surface at 8 weeks after planting (before imposing the drought treatment), and allowed to regrow until the final harvest. Two weeks after the clipping, we applied fertilizer (containing KNO_3 and KH_2PO_4 , resulting in a fertilizer pulse of 30 kg N ha^{-1} and 5 kg P ha^{-1}).

3.2.3. Harvest and morphological traits measurements

Immediately prior to harvest, plant height and number of tillers/stems per pot were measured for both species. Plant height (cm) was measured in each pot from ground level to the tip of the tallest plant; subsequently, the numbers of tillers (grass) and stems (legume) from each pot were counted. The percentage of standing aboveground biomass that was dead was estimated visually and assigned to five classes (0%, 25%, 50%, 75%, 100%), ranging from the entire

plant being healthy to all aboveground plant tissue being senesced. To harvest aboveground biomass, plants were cut at the level of the soil surface, the biomass was weighed, immediately frozen (-18 °C) and later freeze-dried, then weighed again to determine the total dry biomass (g/pot). Dry biomass of lucerne was sorted into leaves (plus flowers when present) and stems, and fractions were weighed to calculate the leaf:stem ratio. Dry leaves of tall fescue could not be distinguished from tillers.

3.2.4. Sample processing

Dried biomass samples were ground to pass through a 1-mm screen in a laboratory mill (Foss Cyclotec Mill, Denmark) and stored in airtight plastic containers in the dark at room temperature prior to collection of near-infrared reflectance spectra and wet chemistry analysis. Prior to nitrogen analysis, subsamples of biomass from each pot were reground to a fine powder using a ball-mill (Retsch® MM200; Hann, Germany). Nutritional analysis was performed on the total aboveground harvested material (a mixture of leaves, stems/tillers and flowers when present), including a mixture of both live and dead material.

3.2.5. Nutritional analysis

All dried and ground samples were each scanned twice and their spectra were collected and averaged using near-infrared reflectance spectroscopy (NIRS; FOSS XDS Rapid Content™ Analyzer) with a spectral range of 400 to 2500 nm (more details are reported in Chapter 2). Half of the samples for each species (4 replicates per treatment and per species) were selected for determining nutrient composition by wet chemistry for all parameters, except for dry matter and crude protein contents (for which all samples were analysed). Samples for wet chemistry were selected using the ‘select’ function in the software WinISI 4.8.0 (FOSS Analytical A/S, Denmark) to represent the range of spectral variation in the population, summarized by a

principal component analysis to minimize redundancy in spectra (Catunda et al., 2021c; see Chapter 2).

For wet chemistry, the selected samples were subjected to analyses of dry matter (DM) and ash (ASH) according to the standard methods and procedures for animal feed outlined by the Association of Official Analytical Chemists (AOAC, 1990). Nitrogen (N) concentration was determined from ~ 100 mg samples using an automated combustion method on a Leco TruMac CN-analyzer (Leco Corporation, USA). Crude protein (CP) concentration was then calculated by applying a 6.25 conversion factor to the N concentration (AOAC, 1990). Ether extract (EE) was determined according to the American Oil Chemists' Society-AOCS high-temperature method using petroleum ether and the Soxhlet method (Buchi 810 Soxhlet Multihead Extract Rack, UK). Fibre fractions were determined with an ANKOM Fibre Analyzer (model 200, ANKOM® Technology, NY, USA) with the use of neutral and acid detergent solutions and corrected for dry matter content (Goering and Van Soest, 1970). Samples were analysed for neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) by the sequential method of Van Soest and Robertson (1980). Sodium sulphite and α -amylase were added to the solution for NDF determination. The concentration of hemicellulose (HEM) was calculated by the difference between NDF and ADF concentrations after sequential analysis, while the cellulose (CEL) concentration was calculated as the difference between ADF and ADL. The values of ASH, EE, CP and NDF were used to calculate non-structural carbohydrates (NSC) according to Sniffen et al. (1992). Estimated digestible dry matter (DDM), expressed as percentage of dry matter, was calculated according to *Equation 1* below (Linn and Martin, 1989):

$$\text{Equation 1. } DDM \% = 88.9 - (0.779 \times ADF \%)$$

For the development of NIRS calibration models, modified Partial Least Squares regression with cross-validation was used to develop predictive equations for each nutritional

parameter to prevent overfitting of models (Shenk and Westerhaus 1991). Standard normal variate and detrend mathematical transformations (Barnes et al., 1989) were applied to raw NIR spectra to reduce the influence of particle size, and a variety of derivative mathematical pre-treatments were employed to decrease spectral noise effects. The best predictive models were selected on the basis of the highest coefficient of determination of calibration (R^2) and the internal cross-validation (one minus the variance ratio, 1-VR), and the lowest standard error of calibration (SEC) and internal cross-validation (SECV), and the smallest difference between SEC and SECV (Norman et al., 2020; Catunda et al., 2021c; see Chapter 2). The best models developed for each nutritional parameter were used to predict the other half of the samples. The mathematical treatment of spectra and descriptive statistics for NIRS calibrations can be found in Catunda et al. (2021c; see Chapter 2).

3.2.6. Calculations and statistical analysis

Plant biomass, morphological traits and nutritional composition (expressed as a percentage of dry matter) were analysed statistically using linear mixed-effects (LME) models in the ‘lme4’ package in R (Bates et al., 2015). Temperatures (T), watering regimes (W), and their interactions (T x W), were defined as fixed effects and the glasshouse chambers were specified as a random factor. Residuals were checked for normality and we applied log-transformation to the percentage of dead material as a continuous response. Data for each species were analysed separately and we were not explicitly interested in contrasting the two species. We calculated the mean effect size due to drought (response ratio) based on the ratio of each mean value in the drought treatments (D) to the mean value in the well-watered treatments (W) at each temperature level ($aT = \text{Equation 2}$; $eT = \text{Equation 3}$) along with 95% confidence intervals (CI). In the effect size figures, positive values indicate that droughted treatments had higher values than in well-watered treatments for the respective temperature level, while

negative values represent the opposite. We expressed effect sizes as percentages (effect size multiplied by 100) in the text throughout the results section.

$$\text{Equation 2. Effect size} = \frac{aT.D}{aT.W} - 1$$

$$\text{Equation 3. Effect size} = \frac{eT.D}{eT.W} - 1$$

To achieve a more holistic overview of changes brought about by climate treatments on plant response variables for each pasture species that accounts for the non-independence of within-plant chemistry, we performed a multivariate, principal components analysis (PCA). To test for the effects of both temperatures and watering regimes on plant biomass, morphological and nutritional responses, we undertook permutational analysis of variance (PERMANOVA) using the ‘vegan’ package in R. All statistical analyses were carried out using the software R version 3.5.2 (R Core Team, 2019).

3.3 RESULTS

3.3.1. Impacts of warming and short-term drought on plant biomass and morphological responses

Drought, but not warming, significantly affected plant dry biomass or morphological traits for both species (**Table 3.1; Supplementary Table S3.2**). For tall fescue, drought alone (aT.D) significantly decreased biomass by 24% ($p < 0.01$; **Figure 3.1A**) and increased the percentage of dead material by 19% ($p < 0.01$; **Figure 3.2A**). There was, however, no effect on plant height or number of tillers (**Figure 3.1A**). For lucerne, drought significantly ($p < 0.01$ for all parameters) decreased biomass (51%; **Figure 3.1B**), plant height (18%), number of stems (28%) and leaf:stem ratio (40%), as well as increased the percentage of dead material (21%; **Figure 3.2B**). Overall, the negative effect of drought on biomass and morphological traits were stronger in lucerne than that in tall fescue.

Table 3.1. Linear mixed effects models with fixed effects for temperatures (T), watering regimes (W) and their interaction (T x W) for plant dry biomass, morphological traits and nutritional parameters of tall fescue (*Festuca arundinacea*) and lucerne (*Medicago sativa*). Growth chamber was included as a random effect.

Variables	Tall fescue						Lucerne					
	T		W		T x W		T		W		T x W	
	F	p	F	p	F	p	F	p	F	P	F	p
Dry biomass (g/pot)	9.73	0.089	8.36	0.008	1.55	0.224	18.2	0.055	177.4	< 0.001	19.4	< 0.001
Plant height (cm)	0.02	0.890	1.40	0.247	1.82	0.189	0.81	0.464	23.6	< 0.001	0.71	0.408
Number of tillers/stems (n/pot)	4.99	0.155	0.57	0.459	0.56	0.459	0.10	0.777	24.5	< 0.001	0.01	0.932
Dead material (%) †	0.15	0.736	32.1	< 0.001	0.40	0.534	0.11	0.772	38.3	< 0.001	6.22	0.020
Leaf:stem ratio	NA	NA	NA	NA	NA	NA	0.15	0.733	26.8	< 0.001	2.65	0.116
Dry matter (%)	3.70	0.194	13.4	< 0.001	1.79	0.193	0.49	0.557	22.6	< 0.001	14.8	< 0.001
Ash (% DM)	10.1	0.086	10.3	0.003	0.74	0.397	13.5	0.067	0.27	0.606	0.19	0.668
Crude protein (% DM)	9.77	0.089	7.71	0.010	2.71	0.111	16.4	0.057	6.61	0.016	3.38	0.078
Non-structural carbohydrates (% DM)	8.66	0.099	24.4	< 0.001	1.03	0.320	14.7	0.062	4.70	0.040	0.58	0.453
Neutral detergent fibre (% DM)	2.77	0.238	15.7	< 0.001	0.33	0.572	0.02	0.911	8.47	0.007	0.19	0.669
Acid detergent fibre (% DM)	4.80	0.160	25.0	< 0.001	0.34	0.563	0.18	0.711	9.40	0.005	0.10	0.758
Acid detergent lignin (% DM)	0.74	0.479	0.02	0.881	0.47	0.499	0.00	0.983	6.74	0.015	1.04	0.316
Hemicellulose (% DM)	1.15	0.395	4.82	0.037	0.20	0.657	0.20	0.701	0.21	0.653	0.16	0.689
Cellulose (% DM)	4.50	0.168	21.8	< 0.001	0.87	0.359	0.28	0.650	8.84	0.006	0.01	0.939
Digestible dry matter (% DM)	4.80	0.160	25.0	< 0.001	0.34	0.563	0.18	0.711	9.40	0.005	0.10	0.758

†The percentage of dead material data was log-transformed; % DM: percentage of dry matter; NA: not applicable; **Bold** values denote statistical significance at the $p < 0.05$ level.

There was not a significant interaction between temperatures and watering regimes (T x W; **Table 3.1**) for biomass or morphological traits in tall fescue, although for lucerne, the interaction significantly affected biomass ($p < 0.01$; **Figure 3.1B**) and the percentage of dead material ($p = 0.02$; **Figure 3.2B**). Specifically, for lucerne, warming (eT) partially offset the negative effect of drought on biomass (eT.D; -40%; **Figure 3.1B**), but exacerbated its effect on the percentage of dead material (+60%; **Figure 3.2B**).

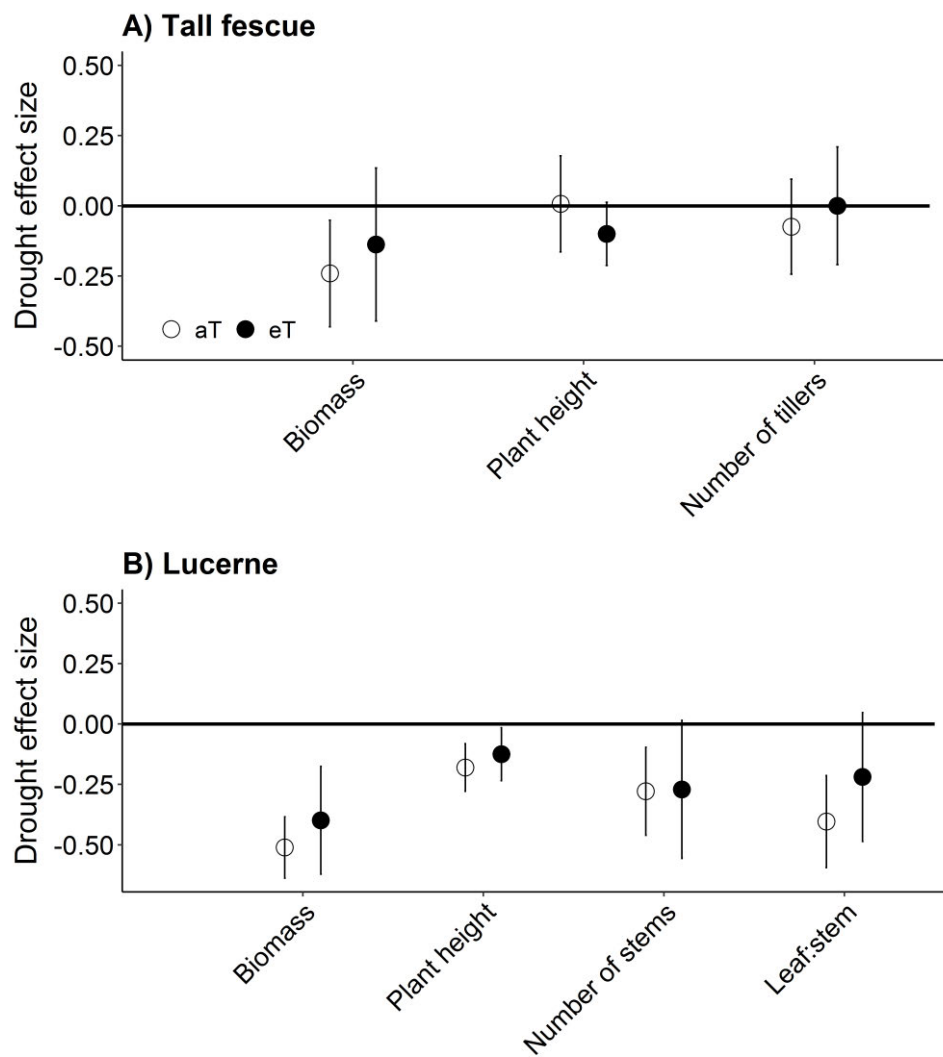


Figure 3.1. Drought effect sizes under ambient (aT, open circle) and elevated (eT, closed circle) temperatures on plant dry biomass and morphological traits of A) tall fescue (*Festuca arundinacea*) and B) lucerne (*Medicago sativa*). Values shown are means with vertical bars representing 95% confidence intervals (n = 8).

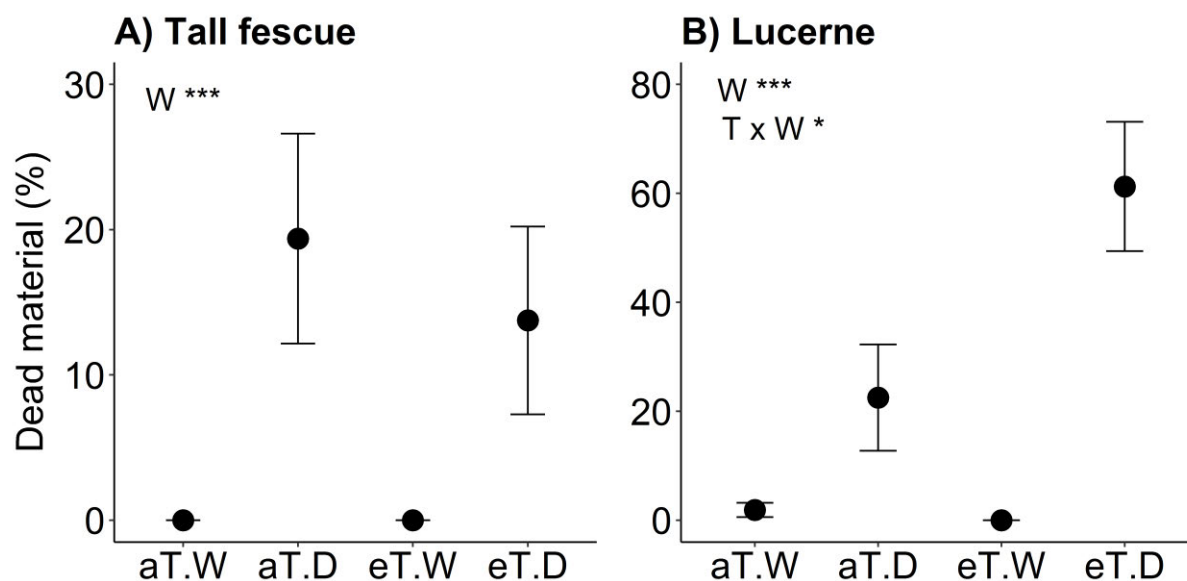


Figure 3.2. Percentage of dead material (%) for A) tall fescue (*Festuca arundinacea*) and B) lucerne (*Medicago sativa*) grown under different temperatures (ambient, aT; elevated, eT) and watering regimes (well-watered, W; droughted, D). Values shown are mean \pm 1 standard error (n = 8). Treatment codes for indicating significance for fixed effects are as follows: T= temperatures, W= watering regimes, and T x W their interaction. Significance levels as follows: * $p < 0.05$, *** $p < 0.001$.

3.3.2. Impacts of warming and short-term drought on nutritional responses

Drought, but not warming, significantly affected nutritional parameters for both species (**Table 3.1; Supplementary Table S3.2**). For tall fescue, drought significantly affected all parameters of nutritional composition, except acid detergent lignin (ADL; $p = 0.88$). Drought resulted in a significant decrease in non-structural carbohydrates (NSC; 12%; $p < 0.01$; **Figure 3.3A**) and digestible dry matter (DDM; 3%; $p < 0.01$), and a significant increase in dry matter (DM; 7%; $p < 0.01$), ash (ASH; 15%; $p < 0.01$), crude protein (CP; 9%; $p = 0.01$), neutral detergent fibre (NDF; 7%; $p < 0.01$), acid detergent fibre (ADF; 11%; $p < 0.01$), hemicellulose (HEM; 4%; $p = 0.04$) and cellulose (CEL; 11%; $p < 0.01$).

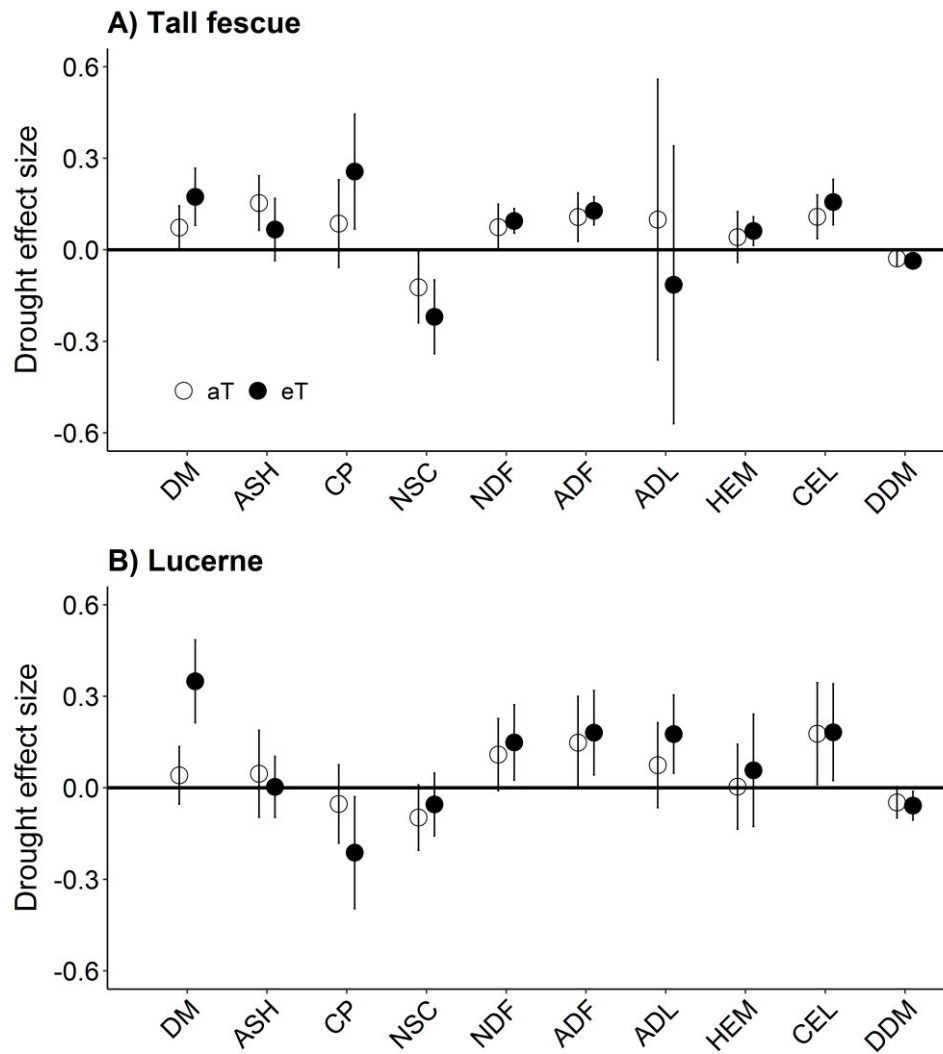


Figure 3.3. Drought effect sizes under ambient (aT, open circle) and elevated (eT, closed circle) temperatures on nutritional parameters of A) tall fescue (*Festuca arundinacea*) and B) lucerne (*Medicago sativa*). Abbreviations are as follows: dry matter (DM), ash (ASH), crude protein (CP), non-structural carbohydrates (NSC), neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), hemicellulose (HEM), cellulose (CEL) and digestible dry matter (DDM). Values shown are means with vertical bars representing 95% confidence intervals (n = 8).

For lucerne, drought significantly affected all nutritional parameters, except ASH ($p = 0.61$) and HEM ($p = 0.65$). In contrast to its effects on tall fescue, drought significantly decreased CP by 5% in lucerne ($p = 0.02$; **Figure 3.3B**) and caused a significant 7% increase in ADL ($p = 0.02$). In addition, compared to the aT.W treatment, drought significantly decreased NSC and DDM by 10% ($p = 0.04$) and 5% ($p < 0.01$), respectively, while significantly increasing DM, NDF, ADF, and CEL by 4% ($p < 0.01$), 11% ($p < 0.01$), 15% ($p < 0.01$) and 18% ($p < 0.01$), respectively.

There was no significant interaction between temperatures and watering regimes for nutritional parameters in either species ($p > 0.05$; **Table 3.1**). However, the combination of warming and drought increased the DM of lucerne by 35% ($p < 0.01$; **Figure 3.3B**), compared with ambient temperature and well-watered plants.

3.3.3. Assessing plant biomass, morphological and nutritional responses to warming and drought in a multivariate context

We used a multivariate approach to assess overall plant responses to the climate treatments. For tall fescue, we found that both temperature (PERMANOVA: $p = 0.018$; **Figure 3.4**) and watering regime (PERMANOVA: $p = 0.001$) significantly influenced plant responses, but there was no interaction (T x W) between treatments (PERMANOVA: $p = 0.600$). However, for lucerne, we found that temperature (PERMANOVA: $p = 0.008$; **Figure 3.5**), watering regime (PERMANOVA: $p = 0.001$) and their interaction (PERMANOVA: $p = 0.020$) significantly influenced plant responses. The ellipses in **Figure 3.4A** and **Figure 3.5A** show statistically significant treatment separation in the trait-space for both species. In tall fescue (**Figure 3.4A**), the most significant separation of these plant responses occurred when under both warming and drought scenarios, while in lucerne (**Figure 3.5A**), the driver separating plant responses were mainly from drought rather than warming.

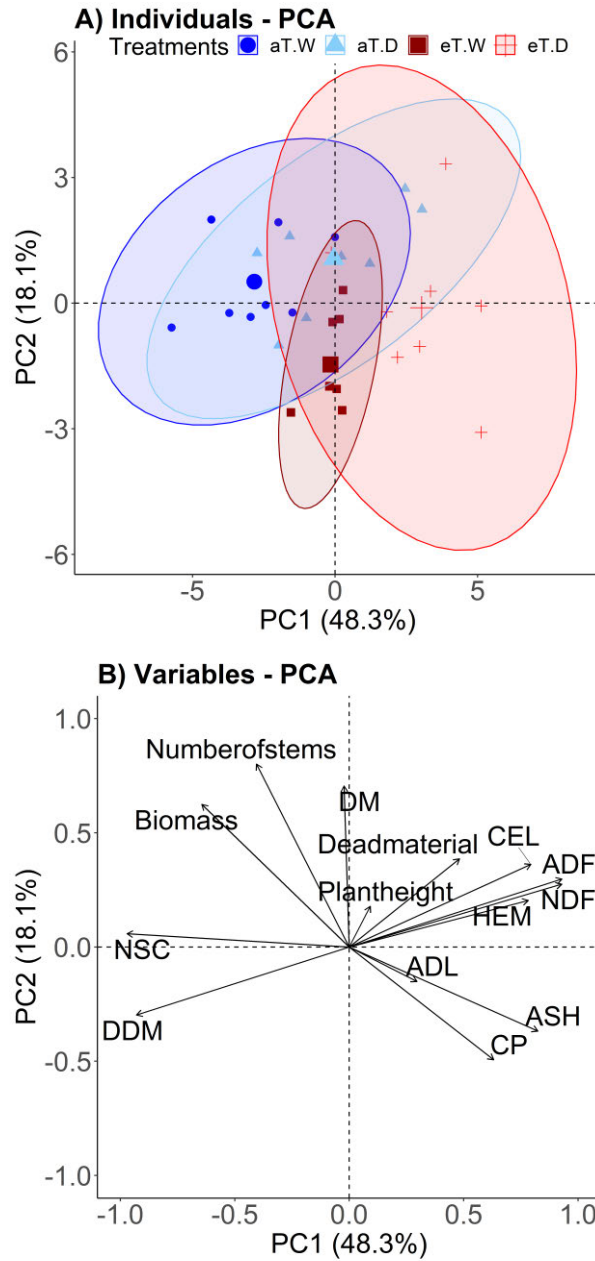


Figure 3.4. Principal component analysis biplot illustrating A) scores for plant individuals, grouped by treatment (ambient, aT; elevated, eT; well-watered W; droughted, D) with 95% confidence ellipses (PERMANOVA: Temperatures $p = 0.018$, Watering regimes $p = 0.001$, Temperatures x Watering regimes $p = 0.600$) and B) variables loadings for tall fescue (*Festuca arundinacea*). Morphological traits include plant height, number of tillers and percentage of dead material. The nutritional parameters follow the abbreviations in Figure 3.3. The symbol shape and colour of each point correspond to climate treatments.

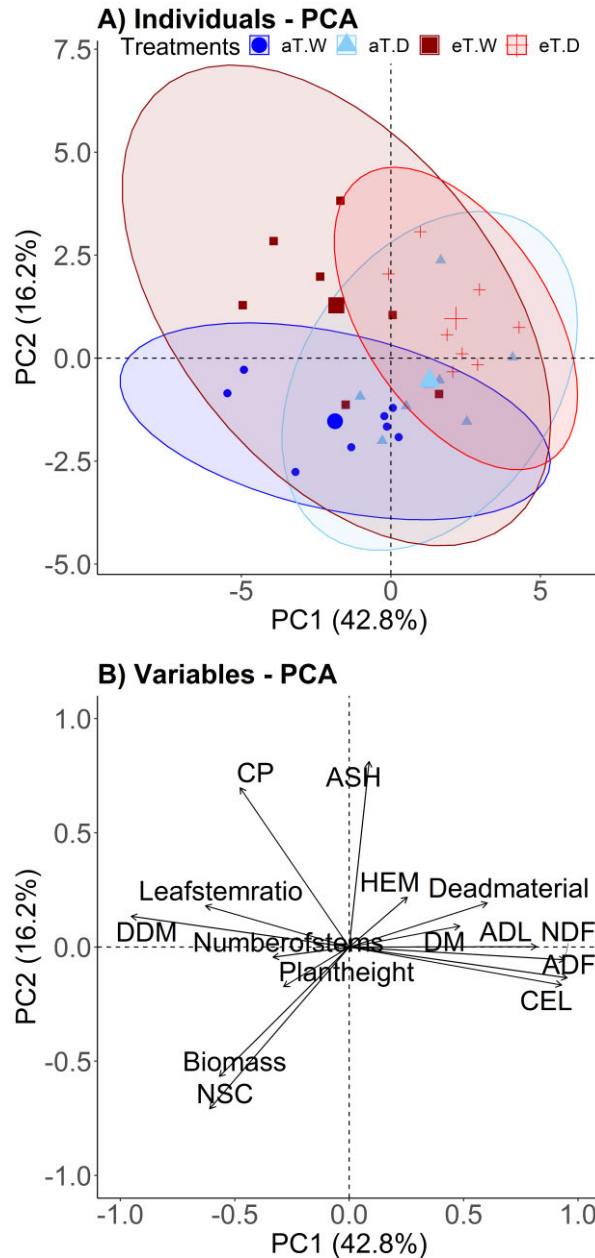


Figure 3.5. Principal component analysis biplot illustrating A) scores for plant individuals, grouped by treatment (ambient, aT; elevated, eT; well-watered, W; droughted, D) with 95% confidence ellipses (PERMANOVA: Temperatures $p = 0.008$, Watering regimes $p = 0.001$, Temperatures x Watering regimes $p = 0.020$) and B) variables loadings for lucerne (*Medicago sativa*). Morphological traits include plant height, number of stems, percentage of dead material and leaf:stem ratio. The nutritional parameters follow the abbreviations in Figure 3.3. The symbol shape and colour of each point correspond to climate treatments.

In tall fescue, the first two principal components explained 66% of the variation across treatments (**Figure 3.4B**). The first principal component (PC1, explaining 48.3% of the data variance) had positive loadings of fibre (NDF, ADF) and ASH, and negative loadings with DDM and NSC (**Figure 3.4B**). The second PC axis (PC2, explaining 18.1% of the data variance) was associated with plant morphological traits and nutritional parameters, with positive loadings for biomass and number of tillers, and negative loadings for CP. In addition, we found that biomass and the number of tillers were negatively correlated with CP, while the percentage of dead material was positively correlated with fibre fractions (ADF particularly) and consequently negatively correlated with DDM (**Figure 3.4B**; **Supplementary Figure S3.1**).

In lucerne, PC1 and PC2 explained respectively 42.8% and 16.2% of the variation in sample biomass, morphological traits and nutritional parameters (**Figure 3.5B**). PC1 (**Figure 3.5B**), had positive loadings for fibre (NDF, ADF, CEL, ADL) and the percentage of dead material, and negative loadings for DDM and leaf:stem ratio. PC2 was associated with higher CP and ASH, and negatively associated with biomass and NSC. Lucerne biomass is positively correlated with NSC and negatively correlated with fibre (CEL particularly), and that fibre (CEL particularly) is negatively correlated with leaf:stem ratio and positively correlated with the percentage of dead material (**Figure 3.5B**; **Supplementary Figure S3.2**).

In addition, for both pasture species (**Figure 3.4**; **Figure 3.5**), the biomass, DDM and NSC were higher for those plants grown under the aT.W treatment, while high fibre concentrations and a greater percentage of dead material were associated with the eT.D treatment. Particularly in lucerne (**Figure 3.5**), eT.W treatment was associated with high leaf:stem ratio and CP. Overall, PC1 showed that the nutritional parameters explained the highest percentage of variance in tall fescue (**Figure 3.4B**), while both the morphological traits and nutritional parameters explained the variance in lucerne (**Figure 3.5B**).

3.4 DISCUSSION

Here, we have determined the effects of warming, short-term drought and their interaction on plant biomass, morphological traits and nutritional composition of two common pasture species (tall fescue and lucerne). We found that exposure of these species to short-term drought resulted in a significant negative impact on biomass production, morphology and nutritive value; warming did not significantly affect individual growth or nutritional parameters, but did have a significant overall effect on both species when assessed in a multivariate context. In addition, we found a significant interaction between warming and drought in lucerne, which resulted in greater differences between well-watered and droughted treatments at elevated than at ambient temperature for dead material and dry matter. These findings demonstrate that drought had far bigger impacts than warming overall, the effects of warming were greater when combined with drought – conditions that more closely reflect predicted climates under which grazing systems will function in the future.

3.4.1. Impacts of warming on plant biomass, morphological and nutritional responses

For both pasture species, we found limited evidence of shifts in plant biomass, morphological, or nutritional responses associated with continuous warming. Previous studies have shown species-specific positive (Bloor et al., 2010; Dieleman et al., 2012), negative (Cantarel et al., 2013; Lee et al., 2017) and neutral (Dukes et al., 2005; Dumont et al., 2015) warming effects on productivity and/or nutritive value in forage species associated with regional climatic differences. A widely anticipated mechanism by which warming can indirectly affect plants is via increased evapotranspiration and consequent reductions in soil water content (Rustad et al., 2001). In our experiment, the eT.W and aT.W treatments were maintained at similar WHC, to be able to isolate the direct effects of air temperature, while minimizing the interactive influence of warming on soil water content. This approach and the resultant lack of significant

warming effects on biomass, morphological traits and nutritional composition suggests that evapotranspiration-mediated indirect effects might be responsible for many of the observed changes in forage productivity and nutritive value attributed to warming under field conditions (Cantarel et al., 2013). In general, in our study, warming was also the minor contributor to interaction effects with drought. Our results suggest that if soil water availability can be maintained under field conditions, then it may be possible to minimise the anticipated negative impacts of rising temperatures on forage nutritive value, at least for the species and temperatures in this study, if not more widely.

3.4.2. Impacts of short-term drought on plant biomass, morphological and nutritional responses

In our experiment, short-term drought significantly decreased biomass while increasing the percentage of dead material for both species, and particularly for lucerne, negatively affecting all morphological traits. In tall fescue, the short-term drought did not alter plant height and the number of tillers, instead, drought influenced plant phenology through accelerated senescence of existing plant tissue. In particular for lucerne, the negative effect of drought on plant height and leaf:stem ratio can be explained by water deficiency having reduced plant growth and accelerated senescence, resulting in relatively more stem material compared to leaves, as also reported in previous studies with pasture species (Bruinenberg et al., 2002; Ren et al., 2016). It is important to mention that immediate responses of rhizobia to water stress may have contributed, to some extent, to changes in the morphology of lucerne in our study, as described by Zahran (1999). These findings suggest that morphological changes in lucerne play a major role in plant adaptation responses under drought stress. Overall, the morphological responses found in our study suggest that these responses must be related to different abilities to tolerate and adapt to drought that are species-specific (Cranston et al., 2016; Lee et al., 2013).

In our study, although drought produced different morphological changes in these two species, a decrease in nutritive value, including digestibility, was common to both. The significant reductions in NSC and increases in fibre (especially cellulose) may be explained by the high percentage of dead material and for lucerne, decreases in leaf:stem ratio that were found in this study. In support of our results, previous studies found that stems are associated with more fibre, higher forage toughness and consequently lower digestibility (Bruinenberg et al., 2002; Buxton, 1996; Durand et al., 2010; Ren et al., 2016). Here, some nutritional responses to drought did differ between species, for example, in terms of fibre fractions such as hemicellulose (increased only in tall fescue) and lignin (increased only in lucerne) concentrations. According to previous studies with forage species, the differences in fibre fractions responses to drought between species may reflect differences in plant structure (Amiri et al., 2012; Pontes et al., 2007). For example, a study showed that legume stem tissue is thick with a high bulk density and is comprised of a considerably larger undegradable fraction like lignin compared to grass species (Amiri et al., 2012). This may have contributed to the bigger drought response on the lignin concentration of lucerne found in our study. Supporting this, lignin concentrations found in our study in lucerne were four times those found in tall fescue.

Also found were species differences in crude protein responses to drought. In tall fescue, CP concentration increase under drought can be explained by trade-offs between concentration and growth, such that lower biomass production increased the tissue concentration of plant CP, as also reported by previous studies investigating the water stress effects on forage nutritive value in grasslands (Dumont et al., 2015; Grant et al., 2014). In lucerne, the decrease found in CP under drought can be linked to reduced rhizobia activity, nitrogen fixation and/or lower nutrient uptake, as observed by Aranjuelo et al. (2011) and Zahran (1999). Studies on forage legumes have found that drought-stressed plants reduce the biological nitrogen fixation activity for root-associated rhizobia (Kuchenmeister et al., 2013;

Liu et al., 2018a; Zahran, 1999). Additionally, a meta-analysis of forage species observed that plant nutrient uptake was lower under dry soils (Dumont et al., 2015). Under severe drought, reduced nutrient uptake is typically driven by reduced diffusion of nutrients through the soil as well as reduced root ability to transfer nutrients to aboveground tissue, thereby contributing to lower CP concentrations (Evans and Burke, 2013; Durand et al., 2010; Gonzalez-Dugo et al., 2005). Finally, previous studies have reported that senescence of aboveground materials promotes nutrient translocation (mainly nitrogen and soluble carbohydrates) from leaves to roots (Durand et al., 2010; Buxton, 1996), which may explain in our study the reduction of CP in lucerne, and NSC in both species, under drought conditions.

3.4.3. Assessing plant biomass, morphological and nutritional responses to warming and drought in a multivariate context

By adopting a multivariate framework to capture a holistic view of plant responses, we detected significant effects of both warming and drought and, for lucerne, a significant interaction effect. The latter was seen as a strengthening of warming effects under drought. This additive effect of warming on drought treatment in lucerne is in line with results reported by other recent studies who also showed an additive effect on plant growth and nutrient responses (Dellar et al., 2018; Oriens et al., 2019). Multivariate analysis is not widely applied in agricultural research, particularly in feed evaluation, however, our findings suggest that this oversight may underestimate the consequences of climate change on forage nutritive value. Statistical ordination techniques like PCA can usefully reduce the complexity of large forage data sets, aiding interpretation (Gallo et al., 2013; Pezzopane et al., 2020) while also avoiding the issue of multiple comparisons posed by numerous univariate analyses and non-independence of the chemical constituents in individual plants. In this study, for tall fescue, the majority of variability under climate change treatments was first associated (PC1) with nutritional

parameters and secondarily (PC2) associated with morphological parameters. For lucerne, shifts in morphological and nutritional parameters contributed similarly to treatment differences. Our findings suggest that nutritional composition should be an essential component of studies aimed at evaluating the impacts of climate change on pasture species. Although in this study the responses of individual compounds were informative, our multivariate analyses of pasture morphology and nutritive value provided a more comprehensive perspective of climate change impacts for future field conditions where the effects of multiple factors occur simultaneously across many aspects of plant biology and nutritional chemistry.

3.5 CONCLUSIONS

In conclusion, drought, even in the short-term, can be a strong driver of change in many individual morphological traits and the nutritional composition of pasture species. However, when considered in a multivariate framework, warming also has a significant impact on plant morphology and nutritive value, although to a lesser degree than drought. We found that exposure of these pasture species to warmer and drier conditions resulted in less forage produced and a decline in nutritive value. Furthermore, the potential negative impacts on nutritive value will have implications for pasture species choice, animal production, and methane emissions. Improved understanding of changes in morphology which might, in turn, affect forage nutritive value among several pasture species under climate change can lead to more efficient use of resources, better economic outcomes, and consequently, an improvement in the future of sustainable livestock production around the globe.

3.6 SUPPLEMENTARY DATA

Table S3.1. Average daily temperature and relative humidity inside the four chambers during the experimental period. Temperature treatments were ambient and elevated, and each treatment had two chambers.

Treatment	Air Temperature (°C)	Relative Humidity (%)
Ambient temperature	23.1 ± 3.8	60.7 ± 16.3
Elevated temperature	26.3 ± 4.0	61.1 ± 7.2

Values represent mean ± standard deviation. Averages include daytime and nighttime.

Table S3.2. Mean values (n = 8) and standard errors (SE) for plant dry biomass, morphological traits and nutritional parameters of tall fescue (*Festuca arundinacea*) and lucerne (*Medicago sativa*) grown under different temperatures (ambient, aT; elevated, eT) and watering regimes (well-watered, W; droughted, D).

Variables	Tall fescue								Lucerne							
	aT.W		aT.D		eT.W		eT.D		aT.W		aT.D		eT.W		eT.D	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Dry biomass (g/pot)	9.64	0.55	7.31	0.57	6.69	0.43	5.76	0.71	25.9	0.63	12.6	0.76	16.9	0.58	10.1	1.10
Plant height (cm)	55.3	3.28	55.7	3.58	59.6	2.95	53.6	1.55	72.6	2.53	59.5	2.16	75.0	1.97	65.6	3.24
Number of tillers/stems (n/pot)	30.1	2.01	27.9	1.53	21.4	1.43	21.4	1.79	25.1	1.98	18.1	0.89	27.4	2.56	20.0	2.24
Dead material (%)	0	0	19.4	7.22	0	0	13.7	6.46	1.87	1.31	22.5	9.77	0	0	61.2	11.9
Leaf:stem ratio	NA	NA	NA	NA	NA	NA	NA	NA	1.09	0.04	0.65	0.06	1.01	0.06	0.79	0.10
DM (%)	31.7	0.69	34.0	1.00	28.5	0.55	33.4	1.46	32.6	0.73	34.0	1.44	30.8	0.94	41.5	2.58
ASH (% DM)	6.31	0.19	7.29	0.24	8.39	0.19	8.95	0.42	6.16	0.32	6.44	0.33	7.40	0.25	7.42	0.29
CP (% DM)	3.48	0.17	3.78	0.20	4.57	0.29	5.74	0.42	13.4	0.74	12.7	0.44	18.6	1.43	14.7	0.78
NSC (% DM)	41.7	1.42	36.6	1.78	35.4	0.66	27.7	1.62	42.3	1.49	38.1	1.60	35.7	1.69	33.7	0.76
NDF (% DM)	48.4	1.33	52.0	1.40	50.6	0.61	55.4	0.92	38.2	2.02	42.4	1.23	37.7	2.19	43.3	1.06
ADF (% DM)	23.8	0.67	26.4	0.76	25.3	0.42	28.5	0.46	27.8	1.88	32.0	1.20	28.0	1.83	33.0	0.87
ADL (% DM)	1.97	0.20	2.17	0.46	2.66	0.29	2.36	0.48	7.93	0.49	8.52	0.29	7.54	0.48	8.86	0.14
HEM (% DM)	24.6	0.78	25.6	0.73	25.3	0.32	26.9	0.54	10.4	0.49	10.4	0.56	9.70	0.82	10.3	0.42
CEL (% DM)	21.9	0.59	24.2	0.60	22.6	0.53	26.2	0.78	19.9	1.45	23.4	1.03	20.5	1.50	24.2	0.81
DDM (% DM)	70.3	0.52	68.3	0.59	69.2	0.33	66.7	0.36	67.2	1.46	64.0	0.93	67.1	1.43	63.2	0.68

DM: dry matter; % DM: percentage of dry matter; CP: crude protein; NSC: non-structural carbohydrates; NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; HEM: hemicellulose; CEL: cellulose; DDM: digestible dry matter; NA: not applicable

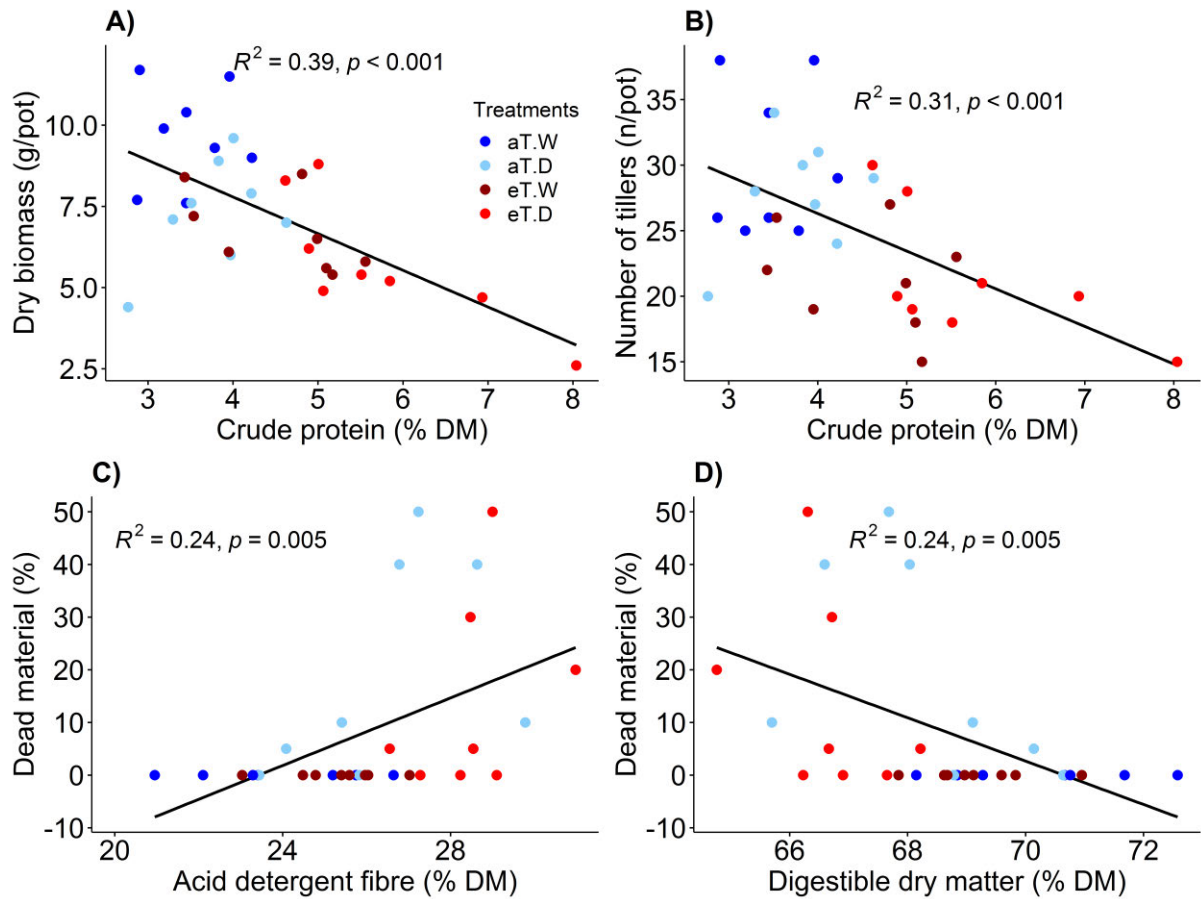


Figure S3.1. Correlations between A) plant dry biomass, morphological traits [B) number of tillers; C) and D) percentage of dead material] and nutritional composition [A) and B) crude protein; C) acid detergent fibre; D) digestible dry matter; all parameters as % of dry matter] for tall fescue (*Festuca arundinacea*) grown under different treatments (ambient, aT; elevated, eT; well-watered, W; droughted, D). Correlations were tested with Pearson correlation, with R^2 and p values shown in each panel.

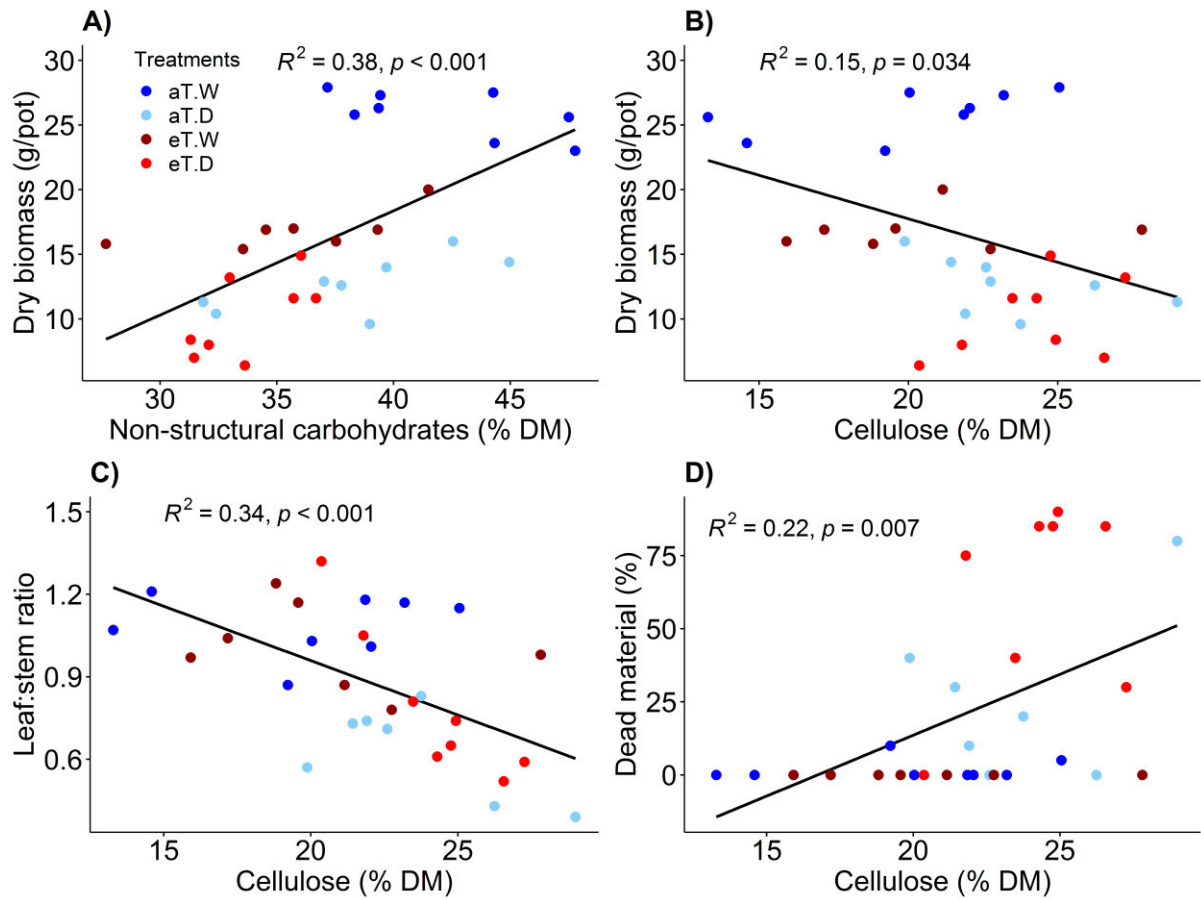


Figure S3.2. Correlations between plant dry biomass [A) and B)], morphological traits [C) leaf:stem ratio; D) percentage of dead material) and nutritional composition [A) non-structural carbohydrates; B), C) and D) cellulose; all parameters as % of dry matter) for lucerne (*Medicago sativa*) grown under different treatments (ambient, aT; elevated, eT; well-watered, W; droughted, D). Correlations were tested with Pearson correlation, with R^2 and p values shown in each panel.

CHAPTER 4: Productivity of temperate pasture species is impacted more than nutritive value by two years of simulated climate extremes

4.0 ABSTRACT

Global warming is predicted to cause more extreme events such as heatwaves and severe droughts, resulting in significant effects on forage production in many regions of the world. However, studies that have focused on productivity responses to climate change have relatively little information on how forage nutritive value will be impacted. This is especially true for studies considering the impact of co-occurring changes in multiple climate factors. In this context, we conducted a field experiment from 2018 to 2020 simulating multi-factor climate extremes to investigate the effects on forage productivity and nutritive value of two common temperate pasture species, tall fescue (*Festuca arundinacea*; a grass) and lucerne (*Medicago sativa*; a legume). Climate extreme treatments included a continuous warming (+3 °C above ambient temperature) and two winter/spring periods of severe drought (60% rainfall reduction), alone and in combination. We found that climate extreme treatments strongly decreased forage production of both species and that impacts on nutritive value varied across individual harvests throughout the study duration, with comparatively marginal improvement in overall forage nutritive value in tall fescue and almost no effects on lucerne. The impacts on forage quantity and nutritional quality were greater under severe drought than under warming, and the effects of drought in combination with warming were not always greater than the effects of either treatment alone. Forage harvested during the spring drought periods (2018 and 2019) was the most affected by the climate treatments. Since treatment impacts on forage nutritive value were minor relative to the observed productivity declines, our findings highlight that planting

decisions around minimising productivity impacts will be of major importance for future climate change scenarios.

4.1 INTRODUCTION

As a result of increased atmospheric concentrations of greenhouse gases, the global climate is changing, with altered regional precipitation and temperature patterns and increased intensity and frequency of extreme climate events apparent in many parts of the world (IPCC, 2013). Extreme climate events, such as heatwaves and droughts, are responsible for large impacts on agriculture, and the consequences can be severe and irreversible in the short-term (IPCC, 2012; IPCC, 2014). Since managed grasslands, such as pastures, are often the primary food source for livestock grazing (e.g. cattle and sheep), future food security will depend on how pastures respond to the changing climate (ABARES, 2016; MLA, 2017). Increases in heatwaves and severe droughts have been identified as one of the main future concerns for pasture-based systems, with major impacts on plant productivity and nutritional status predicted (Howden et al., 2008; McKeon et al., 2009; Chang-Fung-Martel et al., 2017; Harle et al., 2007). Despite a recent increase in the number of studies focusing on climate extremes (Chang-Fung-Martel et al., 2017; De Boeck et al., 2020; Knapp et al., 2017), relatively few have simultaneously addressed the implications of multiple climate variables on the nutritive value (i.e. nutritional composition and digestibility) of forage in a field setting (Catunda et al., 2021b; AbdElgawad et al., 2014). Knowing how forage nutritive value will change is vitally important because it influences digestibility of food, animal physiological condition, reproduction, and production (e.g. growth, milk or wool production; Ball et al., 2001; Coleman and Moore, 2003).

The nutritive value of forage varies throughout the year according to the phenology or growth stage of the plant, as well as the climate and soils in which the plant grows (Patton et al., 2000; Buxton, 1996). For instance, forage nutritive value decreases as plant maturity and

age increase within a given growing season (Ball et al., 2001; Grant et al., 2014) due to the increased proportion of senescent biomass and decreased leaf:stem biomass ratios. In turn, these plant changes affect key nutritional parameters in plant tissue, for example by increasing the concentration of fibre and decreasing that of crude protein (CP) and non-structural carbohydrates (NSC), and thereby reducing forage digestibility (Bruinenberg et al., 2002; Grant et al., 2014). Reduced forage digestibility results in poor animal performance, such as decreased daily weight gain rates, milk production and reproduction efficiency (Ball et al., 2001; Coleman and Moore, 2003).

Plant phenological development and forage nutritive value can also vary across plant functional groups; for example, legumes are often of greater nutritive value, and their digestibility declines at a slower rate than for grasses (Buxton, 1996; Grant et al., 2014; Sanderson, 2010). Furthermore, plant phenology can be affected by changes in temperature and rainfall at both ends of the growing season, with the potential for a delay or advance in initiation of new growth, just as the timing of senescence may change. Together, these two factors affect the length of the growing season (Cleland et al., 2007; Fitchett et al., 2015; Sherry et al., 2007). Under stressful conditions, such as high temperatures and/or drought, plants may speed up maturation or slow it down as a strategy to maximise fitness (either by promoting survival or maximising the likelihood of successful reproduction) that can depend on both the plant developmental stage and the severity and duration of the stressor (Buxton, 1996; Collins and Newman, 2017; Gray and Brady, 2016). The response of forage nutritive value to climate variability has been shown to depend on the precise timing of extreme weather events in relation to plant maturity (Grant et al., 2014). Therefore, there are likely to be implications for the seasonality of forage nutritive value of pasture species when faced with extreme climate conditions.

Plant responses to warming depend on the optimal temperature of the species for growth, with both positive (Bloor et al., 2010; Dieleman et al., 2012) and negative (Cantarel et al., 2013; Lee et al., 2017) responses reported for plant growth and forage nutritive value. Often, warming effects are a consequence of changes in soil water availability, and in the absence of changes to soil water, fewer consequences are detected. For example, positive effects can be driven by increased rates of soil mineralization and nutrient availability, most commonly in colder environments (Bloor et al., 2010; Dellar et al., 2018) and in areas where ambient conditions are below the optima growth temperature of the species (Martinez et al., 2014). Negative effects on plant growth and forage nutritive value are often reported as a result of heat stress on plant physiology and metabolism, high levels of evapotranspiration, and decreases in soil moisture and nutrient availability (Habermann et al., 2019; Rustad et al., 2001; Wilson et al., 1991). The reduced nutritive value under these extreme warming conditions is often associated with increases in fibre, and decreases in CP (Waghorn and Clark, 2004) and NSC (Habermann et al., 2019).

Limited water availability can cause plant stress and, ultimately, mortality (Bruinenberg et al., 2002; Emadodin et al., 2021; Ren et al., 2016). Severe plant water stress inhibits growth, speeds maturation and can induce rapid senescence, resulting in lower forage nutritive value (Durand et al., 2010, Buxton, 1996). Further, leaf loss or tissue death can increase fibre fractions of forage and, consequently, reduce forage digestibility under severe drought conditions (Bruinenberg et al., 2002; Ren et al., 2016). Reduced digestibility under drought stress has also been shown to be associated with increases in lignin and sometimes silica concentrations as plants can accumulate these compounds in order to reduce water loss through evapotranspiration (Debona et al., 2017; Feng Ma, 2004; Liu et al., 2018b; Moura et al., 2010). Importantly, the impacts of severe drought on forage production and nutritive value may also

differ between species, reflecting their sensitivity and/or resistance to declining water availability (Evans et al., 2011, McGranahan and Yurkonis, 2018).

In the coming decades, extreme temperatures and severe droughts are expected to co-occur with greater frequency (IPCC 2014). The combined effects of warming and drought may be more detrimental than individual stresses, with temperature-associated increases in evapotranspiration exacerbating the effects of low soil water availability (Cantarel et al., 2013; De Boeck et al., 2016). The lack of studies in this area means there is considerable uncertainty about how the complex interactive effects of warming and drought affect forage productivity and nutritive value, especially under long-term field conditions (Dellar et al., 2018; Habermann et al., 2021; McGranahan and Yurkonis, 2018). Long-term trials investigating the effects of climatic extremes can advance knowledge of seasonal implications for forage nutritive value and potential adaptation options (Brookshire and Weaver, 2015; Evans et al., 2011; Hoover et al., 2014; Reyer et al., 2013). Furthermore, understanding how climate stress will affect the ability of plants to recover when drought is alleviated is an important knowledge gap in the context of perennial pasture systems, where grazing and cutting are maintained over multiple years. There is evidence that for some species, the capacity to accumulate nutrients reserves in taproots while under water stress facilitates regrowth when drought stress is alleviated (Erice et al., 2011; Saglam et al., 2008).

The aims of this study were to investigate the effects of year-round/continuous warming (including intensification of heatwaves) and severe drought, alone and in combination, on (1) forage productivity and nutritive value, and (2) the relationship between antecedent drought conditions on the nutritive value of post-drought regrowth. To do this, we conducted a 2-year study at the Pastures and Climate Extremes (PACE) experimental field facility in southeastern Australia, exposing two widely used temperate pasture species, tall fescue (*Festuca arundinacea*; a grass) and lucerne (*Medicago sativa*; a legume), to continuous warming (+3

°C) and two consecutive winter/spring 6-month periods (2018 and 2019) of simulated severe drought (60% rainfall reduction) followed by two summer/ autumn periods (2019 and 2020) of post-drought re-growth. The climate treatments represented the climate model predictions for end-of-century Australia in increases in average surface air temperatures and winter/spring droughts for the study region (CSIRO and BOM, 2015; CSIRO, 2020). We hypothesized that (1) long-term warming and severe drought will reduce productivity and nutritive value of both species, with greater impacts in tall fescue than in lucerne, and (2) the combination of warming and drought will intensify plant stress, resulting in still greater negative effects on productivity and nutritive value. We further predicted that after the drought period, droughted plants would show greater regrowth than those previously grown under control (non-droughted) conditions.

4.2 MATERIALS AND METHODS

4.2.1 Site description

The experiment was conducted at the Pastures and Climate Extremes (PACE) field facility (**Figure 4.1**) located on the Hawkesbury Campus of Western Sydney University, Richmond (S33.610, E150.740, elevation 25 m), New South Wales, Australia, between early April 2018 and March 2020. In this region, the mean annual temperature is 17.2 °C, with the monthly maximum and minimum occurring in January (22.9 °C) and July (10.2 °C), respectively. Mean annual precipitation is 800 mm (Australian Government Bureau of Meteorology, Richmond - UWS Hawkesbury Station 1980-2010); however, there is large inter-annual variability (between 500 mm and over 1400 mm over the past 30 years). Winter/spring precipitation accounts for 40% of annual rainfall and plays an essential role in crop production. Soils at this site have a sandy-loam texture (81% sand, 7% silt and 11% clay) with an average pH of 5.7, volumetric water holding capacity of 15-20%, plant available N of 46 mg/kg, plant available (Bray) P of 26 mg/kg and 1% soil organic carbon (more details are reported in Churchill et al.,

2021). Before seeding, we applied fertilizer of diammonium phosphate (DAP; 110 kg ha⁻¹). The PACE field facility is comprised of six replicate polytunnel rainout shelters (48 m x 8 m; **Figure 4.1A**) with eight plots (4 m x 4 m) per shelter. These plots were further subdivided into four subplots (2 m x 2 m) planted with pasture species either in monoculture or mixtures (total 192 subplots) during the austral spring of 2017 and summer 2018. A detailed overview of the experimental facility descriptions was reported in Churchill et al. (2021).



Figure 4.1. The Pastures and Climate Extremes (PACE) field facility located at Western Sydney University, Richmond, New South Wales, Australia. A) There were six open-sided polytunnels, each with eight experimental plots; B) Infra-red (IR) heater (indicated by the

arrow) arrays were mounted above the vegetation canopy and warmed the plot surface an average of 3 °C above paired ambient temperature plots.

4.2.2 Selection and establishment of pasture species

For this experiment, we focused on two monocultures that were exposed to four climate treatments, for a total of 48 subplots (6 subplots per treatment/species). The two pasture species used in this experiment were: the C₃ grass, tall fescue (*Festuca arundinacea* Schreb., Cultivar: Quantum II MaxP) and the C₃ legume, lucerne (also known as alfalfa; *Medicago sativa*, Cultivar: SARDI 7 Series 2). These pasture species were chosen as they are high-quality, productive perennial forage species adapted to a wide range of growing conditions and with a potential to tolerate drought (Bouton, 2012; Gibson and Newman, 2001). Additionally, both species are commonly grown in pasture systems in Australia and many countries around the world (Bouton, 2012; Gibson and Newman, 2001; Hill et al., 1985; McDonald et al., 2003). In this experiment, both species were seeded in August 2017 and established under well-watered conditions prior to winter (tall fescue) or spring (lucerne) of 2018 (Churchill et al., 2021). Swards were managed with seasonal fertilizer application to replace nutrients removed from the soil (55 kg/ha; Cal-Gran Aftergraze, Incitec Pivot Fertilisers, Australia) and hand-weeding to maintain target species. Lucerne was supplied with appropriate rhizobia (Easy Rhiz soluble legume inoculant, Group AL, New Edge Microbials, New South Wales, Australia) necessary for nodulation.

4.2.3 Experimental treatments and environmental monitoring

Both species were exposed to two levels of canopy temperature: ambient (aT) and elevated (eT, +3 °C warming) and two watering regimes: control (C) and drought (D, reduced irrigation) in a full factorial design resulting in four treatment combinations (aT.C, aT.D, eT.C, eT.D),

each with six replicates per species. The experiment included two years of continuous warming and two consecutive winter/spring 6-month periods (2018 and 2019) of simulated severe drought followed by two summer/autumn periods (2019 and 2020) of post-drought.

Temperature manipulation of the warming treatment commenced in April 2018 and was maintained continuously for 24 hours a day until the end of the second drought period (30 November 2019). The plants harvested in 2020 were, therefore, not exposed to the warming treatment as a result of storm damage to heating arrays at this time. Manipulation of canopy temperature was achieved using a heating array of infra-red (IR) heaters (FTE 1000W, Ceramicx, Ireland) in an octagonal arrangement mounted on an aluminium frame (4 m x 4 m) suspended 1.4 m above ground level (**Figure 4.1B**). Target temperatures for these plots were controlled via feedback from IR-sensors (SI-100, Apogee Instruments, Logan, UT, USA) mounted at a height of 3.8 m, recording canopy surface temperatures every five minutes. The +3 °C warming was chosen to represent a mid-range average for end of century temperature estimates under high-emissions scenarios across Australia (CSIRO and BOM, 2015).

Irrigation was applied at the plot level as described in Churchill et al. (2021). The control treatment represented a typical precipitation regime for the local area during years with annual precipitation between 650-750 mm, accounting for long-term patterns in seasonality and in the statistical distribution of event sizes and timing within seasons. The drought treatment consisted of a 60% reduction in daily event size during the 6-month austral winter/spring period from 1 June to 30 November each year (2018 and 2019). In the first year of the experiment (2018), the drought treatment started in August 2018 for lucerne, to accommodate sward development during the early winter period. The drought treatment represented the drier end of climate model predictions for end-of-century seasonal rainfall change for southeastern Australia (CSIRO, 2020). In addition, this 60% reduction in winter/spring is in line with historical climate extremes for key pasture growing regions across

southeastern Australia, which are predicted to increase in both frequency and duration (Australian Bureau of Meteorology, 2019).

The effects of the warming and drought treatments on soil moisture were monitored continuously (0-15 cm; 16 per shelter; Time Domain Reflectometers; CS616, Campbell Scientific) in all subplots of both species. Additionally, air temperature and relative humidity sensors (Series RHP-2O3B, Dwyer Instruments Inc, USA) were mounted in force-ventilated radiation shields (Churchill et al., 2021). The amount of irrigation applied in each treatment, soil moisture and air temperature averaged across the shelters during the entire experimental period can be seen in **Supplementary Figure S4.1**.

4.2.4 Plant sampling during the experimental period

Both species were managed and regularly harvested based on grazing system recommendations practiced in the study region (Clements et al., 2003). Harvesting involved use of hand shears and a sickle mower. Throughout the experimental period, we determined the total aboveground productivity and collected samples for nutritional analyses in all harvests (7 for tall fescue and 8 for lucerne). Harvested plants were cut to 5 cm above the soil surface and weighed (fresh mass), then a representative sub-sample of material (100g, including a mixture of both live and dead material), excluding weeds, of each subplot of each species was immediately microwaved at 600W for 90 seconds to stop enzymatic activity (Landhäusser et al., 2018) followed by oven-drying at 65 °C for at least 48 hours until constant weight. Samples were then weighed (dry mass) to determine total dry matter productivity (kg DM ha⁻¹/harvest) per species for all treatment combinations. While most results are reported for individual harvests, annual productivity is also reported as the sum of all harvests across the two years divided by two (**Supplementary Table S4.1; Supplementary Figure S4.2A**).

4.2.5 Plant sample processing

To fully homogenize samples, dried samples were ground through a 1-mm screen in a laboratory mill (Foss Cyclotec Mill, Denmark) and stored in airtight plastic containers in the dark at room temperature prior to collection of near-infrared reflectance (NIR) spectra and wet chemical analysis. In particular, for the nitrogen and silicon analysis, plant samples were further homogenized using a ball-mill to produce a fine powder (Retsch® MM200; Hann, Germany).

4.2.6 Nutritional analysis

Two sub-samples of each dried and ground sample were scanned using near-infrared reflectance spectroscopy (NIRS; FOSS XDS Rapid Content™ Analyzer) and their spectra (spectral range from 400 to 2500 nm) were collected and averaged (each spectrum was the average of 32 scans per sample). Representative samples were selected in the software WinISI 4.8.0 (FOSS Analytical A/S, Denmark) for determining nutrient composition by wet chemistry for all parameters, except for dry matter and silicon (for which all samples were analysed), in order to calibrate and validate the NIR. Details associated with mathematical treatment of spectra and descriptive statistics for NIRS calibration can be found in Catunda et al. (2021c; see Chapter 2). Calibration equations were developed based on wet chemistry for a selected number of samples (Catunda et al., 2021c; see Chapter 2), with the associated methodologies outlined in brief here. Analyses of dry matter (DM) and ash followed the standard methods and procedures for animal feed outlined by the Association of Official Analytical Chemists (AOAC, 1990). Nitrogen (N) concentration was determined by the combustion method (Leco TruMac CN-analyzer, Leco Corporation, USA). Crude protein (CP) concentration was then calculated by applying a 6.25 conversion factor to the N concentration (AOAC, 1990). Ether extract was determined according to the American Oil Chemists' Society-AOCS high-

temperature method using petroleum ether (B.P. 40-70 °C) and the Soxhlet method (Buchi 810 Soxhlet Multihead Extract Rack, UK). Silicon (Si) concentrations were determined using an X-ray fluorescence spectrometer (Epsilon-3×, PANalytical, EA Almelo, The Netherlands) as described in Reidinger et al. (2012). Fibre fractions were determined with an ANKOM Fibre Analyzer (model 200, ANKOM® Technology, NY, USA) with the use of neutral and acid detergent solutions and corrected for dry matter content (Goering and Van Soest, 1970). Samples were analysed for neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) by the sequential method of Van Soest and Robertson (1980). Sodium sulphite and α -amylase were added to the solution for NDF determination. The values of ash, EE, CP and NDF were used to calculate non-structural carbohydrates (NSC) according to Sniffen et al. (1992). The concentrations of CP, NSC, NDF, ADL and Si were expressed as a percentage of DM. Estimated digestible dry matter (DDM) was calculated according to *Equation 1* below (Oddy et al., 1983):

$$\text{Equation 1.} \quad \text{DDM \%} = 83.58 - 0.824 \text{ ADF \%} + 2.626 \text{ N \%}$$

4.2.7 Calculations and statistical analysis

Statistical analyses of treatment effects were carried out using linear mixed-effects (LME) models in the ‘lme4’ package in the R software package (Version 4.0.0; R Core Team, 2020; Bates et al., 2015) and individual response measurements were checked to ensure underlying statistical assumptions were met. All models included temperature (T; ambient and elevated) and watering regimes (W; control and drought) as fixed effects; random effects included ‘subplot nested within plot’ to account for non-independence among continuous measurements, and these were nested within ‘shelter’ to account for the blocked design of the experiment. In addition, the statistical model also included harvest date as a fixed effect to account for variability among harvests across the experimental period. Pairwise comparisons

to determine individual treatment effects during individual harvests were conducted using the R package emmeans (Lenth, 2019).

4.3 RESULTS

4.3.1 Forage productivity responses to warming, drought and their interaction

4.3.1.1 Tall fescue

During the two-year experimental period, the mean value of dry matter productivity under control conditions was 1,601 kg DM ha⁻¹ per harvest and ranged from 612 (Nov-19, spring) to 2,853 (Mar-20, autumn) kg DM ha⁻¹ per harvest (**Figure 4.2A**). Overall, warming, drought and warming + drought treatments reduced the total annual productivity of tall fescue by 42%, 48% and 64%, respectively, relative to control (**Supplementary Table S4.1; Supplementary Figure S4.2A**).

The drought treatment significantly reduced productivity in most of the individual harvests, including both the drought and post-drought (recovery) periods, with reductions of up to 79% (**Table 4.1; Figure 4.2A**). Warming alone significantly reduced productivity in May-19 harvest (-85%), representing total growth during the austral summer, and Aug-19 harvest (-22%), which included accumulated productivity during a relatively warm austral winter (**Figure 4.2A; Supplementary Figure S4.1**). Warming + drought reduced productivity during the spring drought period of the first year and the succeeding drought recovery interval ($p < 0.05$; **Figure 4.2A**). Evidence for amplifying effects of drought and warming was only found at the end of the first drought-recovery period, suggesting that the winter/spring drought conditions were sufficiently extreme to limit regrowth of the pasture sward during the summer months (**Figure 4.2A**). Furthermore, these amplified effects were sustained with warming + drought treatment into the first harvest of the second drought period. Despite this pattern, in general, the effects of warming + drought treatment on productivity in tall fescue were not

always greater than the effects of both warming or drought treatments in each harvest (**Figure 4.2A**).

4.3.1.2 Lucerne

During the experimental period, the mean value of dry matter productivity under control conditions was 2,037 kg DM ha⁻¹ per harvest and ranged from 1,033 (Oct-18, spring) to 2,732 (Nov-19, spring) kg DM ha⁻¹ per harvest. Overall, warming, drought and warming + drought treatments reduced the total annual productivity of lucerne by 21%, 32% and 34%, respectively, relative to control (**Supplementary Table S4.1; Supplementary Figure S4.2A**). Importantly, the productivity of lucerne varied with time, generally increasing in production per harvest as the sward matured through the two-year interval (**Figure 4.2B**). The impacts of drought and warming varied among individual harvest dates (two-way interactions $p < 0.05$; **Table 4.1**).

For individual harvests, drought reduced productivity in most of the harvests, while warming alone significantly reduced productivity only during the post-drought harvest in May-19 and the drought period in Oct-19 harvest ($p < 0.05$; **Figure 4.2B**). In both years, the first harvests of the drought periods (Oct-18 and Aug-19) were not significantly affected by any climate extreme treatments, while the greatest reductions in productivity were seen during the spring drought period harvests in 2018 (Nov) and 2019 (Oct and Nov), with reductions of up to 57% under drought and 26% under warming (**Figure 4.2B**). Among individual harvests, the effects of drought were greater than the effects of both warming or warming + drought. However, we found compounding effects of warming and drought as the sward aged, with warming + drought plots producing the least forage during the last three harvests (-46% Oct-19, -52% Nov-19, and -32% Jan-20; **Figure 4.2B**).

Table 4.1. Linear mixed effects model output for productivity and nutritional parameters of tall fescue (*Festuca arundinacea*) and lucerne (*Medicago sativa*) in response to temperature (T) and watering (W) treatments, and their interaction (T x W), among harvests (H) during the experimental period.

Response	Fixed Effects	Tall fescue				Lucerne			
		F value	p value	R ² m [#]	R ² c [†]	F value	p value	R ² m [#]	R ² c [†]
Productivity (kg DM ha ⁻¹)	Temperature	10.9	<0.01	0.46	0.52	3.3	0.09	0.50	0.77
	Watering	15.5	<0.01			11.5	<0.01		
	Harvests	11.5	<0.01			33.8	<0.01		
	T x W	2.1	0.16			2.6	0.13		
	T x H	2.8	0.01			2.1	0.04		
	W x H	1.8	0.10			4.4	<0.01		
	T x W x H	1.7	0.13			0.9	0.46		
CP (% DM)	Temperature	16.7	<0.01	0.71	0.80	0.3	0.59	0.51	0.54
	Watering	7.5	0.01			10.0	<0.01		
	Harvests	66.6	<0.01			18.9	<0.01		
	T x W	3.5	0.8			0.1	0.75		
	T x H	3.3	<0.01			2.2	0.04		
	W x H	5.9	<0.01			4.4	<0.01		
	T x W x H	2.0	0.07			2.2	0.04		

NSC (% DM)	Temperature	1.4	0.25	0.71	0.72	1.2	0.28	0.56	0.57
	Watering	6.6	0.02			6.6	0.02		
	Harvests	57.3	<0.01			30.8	<0.01		
	T x W	2.1	0.16			2.8	0.12		
	T x H	2.0	0.08			1.4	0.19		
	W x H	3.8	<0.01			1.6	0.12		
	T x W x H	0.3	0.95			0.3	0.94		
NDF (% DM)	Temperature	11.9	<0.01	0.83	0.84	0.4	0.52	0.37	0.40
	Watering	0.3	0.58			0.0	0.86		
	Harvests	117.0	<0.01			12.4	<0.01		
	T x W	7.2	0.02			3.5	0.08		
	T x H	1.3	0.28			1.7	0.10		
	W x H	8.9	<0.01			1.1	0.35		
	T x W x H	1.2	0.29			0.7	0.67		
ADL (% DM)	Temperature	0.4	0.52	0.56	0.61	0.0	0.85	0.60	0.62
	Watering	9.5	<0.01			5.2	0.04		
	Harvests	22.9	<0.01			40.7	<0.01		
	T x W	1.8	0.19			1.2	0.29		
	T x H	3.1	<0.01			0.6	0.79		
	W x H	3.2	<0.01			0.2	0.97		
	T x W x H	2.8	0.01			0.4	0.87		

Si (% DM)	Temperature	10.0	< 0.01	0.64	0.69	1.2	0.29	0.16	0.21
	Watering	17.3	< 0.01			1.5	0.25		
	Harvests	25.6	< 0.01			3.7	< 0.01		
	T x W	9.2	< 0.01			0.1	0.82		
	T x H	3.5	< 0.01			0.6	0.72		
	W x H	8.5	< 0.01			0.4	0.91		
	T x W x H	1.2	0.33			0.3	0.96		
DDM (% DM)	Temperature	8.3	0.01	0.77	0.84	1.0	0.32	0.53	0.57
	Watering	1.0	0.34			2.4	0.14		
	Harvests	105.1	< 0.01			26.3	< 0.01		
	T x W	4.4	0.05			2.4	0.14		
	T x H	1.3	0.25			2.0	0.05		
	W x H	7.2	< 0.01			2.3	0.03		
	T x W x H	1.4	0.23			1.5	0.15		

[#] R^2m indicates marginal error associated with linear model fixed effects.

[†] R^2c indicates conditional error or the total variation described by the full model, including nested random effects (plots within a shelter).

Notes: **Bold** values denote statistical significance at the $p < 0.05$ level.

Abbreviations: DM: dry matter; CP: crude protein; NSC: non-structural carbohydrates; NDF: neutral detergent fibre; ADL: acid detergent lignin; Si: silicon; DDM: digestible dry matter.

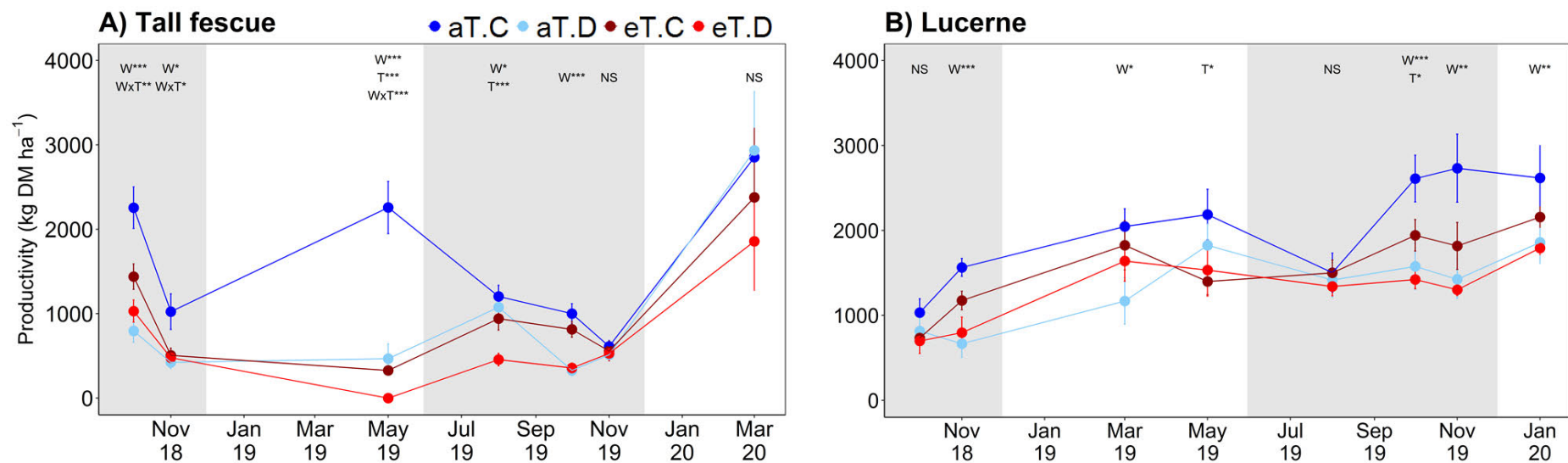


Figure 4.2. Total dry matter productivity (kg DM ha^{-1}) of tall fescue (*Festuca arundinacea*; A) and lucerne (*Medicago sativa*; B) grown under different temperature and watering treatments, across harvests during the experimental period: drought periods 2018/2019 (grey background) and subsequent post-droughts 2019/2020 (white background). Treatments: aT.C (ambient temperature and control), aT.D (ambient temperature and drought), eT.C (elevated temperature and control), eT.D (elevated temperature and drought). Values are means \pm SE. Significant comparisons for the effect of temperature (T) and watering (W) treatments for each harvest are indicated as follows: NS = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3.2 Forage nutritional responses to warming, drought and their interaction

4.3.2.1 Tall fescue

Across harvests during the experiment, the mean values of the nutritional parameters under control conditions in tall fescue were: CP: 11.6% (ranged from 9.6 to 13.2% DM), NSC: 16.5% (10.1-21.7% DM), NDF: 58.4% (54.2-66.3% DM), ADL: 5.1% (4.3-6.0% DM), Si: 1.0% (0.9-1.4% DM), and DDM: 65.4% (61.5-67.8% DM; **Figure 4.3**). Overall, across two years, drought treatment increased CP, ADL and DDM and warming treatment also increased CP and DDM. Warming + drought treatment increased CP, ADL, Si and DDM (**Supplementary Table S4.1; Supplementary Figure S4.2**). Additionally, the effect of watering regime was modified by the timing of individual harvests for all nutritional parameters ($p < 0.05$; **Table 4.1**). The effects of temperature varied among harvests for CP, ADL and Si. There were also significant three-way interactions between watering regime, temperature and individual harvest dates for ADL ($p < 0.05$; **Table 4.1**).

The harvests when most nutritional parameters were significantly affected occurred during the winter/spring drought periods, while harvests conducted during the summer/autumn post-drought periods were less affected by climate extreme treatments (**Figure 4.3**). In general, there were no consistent responses across harvests for most of the nutritional parameters, suggesting an important interaction with plant phenology and the timing of drought or impacts from warming. By way of example, the concentration of CP increased under the climate extreme treatments with the greatest increases during the post-drought and drought periods of 2019, presenting a percent increase of up to 31% under both drought and warming treatments (**Figure 4.3A**). In contrast, NSC was not affected in most of the harvests (**Figure 4.3B**). Under climate extreme treatments, NDF decreased in most of the harvests, with the greatest decreases under drought treatment (up to 10%; **Figure 4.3C**). ADL and Si were significantly impacted during the spring drought periods, with increases of up to 35% in ADL under drought and

warming + drought and 153% in Si under warming + drought (**Figure 4.3D-E**). Lastly, drought and warming + drought increased DDM (ranging from +2% to +6%) in most of the harvests, with the highest increases during the post-drought and winter drought periods of 2019 (**Figure 4.3F**).

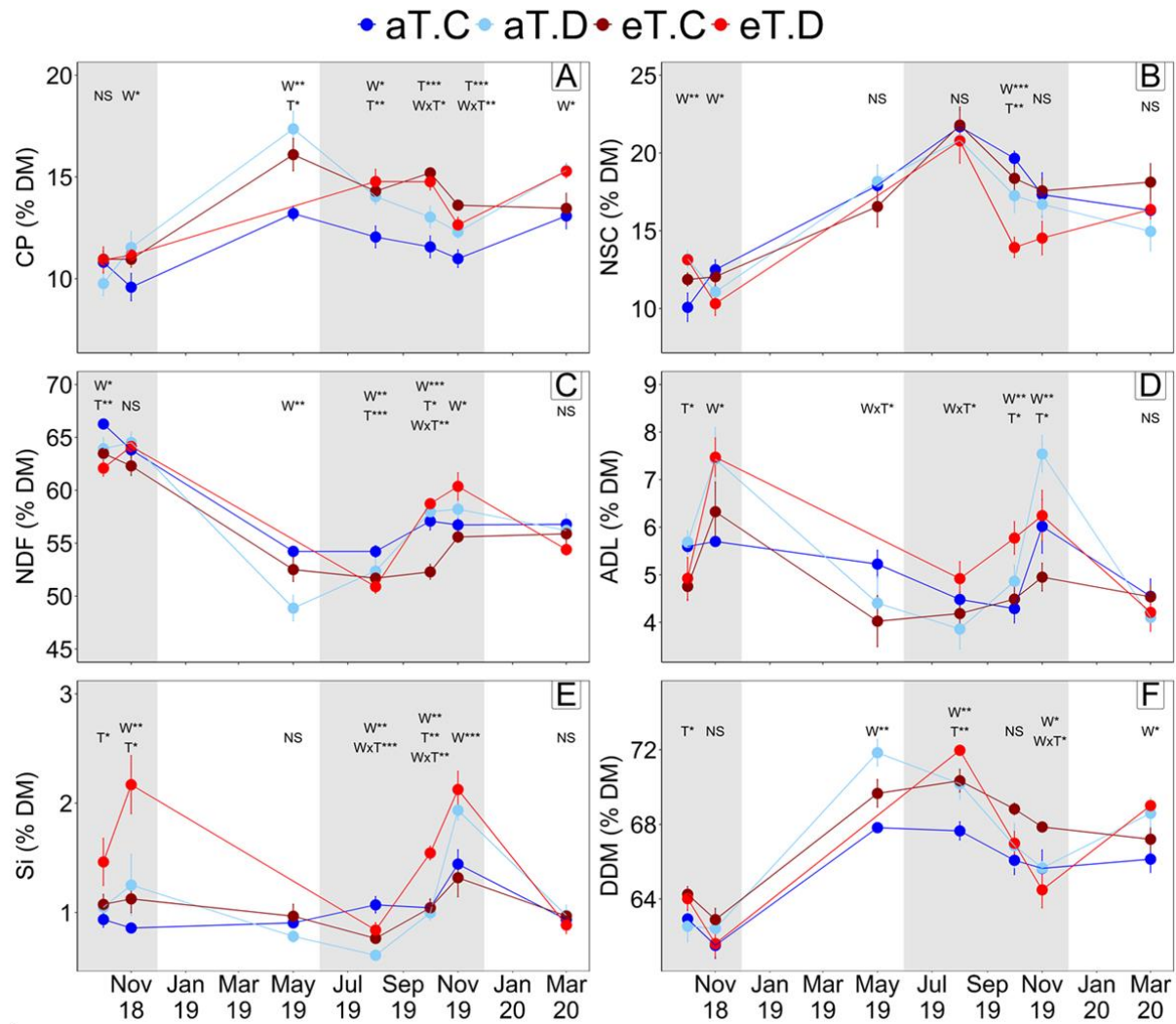


Figure 4.3. Nutritional parameters (A- crude protein %, B- non-structural carbohydrates %, C- neutral detergent fibre %, D- acid detergent lignin %, E- silicon %, F- digestible dry matter %) of tall fescue (*Festuca arundinacea*) grown under different temperature and watering treatments, across harvests: drought periods 2018/2019 (grey background) and subsequent post-droughts 2019/2020 (white background). Treatments: aT.C (ambient temperature and control), aT.D (ambient temperature and drought), eT.C (elevated temperature and control), eT.D (elevated temperature and drought). Values are means \pm SE. Significant comparisons for the effect of temperature (T) and watering (W) treatments for each harvest are indicated as follows: NS = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Note: during the May

2019 harvest, there was not enough biomass to perform nutritional analysis for tall fescue under warming + drought treatment.

4.3.2.2 *Lucerne*

Across harvests during the experiment, the mean values of the nutritional parameters under control conditions in lucerne were: CP: 16.7% (ranged from 14.1 to 20.6% DM), NSC: 25.8% (19.4-30.7% DM), NDF: 45.2% (42.6-49.9% DM), ADL: 9.5% (8.4-11.1% DM), Si: 0.2% (0.1-0.3% DM), and DDM: 65.9% (62.2-68.8% DM; **Figure 4.4**). Overall, across two years, drought and warming + drought decreased CP, while the other nutritional parameters were not affected (**Supplementary Figure S4.2**). Additionally, the effect of watering regime varied among harvests for CP and DDM ($p < 0.05$; **Table 4.1**). The effects of temperature also varied by harvest for CP; and there was also a three-way interaction between watering regime, temperature and individual harvest dates for CP ($p < 0.05$; **Table 4.1**).

Nutritional parameters were affected in very few harvests and there were not consistent responses across harvests for most parameters (**Figure 4.4**). By way of example, CP was the parameter most affected during the experiment, and increased during the post-drought period of 2019 (up to 14% under eT.D), but decreased during the two spring drought periods (up to 16% under all climate extreme treatments; **Figure 4.4A**). The other parameters, including NSC, NDF, ADL, Si and DDM, were not significantly affected by climate extremes in most of the harvests (**Figure 4.4B-C-D-E-F**).

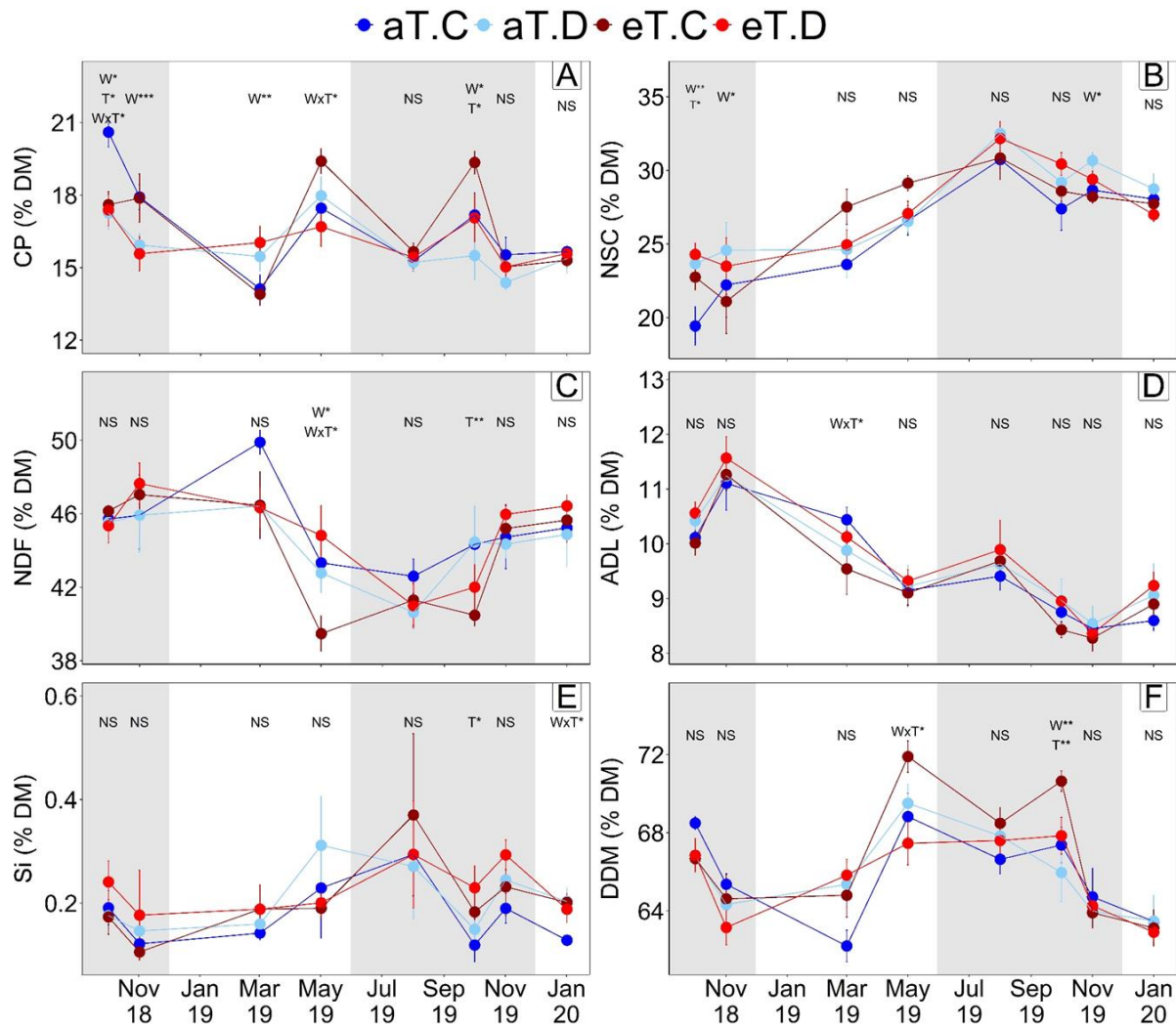


Figure 4.4. Nutritional parameters (A- crude protein %, B- non-structural carbohydrates %, C- neutral detergent fibre %, D- acid detergent lignin %, E- silicon %, F- digestible dry matter %) of lucerne (*Medicago sativa*) grown under different temperature and watering treatments, across harvests: drought periods 2018/2019 (grey background) and subsequent post-droughts 2019/2020 (white background). Treatments: aT.C (ambient temperature and control), aT.D (ambient temperature and drought), eT.C (elevated temperature and control), eT.D (elevated temperature and drought). Values are means \pm SE. Significant comparisons for the effect of temperature (T) and watering (W) treatments for each harvest are indicated as follows: NS = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.4 DISCUSSION

Temperature and water availability are two of the main abiotic factors that influence plant development and, in turn, forage production and nutritive value (Cantarel et al, 2013; Fay, 2009; Patton et al., 2000). Here, we sought to investigate the effects of climate extremes across two years of plant development on forage production and nutritive value of two widely cultivated pasture species. Key findings from this study are: (1) warming and severe drought affected forage productivity much more strongly than the nutritional composition and digestibility of both pasture species; (2) severe drought had a more significant impact than continuous +3 °C warming on productivity and nutritive value, with these impacts greater in tall fescue than in lucerne; and (3) the combination of warming and severe drought resulted in effects not always greater than the isolated effects of warming or drought conditions. Our first hypothesis was supported with both species experiencing strong reductions in productivity under all combinations of treatments. Conversely, our hypothesis that the nutritive value of both species would be reduced under climate extremes was not supported, as we found that tall fescue slightly increased nutritive value due to overall increases in CP and digestibility, while lucerne was largely unchanged. The magnitude and sometimes direction of these effects varied among harvests across the two-year period, with spring (drought period) harvests being the most affected.

4.4.1 Climate extremes decrease forage productivity

Transitory or consistently high temperatures and/or severe water deficits throughout the year are likely to be crucial stressors limiting plant growth in the future (Chang-Fung-Martel et al., 2017; Deléglise et al., 2015; Le Gall et al., 2015). Here we observed that future extreme climate scenarios of winter/spring severe drought and continuous warming, both alone and in combination, significantly decreased the pasture productivity of both species. For instance, we

found decreases in annual productivity of up to 64% in tall fescue and 34% in lucerne under combined scenarios of warming and drought relative to control. These findings are consistent with other long-term studies manipulating temperature and/or seasonal drought that have found significant reductions in annual productivity in temperate grassland ecosystems (Cantarel et al., 2013; Dellar et al., 2018; Grant et al., 2014). For instance, Cantarel et al. (2013) 4-year study in extensively managed upland semi-natural grassland (French Massif Central region) reported reductions in annual productivity of up to 44% under a warming + drought scenario. Plant stress associated with low soil moisture and high evapotranspiration levels inhibits plant growth, resulting in large losses in forage production (Lee et al., 2017; Emadodin et al., 2021; Rustad et al., 2001). Overall, our findings suggest that negative impacts on biomass production generated by the combination of warming and drought scenarios are likely associated with changes in soil water availability, as +3 °C warming may result in an effect on soil water content through increases in evapotranspiration, as reported by previous studies (Cantarel et al., 2013; De Boeck et al., 2016). That is the potential reason why we have found predominant negative effects of warming on productivity throughout the year in our temperate species. In addition, we found that continuous warming produced more frequent and hotter temperature extremes, particularly summer heatwave events (Churchill et al., 2021). Although previous studies have reported positive effects of warming on the productivity of forage species, especially in colder environments (Bloor et al., 2010; Dellar et al., 2018), our results align with model simulations of the effects of +3 °C warming on Australian rangelands (McKeon et al., 2009). The efforts of breeding programs to deliver particularly drought-resistant cultivars will be crucial to overcoming this expected productivity impact on animal feed under future extreme climate scenarios (Fernández-Habas et al., 2021; Habermann et al., 2021).

Our study additionally allowed a comparison between two widely used pasture species, although the negative impacts of climate extremes on productivity were common for both grass

and legume species in our study, tall fescue emerged as the more sensitive species. The magnitude of differences in impact on productivity due to drought and/or warming between these species suggests important trade-offs in mechanisms dictating resistance and resilience. Here, tall fescue presented biomass reductions of up to 100% (warming + drought treatment) relative to control. While the largest losses in biomass of lucerne were of up to 57% (drought treatment). For instance, for tall fescue, the sward was severely impacted by the first drought period, and the plants under climate extreme treatments struggled to recover. Although during the second year, the magnitude of drought effects was smaller, possibly due to more frequent harvests earlier in the drought period interval (e.g. August was the largest harvest, rather than October). Thus, allowing the plants to take advantage of regrowth while water was available. These findings support our predictions about plant recovery when drought stress is alleviated, particularly for older more established pastures, as shown in the second year. The capacity of plants to recover is often due to a survival strategy of accumulating nutrient reserves (e.g. soluble sugars, proline, and vegetative storage proteins) during severe plant stress events such as water deficits, which may act as a carbon source for regrowth, as reported by previous studies (Erice et al., 2007; Foster et al., 2015; Saglam et al., 2008).

Our findings suggest that the climate change implications for the seasonality of plant productivity may also be related to the different abilities of plants to tolerate and adapt to stress that can be species- or functional group-specific (Cranston et al., 2016; Lee et al., 2013). For instance, the effects on lucerne productivity during periods of drought in our study may have been smaller when compared to tall fescue, due to this legume having deep taproots (ranging from 3 to 5 metres) that may have allowed access to deep soil water (Bouton, 2012; Li et al., 2012). Also, it is reported in previous studies that the capacity of lucerne to accumulate and salvage N compounds in taproots under drought conditions may help to improve regrowth after the stress is alleviated (Erice et al., 2007; Erice et al., 2011).

4.4.2 Climate extremes slightly change the forage nutritive value

In our study, the strong impacts on plant growth might be one of the main drivers of the changes in nutritional composition and digestibility reported here. Our results showed that climate treatments had relatively small impacts on the nutritional composition and digestibility of both species, albeit with somewhat greater and more consistent effects on tall fescue compared to lucerne. Overall, the nutritive value of both pasture species responded to drought conditions more than to warming, as also found in previous studies with forage grass (Catunda et al., 2021b; Habermann et al., 2019).

We expected a general reduction in crude protein concentration due to the potential negative effects of severe drought and/or warming conditions on soil nitrogen availability, as reported in previous studies (Buxton, 1996; Durand et al., 2010). However, we found a predominant increase in CP concentration in tall fescue, which may have contributed to increased digestibility, and in lucerne increase during the post-drought period of 2019. Previous studies that reported increases in CP concentration under warming and/or drought scenarios were often associated with colder environments and/or moderate water stress (Deleglise et al., 2015; Dumont et al., 2015). However, in our study, the observed increases in CP are likely associated with a concentration effect, such that low rates of overall biomass increase meant that CP accounted for a greater proportion of the total, as reported in Dumont et al. (2015) and Grant et al. (2014).

Other parameters associated with forage nutritive value are comparatively understudied in the context of climate extremes and are predicted to be principally driven by shifts in plant phenology (maturity) and growth/morphological traits (e.g. leaf:stem ratios; Ball et al., 2001; Buxton, 1996; Collins, 2017). Overall, in lucerne, our imposed climate extreme treatments had little or no effect on nutritional parameters other than CP, across the two-year experiment. These findings are likely to be associated with lucerne's deep roots and ability to fix biological

nitrogen, which may have helped to maintain nutritive value and therefore digestibility across the seasons and years under extreme climate conditions (Erice et al., 2007; Suter et al., 2015). Contrastingly, in tall fescue, increases in ADL and Si, mainly observed in plants under warming + drought treatment, can be explained by plant defense mechanisms designed to alleviate the stress caused by water loss (Debona et al., 2017; Liu et al., 2018b; Moura et al., 2010). Previous research, has shown that plants growing under high temperatures and drought stresses allocate resources for strong structural development within their tissues, including building thick cells that are highly lignified and accumulate silica (SiO_2 ; Kering et al., 2011; Liu et al., 2018b; Moura et al., 2010). This accumulation of lignin and silica can reduce transpiration during plant stress from heat or low-water available conditions as they can act as impermeable films (Debona et al., 2017; Feng Ma, 2004; Liu et al., 2018b; Moura et al., 2010). Importantly, these plant defense mechanisms have strong impacts on plant digestibility. Lignin and silica are plant compounds that are almost totally indigestible by ruminants, and can thus have significant consequences for ruminant nutrition and performance (Buxton et al., 1995; Johnson et al., 2021; Jung et al., 1997; Van Soest and Jones, 1968), despite fewer metrics for digestibility considering Si in calculations. In our study, while we found that lignin increased in response to climate treatments, this did not translate into a negative impact on the calculated digestibility of tall fescue. Furthermore, the digestibility index used in our study, quantified based on nitrogen and acid detergent fibre (cellulose and lignin; Oddy et al., 1983), does not account for changes in Si that may, in fact, represent a hidden cost to forage digestibility under more extreme future climates. This hidden cost can come from a couple potential sources, as silica, like lignin, is part of the cell wall matrix and can reduce fermentation and accessibility of cell wall carbohydrates to the attack of digestive microorganisms in ruminants (Jones and Handreck, 1967; Montes-Sanchez and Villalba, 2017; Van Soest and Jones, 1968). Another possibility, which does not exclude the first, is a lower palatability (characteristics of a feed

that invoke a sensory response in the animal) of the forage for the animal, due to the presence of highly silicified cells projected on the edge of the leaves, making the material rough to the touch (Massey et al., 2007).

In our study, the increase in digestibility under climate extremes in tall fescue was associated with a decrease in fibre (NDF) in combination with overall increases in CP concentration. The decreases in fibre here may be explained by the delay in stem elongation and increases in leaf:stem biomass ratios associated with slower rates of maturation and growth under stress conditions (Buxton, 1996; Küchenmeister et al., 2013; Wilson et al., 1983). This suggests that climate extremes may improve the sward structure through decreases in the relative contribution of stems to forage productivity, thus facilitating greater forage intake and gains in performance; such morphology-associated improvements in forage digestibility may also result in lower ruminant methane emissions due to more efficient digestion processes (Lee et al., 2013; 2017). In contrast to our findings, other studies have reported that severe droughts or extremely high temperatures may result in faster maturation and severe leaf senescence, decreasing leaf:stem ratios, consequently increasing fibre and decreasing forage digestibility (Bruinenberg et al., 2002; Habermann et al., 2019; Ren et al., 2016; Waghorn and Clark, 2004). These differences in fibre and digestibility responses to climate extremes across studies may depend on the forage species itself as well as the timing, severity and duration of climate stress in relation to the plant developmental stage (Buxton, 1996; Collins and Newman, 2017; Gray and Brady, 2016).

Overall, there is still a great level of uncertainty, particularly in the context of field studies, in relation to forage production and nutritional response patterns across seasons to combined scenarios of water stress and warming in a range of pasture species. In grassland ecology, most experiments investigating aboveground productivity collect biomass measurements once/twice per year (Cantarel et al., 2013; Grant et al., 2014). Managed systems

are, however, typically grazed or mown much more frequently (Deleglise et al., 2015; Emadodin et al., 2021) and seasonal responses to climate extremes as well as changes in sward development are fundamental in predicting and maintaining productive pastures into the future. Our study found that the effects of warming and/or drought on productivity and nutritional composition were modified by the timing of individual harvests across the years on both pasture species (**Table 4.1**). The novelty of our study approach in terms of both longevity (2 years) and multi-harvest (up to 5 harvests per year) was able to capture the range of variation in pasture productivity and nutritive value under extreme climate scenarios associated with differences in phenology.

4.5 CONCLUSIONS

Throughout two successive years of field experiment, warming and severe drought significantly decreased productivity of both pasture species, and slightly increased nutritive value in tall fescue and largely unchanged the nutritive value in lucerne. These effects of severe drought were greater than warming, and their combination resulted in effects not always greater than the isolated effects of warming or drought conditions. Overall, the climate treatments effects were stronger during the spring drought period compared to the post-drought period. Our results suggest that under climate extremes, positive changes to nutritive value, where they occur, are insufficient to counter the great decrease in pasture productivity and associated animal productivity, in terms of livestock production per hectare. In extensive grazing situations, if stocking densities can be rapidly reduced during drought, then the maintenance of, or small increase in, nutritive value is reassuring, and may allow animal condition to be maintained, but for fewer animals. Finally, a valuable goal would be to identify pasture species and cultivars with greater resistance to climate extremes, especially to severe drought, that can

maintain or experience smaller reductions in productivity and nutritive value in order to minimize/buffer future livestock industry exposure to climate-related risk.

4.6 SUPPLEMENTARY DATA

Table S4.1. Linear mixed effects model output for total annual productivity and nutritional parameters of tall fescue (*Festuca arundinacea*) and lucerne (*Medicago sativa*) in response to temperature (T) and watering (W) treatments, and their interaction (T x W), across the two years of the experiment.

Response	Fixed Effects	Tall fescue				Lucerne			
		F value	<i>p</i> value	R ² m [#]	R ² c [†]	F value	<i>p</i> value	R ² m [#]	R ² c [†]
Annual Productivity (kg DM ha ⁻¹)	T	10.0	< 0.01	0.15	0.15	7.3	< 0.01	0.15	0.27
	W	14.9	< 0.01			26.6	< 0.01		
	T x W	3.3	0.07			5.8	0.02		
CP (% DM)	T	7.9	< 0.01	0.11	0.12	0.1	0.73	0.03	0.04
	W	4.1	0.04			6.7	0.01		
	T x W	7.7	< 0.01			0.1	0.72		
NSC (% DM)	T	0.9	0.35	0.03	0.03	0.5	0.46	0.02	0.02
	W	2.8	0.10			2.9	0.09		
	T x W	1.1	0.29			1.2	0.27		
NDF (% DM)	T	0.6	0.43	0.03	0.03	0.3	0.58	0.02	0.02
	W	0.4	0.51			0.1	0.82		
	T x W	3.7	0.06			2.8	0.09		
ADL (% DM)	T	0.1	0.78	0.07	0.07	0.0	0.87	0.01	0.01
	W	9.2	< 0.01			2.2	0.14		
	T x W	2.3	0.13			0.4	0.51		
Si (% DM)	T	9.6	< 0.01	0.18	0.18	1.3	0.25	0.01	0.04
	W	14.6	< 0.01			1.6	0.20		
	T x W	9.6	< 0.01			0.1	0.81		
DDM (% DM)	T	1.9	0.17	0.04	0.04	0.5	0.48	0.02	0.03
	W	0.2	0.67			1.5	0.22		
	T x W	5.2	0.02			1.2	0.28		

[#]R²m indicates marginal error associated with linear model fixed effects.

[†]R²c indicates conditional error or the total variation described by the full model, including nested random effects (plots within a shelter).

Notes: **Bold** values denote statistical significance at the $p < 0.05$ level.

Abbreviations: DM: dry matter, CP: crude protein, NSC: non-structural carbohydrates, NDF: neutral detergent fibre, ADL: acid detergent lignin, Si: silicon, DDM: digestible dry matter.

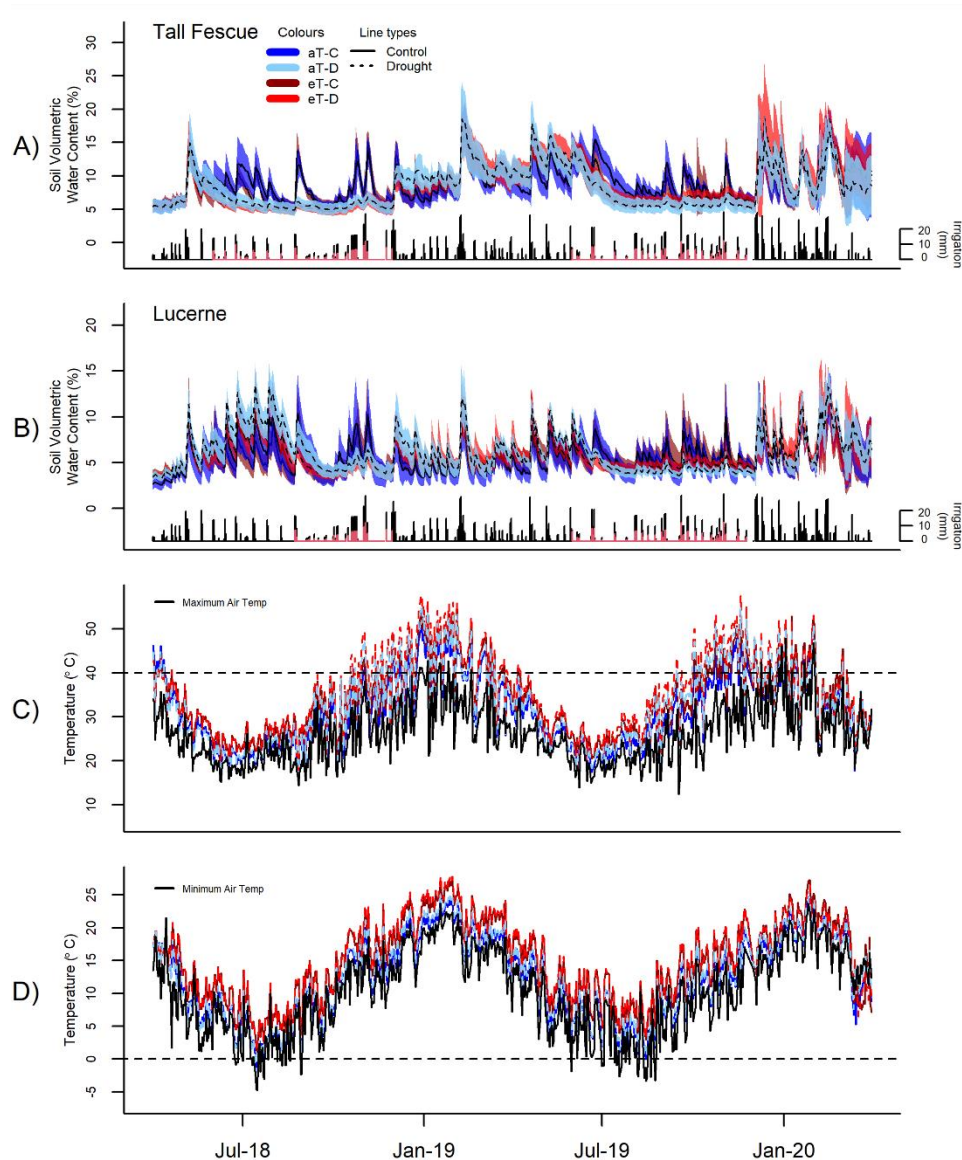


Figure S4.1. Effects of winter/spring drought [applied 1 June (tall fescue) or August (lucerne) to 30 November 2018 and 1 June to 30 November 2019 for both species] and warming (from 16 April 2018 to 30 November 2019) on soil moisture (panels A and B) and plot surface temperature (panels C and D) averaged across the six shelters. Average soil moisture content in A) tall fescue and B) lucerne subplots with 95% confidence intervals as well as individual irrigation events as daily rainfall totals for control (black) and droughted (red) plots over the entire experimental period; C) Daily maximum plot surface temperature, relative to maximum air temperature (black line) and 40 °C (representing extreme temperatures, dashed lines); D) Daily minimum temperature compared with minimum air temperature (black line) and 0 °C (dashed lines). Treatments: aT.C (ambient temperature and control), aT.D (ambient temperature and drought), eT.C (elevated temperature and control), eT.D (elevated temperature and drought).

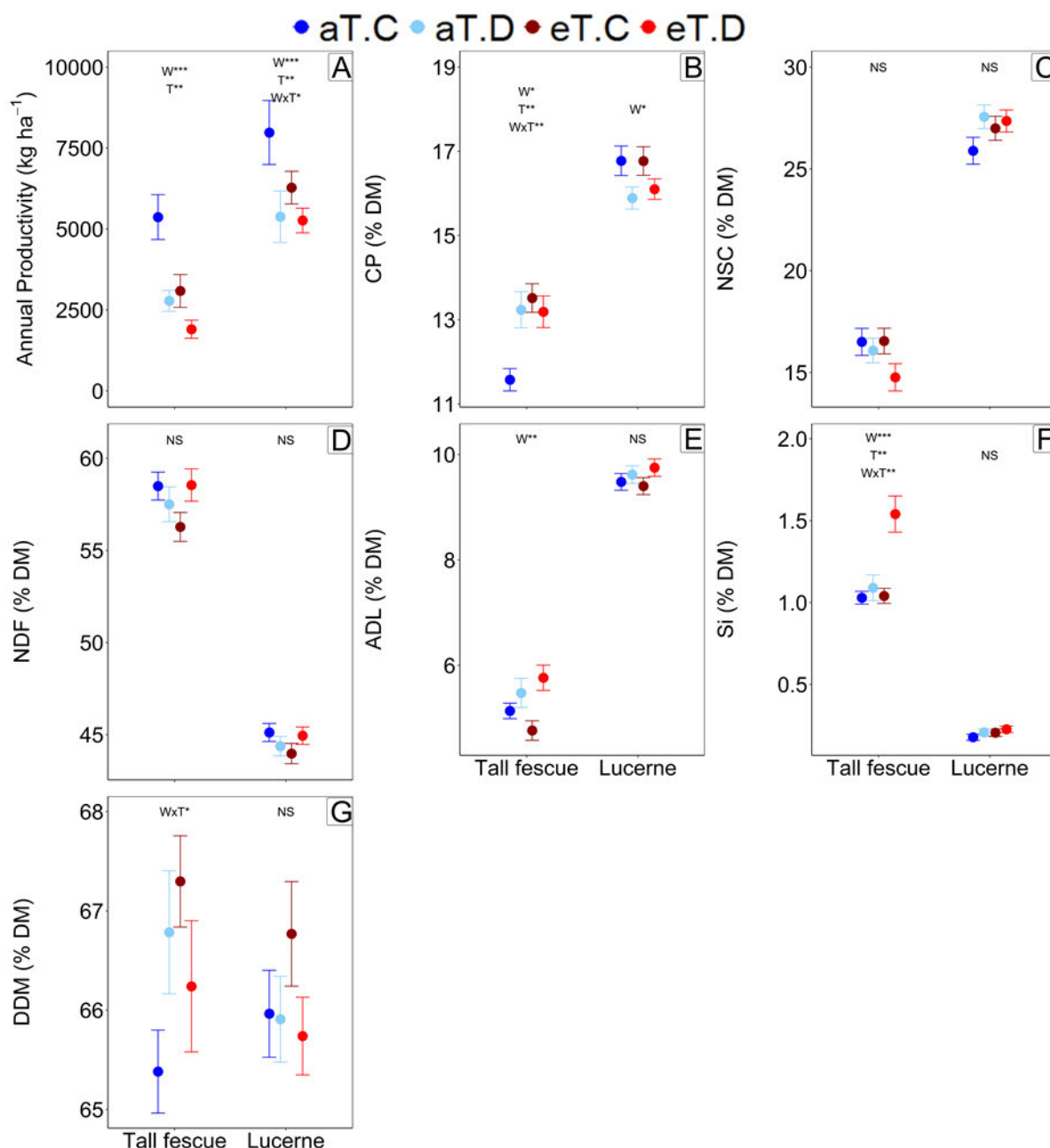


Figure S4.2. Total annual productivity (A) and averages of nutritional parameters (B- crude protein %, C- non-structural carbohydrates %, D- neutral detergent fibre %, E- acid detergent lignin %, F- silicon %, G- digestible dry matter %) of tall fescue (*Festuca arundinacea*) and lucerne (*Medicago sativa*) grown under different temperature and watering treatments during the entire experimental period (from April 2018 to March 2020). Treatments: aT.C (ambient temperature and control), aT.D (ambient temperature and drought), eT.C (elevated temperature and control), eT.D (elevated temperature and drought). Values are means \pm SE. Significant pairwise comparisons for the effect of temperature (T) and watering (W) treatments for each harvest are indicated as follows: NS = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

CHAPTER 5: Plant structural and nutritional responses to drought differ among common pasture species

5.0 ABSTRACT

In the face of a changing climate, research indicates that more frequent and severe drought is a critical problem that will constrain production of forage of high nutritive value in the future. Potential trade-offs in plant morphology and nutritional composition may influence plant drought adaptation strategies, with consequences for forage nutritive value and the performance of grazing animals, in terms of milk and meat production. Here we present the results of a study investigating the effects of drought on biomass productivity, dead material, leaf:stem biomass allocation and nutritional composition (whole-plant and tissue-specific) across nine diverse pasture species. For this, we conducted a field experiment exposing species to a 6-month period of simulated severe drought (60% rainfall reduction during winter and spring). We found that drought had different, harvest-specific effects on plant biomass structure and nutritional composition among pasture species. In general, drought strongly reduced productivity, increased the percentage of dead material and had mixed effects (both increases/decreases and no effect) on leaf:stem ratio and concentrations of crude protein, non-structural carbohydrates, neutral detergent fibre and lignin. Changes in plant-level nutritive value were driven by simultaneous changes in both leaf and stem tissues for most, but not all, species. Overall, the negative impacts of short-term severe drought on pasture were more significant on productivity than on nutritional composition. Our findings may be especially helpful for selection of adapted species/cultivars that could minimize potential drought risks on forage, thereby optimising pasture performance under future drought scenarios.

5.1 INTRODUCTION

Grassland ecosystems, which include managed pastures and rangelands, account for approximately 40% of the Earth's land area and play a key role in food security due to their important service in supplying feeding sources for grazing livestock (ABARES, 2016; Gibson, 2009; Masters et al., 2019; MLA, 2017). However, efficient feeding of livestock is complicated by seasonal and inter-annual changes in plant growth and production (Chapman et al., 2009; Perera et al., 2020). Regional plant productivity is determined by responses to long-term climate patterns, however, local-scale forage availability can be driven by rainfall and associated soil moisture (Brown et al., 2019; McKeon et al., 2009; Murray-Tortarolo and Jaramillo, 2020). As global warming proceeds, many regions of the world will become unable to fulfill requirements for forage quantity and quality, due to more frequent and intense periods of drought (Perera et al., 2020; Rojas-Downing et al., 2017). This will have important implications for the success of the livestock industry and global food production (Dumont et al., 2015; Grant et al., 2014).

Reduced soil moisture increases plant water stress and alters plant physiology (Fay, 2009; Heisler-White et al., 2008), which in turn can change plant structural allocation, morphology and nutritional composition. All of these ultimately impact forage nutritive value (i.e. nutritional composition and digestibility) and, consequently, animal nutrition and performance (AbdElgawad et al., 2014; Herrero et al., 2015; Howden et al., 2008). Although many studies have addressed the effects of drought on aboveground biomass production (Churchill et al., 2021; Perera et al., 2019, 2020), a study gap remains in relation to drought impacts on plant biomass structural allocations and nutritional composition.

Drought can affect forage nutritive value via changes in structure (e.g. proportional allocation to leaves, stems and flowers), nutritional composition and digestibility of said plant parts, with the magnitude of impacts dependent on plant developmental stage and the severity

and duration of drought (Gray and Brady, 2016; IPCC, 2014). Moderate drought stress can delay plant maturation and growth, causing mild or moderate senescence and increases in leaf:stem ratio (Buxton, 1996). However, whole-plant nutritional responses to moderate drought conditions are inconsistent across studies; these include no change or reductions in fibre concentration and no change or slight improvements in both crude protein concentration and digestibility of forage species (Deleglise et al., 2015; Dumont et al., 2015; Kuchenmeister et al., 2013; Staniak and Harasim, 2018). This inconsistency in studies may be explained by differences between plant species, the growing stage of the plant when the drought was imposed, and by differences in the nature of drought treatments. In contrast, studies of prolonged and/or severe drought stress have reported growth inhibition (lower productivity), accelerated maturation, death of plant tissue and decreased leaf:stem ratios (Bruinenberg et al., 2002; Ren et al., 2016). Accompanying these responses are increases in whole-plant fibre concentrations, especially for the lignin fraction, and increased cell-wall thickness and forage toughness, thus reducing the forage nutritive value (Bruinenberg et al., 2002; Deetz et al., 1996; Dumont et al., 2015; Ren et al., 2016). Other studies have also reported reduced nutritive value through the decreased concentrations of crude protein and non-structural carbohydrates due to increased translocation of nitrogen and soluble carbohydrates from leaves to roots as senescence proceeds (Buxton, 1996; Durand et al., 2010). While these changes to plant structure and nutritional composition are generally reported separately (Deleglise et al., 2015; Dumont et al., 2015; Ren et al., 2016), herbivores and graziers experience their consequences in combination. The net impact of these changes upon the nutrition of grazers is relatively unknown.

Severe drought generally results in a decrease of forage nutritive value at the whole-plant level (Buxton, 1996; Deleglise et al., 2015; Durand et al., 2010; Ren et al., 2016), and patterns of resource allocation among plant parts likely underlie many of these changes (Grev

et al., 2020). Because some grazers can forage selectively on different plant parts to various extents, changes to the nutritive value of particular tissues will directly impact herbivores in different ways. There is some evidence that nutritive value responds differently to drought for leaves and stems (Pecetti et al., 2017; Wilson et al., 1983). Changes to the relative proportions of plant fractions and to nutritional composition within leaf and stem tissues may reflect diverse adaptation strategies of plants to water stress and strategies to maintain growth (Buxton and Fales, 1994; Le Gall et al., 2015). Understanding these strategies may help to identify plant traits that confer high drought tolerance on plants whilst maintaining structural and nutritional features that ameliorate effects on animal performance under drought conditions (Cavalcante et al., 2014; Tadielo et al., 2017).

The aims of this study were to investigate the effects of severe drought on pasture productivity, nutritional composition at the whole-plant, leaf and stem levels, the percentage of dead plant material and the leaf:stem biomass ratio. To do this, we conducted a field study exposing nine common pasture species to a 6-month period of severe drought (60% rainfall reduction) during winter and spring. We hypothesised that drought would reduce forage production and nutritive value, with species-specific differences in the magnitude of effects due to trade-offs in resource allocation among plant parts, such as shifts in leaf:stem biomass ratios.

5.2 MATERIALS AND METHODS

5.2.1 Site description

This study was conducted at the Pastures and Climate Extremes (PACE) facility at the Hawkesbury Campus of Western Sydney University, Richmond, New South Wales, Australia (S33.610, E150.740, elevation 25 m; Churchill et al., 2021). The mean annual precipitation at this location is 800 mm (Australian Government Bureau of Meteorology, Richmond - UWS

Hawkesbury Station 1980-2010); however, there is large inter-annual variability (between 500 mm and over 1400 mm over the past 30 years). Winter/spring precipitation accounts for 40% of the annual amount. The mean annual temperature is 17.2 °C, with the warmest and coolest months occurring in January (mean temperature of 22.9 °C) and July (10.2 °C), respectively. The soil is a loamy sand with a volumetric water holding capacity of 15-20%, pH of 5.7, plant available N of 46 mg/kg, plant available (Bray) P of 26 mg/kg and 1% soil organic carbon (Churchill et al., 2021). The field facility comprises six replicate polytunnel rainout shelters (48 m x 8 m) with eight treatment plots (4 m x 4 m) per shelter. Individual treatment plots were further subdivided into four subplots, each with a different monoculture or mixed-species sward (total of 192 subplots). This study focused on all monoculture pasture subplots that were exposed to control and drought treatments, for a total of 108 subplots with nine pasture species. A detailed overview of the experimental facility descriptions is reported in Churchill et al. (2021).

5.2.2 Selection and establishment of pasture species

Monoculture subplots encompassed a range of functional diversity (C₃/C₄ grasses, legumes, annuals and perennials) and species' origins (native grasses, tropical and temperate pastures; **Table 5.1**) that are all either commonly used in improved grasslands (pastures) or in rangelands across southern Australia and internationally, with the exception of the grass *Rytidosperma caespitosum*. All pastures were established prior to winter (*Chloris gayana*, *Digitaria eriantha*, *Festuca arundinacea* and *Themeda triandra*) or spring (remaining species) of 2018 (Churchill et al., 2021) and swards were managed with seasonal fertilizer application to replace nutrients removed from the soil (55 kg/ha; Cal-Gran Aftergraze, Incitec Pivot Fertilisers, Australia) and hand-weeding to maintain target species dominance. The two legume species received appropriate rhizobium inoculant during sward establishment: ALOSCA granular inoculant for

Biserrula pelecinus subplots (Group BS; ALOSCA Technologies, Western Australia, Australia) and EasyRhiz™ soluble legume inoculant and protecting agent for *Medicago sativa* subplots (Group AL; New Edge Microbials, New South Wales, Australia).

Table 5.1. Information about pasture species included in the study.

Species (cultivar)	Origin	Growth Form	Photosynthetic pathways	Lifecycle
<i>Biserrula pelecinus</i> (Casbah)	Temperate, introduced	Legume	C ₃	Annual
<i>Chloris gayana</i> (Katambora)	Tropical, introduced	Grass	C ₄	Perennial
<i>Digitaria eriantha</i> (Premier)	Tropical, introduced	Grass	C ₄	Perennial
<i>Festuca arundinacea</i> (Quantum II MaxP)	Temperate, introduced	Grass	C ₃	Perennial
<i>Lolium perenne</i> (Kidman)	Temperate, introduced	Grass	C ₃	Annual [#]
<i>Medicago sativa</i> (SARDI7 series 2)	Temperate, introduced	Legume	C ₃	Perennial
<i>Phalaris aquatica</i> (Holdfast GT)	Temperate, introduced	Grass	C ₃	Perennial
<i>Rytidosperma caespitosum</i> (Evans)	Temperate, native	Grass	C ₃	Perennial
<i>Themeda triandra</i> (Badgerys Creek, NSW)	Tropical, native	Grass	C ₄	Perennial

#Although this species is perennial, the high summer temperatures at the study site meant it functioned as an annual in this study, dying back at the end of spring and requiring re-sowing.

5.2.3 Experimental treatments and environmental monitoring

All nine pasture species were exposed to the same irrigation regime. The control (C) treatment represented a typical precipitation regime for the local area during years with annual precipitation between 650-750 mm, accounting for long-term patterns in seasonality and in the statistical distribution of event sizes and timing within seasons. In the drought (D) treatment, precipitation event sizes were reduced by 60% throughout the 6-month austral winter/spring period from 1 June to 30 November 2019. This drought treatment represented the drier end of climate model predictions for end-of-century seasonal rainfall change for southeastern Australia, under the Representative Concentration Pathway - RCP8.5 (CSIRO, 2020). A 60% reduction in rainfall falls within the range of observed historical rainfall patterns for key pasture growing regions across southeastern Australia, including the study site, and such extremes are predicted to increase in frequency and duration (Australian Bureau of Meteorology, 2019). Target precipitation was applied using an irrigation system installed in each plot (5 irrigation points in each) as described in Churchill et al. (2021). Prior to the start of the winter season, all plots received the same irrigation inputs (1 December 2018 to 31 May 2019; 419.7 mm total amount).

Environmental monitoring of treatment plots included continuous recording of soil moisture (0-15 cm; 16 per shelter; Time Domain Reflectometers; CS616, Campbell Scientific) in four different species subplots (*Biserrula pelecinus*, *Festuca arundinacea*, *Lolium perenne* and *Medicago sativa*). Air temperature and humidity sensors (Series RHP-2O3B, Dwyer Instruments Inc, USA) mounted in force-ventilated radiation shields were installed inside and outside the rainout shelters at 60 cm height, with records collected every 5 min to determine shelter effects on environmental conditions. The amount of irrigation applied in each treatment, air temperature and soil moisture averaged across the shelters during the 6-month experimental period (1 June to 30 November 2019) can be seen in **Supplementary Figure S5.1**.

5.2.4 Plant sampling during the experimental period and measurements

All subplots were managed and harvested regularly before and during this study based on grazing system recommendations practiced in the study region (Clements et al., 2003). Harvesting involved the use of hand shears and a sickle mower. Prior to the start of the winter season, all species were harvested at the end of May 2019. During the 6-month winter/spring experimental drought period, aboveground productivity was determined via three harvests, one in mid-August, one in early October and one in mid-November 2019, for all species, except for *Chloris gayana* and *Digitaria eriantha* that in the August harvest there was no plant biomass in the respective plots (**Supplementary Table S5.1**). In all harvests, plants were cut to 5 cm above the soil surface and weighed (fresh mass), with a representative sub-sample sorted to remove/exclude weeds and to determine the percentage of dead material in the total biomass by weight (fresh mass); thereafter, all plant biomass sub-samples, including live (green) and dead material, were immediately microwaved at 600W for 90 seconds to stop enzymatic activity (Landhäusser et al., 2018) and then oven-dried at 65 °C for at least 48 hours (until constant weight), and weighed to determine total dry matter productivity (kg DM ha⁻¹; live and dead material) per harvest, for each species and treatment.

5.2.5 Plant structural analysis and sample processing

For the nutritional analysis of the whole-plant material, were analysed dry samples from the August, October and November harvests, which were composed of a proportionally representative mixture of live and dead leaves, stems (or culms/tillers) and inflorescences (**Supplementary Table S5.1**). In addition, for the November harvest, the samples were sorted (composed of both live and dead material) into leaves and stems (or culms/tillers), the fractions were weighed to calculate the leaf:stem ratio (**Supplementary Table S5.1**) and analysed separately for nutritional composition. Dried samples were ground through a 1-mm screen in a

laboratory mill (Foss Cyclotec Mill, Denmark) and stored in airtight plastic containers in the dark at room temperature prior to collection of near-infrared reflectance (NIR) spectra and wet chemical analysis. Plant samples were further homogenized using a ball-mill to produce a fine powder prior to nitrogen determination by elemental analysis (Retsch® MM200; Hann, Germany).

5.2.6 Nutritional analysis

All dried and ground samples were scanned twice and their spectra averaged using a near-infrared reflectance spectrophotometer (NIRS; FOSS XDS Rapid Content™ Analyzer) with a spectral range of 400 to 2500 nm (more details are reported in Chapter 2). Representative samples were selected using the software WinISI 4.8.0 (FOSS Analytical A/S, Denmark) for analysis of nutrient composition by wet chemistry for all parameters, in order to calibrate and validate the NIR.

The selected samples were analysed for ash (ASH) according to the standard methods and procedures for animal feed outlined by the Association of Official Analytical Chemists (AOAC, 1990). Nitrogen (N) concentration was determined from ~ 100 mg samples using an automated combustion method on a Leco TruMac CN analyzer (Leco Corporation, USA). Crude protein (CP) concentration was then calculated by applying a 6.25 conversion factor to the N concentration (AOAC, 1990). Ether extract (EE) was determined according to the American Oil Chemists' Society-AOCS high-temperature method using petroleum ether (B.P. 40-70 °C) and the Soxhlet method (Buchi 810 Soxhlet Multihead Extract Rack, UK). Fibre fractions were determined with an ANKOM Fibre Analyzer (model 200, ANKOM® Technology, NY, USA) with the use of neutral and acid detergent solutions and corrected for dry matter content (Goering and Van Soest, 1970). Samples were analysed for neutral detergent fibre (NDF), acid detergent fibre and acid detergent lignin (ADL) by the sequential method of

Van Soest and Robertson (1980). Sodium sulphite and α -amylase were added to the solution for NDF determination. The values of ASH, EE, CP and NDF were used to calculate non-structural carbohydrates (NSC) according to Sniffen et al. (1992). All nutritional parameters were expressed as percentage of total DM.

Details associated with mathematical treatment of spectra and descriptive statistics for NIRS calibration can be found in Catunda et al. (2021c; see Chapter 2). However, in brief, for the development of NIRS calibration models, modified Partial Least Squares regression with cross-validation was used to develop predictive equations for each nutritional parameter to prevent overfitting of models (Shenk and Westerhaus 1991; Catunda et al., 2021c; see Chapter 2). The NIRS calibration equations were considered to be both suitable and robust to estimate all the nutritional parameters of the samples of all pasture species assessed (Catunda et al., 2021c; see Chapter 2).

5.2.7 Calculations and statistical analysis

Average values were calculated across replicate subplots for each species/water regime combination. Were analysed the effects of drought on pasture productivity, percentage of dead material and nutritional composition of the whole-plant separately for each harvest, but only considered changes in leaf:stem ratio and nutritional composition of leaf and stem fractions at the end of the drought period (November). All pasture responses were analysed using linear mixed-effects (LME) models in the ‘lme4’ package in the software R version 4.0.0 (R Core Team, 2020; Bates et al., 2015). Watering regime (Control: C, Drought: D) was included as a fixed effect and the rainout shelter as a random factor; residuals were checked for normality. The mean effect size due to drought (*Equation 1*; for the figures in the results section) was calculated as the ratio of drought to their respective control treatment values, along with 95% confidence intervals (CI).

$$\text{Equation 1. Effect size} = \frac{\text{Drought}}{\text{Control}} - 1$$

In the effect size figures, positive values represent responses that are greater under drought than in control plots, while negative values represent the opposite. Effect sizes were expressed as percentages (effect size multiplied by 100) in the text throughout the results section.

Finally, to produce a more holistic overview of changes brought about by watering regime treatments on plant response variables across all pasture species that accounts for the non-independence of forage production and structural traits along with nutritional composition, we performed a principal component analysis (PCA) of the data from the end of the drought period (November harvest). To test for the effects of the watering regime on plant responses, permutational analysis of variance (PERMANOVA) was undertaken using the ‘vegan’ package (Oksanen *et al.* 2020) in R version 4.0.0 (R Core Team, 2020).

5.3 RESULTS

5.3.1 Effects of 6-months’ drought on productivity, dead material and nutritional composition of the whole-plant

The effect of drought on dry matter productivity, percentage of dead material and nutritional composition varied among the nine pasture species studied and, in some cases, also differed between individual harvests. These effects included either a significant reduction or no effect on productivity and an increase or no effect on the percentage of dead material. Drought had varied effects on the whole-plant concentrations of CP, NSC, NDF and ADL for different species and harvests (**Table 5.2; Figure 5.1**).

Across species and harvests, productivity ranged from 304 (*Biserrula pelecinus*, November) to 3,685 kg DM ha⁻¹ (*Themeda triandra*, October) under control treatment and the peak productivity varied across species, for example, *Themeda triandra* peaked in October and

Biserrula pelecinus in August (**Table 5.2**). Total productivity under the control treatment, across the six-month period, was highest for *Themeda triandra* (8,366 kg DM ha⁻¹) and *Medicago sativa* (6,845 kg DM ha⁻¹) and lowest for *Chloris gayana* (2,822 kg DM ha⁻¹) and *Festuca arundinacea* (2,817 kg DM ha⁻¹). Droughted subplots were significantly less productive, and drought impacts became progressively greater with time across harvests, with the last two harvests being the most affected (**Figure 5.1A, Supplementary Table S5.2**). All pasture species were significantly affected by the drought, but not all were significantly affected at all harvests. The highest reductions in productivity per harvest occurred for *Themeda triandra* (-80% November) and *Digitaria eriantha* (-73% November), and the lowest for *Medicago sativa* and *Chloris gayana* (both -48%, November; **Figure 5.1A**).

The mean percentage of dead material ranged from 1% to 31% in control subplots, and from 1% to 68% in droughted plots (**Table 5.2**). In contrast to productivity responses, the severity of drought impacts on the percentage of dead material was not generally progressive across harvests, and for some species, in fact, was actually reduced in successive harvests. Overall, the percentage of dead material increased under drought for all species, except *Themeda triandra*, which was not affected in any harvest ($p > 0.05$; **Figure 5.1B; Supplementary Table S5.2**). The most strongly affected species were *Lolium perenne* (+886%, November), *Phalaris aquatica* (+429%, October) and *Festuca arundinacea* (+429%, November). And the least affected species were *Biserrula pelecinus* (+153%, October) and *Medicago sativa* (+117%, November; **Figure 5.1B**).

Effects of drought on whole-plant nutritional composition were apparent for seven of the nine study species. The exceptions were *Medicago sativa* and *Phalaris aquatica*, which, in fact, experienced no significant treatment impacts on nutritive value at any time throughout the experiment (**Table 5.2; Supplementary Table S5.2**). Drought effects on nutritional parameters are summarised in **Figure 5.1C-F**. In the August harvest, drought only had impacts

on *Festuca arundinacea* and *Lolium perenne*, which experienced an increase in CP (+16% for both species). In October, the drought increased CP (+11%) in *Biserrula pelecinus* and NSC (+31%) in *Themeda triandra*, but decreased CP (-17%) and slightly increased NDF (+4%) in *Digitaria eriantha*. In the final harvest (November), drought was associated with improved nutritional value in *Digitaria eriantha* (+41% CP and -8% NDF) and *Themeda triandra* (+15% NSC and -20% ADL), but reduced the nutritive value of *Lolium perenne* through an increase in NDF (+9%) and ADL (+63%).

Table 5.2. Mean \pm standard errors (n = 6) for productivity (kg DM ha⁻¹), percentage of dead material, and nutritional composition (in percentage of dry matter) of the whole-plant of pasture species grown under different watering regimes (control, C; drought, D) treatments during the drought period (August, October and November harvests).

Species	Harvest	Productivity		Dead material		CP		NSC		NDF		ADL	
		C	D	C	D	C	D	C	D	C	D	C	D
Bis	August	1737 \pm 376	812 \pm 189*	2.2 \pm 0.7	3.6 \pm 0.9	19.2 \pm 0.6	18.9 \pm 0.8	29.3 \pm 0.6	29.5 \pm 1.3	38.3 \pm 0.7	38.2 \pm 2.0	7.9 \pm 0.2	8.1 \pm 0.3
	October	882 \pm 177	323 \pm 52.1*	1.7 \pm 0.7	4.3 \pm 0.9*	16.1 \pm 0.3	17.7 \pm 0.6*	30.9 \pm 0.9	29.1 \pm 0.4	42.2 \pm 1.1	41.2 \pm 0.8	7.6 \pm 0.5	7.7 \pm 0.4
	November	304 \pm 83.8	124 \pm 35.7	6.9 \pm 1.1	10.3 \pm 3.1	14.6 \pm 0.3	16.3 \pm 0.3*	30.8 \pm 0.7	28.3 \pm 1.5	44.7 \pm 0.9	45.4 \pm 1.4	7.6 \pm 0.2	8.1 \pm 0.3
Chl	October	1176 \pm 137	702 \pm 96.5	7.2 \pm 1.1	11.2 \pm 2.7	6.3 \pm 0.4	6.6 \pm 0.3	17.5 \pm 0.9	17.0 \pm 0.7	67.3 \pm 0.6	66.5 \pm 0.5	4.3 \pm 0.2	4.0 \pm 0.3
	November	1646 \pm 168	844 \pm 12.7*	6.2 \pm 1.6	31.3 \pm 3.1*	6.7 \pm 0.3	7.2 \pm 0.4	17.3 \pm 0.7	15.3 \pm 0.5	65.6 \pm 0.7	66.5 \pm 1.1	2.9 \pm 0.1	5.9 \pm 0.6*
Dig	October	1128 \pm 109	389 \pm 82.5*	9.6 \pm 1.9	53.3 \pm 17*	11.6 \pm 0.8	9.6 \pm 0.9*	12.9 \pm 0.9	12.6 \pm 0.5	62.6 \pm 0.8	65.2 \pm 0.9*	6.2 \pm 0.3	6.4 \pm 0.4
	November	2535 \pm 217	681 \pm 187*	6.7 \pm 1.5	14.6 \pm 2.7*	7.1 \pm 0.3	10.0 \pm 0.2*	15.0 \pm 0.8	15.9 \pm 0.5	67.1 \pm 0.8	62.0 \pm 0.8*	3.8 \pm 0.3	3.3 \pm 0.2
Fes	August	1204 \pm 133	1076 \pm 52.5	7.1 \pm 0.4	10.1 \pm 2.4	12.1 \pm 0.5	14.0 \pm 0.4*	21.7 \pm 1.1	20.8 \pm 1.2	54.2 \pm 0.5	52.4 \pm 1.2	4.5 \pm 0.1	3.9 \pm 0.4
	October	1001 \pm 117	328 \pm 61.1*	6.5 \pm 1.1	24.6 \pm 3.1*	11.6 \pm 0.5	13.0 \pm 0.5	19.7 \pm 0.5	17.3 \pm 1.1	57.1 \pm 0.8	58.0 \pm 1.0	4.3 \pm 0.3	4.9 \pm 0.3
	November	612 \pm 68.3	513 \pm 67.6	12.6 \pm 3.9	66.6 \pm 3.3*	11.0 \pm 0.4	12.3 \pm 0.3*	17.3 \pm 1.4	16.7 \pm 0.9	56.7 \pm 1.5	58.2 \pm 0.8	6.0 \pm 0.6	7.5 \pm 0.4*
Lol	August	2085 \pm 328	2707 \pm 311	2.5 \pm 0.3	3.0 \pm 0.5	8.4 \pm 0.3	9.7 \pm 0.3*	39.1 \pm 0.5	36.1 \pm 1.3	41.9 \pm 0.7	42.4 \pm 0.9	2.8 \pm 0.1	2.8 \pm 0.2
	October	1110 \pm 163	335 \pm 55.0*	3.0 \pm 0.9	16.6 \pm 4.0*	11.8 \pm 0.6	14.3 \pm 0.4*	25.2 \pm 0.3	21.2 \pm 0.9*	51.2 \pm 0.8	52.0 \pm 1.1	3.5 \pm 0.3	4.2 \pm 0.3
	November	599 \pm 108	192 \pm 26.3*	5.1 \pm 0.8	50.3 \pm 6.3*	11.2 \pm 0.4	11.9 \pm 0.4	20.9 \pm 0.9	19.1 \pm 0.8	53.7 \pm 1.0	58.3 \pm 1.0*	3.2 \pm 0.3	5.2 \pm 0.4*
Med	August	1503 \pm 230	1417 \pm 204	1.6 \pm 0.6	2.1 \pm 1.1	15.3 \pm 0.4	15.2 \pm 0.4	30.7 \pm 0.6	32.5 \pm 0.8	42.6 \pm 0.9	40.6 \pm 0.9	9.4 \pm 0.3	9.6 \pm 0.3
	October	2610 \pm 275	1577 \pm 271*	1.0 \pm 0.4	1.0 \pm 0.1	17.2 \pm 0.9	15.5 \pm 1.0	27.4 \pm 1.5	29.2 \pm 1.1	44.3 \pm 1.1	44.5 \pm 1.9	8.8 \pm 0.2	9.0 \pm 0.4

	November	2732 ± 400	1425 ± 226*	1.0 ± 0.2	1.3 ± 0.7*	15.5 ± 0.7	14.4 ± 0.3	28.7 ± 0.9	30.7 ± 0.6	44.7 ± 1.7	44.4 ± 0.8	8.5 ± 0.2	8.5 ± 0.3
Pha	August	1817 ± 284	1148 ± 135*	1.7 ± 0.4	4.5 ± 0.8*	14.1 ± 0.4	14.1 ± 0.3	26.5 ± 1.2	25.4 ± 0.6	45.9 ± 0.5	46.8 ± 0.5	2.9 ± 0.2	2.4 ± 0.3
	October	1299 ± 130	523 ± 54.2*	1.4 ± 0.4	7.4 ± 3.0*	15.3 ± 0.7	16.5 ± 0.3	18.2 ± 0.9	16.0 ± 0.5	53.1 ± 0.8	53.9 ± 0.3	2.9 ± 0.3	3.3 ± 0.1
	November	621 ± 72.8	236 ± 41.0*	5.9 ± 1.5	29.7 ± 4.5*	9.7 ± 0.4	10.6 ± 0.3	16.2 ± 0.4	15.3 ± 1.1	61.9 ± 0.9	62.2 ± 0.7	4.0 ± 0.3	4.9 ± 0.7
Ryt	August	2081 ± 397	1439 ± 169	2.8 ± 0.4	4.2 ± 0.3	11.0 ± 0.5	11.5 ± 0.3	14.7 ± 1.0	13.1 ± 0.9	63.5 ± 0.8	64.2 ± 0.5	4.0 ± 0.3	3.9 ± 0.2
	October	1932 ± 317	727 ± 98.4*	2.1 ± 0.7	9.0 ± 1.6*	10.8 ± 0.2	12.0 ± 0.3	10.1 ± 0.4	10.8 ± 0.8	69.8 ± 0.3	67.3 ± 0.5*	2.2 ± 0.2	3.4 ± 0.3*
	November	618 ± 123	323 ± 52.8	31.0 ± 5.7	67.7 ± 8.4*	9.9 ± 0.3	10.1 ± 0.2	13.2 ± 0.6	13.9 ± 0.6	68.4 ± 0.9	66.3 ± 0.5	5.3 ± 0.6	6.4 ± 0.3*
The	August	2764 ± 241	1972 ± 213	2.9 ± 0.8	4.0 ± 1.7	9.5 ± 0.3	9.1 ± 0.2	16.0 ± 0.7	16.9 ± 0.5	65.8 ± 0.9	64.8 ± 0.5	4.9 ± 0.2	5.0 ± 0.2
	October	3685 ± 272	1136 ± 214*	1.0 ± 0.1	1.0 ± 0.1	9.1 ± 0.4	8.1 ± 0.3	13.3 ± 0.8	17.4 ± 1.0*	70.2 ± 0.7	67.7 ± 1.3	3.7 ± 0.3	3.2 ± 0.5
	November	1917 ± 130	379 ± 46.9*	3.3 ± 0.4	4.5 ± 0.4	7.2 ± 0.1	7.4 ± 0.2	15.8 ± 0.4	18.2 ± 0.5*	69.7 ± 0.5	67.9 ± 0.4	4.4 ± 0.1	3.5 ± 0.3*

Note: Asterisks () and **bold** values denote statistical significance at the $p \leq 0.05$ level. During the harvest in August, there was no biomass for *Chloris gayana* and *Digitaria eriantha* under both treatments.*

Abbreviations: CP: crude protein; NSC: non-structural carbohydrates; NDF: neutral detergent fibre; ADL: acid detergent lignin; Bis: Biserrula pelecinus; Chl: Chloris gayana; Dig: Digitaria eriantha; Fes: Festuca arundinacea; Lol: Lolium perenne; Med: Medicago sativa; Pha: Phalaris aquatica; Ryt: Rytidosperma caespitosum; The: Themeda triandra.

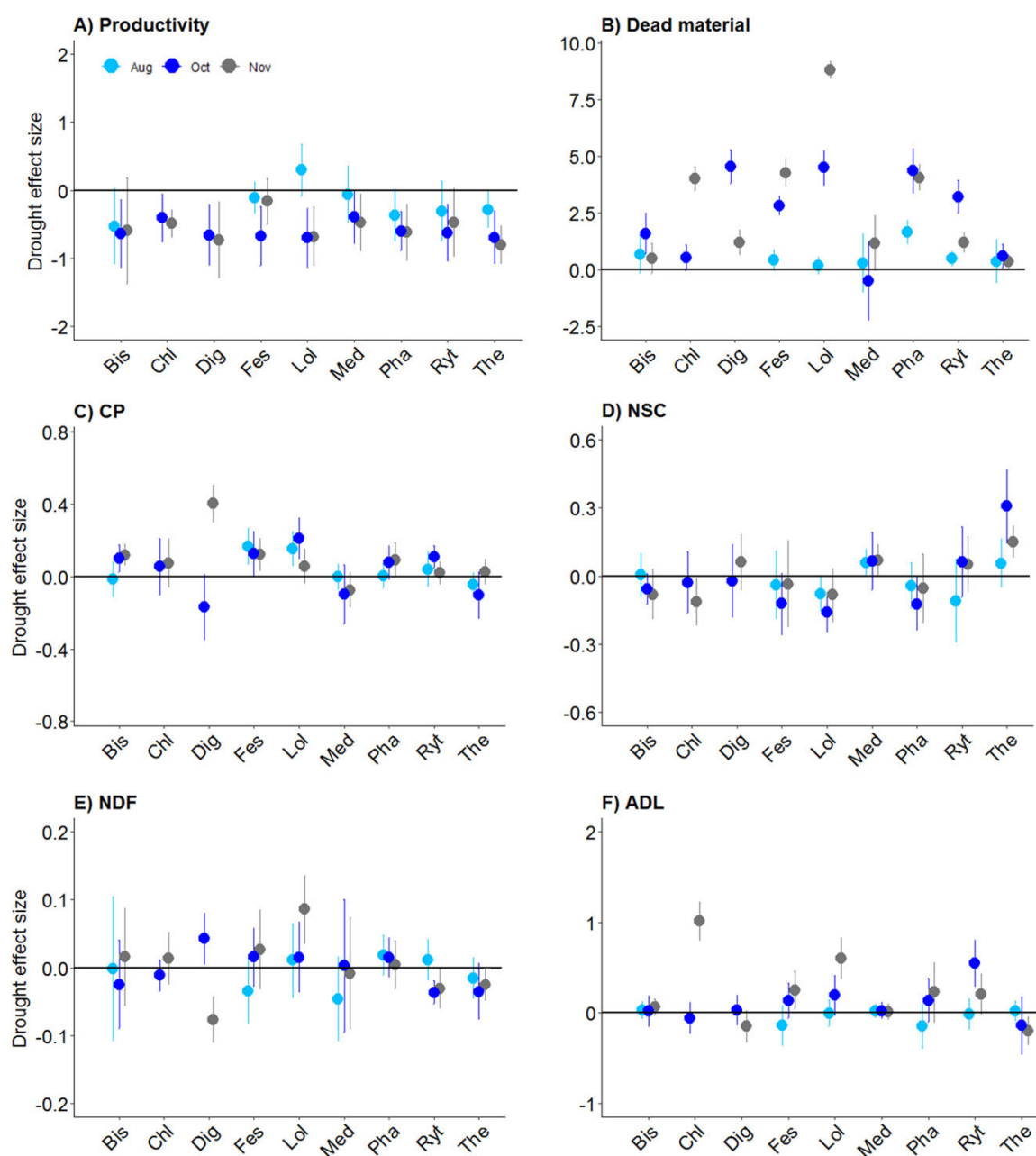


Figure 5.1. Drought effect sizes on A) productivity, B) percentage of dead material, and whole-plant nutritional composition [C) crude protein, D) non-structural carbohydrates, E) neutral detergent fibre, F) acid detergent lignin] of pasture species during the drought period (August, October and November harvests separately). Values shown are means with vertical bars representing 95% confidence intervals (n = 6). Species abbreviations are as follows: *Biserrula pelecinus* (Bis), *Chloris gayana* (Chl), *Digitaria eriantha* (Dig), *Festuca arundinacea* (Fes), *Lolium perenne* (Lol), *Medicago sativa* (Med), *Phalaris aquatica* (Pha), *Rytidosperma*

caespitosum (Ryt), *Themeda triandra* (The). Note: during the harvest in August, there was no biomass for *Chloris gayana* and *Digitaria eriantha*.

5.3.2 Effects of drought on leaf:stem ratio and nutritional composition of leaf and stem tissues

At the end of the 6-month period of drought (November harvest), the mean leaf:stem ratio ranged from 0.7 to 7.9 under control conditions across pasture species, reflecting the range of plant growth forms and differences in phenology; under drought, leaf:stem ratios ranged from 0.8 to 9.5 (**Table 5.3**). The drought treatment significantly increased the leaf:stem ratio of *Phalaris aquatica* by 129%, *Themeda triandra* by 102%, and *Digitaria eriantha* by 80%, and decreased that of *Chloris gayana* by 50% (**Figure 5.2A**). However, drought had no effect on the leaf:stem ratios of the remaining species ($p > 0.05$; **Supplementary Table S5.3**).

Tissue-specific responses to drought varied in both magnitude and direction across pasture species (**Table 5.3**; **Supplementary Table S5.3**). For instance, drought increased CP in *Biserrula pelecinus* and *Festuca arundinacea* leaf tissue, in both leaves and stems in *Digitaria eriantha* and in the stems only in *Lolium perenne* (**Figure 5.2B**). In contrast, drought decreased NSC in *Lolium perenne* stems while increased it in both plant parts in *Themeda triandra* (**Figure 5.2C**). Drought also decreased NDF in *Digitaria eriantha* stems and leaves, while increased it in *Lolium perenne* leaves (**Figure 5.2D**). In both plant parts of *Chloris gayana*, *Festuca arundinacea* and *Lolium perenne*, as well as in *Rytidosperma caespitosum* stems, the drought increased ADL, while in *Themeda triandra* stems it decreased. (**Figure 5.2E**). Interestingly, *Medicago sativa* and *Phalaris aquatica* were the only species where drought affected the nutritional composition of individual plant parts but not the whole-plant. Specifically, in *Medicago sativa*, drought increased NSC in the stem tissue, with no other changes detected. For *Phalaris aquatica*, drought affected plant parts in opposite directions for NSC (**Figure 5.2C**) and NDF (**Figure 5.2D**).

Table 5.3. Mean \pm standard errors (n = 6) for leaf:stem ratio and nutritional composition of plant parts (leaves and stems) of pasture species grown under different watering regimes (control, C; drought, D) treatments at the end of the drought period (November harvest).

Species	Leaf:stem		Plant parts	Parameters (in percentage of dry matter)							
				CP		NSC		NDF		ADL	
	C	D		C	D	C	D	C	D	C	D
Bis	1.6 ± 0.1	1.3 ± 0.1	Leaves	17.1 ± 0.4	19.7 ± 0.3*	38.1 ± 0.5	35.4 ± 1.4	32.7 ± 1.0	31.9 ± 1.4	8.1 ± 0.3	7.8 ± 0.3
			Stems	10.6 ± 0.7	11.8 ± 0.7	28.7 ± 1.3	26.1 ± 0.9	51.5 ± 1.2	52.9 ± 0.7	9.4 ± 0.4	9.9 ± 0.5
Chl	4.1 ± 0.9	2.0 ± 0.2*	Leaves	7.3 ± 0.4	8.1 ± 0.4	17.1 ± 0.7	14.9 ± 1.0	64.1 ± 0.7	64.2 ± 1.5	2.5 ± 0.2	6.0 ± 0.9*
			Stems	4.7 ± 0.5	5.5 ± 0.5	17.7 ± 1.3	16.5 ± 0.9	70.9 ± 1.1	70.8 ± 0.6	4.2 ± 0.4	5.9 ± 0.4*
Dig	4.8 ± 1.1	8.7 ± 1.8*	Leaves	7.6 ± 0.4	10.4 ± 0.2*	14.7 ± 1.1	16.1 ± 0.7	65.5 ± 0.9	60.9 ± 0.7*	3.9 ± 0.3	3.2 ± 0.2
			Stems	4.9 ± 0.3	7.6 ± 0.6*	14.5 ± 0.9	15.8 ± 1.1	74.2 ± 1.2	69.0 ± 1.5*	3.4 ± 0.2	4.4 ± 0.5
Fes	7.7 ± 1.3	9.5 ± 1.7	Leaves	11.5 ± 0.4	12.6 ± 0.3*	17.2 ± 1.5	16.2 ± 1.0	57.4 ± 1.6	58.1 ± 0.9	6.3 ± 0.7	7.7 ± 0.4*
			Stems	8.9 ± 0.9	9.5 ± 0.5	20.3 ± 0.9	20.8 ± 0.7	61.7 ± 0.9	58.7 ± 0.7*	4.8 ± 0.3	6.4 ± 0.5*
Lol	7.9 ± 0.5	8.1 ± 2.2	Leaves	11.8 ± 0.5	12.2 ± 0.4	20.0 ± 1.0	18.4 ± 1.0	53.9 ± 0.7	58.5 ± 1.1*	3.3 ± 0.3	5.3 ± 0.5*
			Stems	8.2 ± 0.5	9.6 ± 0.2*	28.8 ± 1.8	24.3 ± 1.3*	54.5 ± 1.1	57.4 ± 1.2	2.5 ± 0.4	4.3 ± 0.3*
Med	0.7 ± 0.1	0.8 ± 0.1	Leaves	22.3 ± 0.1	21.1 ± 0.2	33.7 ± 0.6	36.0 ± 0.9	30.7 ± 0.5	30.7 ± 0.6	9.0 ± 0.2	8.8 ± 0.5
			Stems	8.9 ± 0.3	8.8 ± 0.3	22.9 ± 0.9	26.4 ± 1.1*	60.1 ± 1.7	57.2 ± 1.4	8.1 ± 0.3	8.6 ± 0.4

Pha	1.4 ± 0.1	3.1 ± 0.4*	Leaves	12.2 ± 0.3	12.0 ± 0.4	16.7 ± 0.5	13.7 ± 1.3*	56.4 ± 0.5	60.7 ± 1.1*	4.8 ± 0.4	5.3 ± 0.9
			Stems	5.8 ± 0.2	6.5 ± 0.4	16.0 ± 0.8	20.2 ± 1.1*	71.1 ± 0.5	66.0 ± 0.6*	3.1 ± 0.4	3.7 ± 0.3
Ryt	4.1 ± 1.3	4.4 ± 0.6	Leaves	11.0 ± 0.3	11.0 ± 0.3	13.3 ± 1.0	14.5 ± 0.6	65.5 ± 1.4	64.6 ± 0.8	6.5 ± 0.7	6.5 ± 0.3
			Stems	5.9 ± 0.5	6.5 ± 0.4	15.0 ± 1.3	12.9 ± 1.2	73.3 ± 0.9	73.1 ± 0.8	4.9 ± 0.3	6.6 ± 0.4*
The	1.7 ± 0.3	3.4 ± 0.6*	Leaves	9.6 ± 0.1	8.8 ± 0.1	16.4 ± 0.5	18.4 ± 0.5*	64.5 ± 0.4	65.2 ± 0.4	4.1 ± 0.2	3.4 ± 0.4
			Stems	3.3 ± 0.3	3.4 ± 0.4	11.8 ± 1.0	15.0 ± 0.9*	80.5 ± 1.5	78.0 ± 1.3	4.9 ± 0.4	4.0 ± 0.1*

Note: Asterisks (*) and **bold** values denote statistical significance at the $p \leq 0.05$ level.

Abbreviations: CP: crude protein; NSC: non-structural carbohydrates; NDF: neutral detergent fibre; ADL: acid detergent lignin; Bis: Biserrula pelecinus; Chl: Chloris gayana; Dig: Digitaria eriantha; Fes: Festuca arundinacea; Lol: Lolium perenne; Med: Medicago sativa; Pha: Phalaris aquatica; Ryt: Rytidosperma caespitosum; The: Themeda triandra.

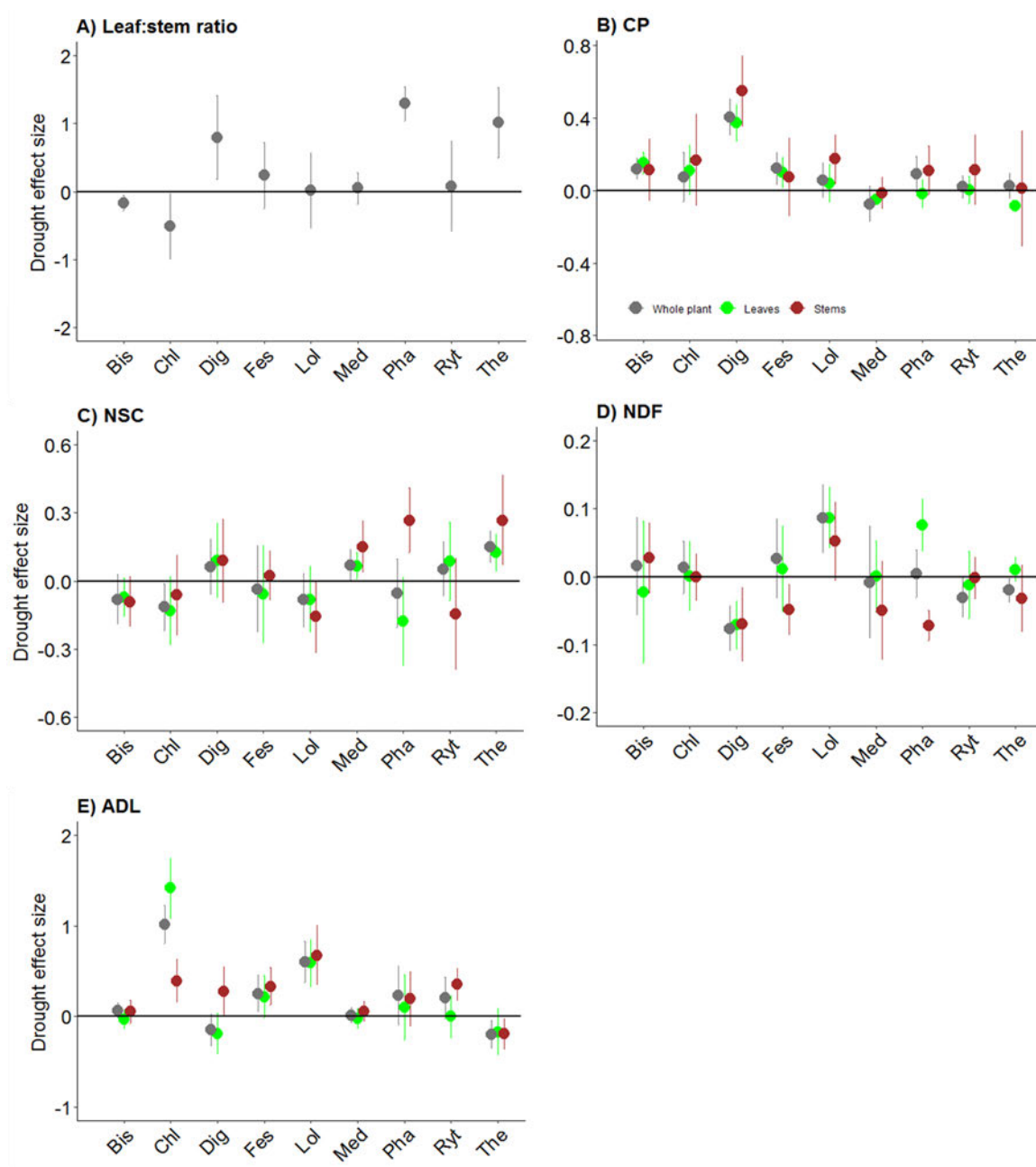


Figure 5.2. Drought effect sizes on leaf:stem ratio and nutritional composition [A) crude protein, B) non-structural carbohydrates, C) neutral detergent fibre, D) acid detergent lignin] of whole-plant (grey circle), leaves (green circle) and stems (brown circle) of pasture species at the end of the drought period (November harvest). Values shown are means with vertical bars representing 95% confidence intervals (n = 6). Species abbreviations follow Figure 5.1.

5.3.3 Assessing plant responses to drought in a multivariate context

The first two principal components explained 73% of the variation in plant responses across treatments (**Figure 5.3**). Drought had a significant effect (PERMANOVA: $p < 0.01$) across all the species (**Figure 5.3A**). Differences in multivariate plant responses among individual species were further apparent with clear separation between responses of legumes (*Biserrula pelecinus* and *Medicago sativa*) and grasses (PC1; **Figure 5.3B**), and within grasses, with C₃ grasses differing from C₄ grasses along PC2. The first principal component (PC1, 47.9% data variance) was associated with nutritional composition and had positive loadings for CP, ADL and NSC, and negative loadings for NDF (**Figure 5.3C**). The second component (PC2, 24.7% data variance) was associated with plant structural characteristics, including positive loadings for the percentage of dead material and leaf:stem ratio, and negative loadings for total biomass production. Overall, nutritional parameters explained a greater proportion of the variance in data than morphological parameters across treatments and all studied pasture species.

In general, the percentage of dead material and leaf:stem ratio were negatively associated with total biomass production, while CP, ADL and NSC were negatively associated with NDF. The control treatment was associated with higher biomass and the drought treatment with more dead material and a high leaf:stem ratio. Furthermore, the percentage of dead material and leaf:stem ratio were higher for C₃ grasses; high concentrations of ADL, CP and NSC were associated with legumes, while high values of NDF were associated with C₄ grasses.

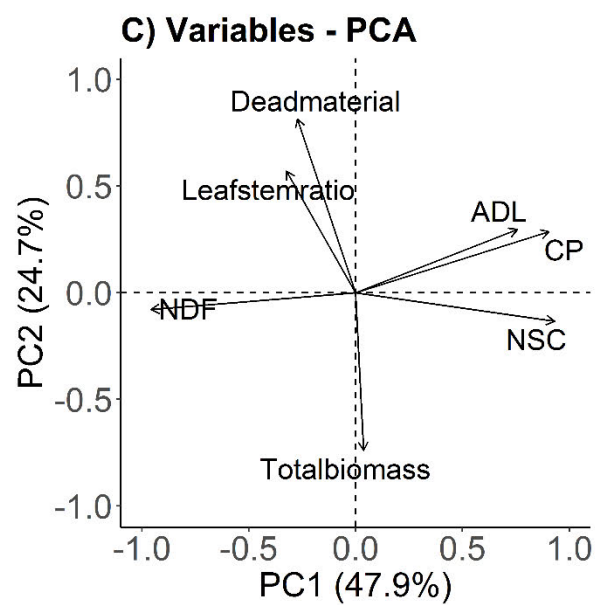
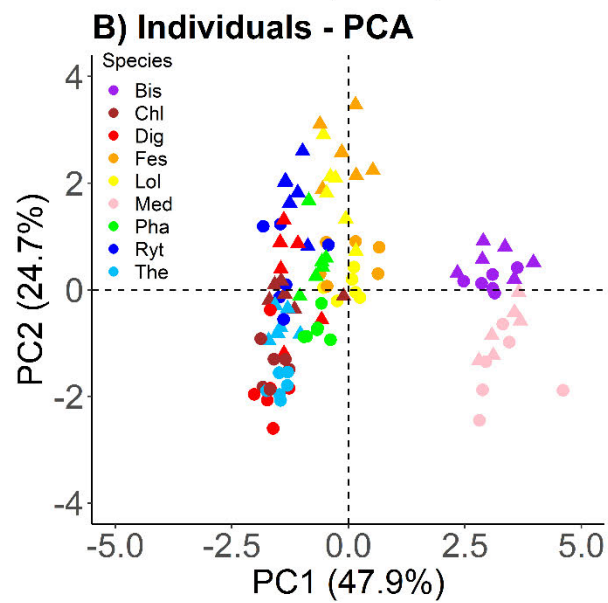
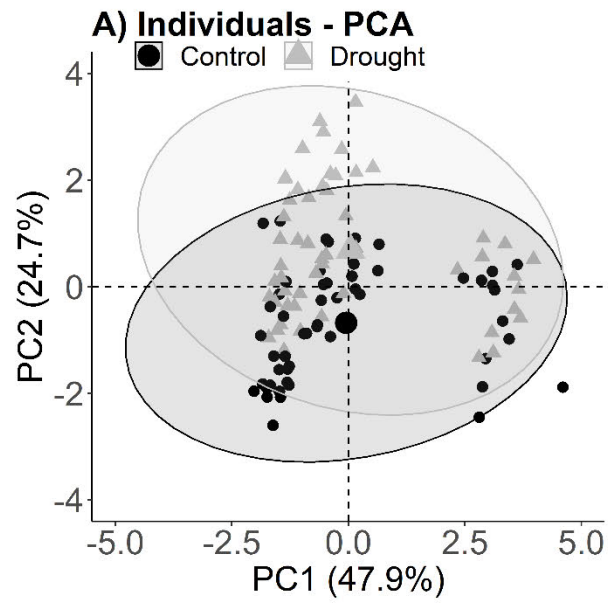


Figure 5.3. Principal component biplots illustrating variation in total biomass production, structural and nutritional traits across pasture species: A) scores for plant individuals grouped by treatment (control and drought; shapes) with 95% confidence ellipses, B) scores for species individuals (colours) and C) variables loadings. Nutritional parameters abbreviations are as follows: crude protein (CP), non-structural carbohydrates (NSC), neutral detergent fibre (NDF), acid detergent lignin (ADL). Species abbreviations follow Figure 5.1.

5.4 DISCUSSION

Here, we presented the effects of a 6-month winter/spring drought on productivity as well as aboveground plant structure and nutritional composition for a diverse range of globally-important pasture species. Consistent with our hypothesis, we found that drought reduced pasture productivity and increased dead material across multiple time points, although some species were unaffected during individual harvests. In most species, ongoing drought amplified the negative effects on productivity from one harvest to the next. There were large differences in the magnitude and direction of the responses of species to water stress in terms of leaf:stem biomass ratios and nutritive value. In some cases, these findings were contrary to our expectations of reduced nutritional value under drought. Significant changes to whole-plant nutritive value were generally driven by simultaneous changes to both leaf and stem tissues, but in a few species were driven by changes in only one tissue. Overall, the effect of drought on nutritive value was considerably less pronounced than that on productivity and the percentage of dead material. Across the entire experiment, *Chloris gayana*, *Lolium perenne* and *Rytidosperma caespitosum* were the species most adversely impacted by cool-season drought in terms of productivity, dead material and nutritive value, while *Biserrula pelecinus* and *Themeda triandra* were the least affected. *Medicago sativa* and *Phalaris aquatica* were the only species with no change in nutritive value under drought conditions. The species-specific

nature of morphological and nutritional responses to drought highlights the importance of carrying out studies across multiple plant species, with diverse traits, to better understand climate change impacts on pastures.

5.4.1 Productivity and dead material

Change in aboveground productivity is a fundamental plant response to environmental change (Wang et al., 2007). Studies have highlighted the impacts of drought on biomass reduction across pasture species, however, uncertainty remains in terms of the nature and magnitude of the effects, and the consequences for production systems and/or ecosystem function (Cantarel, et al., 2013; Deleglise et al., 2015; Grant et al., 2014). Declining soil water content reduces the ability of plants to acquire sufficient water and nutrients for normal functioning, resulting in lower rates of plant growth and, in severe cases, tissue death (Buxton, 1996; Bruinenberg et al., 2002; Durand et al., 2010; Ren et al., 2016). The physiological mechanisms underpinning growth responses are often species-specific and reflect different strategies associated with drought resistance and drought survival (Baruch, 1994; Guenni et al., 2002; Munné-Bosch and Alegre, 2004).

Our observed reductions in productivity of up to 80% in individual harvests and up to an 8-fold increase in the percentage of dead material align with previous studies exposing grassland species to short/long-term or moderate/severe drought conditions, which have reported large declines in biomass production (Cantarel et al., 2013; Deleglise et al., 2015) and increases in dead biomass (Power et al., 2016; Skinner et al., 2004). The large increase in the percentage of dead material in most of the species in this study may be due to both advanced senescence and a more rapid life cycle, as previously reported in severe drought stress scenarios (Bruinenberg et al., 2002; Ren et al., 2016). In our study, *Digitaria eriantha* and *Phalaris aquatica* showed consistent reductions in production and increases in dead material across the

6-month drought treatment, whereas other species had responses that differed between harvests. There are a number of mechanisms that might drive such differences, including different drought sensitivities at various stages in the life cycle of the plant or different degrees of realised water stress—reflecting the actual timing of rain (irrigation) events and temperature differences driving potential evapotranspiration, at different stages in winter and spring. In addition, these temporally variable effects of drought align with research emphasizing plant species adjustments in growth and resource allocation during exposure to drought conditions (Eziz et al., 2017; Gray and Brady, 2016). For example, unaffected species may accumulate nutrients that were not used for growth during a drought event, but then are available for a rapid increase in leaf growth during any rewatering event that preceded a specific harvest, as reported by Guenni et al. (2002) in a study with forage grass species. Overall, our findings highlight species differences in ability to tolerate and adapt to drought, as well as seasonal/phenology effects on the extent of drought sensitivity (Gray and Brady, 2016; Lee et al., 2013).

5.4.2 Nutritional composition and structural biomass allocation

Reduced growth and increased senescence and/or death of biomass during drought have been reported to significantly affect the nutritive value of forage species (Deleglise et al., 2015; Dumont et al., 2015; Ren et al., 2016). The proportion of aboveground biomass that is dead influences forage nutritive value, as dead herbage is always associated with low forage energy value and digestibility (Hodgson et al., 1990; Shakhane et al., 2013). We found a significant negative correlation between the percentage of dead material and digestibility across all pasture species from both watering regimes throughout the experimental period (**Supplementary Figure S5.2A**). While lower forage nutritive value, is often reported in severe drought conditions (Buxton, 1996; Deleglise et al., 2015; Durand et al., 2010; Ren et al., 2016), no change or slight improvements in nutritive value are commonly reported in response to

moderate drought (Dumont et al., 2015; Kuchenmeister et al., 2013; Staniak and Harasim, 2018). Here, we found that drought decreased nutritive value in a few species, while increasing it in the majority of the species and having no effect on a few others.

In this study, the drought-related decrease in nutritive value associated with increased fibre, mainly the lignin fraction, may be explained by plant maturation, leaf senescence and anatomical or cellular modifications that certain species develop to prevent water losses and to improve plant stress resistance (Habermann et al., 2021; Le Gall et al., 2015). Previous studies have reported that under severe drought stress, as plant maturation accelerates, stem growth advances, thereby decreasing the leaf:stem ratio and increasing the accumulation of fibrous components, which may result in forage toughness and lower digestibility (Bruinenberg et al., 2002; Deetz et al., 1996; Dumont et al., 2015; Ren et al., 2016). Our hypothesis that the reduction in nutritive value due to severe drought would be associated with a decrease in leaf:stem ratio was confirmed only for one species (*Chloris gayana*). In addition, no correlation was found between leaf:stem ratio and digestibility under drought conditions among the pasture species throughout the experimental period, although there was a positive correlation under control conditions (**Supplementary Figure S5.2B**). In addition, severe drought causes plant cells to lose water or even die, and accumulation of lignin (an important component of the plant cell wall) can reduce plant cell wall water penetration and transpiration, helping to maintain cell osmotic balance and protect membrane integrity under drought stress (Liu et al., 2018b, Moura et al., 2010). These changes may have important implications for animal nutrition as lignin acts as a barrier to fibre degradation by rumen microbes, making energy from fibre unavailable for ruminants and ultimately decreasing forage digestibility (Amiri et al., 2012; Buxton et al., 1995; Grev et al., 2020; Jung et al., 1997).

In our study, although we found that under drought, *Chloris gayana* and *Lolium perenne* significantly increased lignin (up to +103% and +63%, respectively; November harvest), only

Lolium perenne decreased digestibility (2 units) when compared to the control treatment (**Supplementary Figure S5.3**). However, despite this, *Lolium perenne* digestibility was still within the digestibility range (60-70%) required for maintaining moderate livestock production (DPI, 2020). These findings indicate that drought-induced declines in nutritive value may not preclude the continued ability of some species to provide sufficient nutrients for maintaining the digestive process and moderate animal production. The only exception to this was for *Chloris gayana*, a C₄ grass, in which CP (~ 6.5%) was insufficient, even under control conditions, to ensure adequate fermentation and thus might reduce nutrient utilization efficiency by the ruminal microbiota and negatively affect animal production (NRC, 2001; Van Soest, 1994). If *Chloris gayana* is used as the principal pasture species, it must therefore be used in conjunction with high-protein food, such as legume species or urea supplementation, to optimize nutrient use efficiency and production goals (e.g. liveweight gains or milk production), even when grown under higher rainfall conditions.

Importantly, for a subset of our species, we found an increase in nutritive value under drought through an increase in CP and NSC, and decrease in NDF and ADL. Previous studies have reported that moderate drought stress can induce a delay in plant maturation and growth, resulting in plants with fewer, shorter stems and flowering parts, and increases in leaf:stem ratio, which explained much of the improved CP concentrations and digestibility (Buxton, 1996; Dumont et al., 2015; Kuchenmeister et al., 2013; Staniak and Harasim, 2018). In this context, selection of pasture species and/or varieties with delayed onset of flowering may allow for improved digestibility in drought conditions by increasing the leaf:stem ratios (Power et al., 2020). In our study, *Digitaria eriantha* and *Themeda triandra* (both C₄ grasses) increased allocation to leaves relative to stems under drought, and whole-plant digestibility subsequently increased when compared to the control treatment (November harvest; **Supplementary Figure S5.3**). Furthermore, the increase in CP concentrations of some species under drought may be

explained by trade-offs between nutrient accumulation and growth dilution, such that lower biomass production increased the tissue nitrogen concentration, as has been reported in previous studies (Dumont et al., 2015; Grant et al., 2014).

In relation to the increases in non-structural carbohydrates, studies with grasses suggested that this may alter the leaf osmotic potential, helping to maintain the uptake of soil water and thus resulting in increased drought tolerance and survival (DaCosta and Huang 2006; Fariaszewska et al., 2020; Volaire and Lelièvre 1998). In our study, the reduced fibre and lignin concentrations found in some species (e.g. *Themeda triandra*) can be explained by delayed stem elongation associated with slower rates of maturation and growth under water stress, as previously reported (Buxton, 1996; Küchenmeister et al., 2013; Wilson et al., 1983). Such reduced stem elongation of some species under drought may result in higher leaf:stem biomass ratios, improving forage digestibility and sward structure for ease of grazing and forage intake (Buxton, 1996; Wilson et al., 1983).

In general, we found that while the direction and magnitude of drought impacts on forage nutritive value varied across species and harvests, most of the pasture species were still able to provide sufficient nutrients to support ungulate digestion and, subsequently, maintain moderate animal production. However, a significant reduction in biomass production was common for all pasture species. This suggests that even with desired forage nutritive value, the amount of available forage may be insufficient to support the high performance of grazing ruminants in drought scenarios. In this case, reduced stocking densities could be an appropriate management strategy to be verified in future research.

5.5 CONCLUSIONS

The 6-month period of severe drought resulted in divergent responses in forage production, structural traits and nutritional composition among the nine pasture species tested. In general,

productivity and percentage of dead material were more strongly and adversely impacted by drought than nutritive value across all species. The changes in nutritional composition appeared to be related to either shifts in plant morphology (leaf:stem biomass ratios) or reduced growth, both of which were species-dependent, reflecting diverse drought adaptation strategies among species. Long-term field studies (for at least two consecutive years) to evaluate the patterns of response of these pasture species to drought should be explored. Identification of the factors that drive changes in nutritive value across different pasture species in response to various drought scenarios (e.g. short/long-term and moderate/severe drought) is essential to generating information about potential risks for farmers and industries in the face of climate change. This knowledge can inform management strategies in relation to the timing of grazing or cutting, selection of drought-tolerant species/cultivars, and optimization of forage resources to support animal performance. Future research with animal trials is needed to determine the extent to which observed changes in the nutritive value of pasture species affect forage intake and animal production (e.g. milk and meat), as well as the incidental environmental impacts of consuming forage produced under drought conditions, such as altered ruminant methane emissions, a key industry consideration.

5.6 SUPPLEMENTARY DATA

Table S5.1. Plant biomass harvests by pasture species performed during the drought period of 2019 (August, October, November); measurements taken (productivity, percentage of dead material, leaf:stem ratio) and plant materials (whole-plant, leaves and stems) used for nutritional analysis from each harvest.

Species	Months		
	August	October	November
<i>Biserrula pelecinus</i>	Harvest	Harvest	Harvest
<i>Chloris gayana</i>	NA	Harvest	Harvest
<i>Digitaria eriantha</i>	NA	Harvest	Harvest
<i>Festuca arundinacea</i>	Harvest	Harvest	Harvest
<i>Lolium perenne</i>	Harvest	Harvest	Harvest
<i>Medicago sativa</i>	Harvest	Harvest	Harvest
<i>Phalaris aquatica</i>	Harvest	Harvest	Harvest
<i>Rytidosperma caespitosum</i>	Harvest	Harvest	Harvest
<i>Themeda triandra</i>	Harvest	Harvest	Harvest
Measurements taken:	Productivity	Productivity	Productivity
	Dead material	Dead material	Dead material
			Leaf:stem ratio
Plant materials used for nutritional analysis:	Whole-plant	Whole-plant	Whole-plant
			Leaves
			Stems

NA: no harvest due to lack of plant biomass in the plots of the respective species.

Table S5.2. *P* values for productivity, percentage of dead material, and nutritional composition of the whole-plant of pasture species in response to drought treatment during the drought period (August, October and November harvests).

Species	Harvest	Productivity	Dead material	CP	NSC	NDF	ADL
Bis	August	0.04	0.29	0.59	0.91	0.95	0.47
	October	0.02	0.04	0.05	0.15	0.43	0.73
	November	0.38	0.49	<0.01	0.17	0.61	0.35
Chl	October	0.05	0.52	0.68	0.68	0.56	0.61
	November	<0.01	<0.01	0.31	0.06	0.50	<0.01
Dig	October	<0.01	<0.01	0.05	0.81	0.05	0.67
	November	<0.01	0.02	<0.01	0.43	<0.01	0.27
Fes	August	0.67	0.69	<0.01	0.51	0.15	0.06
	October	<0.01	<0.01	0.08	0.05	0.50	0.22
	November	0.63	<0.01	<0.01	0.59	0.28	<0.01
Lol	August	0.09	0.75	0.03	0.18	0.72	1.00
	October	<0.01	0.03	<0.01	<0.01	0.55	0.14
	November	<0.01	<0.01	0.19	0.13	<0.01	<0.01
Med	August	0.78	0.75	0.93	0.18	0.13	0.50
	October	<0.01	0.97	0.13	0.14	0.93	0.65
	November	<0.01	0.04	0.12	0.09	0.78	0.86
Pha	August	0.03	0.01	0.97	0.40	0.51	0.20
	October	<0.01	0.01	0.16	0.06	0.54	0.39
	November	<0.01	<0.01	0.07	0.45	0.85	0.08
Ryt	August	0.10	0.32	0.47	0.22	0.57	0.89
	October	<0.01	<0.01	0.06	0.59	0.05	0.01

	November	0.15	<0.01	0.70	0.55	0.12	0.04
The	August	0.08	0.44	0.45	0.50	0.43	0.74
	October	<0.01	0.95	0.25	<0.01	0.06	0.28
	November	<0.01	0.81	0.72	0.04	0.06	0.02

*Note: **Bold** values denote statistical significance at the $p \leq 0.05$ level. During the harvest in August, there was no biomass for *Chloris gayana* and *Digitaria eriantha*.*

*Abbreviations: CP: crude protein; NSC: non-structural carbohydrates; NDF: neutral detergent fibre; ADL: acid detergent lignin; Bis: *Biserrula pelecinus*; Chl: *Chloris gayana*; Dig: *Digitaria eriantha*; Fes: *Festuca arundinacea*; Lol: *Lolium perenne*; Med: *Medicago sativa*; Pha: *Phalaris aquatica*; Ryt: *Rytidosperma caespitosum*; The: *Themeda triandra*.*

Table S5.3. *P* values for leaf:stem ratio and nutritional composition of plant parts (leaves and stems) of pasture species in response to drought treatment at the end of the drought period (November harvest).

Variables	Species								
	Bis	Chl	Dig	Fes	Lol	Med	Pha	Ryt	The
Leaf:stem ratio	0.47	0.01	0.03	0.44	0.67	0.84	<0.01	0.35	<0.01
CP									
Leaves	<0.01	0.11	<0.01	0.02	0.35	0.06	0.64	0.96	0.10
Stems	0.08	0.25	<0.01	0.35	0.04	0.84	0.35	0.34	0.96
NSC									
Leaves	0.06	0.08	0.30	0.43	0.21	0.08	0.02	0.37	0.05
Stems	0.09	0.48	0.41	0.76	0.01	0.03	<0.01	0.17	0.05
NDF									
Leaves	0.60	0.99	<0.01	0.63	<0.01	0.98	<0.01	0.56	0.62
Stems	0.39	0.97	<0.01	0.05	0.08	0.07	<0.01	0.93	0.12
ADL									
Leaves	0.69	<0.01	0.28	0.04	<0.01	0.79	0.47	0.98	0.30
Stems	0.41	<0.01	0.13	0.01	<0.01	0.44	0.34	<0.01	0.05

Note: **Bold** values denote statistical significance at the $p \leq 0.05$ level.

Abbreviations follow Table S5.2.

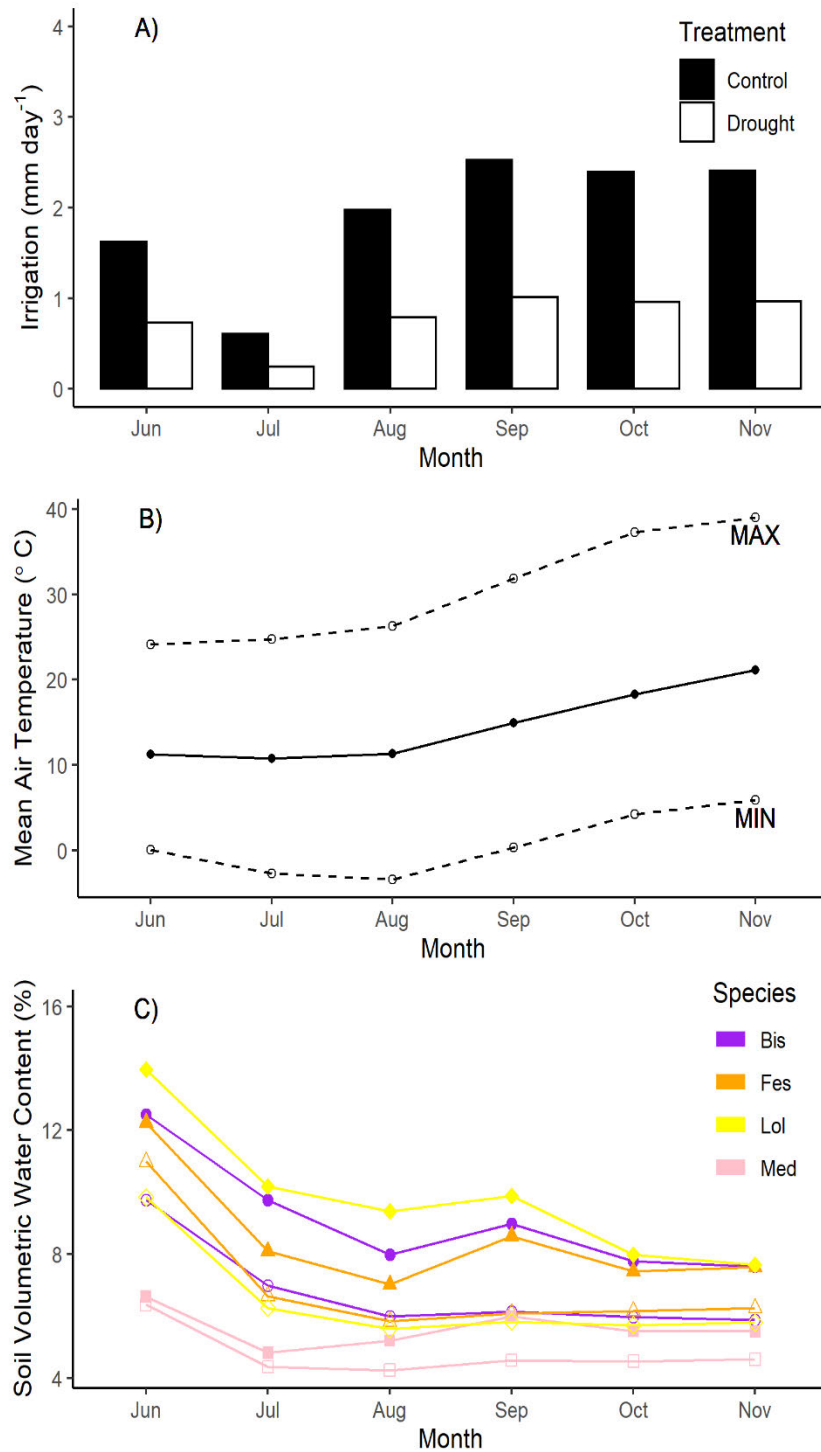


Figure S5.1. Amount of irrigation (mm day⁻¹; panel A) applied in each treatment (control, drought), mean air temperature (maximum and minimum; panel B) and soil moisture content in four species subplots (Bis: *Biserrula pelecinus*, Fes: *Festuca arundinacea*, Lol: *Lolium perenne* and Med: *Medicago sativa*; panel C) under both treatments (control: filled shape and drought: empty shape) during the experimental period (1 June to 30 November 2019).

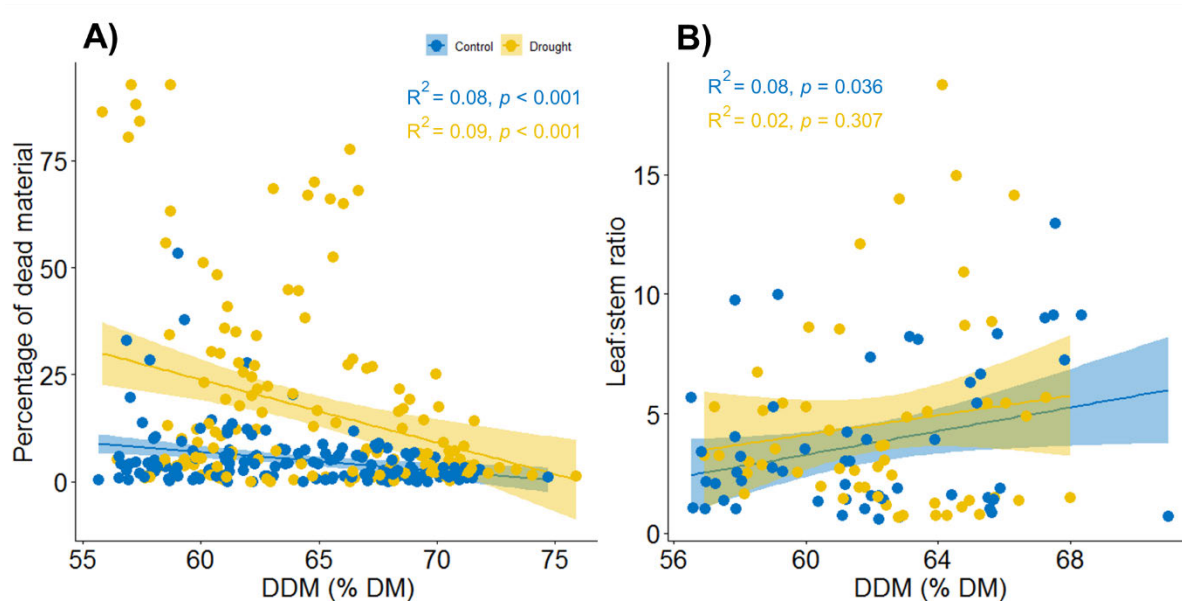


Figure S5.2. Correlations between A) percentage of dead material, B) leaf:stem ratio and estimated digestible dry matter (DDM in % of dry matter; whole-plant) for the nine pasture species studied under different watering regime treatments (control and drought) during the 6-month experimental period (August, October and November harvests). Correlations were tested with Pearson correlation, with R^2 and p values for each treatment shown in different colors in each panel (blue = control, yellow = drought). Digestible dry matter was calculated according to Oddy et al. (1983) as follows: $\text{DDM \%} = 83.58 - (0.824 \times \text{Acid detergent fibre \%}) + (2.626 \times \text{Nitrogen \%})$.

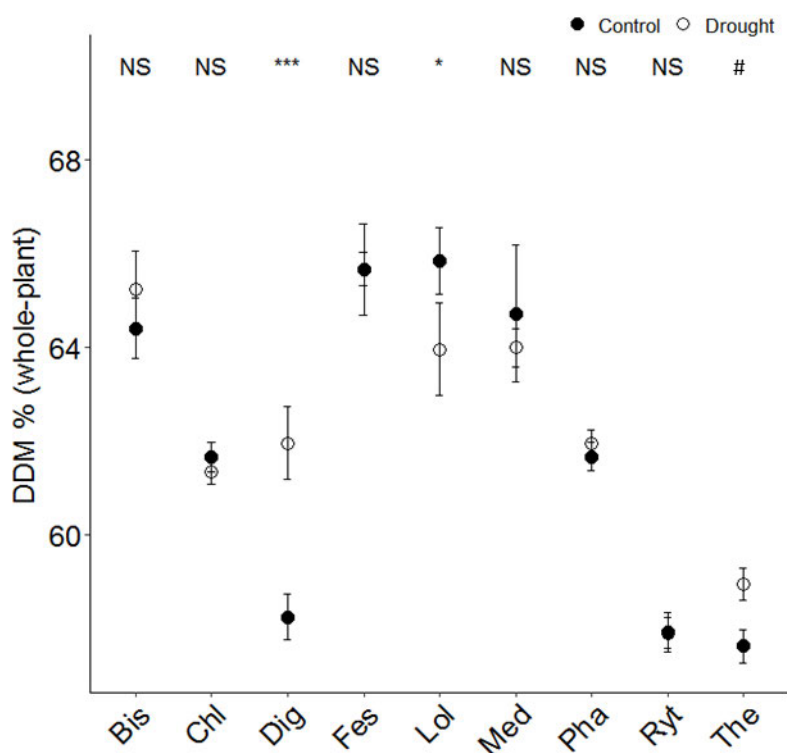


Figure S5.3. Estimated digestible dry matter (DDM %; whole-plant) for the nine pasture species studied under different watering regimes treatments (closed circle = control, open circle = drought) in November harvest. DDM calculation follows Figure S5.2. Significant comparisons for the effects of treatments are indicated as follows: NS = not significant, # $p \leq 0.1$, * $p < 0.05$, *** $p < 0.001$. Species abbreviations are as follows: *Biserrula pelecinus* (Bis), *Chloris gayana* (Chl), *Digitaria eriantha* (Dig), *Festuca arundinacea* (Fes), *Lolium perenne* (Lol), *Medicago sativa* (Med), *Phalaris aquatica* (Pha), *Rytidosperma caespitosum* (Ryt), *Themeda triandra* (The).

CHAPTER 6: General Discussion

6.1 Thesis summary and key findings

My thesis aimed to address the current lack of knowledge regarding the impacts of future climate extremes, namely high temperatures and severe droughts, on a diverse range of globally important pasture species. The main focus of my research was the consequences of these events on forage nutritive value, which I addressed through the analysis of nutritional composition (e.g. crude protein, non-structural carbohydrates and fibre fractions) and calculations to estimate digestibility. To understand the mechanisms underlying the consequences of climate extremes, I also investigated effects on plant growth and morphological traits, including productivity and structural allocation (e.g. dead material and leaf:stem biomass ratios). I took this comprehensive approach in three original research chapters (Chapters 3, 4 and 5). In Chapter 3, I report the results of a 4-month glasshouse-based study that investigated the effects of warming and short-term drought scenarios on forage production, nutritional composition and digestibility, as well as on plant morphology traits of temperate pasture species. In Chapter 4, I present the results of a 2-year field experiment that also investigated the effects of warming and seasonal drought scenarios on forage production, nutritional composition and digestibility of temperate pasture species. Lastly, in Chapter 5, I report the results of a 6-month field experiment that investigated the effects of seasonal drought on forage production, structural allocation, and nutritive value of nine pasture species, including C₃ legumes and C₃/C₄ grasses.

There are a number of common threads connecting the outcomes of these chapters and the following discussion synthesizes my key overall findings in three main topics.

6.1.1 Are the consequences of climate extremes for forage production and nutritive value closely related to water availability?

As climate change proceeds, warmer future temperatures will be ubiquitous, while changes to rainfall patterns are less predictable and expected to be more variable, both spatially and temporally (IPCC, 2019, IPCC, 2021). For the region where I undertook my research, southeastern Australia, future climate projections indicate increases in the duration, frequency and intensity of climate extremes, including heatwaves and cool-season droughts (during winter and spring; CSIRO and BOM, 2015, IPCC, 2021). Changes to climate that have occurred over recent decades already pose a significant challenge to both the productivity and nutritive value of the pasture feed base that traditionally supports the diets of domestic livestock in Australia (Brown et al., 2019; Chang-Fung-Martel et al., 2017; Perera et al., 2020; McKeon et al., 2009); predicted increases in the frequency and extent of climate extremes are expected to exacerbate this issue.

Forage production and nutritive value respond in a magnitude-dependent way to the severity of warming and soil moisture limitation (Cantarel et al., 2013; De Boeck et al., 2016; Heisler-White et al., 2008). The combined effects of warming and drought may be more detrimental than individual stresses, with temperature-associated increases in evapotranspiration exacerbating plant water stress (Cantarel et al., 2013; De Boeck et al., 2016; Rustad et al., 2001). In this thesis, the examined scenarios of climate extremes affected both the productivity and nutritive value of pasture species and the magnitude of effects was often greater under drought and warming + drought than under warming alone. Drought, on its own and in combination with warming, significantly reduced soil moisture, which, in turn, affected forage production, structural allocation, nutritional composition and digestibility of pasture species (Chapters 3, 4 and 5). The substantial decreases in pasture productivity and increases in dead material under drought and warming + drought scenarios illustrate the severity of the

plant stress imposed in this research. Despite this, I observed few impacts on forage nutritive value, along with variability in the magnitude and directionality of impacts that did occur (increase, decrease and no change), depending on pasture species and the timing of stressors relative to plant development stage. Similar findings have been reported from previous studies with elevated temperature and drought scenarios in temperate grasslands (Cantarel et al., 2013; Grant et al., 2014), and meta-analyses of European pastures (Dellar et al., 2018) and grasslands from Mediterranean and montane areas (Dumont et al., 2015).

Plant productivity and nutritive value can respond to both direct effects of temperature on plant physiology and biochemistry (including increased rates of transpiration) and its associated indirect effects via the depletion of soil moisture (Mitchell, 1956; Rustad et al., 2001; Wilson et al., 1991). Significant warming effects commonly occur as a consequence of indirect effects on vapour pressure deficit and soil water availability and, in the absence of such changes, fewer consequences may be observed. For instance, negative effects on plant growth and forage nutritive value are often reported as a result of direct effects of heat stress on plant physiology and indirectly through high levels of evapotranspiration and decreases in soil moisture and nutrient availability (Habermann et al., 2019; Rustad et al., 2001; Wilson et al., 1991). My results from Chapters 3 and 4 suggest that the overall consequences of climate warming alone on forage production and nutritive value can be associated with reduced soil moisture through increased levels of evapotranspiration due to the extremely high temperatures experienced. My approach in this thesis allowed me to explore the effects of high temperatures (an increase of 3°C - 4°C above ambient temperature) on two common temperate pasture species (*Festuca arundinacea* and *Medicago sativa*) in both glasshouse and field experiments. In Chapter 3, the lack of temperature effects is likely due to the maintenance of the pots under well-watered conditions. This approach in the glasshouse was able to isolate the direct effects of temperature while minimizing the interactive influence of warming on soil water content. In

contrast, in Chapter 4, warming decreased pasture productivity and slightly changed nutritional composition and digestibility, which might have been due to decreased soil moisture under the warming treatment in the field. Additionally, the occurrence of exacerbated heatwaves in the field for plots under warming suggested that more extreme heat might have exacerbated plant water stress via increases in evapotranspiration. Furthermore, these extreme events are also particularly important as excessive heat can cause physiological and biochemical stress, especially at the cellular level, by stopping enzyme systems, resulting in tissue and even plant death (Williams et al., 2016).

Based on the soil moisture data, my findings suggest that evapotranspiration-mediated indirect effects in warming scenarios might be responsible for much of the observed changes, particularly in pasture productivity of temperate species, in the field. Similar findings were reported in a long-term study of temperate grasslands (French Massif Central region; Cantarel et al., 2013) and in a study investigating heatwaves in Swiss alpine grassland (De Boeck et al., 2016). Heatwaves are problematic for grazing systems in regions that rely on temperate pasture species, as these species often have low heat tolerance (thermotolerance; Langworthy et al., 2020). In this context, an important research question that can arise is whether increases in irrigation can mitigate the detrimental effects of heatwaves on pasture systems (Langworthy et al., 2020; Langworthy et al., 2018). There is some evidence that in temperate grasslands, the effects of heatwaves tend to be limited as long as water is available to the plants (De Boeck et al., 2011; De Boeck et al., 2016). However, this can only be applied to systems where irrigation water availability is not limiting. In Australia, just 0.08% of the 54% of land used for livestock grazing is irrigated pasture (Australian Bureau of Agricultural and Resource Economics and Sciences), and despite the potential benefits of irrigation, climate change and competing demands are likely to reduce the availability of water for irrigation in the future. The overall decline in rainfall and competing demands for irrigation of other crops may force farmers to

reduce the areas of their pasture or/and switch to species with less reliance on water (Rogers et al., 2017).

In summary, my findings suggest that water availability may be a key driver for pasture productivity and nutritional responses to future extreme scenarios of droughts and/or high temperatures. Therefore, if soil water availability can be maintained under field conditions, it may be possible to alleviate the potential negative indirect impacts of temperature increases on forage production and nutritive value. Some adaptation strategies that farmers and land managers can adopt to optimize soil water availability under these future climate scenarios include: 1) investing in irrigation systems and ensuring adequate water supply throughout the year, albeit adding a cost to farm production systems (Lee et al., 2013); 2) Maintaining ground cover by leaving adequate remaining herbage (e.g. stem and crowns) – allowing species to maintain healthy root systems – to optimize water infiltration into the soil, reduce evaporation losses, and protect the soil against hot and drying winds (Lee et al., 2013; NDMC, 2021; Shaxson and Barber, 2003); and 3) controlling weeds, because weed transpiration can significantly reduce the amount of water available in the soil to pasture (Shaxson and Barber, 2003).

6.1.2 Forage nutritive value under climate extremes varied with individual pasture species

A particular concern for grazing systems is the relative abundance of C₃/C₄ and grass/legume plants, since these respond differently to heat and drought stress and are key drivers of forage nutritive value and biomass changes (Grant et al., 2014; Lee et al., 2017; McGranahan and Yurkonis, 2018; Wilson et al., 1991). For instance, higher temperatures can affect grasses more than legumes (Cantarel et al., 2013), and C₃ species more than C₄ species due to their higher temperature optima (Howden et al., 2008). Legume responses to warming are likely to vary among species and the effects can be associated with thermal constraints on rhizobial nitrogen

fixation (Aranjuelo et al., 2007; Whittington et al., 2012). In addition, C₄ grasses are generally more resistant to drought than C₃ grasses, partly as a result of their higher water use efficiency (Evans et al., 2011; McGranahan and Yurkonis, 2018). Also, legumes tend to be more drought-resistant than grasses (Adams et al., 2016).

I have found that the forage nutritional responses to high temperature and drought scenarios vary widely across pasture species. The results of Chapter 4 showed that warming, drought and warming + drought scenarios slightly increased the nutritive value of *Festuca arundinacea*, but had no effect on *Medicago sativa*. This lack of effects observed in *Medicago sativa* might be associated with its high water use efficiency and deep taproots (Erice et al., 2007; Suter et al., 2015). Deep-rooted species are likely to maintain greater nutritive value as they can access/draw deep soil moisture and nutrients, reducing the effects at the sward level, which makes them drought resistant (Dellar et al., 2018; DPI, 2003; Erice et al., 2007).

The impacts of drought on forage nutritive value can differ between species, reflecting their sensitivity and/or resistance to declining water availability (Evans et al., 2011; Gray and Brady, 2016; McGranahan and Yurkonis, 2018). In Chapter 5, I evaluated the forage nutritive value responses to drought across nine pasture species from a range of functional diversity (C₃ legumes and C₃/C₄ grasses). For certain species, the observed changes in nutritive value were associated with the development of morphological (e.g. leaf:stem ratios) or cellular modifications (e.g. accumulation of lignin; Chapter 5). These modifications are related to plant adaptation strategies to cope with stressful conditions and prevent water loss, and are associated with drought resistance and drought survival (Habermann et al., 2021; Le Gall et al., 2015; Munné-Bosch and Alegre, 2004). For example, under water stress, plants can increase senescence and leaf loss, thus decreasing leaf:stem biomass ratios as a strategy to avoid losing water through transpiration and maintain growth (e.g. *Chloris gayana* – C₄ grass; Bruinenberg et al., 2002; Le Gall et al., 2015; Ren et al., 2016). Furthermore, certain species (e.g. *Chloris*

gayana and *Lolium perenne* – C₃ grass) can accumulate lignin in vascular tissues, to reduce plant cell wall water penetration and transpiration under water stress conditions (Liu et al., 2018, Moura et al., 2010), although it may result in forage toughness and lower digestibility (Bruinenberg et al., 2002; Dumont et al., 2015; Ren et al., 2016). Besides impacts on animal production, such decreases in forage digestibility may also result in increases in ruminant methane (CH₄) emissions due to less efficient digestion processes, and thus detrimentally affect the rate of climate change (Lee et al., 2013; 2017).

The findings of my research indicate that, in terms of forage nutritive value (nutritional composition and digestibility), the best performing species under drought were *Digitaria eriantha* and *Themeda triandra*, both C₄ grasses, which increased digestibility, and *Medicago sativa* – C₃ legume and *Phalaris aquatica* – C₃ grass, which were able to maintain their nutritional composition and digestibility. Furthermore, *Digitaria eriantha*, *Themeda triandra* and *Phalaris aquatica* also increased leaf:stem biomass ratios, which may contribute to improving sward structure for ease of grazing and forage intake (Buxton, 1996; Wilson et al., 1983). These species are important components of perennial pastures in Australia and many parts of the world, which make these findings to be broadly applicable to a wide range of grasslands. For instance, *Phalaris aquatica* (good nutritive value) and *Medicago sativa* (high nutritive value) are considered species that are persistent and productive in many parts of the world; *Digitaria eriantha* is often considered to be one of the species among tropical grasses with high nutritive value, which are widespread in many humid tropical and subtropical regions; all of them are also used as hay and silage. *Themeda triandra* (often poor nutritive value) is the most common grass in the natural grasslands of Africa, but can also be found in mainland Australia, Tasmania, South-East Asia, Papua New Guinea and India, it is also an important grass for grazing wildlife (e.g. kangaroos and wild ruminants; DPI, 2012).

In addition, the tropical grasses in this study also demonstrated good persistence under drought, which suggests that these species might play an important role in future grazing systems by contributing to an enhanced supply of forage throughout the year. However, as the nutritive value (e.g. digestibility) of tropical grasses is generally lower (e.g. due to high fibre and low crude protein) than that of temperate grasses, good grazing management is needed to maintain the pasture in a vegetative state, as nutritive value decreases once stem elongation starts, especially due to the increase in fibre (Boschma, et al., 2010). Also, other management strategies, such as companion planting with legumes, especially to improve crude protein concentration, and use of additional fertiliser (including nitrogen) and high-protein supplementation (e.g. protein meals and urea), would be required to take full advantage of the productivity and climate resilience of tropical species (Boschma, et al., 2010; Power et al., 2020).

It is important to mention that climate-related shifts in above-belowground allocation, as adaptation strategies for drought and warming, can also affect both water uptake capacity and access to nutrients, which in turn can affect forage nutritive value (Power et al., 2020). Overall, the species-specific nature of nutritional responses to water and heat stress observed in this thesis highlights the importance of conducting studies on different pasture species with different shoot and/or root characteristics to better understand the impacts of climate extremes on pasture systems. For example, desirable traits in pasture species in the future include: high water use efficiency, drought and heat tolerance, deep taproots (Lee et al., 2013; NDMC, 2021; Shaxson and Barber, 2003), continued leaf growth under stress, and stomatal closure at low relative water content (Abberton et al., 2008). Furthermore, species with different growth phenologies are required to support year-round grazing (Churchill et al., 2021; Power et al., 2020). A better understanding of the relationships between plant traits and climate sensitivity will contribute to identifying trait combinations associated with climate resilience across

pasture species. Such information can help guide species and cultivar selection and breeding programmes for cultivars that can perform well in future climates (Power et al., 2020). Identifying the best performing species in extreme climate scenarios is essential to alleviating the potential climate-related risk to livestock production.

6.1.3 Forage nutritional responses varied with the timing of water stress in relation to plant developmental stage

Water stress is widely recognized as a limiting factor that alters multiple aspects of plant growth and development. Drought effects on forage nutritive value can vary depending on the plant developmental stage due to changes in plant structure (e.g. leaf:stem biomass allocation; Bruinenberg et al., 2002; Gray and Brady, 2016; Grev et al., 2020; Ren et al., 2016). The findings of my thesis show that the water stress imposed in all experiments (Chapters 3, 4, and 5) inhibited plant growth (lower productivity) and accelerated the death of plant tissue in both vegetative and reproductive plant development stages. However, I found evidence that the direction of the effect of drought on nutritive value might be strongly associated with the developmental stage of the plant when the drought was imposed. For example, in *Festuca arundinacea* and *Medicago sativa*, the water deficit imposed during the reproductive stage resulted in decreases in leaf:stem ratio and forage nutritive value (including digestibility; Chapter 3); while drought imposed during the vegetative growth stage left the leaf:stem ratio unchanged and slightly increased or created no change in the forage nutritive value of these species (Chapter 4 and 5). The decrease in forage nutritive value observed during the reproductive development stage may be associated with the high sensitivity of this stage to water stress, forcing the plants to use dehydration avoidance strategies, such as severe leaf loss and increased cell-wall thickness of the existing stems (Dumont et al., 2015; Gray and Brady, 2016; Ren et al., 2016). Also, increased cell-wall thickness due to increases in fibre, especially

lignin, increases forage toughness and reduces digestibility (Bruinenberg et al., 2002; Dumont et al., 2015; Ren et al., 2016). Despite having received little attention, the accumulation of silica in some grasses as a strategy to reduce transpiration during plant water stress can also contribute to decreases in digestibility (Montes-Sanchez and Villalba, 2017; Van Soest and Jones, 1968).

During the early plant development stage, the lack of water substantially impacted the growth of vegetative structures, which may have caused delays in stem elongation that contributed to fibre decrease and, hence, less drought impact on forage nutritive value (Buxton, 1996; Gray and Brady, 2016; Küchenmeister et al., 2013). However, even with these minor impacts on the nutritional side, the palatability of herbage during drought conditions may be reduced due to the increase in the proportion of dead material, which can reduce opportunities for selective grazing (Longland, 2013; Shakhane et al., 2013). Selective grazers choose the plants in order of their preference; for example, sheep can pick up small leaves with their lips, nibble just one leaf and not the one next to it and will revisit the plant after grazing it to get the fresh regrowth (Burlace, 2013). During drought, if there is a lot of dead material, selective grazers may remove desirable species through overgrazing and cause pasture degradation, or even if there is not enough green pasture, they may reduce their intake of forage.

These findings can help farmers and land managers improve decision-making during drought conditions in individual circumstances associated with pasture growth stages, and further develop grazing management strategies. Although under drought conditions, forage nutritive value can vary, the biggest challenge will be to feed the animals with very low pasture production. In order to minimize the negative effects of drought on forage production and nutritive value, pasture management adaptation strategies may depend on the severity of drought impacts on the plants, but it is likely that, in all cases, stretching available forage with hay or supplemental feedstuffs may be required. However, the cost of supplemental feeding

must be considered against the benefits. Management grazing strategies that can be adopted include lowering stocking densities temporarily (fewer grazing animals per hectare) and, in severe cases, which is the expected scenario, advice will be not to graze pastures, and instead leaving them fallow in order to maintain pasture health for recovery when rains come. If the pasture has not grown back sufficiently to support viable grazing, a strategy could be confining animals in a small sacrifice paddock that is already in poor condition – although this may destroy the plants in that sacrifice paddock; renovating the small area will be less costly than renovating the entire pasture after the drought ends (Cassida and Thurlow, 2021). Importantly, it will be necessary to ensure that regrowth is not grazed until enough new leaves have formed/appeared on grass tillers to support the root system or new flowers have appeared on legume species (Cassida and Thurlow et al., 2021).

6.2 Future research directions

The findings from my thesis have considerable implications for pasture-based systems in southeastern Australia as well as globally. Climate extremes have resulted in multiple impacts on forage production and nutritive value, and usually do not impact every pasture species to the same extent, which makes the development of management strategies complex. Because the unpredictability of naturally occurring climate extremes limits assessment of their ecological impacts, future studies should consider different climatological regions (e.g. temperate, tropical and dry) with different combinations of temperature and rainfall, including heatwaves, floods, droughts and frosts, to better understand the impacts of future climate scenarios on grazing livestock systems. In this context, a holistic approach, taking into account the different pasture management options and plant species, along with long-term studies, and animal trials, needs to be further investigated and emphasized in the research of climate change, particularly the impact of extreme climate events.

For example, field research is needed to investigate the efficacy of pasture management strategies to cope with a significant reduction in pasture productivity during heat and water stress, such as adjustments in stocking rates and rotational grazing plans to allow longer rest periods for plants to recover (Raynor et al., 2021; Derner et al., 2016). An alternative line of focus includes studies addressing the performance of new genotypes/varieties of pasture under future climate extreme scenarios. For this, the efforts of breeding programs will be crucial to delivering heat and drought-resistant and/or resilient cultivars that can maintain or experience smaller reductions in productivity and nutritive value. In addition, studies to investigate the performance of mixed perennial pasture species under extreme climate conditions, particularly the nutritional responses to the combination of warming and drought scenarios, are required. Research involving a consortium of species with diverse traits (e.g. morphology and phenology), such as legumes and grasses, could be a good management strategy to improve pasture systems' productivity and nutritive value under future scenarios. In mixed pastures, the nitrogen-fixing ability of legumes can facilitate grass growth and improve sward nutritive value through niche differentiation and facilitation, and also reduce fertiliser nitrogen requirements in the pasture (Power et al., 2020). Associated research from the PACE project showed that legume-grass mixtures (*Phalaris aquatica* + *Trifolium subterraneum* and *Biserrula pelecinus* + *Digitaria eriantha*) were more drought-resistant in terms of maintaining production than their respective monocultures, significantly so for one species pair (Churchill et al., 2021; Power et al., 2020). Also, under both control and drought treatments, the grasses presented higher crude protein concentrations and digestibility when grown with the legumes, compared to monoculture – although the opposite was true for the legumes (Power et al., 2020). However, differences in growth phenology and sensitivity to drought in mixture pastures will be a strong determinant of their ability to facilitate the growth of neighboring species (Power et al., 2020; Sherry et al., 2007).

Few studies have imposed longer-term drought and/or warming under field conditions, where plants can have time to acclimatise to stressors. For instance, pasture productivity data from PACE project-related studies suggested the possibility of plant acclimation of some species to water stress associated with warming, which may be crucial for the persistence and profitability of some species (e.g. perennial grasses and legumes) in the future (Churchill et al., 2021; Power et al., 2020). Such acclimation might require trade-offs between structural and nutritional traits, which may not be observed in shorter studies, and this is an area that has received little attention (Deleglise et al., 2015; Grant et al., 2014; McGranahan and Yurkonis, 2018). Therefore, long-term trials investigating the impacts of climatic extremes on different pasture species should be considered, since these studies can advance knowledge of seasonal implications for forage productivity and nutritive value, as well as potential adaptation options for grazing management under future climatic conditions (Brookshire and Weaver, 2015; Evans et al., 2011; Hoover et al., 2014).

Pasture studies with climate manipulation usually do not involve animals in the experiment as this includes high costs, labour and time investment. However, in order to accurately understand the impacts of climate extremes on ruminant livestock performance, studies with animal trials should occur. This includes investigations in relation to the impacts on forage intake, palatability and digestibility (e.g. *in vivo*, *in sacco* or *in vitro* essay), animal production (e.g. milk and meat), heat stress on animals, and methane emissions-a key industry consideration. Importantly, animal nutritional requirements may also change subtly under climate change and especially under heat stress conditions and research is needed to look at how this translates to animal performance (Joy et al., 2020; Lacetera, 2019; Moore et al., 2015).

These investigations are particularly important as they can provide the scientific basis for decision-making while providing knowledge to stakeholders to minimize the detrimental

impacts of climate extremes on pasture-based systems, livestock performance, and, consequently, food production globally.

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