Unveiling the dynamics of livestock behaviour, pasture growth, and livestock saliva

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Doctor of Philosophy

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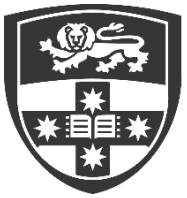

THE UNIVERSITY OF SYDNEY

School of Life and Environmental Sciences

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Abstract

UNVEILING THE DYNAMICS OF LIVESTOCK BEHAVIOUR, PASTURE GROWTH, AND LIVESTOCK SALIVA

Grazing practices must consider a range of variables to enable efficient and sustainable management of animals, plants, soils, water, and nutrients. Extensive research has detailed changes in these variables, but a multidisciplinary agroecological approach is critical to understand how they interact to ensure best management practices. This thesis investigated three areas within agroecological sphere: the role of grazing on plant growth (Chapters 2 and 3), constituents in livestock saliva that may affect plant growth (Chapter 4), and grazing decisions of livestock on pasture using predictive modelling (Chapters 5 and 6). The aim of my thesis is to weave a narrative that explores the complex dynamics of these variables in an agricultural ecosystem and provide a pathway for how they can be analysed. Grazing management is the relationship between animals and plants, and as such, the ability to qualify and quantify the intricacies of the art and science will benefit researchers in their studies, and pastoralists in making management decisions on farm.

Sydney Institute of Agriculture

School of Life and Environmental Sciences

Faculty of Science

The University of Sydney

For Aston, the dog, who was no help at all.

Acknowledgements

As with any *good* thesis, there is always a section to acknowledge those who have helped me along my journey in science and make a shout out to those who have constantly supported me during my PhD, and hopefully will continue to support me for the rest of my life.

I would like to start this by thanking **Andrew** and **Malcolm** for their encouragement and support as my supervisors during hons. in 2013. If it weren't for these two, I don't think I would have truly enjoyed or appreciated research as much as I do now, fart jars and all. I am also grateful for both **Malcolm** and **Tina** taking me under their wing as a research assistant and teaching me invaluable valuable skills like being able to identify a double space from a mile away and learning the ins and outs of SWPs. I also need to give a shout out for my friends who were constantly cheering me on from the side-lines. These friends have consistently provided me with an outlet to rant to when things did not make sense, to play video games (**Olivia**), to take creative breaks (**Rachel**), told me to get out of the house and touch grass (**Mandy**), and reminded me to breathe and keep going (**Gemma**). I also am grateful for my sister (**Erin**) and siblings-in-law (**Emily** and **Shaun**), for their constant motivation, and especially for my in-laws, **Paula**, and **Steve**, for their endless life advice. A big thank you should similarly be given to my brother, **Jacob**, for practically being an in-house statistician, always helping me when I needed it, and having rants about our PhDs together.

My two PhD supervisors, **Andrew**, and **Lachlan**. There are honestly no words that could *ever* amount to the level of gratitude and respect I have for them, let alone vats of beer and bottles of gin that I could repay them with. They took me onboard as a PhD student and remained so open minded with where I have navigated this thesis to. They were also extremely supportive with navigating my candidature and well, life too, around my least favourite C-word, COVID. **Lachy**, thank you for always picking up that phone call, attempting to fix any and every coding error I had in R, and jumping on zoom to 'discuss work' even though it always ended up being a two-hour yarn. I can definitely say I have enjoyed every moment so far, with special mention of the time I threw a snowball from behind and over a car, somehow managing to perfectly hit you square on the head with it in Yellowstone. **Andrew**, I am not sure how you have managed to put up with me being around for the last six or so years as both an undergrad and postgrad, but I have learnt so much from you as a mentor. These learning events are not strictly limited to the ins and outs of life as a tertiary educator and 'gooder' scientific prose, but also included staying humble in a world of chaos, jar sauce, Randy FeltFace, beer making, and most importantly, learning to "keep *qualm* and carry on".

Although there is a sole page for my dog, whose constant companionship I could never repay (though, maybe with a lifetime supply of tennis balls), this thesis is dedicated to my parents, **Nigel,** and **Carol** whom I owe great thanks to, with a very competitive rate of interest. Your constant support has kept me believing that *anything I put my mind to was possible.* Alongside my parents, I also dedicate this document of blood, sweat, and ~~beers~~ tears, to my husband, **Liam**. He has been there for the last nine and a half years of our lives together doing crazy things (like trying to plan a wedding during a global pandemic). In the last four alone, he has provided much needed humour breaks, has been my number one supporter, and has constantly maintained the last strand of sanity in the household. Thank you for *making everything possible.*

Declaration of authorship

This thesis includes two original papers (Chapters 5 and 6) which have been published in peer reviewed journals, with the published version being included in this thesis for Chapter 5, and a formatted version to fit within this thesis being provided for Chapter 6.

One chapter has been recently accepted for publication within a journal (Chapter 2) and is currently undergoing proofing, and two chapters are standalone manuscripts (Chapter 3 and 4), written with intent to publish within a journal, and subsequently include an author attribution statement at the start of the chapter.

This thesis has been written in publication style, and as such, all papers presented in this thesis have been formatted as per the respective journal guidelines they have been published within, or are intended to be published in.

I hereby certify that the intellectual content of this thesis is the product of my work, and the assistance received for preparing this thesis and sources have been acknowledged either in the author list in each chapter/publication, or within the acknowledgement section of the respective chapter.

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

Danica Parnell

5th December, 2023

Research work and authorship

The organisation, analysis, and writing of all chapters and publications in this thesis are the principal responsibility of the candidate, Danica Parnell, who worked independently under the supervision of Dr Lachlan Ingram (The University of Sydney/NSW Department of Primary Industries) and Associate Professor Andrew Merchant (The University of Sydney).

The inclusion of co-authors in specific chapters, reflects the collaboration between researchers and does acknowledge input into team-based research. Author attribution statements are included at the start of Chapters 2, 3, and 4, and have been included with publication for Chapters 5 and 6. Any technical fieldwork and staff assistance are recognised in the acknowledgement section of the respective chapter.

Danica Parnell

5th December, 2023

As the primary supervisor for the candidature, I can confirm that the authorship attribution statement above is correct.

Associate Professor Andrew Merchant

15th December, 2023

COVID-19 Impact Statement

This thesis began its journey (part time) in 2019, remaining hopeful for a slew of fieldwork to be achieved after moving to full time in June 2020. Plans were not thwarted by a lack of student coordination, but instead, was impacted by a global pandemic that shut down **everything**. Since thesis inevitably becomes a period piece, it is important to reflect on the wild events that occurred during 2020 and 2021… two important years during my candidature. There was a toilet paper crisis in all Australian supermarkets, and a mask mandate that certain people took to an extreme by wearing pan lids inside their hoodies. There were border closures to countries worldwide, and closures interstate that left Western Australia as basically its own country, Victoria in permanent isolation, and NSW with the taxi driver from Bondi which shafted all of southwest Sydney.

Ultimately, the continuous lockdown that occurred, impacted the ability to perform planned fieldwork during 2020. Restrictions placed within the faculty impacted planned lab work during 2021. The intended body of work to be included had to be altered, and instead had larger focuses placed on data previously collected (GPS behavioural studies in Chapter 5 and 6), in 2017, and 2014 respectively. The end of lockdowns and social distancing requirements enabled the ability to perform lab work that was planned for 2021, in 2022. Unfortunately, the level of detail in data collected was less than originally intended for this aspect of the study. As such, a significant portion of detail is placed in the general discussion (Chapter 7) that considers future work based around original plans for this thesis.

Publications during candidature

Publications in Journals

Parnell, D., Kardailsky, I., Parnell, J., Badgery, W.B., & Ingram, L. (2022). Understanding sheep baa-haviour: Investigating the relationship between pasture and animal grazing patterns. Grassland Research, 1(3), 143–156. https://doi.org/10.1002/glr2.12026

Parnell, D., Edwards, J., Ingram, L. (2023) Exploring 'wether' grazing patterns differed in native or introduced pastures in the Monaro region of Australia. *Animals*, 13, 1500. https://doi.org/10.3390/ani13091500

Parnell, D., Merchant, A., Ingram, L. (2023) Is animal saliva prominent factor in pasture regrowth? *Crop and Pasture Science*, CP23201. https://www.publish.csiro.au/CP/ CP23201

Conference Publications

Parnell, D., Kardailsky, I., Parnell, J., Badgery, W., Ingram, L. (2022) Understanding sheep baa-haviour. *Flourish! Interdisciplinary solutions for a thriving planet symposium* (Australian Academy of Science), 6th – 7th June 2022, Brisbane, Queensland [Poster].

Parnell, D., Ingram, L., Edwards, J. (2023) Determining 'wether' sheep behaviour, environment, or pasture drives sheep grazing patterns. A paper presented at the *International Grasslands Congress Conference,* 14th – 20th May 2023, Kentucky, USA. https://doi.org/10.52202/071171-0320

Scholarships, awards, and grants

2020:

AW Howard Memorial Trust Study Grant
Awarded $5,000 for a study grant to travel internationally and attend the International Rangelands and Grasslands Congress (IRGC) Conference in Nairobi, Kenya.

IRGC Delegate Support Grant
Awarded $1,500 (USD) in support to attend the IRGC Conference in Nairobi, Kenya, to present research submitted.

2021:

University of Sydney Postgraduate Research Support Scheme (PRSS) Fund
Awarded $3,500 in support from the University for project related costs to assist with purchasing of experimental equipment, textbooks, and software.

2022:

Flourish! Symposium Travel Assistance Grant $1k
Awarded $1,000 in support to attend the Flourish! Symposium, run by the Australian Academy of Science, in Brisbane, Australia. Grant was provided to attend workshops based on undertaking interdisciplinary approaches to research, and to present a poster on current research.

2023:

IGC Delegate Support Grant
Awarded $2,000 in support for accommodation and conference fees to attend the International Grasslands Congress (IGC) Conference in Covington KY., USA, to present research submitted.

AW Howard Memorial Trust Study Grant
Awarded $5,000 for a study grant to travel internationally and attend the IGC Conference in Covington KY., USA.

University of Sydney Postgraduate Research Support Scheme (PRSS) Fund
Awarded $1,800 in support from the University for assistance with publishing costs, and travel expenditure to attend the IGC Conference in Covington K.Y., USA.

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CHAPTER 2 – Is animal saliva a prominent factor in pasture growth?

CHAPTER 3 – Saliva and understanding its impact on *Phalaris aquatica* in rhizoboxes

Chapter 1: General introduction

Grasslands are a major contributor to net primary productivity of terrestrial ecosystems consisting of 37% of the land surface (O'Mara, 2012). Management and conservation of grasslands face significant challenges regarding the impact of animal production, landscape conservation, and other socio-economic activities (Baldi et al., 2001; Saberwal, 1996). For example, pastoralists and livestock farmers depend on grasslands and sown pasture as a source of livestock feed. To ensure that degradation or loss of biodiversity, habitat, and productivity does not transpire, it is critical that they are managed in a manner appropriate for the climatic conditions under which they occur. Grassland focused research is often based on investigating a single aspect of the grassland system and as a result, fails to address the interdependency between biotic (animals, plants) and abiotic components (soil, climate, topography). Interdisciplinary research is therefore needed that investigates the dynamics between all facets of the intra- and inter-field variation of animals, soil, and vegetation to ensure effective management of agroecosystems. An agroecosystem is generated when human alteration occurs on an ecosystem, in order to establish agricultural production for food, fibre, fuel, and other products (Gliessman, 2004). In addressing this gap, this thesis examines some of the multifaceted interactions of grazing, pasture productivity, and pasture management from plants to landscape.

In pasture-based systems, livestock play an integral part in the landscape, influencing the physiological processes of plants through physical (i.e., biting or trampling) or chemical (i.e., saliva deposition, urination) impacts (Frank et al., 2002). Further investigation into the latter alteration uncovered the often-contradictory nature of existing research, whereby the effect of saliva deposition has seen a multitude of responses (i.e., Bergman, 2002; Detling et al., 1981; Gavloski & Lamb, 2000; Howe et al., 1982; Liu et al., 2012), with few mechanisms identified as to why these responses occur. This is highlighted in Chapter 2, 'Is animal saliva a prominent factor in pasture growth', a critical review of current literature. In this review, we speculate what potential compounds may be evident in animal saliva that causes growth promotion and discuss the greater practical applications of this knowledge.

Greater applications of this knowledge include understanding how the 'art' of grazing ultimately influences pasture productivity. In doing so, the result of such work will assist our understanding of how grazing impacts the growth of plants/grasses and contribute towards improving our management of grazed grasslands. As identified in Chapter 2, the resulting effect of grazing at a whole plant level remains unclear. Pasture growth is impacted by the characteristics of grazing timing and intensity (Harradine & Whalley, 1981), and as such, understanding the variable growth characteristics of pasture types *in-situ* is important for one's ability to exploit grazing to encourage plant growth and maintain productivity. My thesis aims to identify this in Chapter 3, 'Saliva and udder-standing its impact on *Phalaris aquatica* in rhizoboxes', whereby the effect of a defoliation event along with the application of saliva is investigated. This chapter identified a growth response by plants to saliva treatment, both as a single and repeated application.

With existing publications and work presented in Chapter 3, it is clear that an understanding of the constituents within saliva should be determined, before speculations on what is the leading mechanism (chemical or physiological) of growth response can be identified. To date,

while a variety of animal biofluids such as urine and blood have been investigated using analytical profiling, however no such analysis has been thoroughly undertaken on livestock saliva. A range of compounds have been identified in saliva and postulated to impart the growth response such as thiamine (Bonner & Greene, 1939) and bovine serum albumin (Vittoria & Rendina, 1960) however no consensus has been reached. Profiling the chemical constituents of saliva can provide insight into what may be imparting this growth response but may also act as an exploratory step in the development of bioindicators of animal health and nutrition. I address this knowledge gap in Chapter 4, 'Moo-ving the knowledge forward on *Bos taurus* saliva using a metabolomic approach'. This study was a non-targeted, metabolomic approach to characterise the polar metabolite constituents of cattle saliva collected from four different breeds of cattle.

The ability to qualify, quantify, and match the location of plant species and biomass with the location of grazed areas has great potential to further our insight into paddock utilisation gaps in livestock enterprises (Taylor et al., 2011). As an example, animal movement is largely influenced by abiotic factors, topography and temperature (Dickson & Beier, 2007; Humphries et al., 2010; Kie et al., 2005; Patterson et al., 2009; Raynor et al., 2021), and these drivers need to take into consideration the spatial and temporal distribution of forage quality and quantity as a critical modulator of understanding space use in livestock (Bailey et al., 1996; Johnston & Bailey, 1972). Pastoralists and livestock farmers depend on grasslands and sown pasture as an economical source of livestock feed. For effective management of nutrition and ecology in these pasture-based systems, graziers must understand the determinants of intake at grazing, as well as the associated dynamics between animal and vegetation (Baumont et al., 2004).
Though we have scraped the surface on how grazing can influence the growth of pasture, knowledge needs to be applied to understanding how animals graze on the landscape, and how grazing preferences may influence location of the herd and associated regrowth rates of the pasture. In Chapter 5, I highlight the complexity of predictive modelling of sheep behaviour in a small scale, year-long study, 'Understanding sheep baa-haviour', and additionally in a larger scale, seasonal study in Chapter 6, 'Exploring 'wether' grazing patterns differed in native or introduced pastures in the Monaro region of Australia'. These two chapters look at vegetation-based characteristics in two different areas in New South Wales, Australia, and how these characteristics alongside other landscape-based variables such as weather, water locations, and presence of shelter/fence lines, drove grazing behaviour for tracked wethers.

Finally, in Chapter 7 I draw upon the findings of the work presented in this thesis to propose theoretical and practical implications of this new knowledge. I then propose new research directions that will likely improve pasture management and grazing systems as well as a number of practical tools to be considered for deployment in this significant and far-reaching agricultural system.

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Chapter 2:

Is animal saliva a prominent factor in pasture growth?

Publication details:

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This chapter has been accepted for publication in the CSIRO Journal, Crop and Pasture Science, and the version presented here has been formatted per journal guidelines, and has been configured to fit this thesis during submission.

AUTHOR ATTRIBUTION STATEMENT

All author contributions and the role of **Danica Parnell**, in preparation of the following manuscript are listed below:

Parnell, D., Merchant, A., Ingram, L. (2023) Is animal saliva a prominent factor in pasture growth?

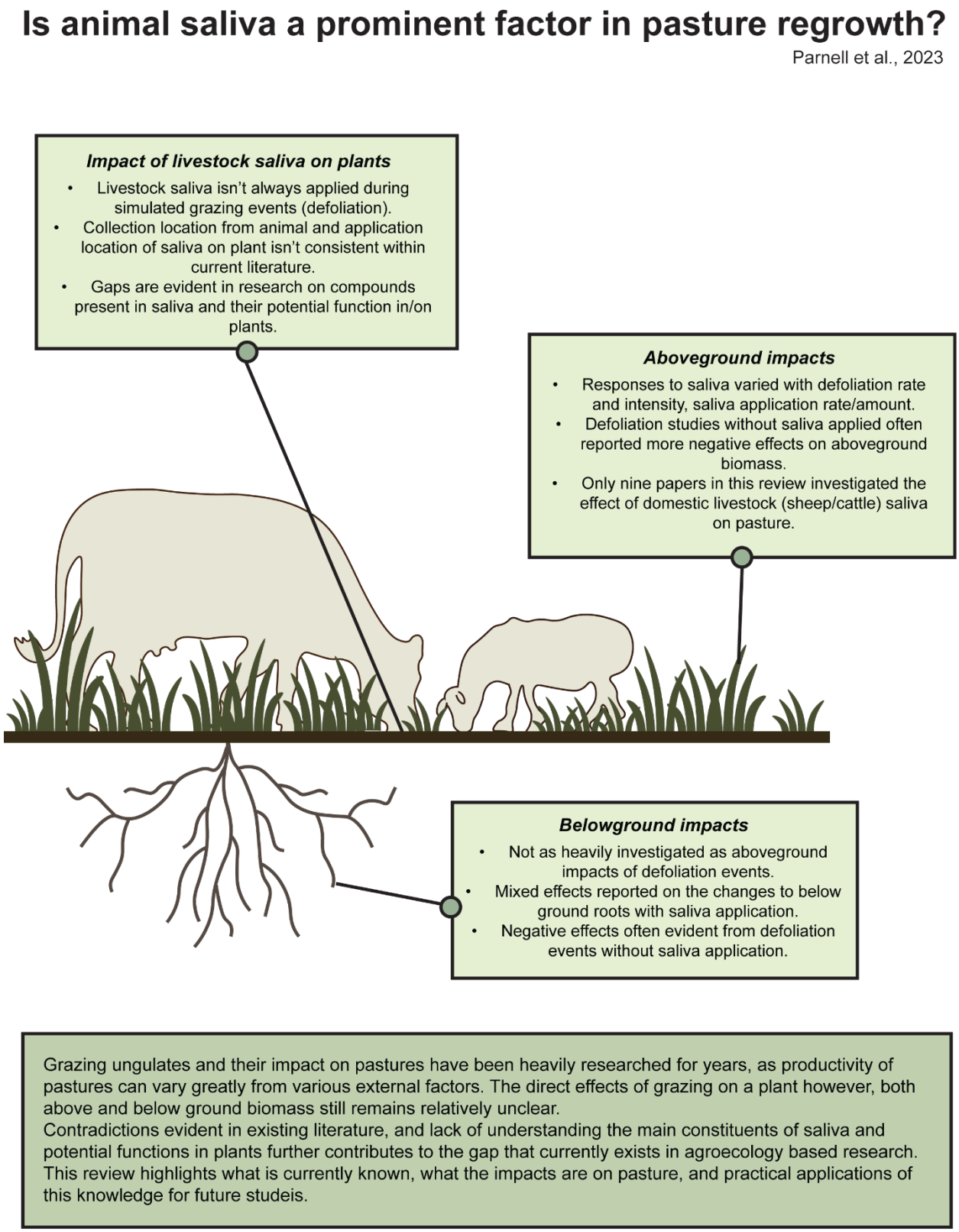
Task	***Author contribution***
Conceptualisation	DP, LI, AM
Investigation of literature	DP
Analysis and interpretation of results	DP
Draft manuscript preparation	DP
Reviewing and edits	DP, LI, AM

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the above authorship attribution statement for this chapter is correct.

Associate Professor Andrew Merchant

15th December, 2023

Graphical Abstract:



Is animal saliva a prominent factor in pasture regrowth?

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Abstract:

Over a period spanning more than 100 years, a substantial amount of research has been undertaken to determine the impact that grazing ungulates have on grassland production systems globally, as they are the primary source of feed for these animals. Productivity of these lands, however, is highly dependent on a variety of factors such as quality and quantity of the forage, regrowth rates, and grazing rates. Expected regrowth rate of pasture, may be more influenced by animals than originally thought, as the direct effect of saliva deposition on plants on both the above and belowground biomass of plants remains relatively unclear. Though research is evident on grazing impacts on pasture, those which have utilised saliva have often found contradictory results, or do not discuss the mechanisms behind the responses in pasture observed. As such, we believe though it is a miniscule aspect of the entire grazing picture, investigating the effect of saliva in further detail may highlight gaps apparent in current research, such as what compounds are evident in saliva, and what those individual components functions are in plants, or what result may occur when applied on to plants. This review discusses what is currently known about animal saliva, the impact on pasture, and the greater practical applications of this knowledge for graziers.

Keywords: animal saliva; pasture ecology; pasture production; multi-omics; plant-animal interactions; plant growth regulators; plant growth and development; plant physiology; grazing; grazing interactions

Introduction:

Grasslands across the globe are primarily used as pasture for livestock grazing however, for effective management of nutrition and ecology, landholders must understand the determinants of intake at grazing alongside associated dynamics between animal and

vegetation (Baumont, Cohen-Salmon et al. 2004). Understanding pasture productivity is highly dependent on both the quantity and quality of forage, as well the associated regrowth rates of plants. The respective growth, or regrowth rate of pasture is presumed to be influenced by the processes of grazing livestock. However, the direct effect of grazing and how it impacts pasture, both above and belowground, remains relatively unclear. As livestock are an integral part of grass-based systems, the effect of physical damage (i.e., biting) and chemical transfer (i.e., saliva deposition) by the animal to the plant, are expected to have a significant effect on various aspects of physiology through changes in plant biomass allocation and chemical elicitation of plant growth responses.

The process of grazing differs across the diversity of plant and animal species according to both the amount of pasture consumed, and the extent of interaction during grazing and mastication. Cattle differ in their interaction with pasture than sheep, and the mechanism in which livestock graze differs between the two species. For example, cattle are known to need more height in plant material as during the grazing process they use their tongues to select and remove material, whilst sheep in contrast, can cope with lower pasture height as they graze with their teeth. This distinct difference alone in the defoliation process (i.e., ripped vs. cut) can potentially lead to a varying growth responses from a grass. While most grazing studies have simulated the grazing (defoliation) processes in-situ by the process of clipping or mowing plants, arguably however, these are not reflective of an actual grazing event in a variety of ways (Howe, Grant et al. 1982, McNaughton 1985). For example, studies that clip plants have had uniformity in cutting of the leaf blade, which may not be accurate due to the various ways and heights that animals defoliate a plant. The results of these types of studies have never been compared to in *situ* grazing and, as such, these studies can only be taken as an approximation of the response by a plant to grazing. Furthermore, saliva may have growth promoting effects beyond that of the concomitant effects elicited by the removal of leaf area. Studies have reported that the addition of saliva can stimulate growth in plants (Young and Schneyer 1981, Dyer, Detling et al. 1982, McNaughton 1985), and others have also examined the use of chemical alternatives to saliva which have resulted in similar growth promoting responses (Reardon, Leinweber et al. 1974, Lamy, Graça et al. 2010, Huang, Peng et al. 2014, Li, Liu et al. 2014). However, little to no studies have explicitly determined what constituents in livestock saliva will stimulate pasture growth.

Plant growth is regulated by a range of metabolic factors however aboveground plant growth can be elicited by application of external plant growth regulators. Based upon our current

knowledge of its biochemical composition, the growth promoting effects of livestock saliva may be realised through a combination of several mechanisms. It is presumed that biological agents with high activity may be transferred from herbivores to plants, known as 'growth factors' or 'growth regulators' during the grazing process, which in turn, has influences on plants and herbivores (Young and Schneyer 1981, Dyer, Detling et al. 1982, McNaughton 1985). Saliva glands are known rich sources of growth promoting chemicals for plants, such as epidermal, nerve, and transforming growth factors (Cohen 1962, Frazier, Boyd et al. 1974). Steroid hormones have also been found alongside growth factors in saliva and has been reported to also have physiological impacts on plant growth (Dyer and Bokhari 1976, Detling, Ross et al. 1981, Teng, Ba et al. 2010, Li, Liu et al. 2014). Growth factors have been reported to intervene directly in cellular metabolism through promotion of differential transcription of genes (Murdoch, Rosenfeld et al. 1982). Vittoria and Rendina (1960) were the first to investigate chemicals being transferred to grasses during grazing and suggest that subsequently, there were positive influences on plant growth.

The impact of herbivory and its effect on plant production has been the subject of research within the last several years (Teng, Ba et al. 2010, Gullap, Erkovan et al. 2011) stating positive, negative, and neutral effects on grass productivity. Some grassland communities may be stimulated by grazing and result in an increase in productivity (McNaughton 1985, Frank, Kuns et al. 2002, Schaffers 2002). Herbivory has been reported to stimulate aboveground and belowground net primary production by 21% and 35% in Yellowstone National Park (Frank, Kuns et al. 2002), with similar values reported in Arizona (Loeser, Crews et al. 2004). Additionally, studies have shown that these events can also affect root development in grasses, due to the change in demand for sucrose in the root sink (Thornton and Millard 1996, Mackie-Dawson 1999, Morvan-Bertrand, Boucaud et al. 1999, Amiard, Morvan-Bertrand et al. 2003). Interestingly however, saliva from other species such as bison, has been found to provide no influence on growth, yield, or activity of plants (McNaughton 1985). The influence of growth by saliva will be heavily dependent on grazing intensity, the transfer of saliva, and may also be influenced when plants are grown in limiting conditions. Therefore, further detail on what is in saliva, any discrepancies between species, and its impacts on a variety of plant species is critical.

The effect of animal saliva on pasture growth may only be a small aspect of the bigger impact of livestock grazing, however, the mechanisms of growth promotion (or inhibition) may offer useful insight and tools for understanding pasture growth. At present, the influential effects of

saliva are not clear, due to the consistency of reported growth promoting effects. Due to the variability across all the studies, it is unclear what the overall mechanistic response is by plants, however, there is evidence that suggests what this response could be. Henceforth, this paper aims to review current evidence regarding the nature and magnitude of saliva application on plant growth, and the chemical composition of saliva and its variation, in order to ascertain current knowledge, and identify research objectives to characterise this important pasture-livestock interaction.

Pasture production, terminology, and the challenges of inter-study comparisons

Grazing management can alter and impact the quality and quantity of forage available for animals. Grazing techniques and controlling stocking density can influence the above ground effects (i.e., trampling) of pasture production. The implementation of good grazing management techniques help optimise both the production of forage for livestock consumption whilst at the same time, maintaining minimum amounts of pasture residue. Pasture that is managed well helps ensure that both ground cover and root growth maintained, which helps maintain soil health and minimise soil erosion. By definition, the process of grazing in grasslands will impact on biomass accumulation and productivity. During grazing, several components can be considered in sequence that impart effects on plant physiological, biophysical, and biochemical systems. This includes defoliation eliciting wound responses, the loss of leaf surface area, changes in the ratio of plant organ sizes (e.g., root: shoot) and the deposition of saliva. All such effects impact the acquisition and allocation of plant resources including water, nutrients and photo assimilates and as such, observations regarding changes in growth rates are not uncommon. Nevertheless, evidence has suggested that application of saliva to the leaf surface elicits growth promotion in addition to these effects, see Table 1 (Reardon, Leinweber et al. 1972, Reardon, Leinweber et al. 1974, Dyer and Bokhari 1976, Reardon and Merrill 1978, Dyer 1980, Howe, Grant et al. 1982, Kato, Nagayama et al. 1993, Rooke 2003, Loeser, Crews et al. 2004, Teng, Ba et al. 2010, Gullap, Erkovan et al. 2011, Liu, Wang et al. 2012, Li, Liu et al. 2014).

Deposition of saliva onto grasses during grazing occurs as animals lick forage to direct biomass into their mouths, or the mouth come into contact with grass; both of which leads to the transfer of chemicals to the un-grazed plant tissue (McNaughton 1985, Liu, Wang et al. 2012, Huang, Peng et al. 2014). It is generally presumed that saliva deposition is a cue for plants to stimulate growth and leading to the initiation of the compensatory growth response

(McNaughton 1979). While it generally hypothesised that herbivory (grazing by livestock) has positive influences on pasture growth, increasing fitness and competition (Vittoria and Rendina 1960, Dyer and Bokhari 1976, McNaughton 1979, Detling, Dyer et al. 1980, Detling, Ross et al. 1981, McNaughton 1985, Matches 1992, Bergman 2002), a number of studies have observed that also suggests either negative, or no impacts at all to plants (Johnston and Bailey 1972, Reardon, Leinweber et al. 1974, Capinera and Roltsch 1980, Detling, Ross et al. 1981). As such, despite the paucity of research regarding the effect of saliva on plant growth, it is generally believed that under certain conditions growth promotion is likely to occur. Its therefore prudent to investigate potential mechanistic explanations of how this might occur.

Growth promotion

The terminology describing the process of plant biomass removal during the grazing process has not been consistently applied among previous studies and has implications for interpreting any in-situ and ex-situ treatment effects. For example, there are papers which have discussed animals 'defoliating' a plant, whilst others mention 'grazing', and few too have discussed 'defoliation' as a form of mechanical/manual clipping only. As such, in this paper we use the term 'grazing' as the process in which livestock licks, bites, rips, and swallow pasture and 'defoliation' as the process by which pasture is mechanically cut to emulate the process of grazing. In either method, leaves are either completely or partially removed. This in turn has impacts on the photosynthetic activity, secondary metabolite activity, and carbohydrate relocation of the plant (Gold and Caldwell 1990, De Visser, Vianden et al. 1997, Macduff, Humphreys et al. 2002). Though responses to grazing/herbivory and defoliation/clipping events may have similar results, it is not feasible to substitute one for the other when discussing the effect of animals grazing on pasture (White 1973, Capinera and Roltsch 1980). Where the effect of mechanical clipping/defoliation is used as a substitute for actual grazing and the subsequent research has focused on e.g., plant photosynthetic capacity, root growth or nutrient uptake, the vast majority of cases does so without the added influence of saliva (Clement, Hopper et al. 1978, Parsons, Leafe et al. 1983, Jarvis and Macduff 1989, Christopher, Miranda et al. 2004) (Table 1). Additionally, while studies have investigated the role of defoliation only (Heady 1961, Jameson 1964, Harris 1978, Stroud, Hart et al. 1985, Lindgren, Klint et al. 2007, Deutsch, Bork et al. 2010, Wang, Zhao et al. 2020, Wang, Ji et al. 2022, Koptur, Primoli et al. 2023) these studies ignore the potential impact that saliva has on stimulating plant growth (Reardon,

Leinweber et al. 1972, Detling, Dyer et al. 1980, McNaughton 1985, Teng, Ba et al. 2010, Liu, Wang et al. 2012) (Table 1).

There are a plethora of plant responses as a result of mechanical clipping and grazing which are not clearly understood, as there are a range of factors in play which may alter the response. For example, the presence of an animal and their influence on nutrient cycling in the ecosystem is one of those factors. Studies have also previously suggested that the vast majority of nitrogen, phosphorous and potassium in forage consumed by graziers is returned to the pasture in the form of faeces or urine, however in greenhouse studies where plants are defoliated this material is typically removed from the system altogether (Sears 1951, Peterson, Lofgreen et al. 1956). Another example is the height at which plants are clipped, compared to what is observed by grazing livestock such as, clipping occurring at a random height in an attempt to emulate livestock bite patterns (Jameson 1964)) or has been performed at levels far more severe than what occurs during grazing events (Heady 1961).

In addition to variability in application of defoliation (vs grazing) treatments, plants have evolved a variety of adaptive mechanisms which makes them more resilient to the grazing/defoliation process and as a result, plants differ in their responses to grazing by herbivores (Harradine and Whalley 1981, Hodgkinson, Ludlow et al. 1989, Gullap, Erkovan et al. 2011). Furthermore, environmental conditions such as plant growth stage, soil, nutrients, weather, and climate play a large factor in forage growth (Reardon and Merrill 1978, Buxton and Mertens 1995, Loeser, Crews et al. 2004, Schacht and Volesky 2010, Gullap, Erkovan et al. 2011), also influence growth responses after a defoliation/grazing event. Finally, plants are rarely grazed only a single time so the frequency, intensity, and timing of defoliation or grazing events will also influence a plant response to grazing/defoliation events (Harris, 1978; Gullap et al., 2011).

Additionally, previous research has reported on saliva and defoliation events on *Bouteloua curtipendula* (Reardon et al., 1974), *Sporbolus ioclados* and *S. pyramidalis* (McNaughton 1985), *Agropyron smithii* Rydb. (Stroud et al., 1985), *Salix caprea* (Bergman, 2002), *Carex bigelowii* (Lindgren et al., 2007), *Leymus chinensis* (Liu et al., 2012). In an Australian context, few plant species have been investigated with regard to their response to saliva and defoliation events. Notably, predominant native Australian pasture species such as *Themeda triandra* (kangaroo grass), have not yet been investigated. As Australian native grasses often respond poorly to grazing, due to their ability to easily be overgrazed and poor responses to

fertilisation treatment (Garden, Dowling et al. 2001, Mokany, Friend et al. 2006, Nie and Zollinger 2012, Mavromihalis, Dorrough et al. 2013), and the generally poor adaptation of grasses to grazing events in general, it is critical to identify how the intricate process of grazing impacts the regrowth of these species, and identify new insights on how grazing impacts not only native grasses, but also on a range of introduced grasses that are a critically important component of much of Australia's extensive livestock production systems. Therefore, to manage pasture in the most appropriate manner to maximise productivity, it is necessary to understand, and quantify the response of grass to grazing activities, as distinct from grass defoliated using artificial methods, often explored *in-situ*.

Chemical constituents of saliva and its physiochemical properties

Saliva is comprised of a series of compounds that assists in masticating and swallowing and is secreted in large quantities and at various rates during resting, eating, and rumination (Kay 1960, Bailey and Balch 1961). Saliva in ruminants contributes water and salts to the rumen and has a pH of approximately 8.4 (Bailey and Balch 1961). Previous research has found that the submaxillary glands secrete saliva during grazing (480 mL h^{-1} by cattle; (Ellenberger and Hofmeister 1887), whilst the parotid glands secrete continuously but at an increased rate during mastication of food (Bailey 1961). Saliva is produced in glands which are present all over the mouth, however, the major glands function through connective ducts to the parotid and mandibular glands, which are innervated parasympathetically (Colin 1886, Bailey 1961, Bailey and Balch 1961). Of note however, is the type of secretions these two major glands produce in livestock; the parotid salivary gland is known to produce serous based secretions (water based, rich in proteins), whilst the mandibular glands produce a meocrine solution (a water and mucous mixed solution) comprised of mucopolysaccharides and glycoproteins (Nonaka and Wong 2022). The mandibular gland is the largest gland found in livestock (Dehghani, Lischer et al. 1994, Dehghani, Tadjalli et al. 2000) and produces more saliva than the parotid gland. At the mandibular level, there are two primary glands which secrete saliva within the oral cavity, the submandibular gland, and the sublingual gland (Dehghani, Lischer et al. 1994, Dehghani, Tadjalli et al. 2000, Ellis 2012). The submandibular gland being the larger gland located near the mandible, releases saliva into the mouth through the ducts, whilst the sublingual glands are located beneath the tongue, which releases saliva produced into the floor of the mouth (Ellis 2012). Buccal salivary glands are minor salivary glands, located along the inner side of cheek within the mouth (Ellis 2012). Essentially, saliva is a result of continuous secretion of parotid, mandibular, sublingual, and buccal salivary glands,

and rate of secretion can depend on factors such as gland type, stimuli, individual animal, diet, and activity (Kay 1960).

Whilst little is known about specific properties of livestock saliva which may induce a growth response in plants, prior exploratory analysis of biomarkers within saliva however has observed common constituents such as water, various proteins, salts, electrolytes, and antimicrobial agents (McDougall 1948, Chauncey, Lionetti et al. 1954, Chauncey 1955, Kay 1960, Chauncey, Henriques et al. 1963, Young and Schneyer 1981, Turunen, Puurunen et al. 2020). Authors have speculated presence of notable compounds such as thiamine (Bonner and Greene 1939, Reardon, Leinweber et al. 1972), and bovine serum albumin (Vittoria and Rendina 1960, Reardon, Leinweber et al. 1974) which may in turn, be responsible for positive growth responses post defoliation. Despite some qualitative understanding of the constituents of livestock biofluids (i.e., urine, ruminal fluid), no descriptive, 'omic' based approach to chemical characterisation of livestock saliva has been conducted to date. Proteomics, metabolomics, and ionomics are studies which can be undertaken to identify the presence of proteins, various metabolites (i.e., peptides, carbohydrates, lipids) and ions, respectively. These studies allow for a deeper understanding of novel molecules, various concentration ranges and presence with biological samples. Little is known about the scope of chemical diversity, nor the quantitative range chemical constituents both of which may contain valuable information regarding the mechanisms of growth promotion. However, a summary of what compounds are known to be present in saliva (regardless of species of animal that they have been obtained from), those which have been speculated to be present in livestock, and the role they may have on influencing grass growth and productivity following a grazing event, would be beneficial. We present below chemical candidates from existing literature, categorised in to compound classes.

Metabolites

Currently within the literature, the metabolite proposed as an active component within saliva for plant growth promotion is thiamine. Thiamine is an essential micronutrient which aids in the growth, development, and function of cells (Addicott 1941). It is a water-soluble vitamin which has a N containing ring based in the centre and is essential in a phosphorylated form for metabolic reactions associated with the breakdown of carbohydrates and amino acids. Thiamine has been classified as a growth factor, often produced in the shoot and is necessary for root and shoot growth in plants (Bonner 1937, Robbins and Bartley 1937, Bonner and

Greene 1939, Bonner 1940, Addicott 1941, Clark 1942, Vittoria and Rendina 1960, Reardon, Leinweber et al. 1972, Reardon, Leinweber et al. 1974, McNaughton 1985). Thiamine is active in root nodule and mycorrhizal symbioses in which it has a profound effect on nitrogen and phosphorous uptake and thus on plant growth and development (Fitzpatrick and Chapman 2020). Previous publications have shown that animal saliva contained thiamine at concentrations that would stimulate a growth response in grasses (Bonner and Greene 1939, Reardon, Leinweber et al. 1972). However, in spite of this, there has been little to no evidence presented in the literature as to why it is considered a stimulator of plant growth following grazing, as the simple presence of thiamine in saliva can in no way be used to conclusively show that it does fact stimulate plant growth. As such its presence in a saliva substitute or an artificial saliva may or may not influence plant growth.

Proteins and their derivatives

Several proteins and their derivatives in saliva have been postulated as having a positive growth promoting effect on plants however, no candidates are explicitly correlated with growth promotion and no study has mechanistically proven such an effect. Understanding their chemical composition, functional groups and in the case of protein activity; substrate affinity and enzyme kinetics, may offer some insight into the functional activity of these chemical compounds. Bovine serum albumen (BSA) is a protein derived from bovines (cattle) and are primarily responsible for providing colloid osmotic-pressure within capillaries, transporting fatty acids, minerals, and hormones (Peters 1996, Chen, Hammo et al. 2021). In addition, BSAs also function as an anti-oxidant and -coagulant (Peters 1996). BSA has been used due to it being a common blood protein found in livestock, often a carrier for steroids, fatty acids, and thyroid hormones, and has been studied in plant research for over decades (Vittoria and Rendina 1960, Reardon, Leinweber et al. 1974). It has been suggested previously that BSA is the protein that interacts in plants, with signs of cell death and various activities (i.e., stress, and transport) being evident when applied (Lamy, Graça et al. 2010, Huang, Peng et al. 2014). Additionally, epidermal growth factors (EGF) are known to enhance growth, promote adventitious root formation, and cell division of epicotyl cuttings (Kato, Nagayama et al. 1993), and have been speculated to be the compounds responsible for how herbivores may aide in regulating plant productivity (Dyer 1980).

The enzyme amylase is a major digestive enzyme (Daja and Treska 2015) and is also present in saliva, which is the chemical elicitor that begins the digestion process as it is responsible for breaking starch into maltose and dextrin (Fried, Abramson et al. 1987). In animals, the

primary form of amylase is alpha-amylase and is produced in the salivary glands. In contrast, plants predominantly have beta-amylase, though both alpha-, and gamma-amylase classes are also present (Azzopardi, Lloyd et al. 2016), however, as in ruminants, amylase in plants breaks down starch into sugar to provide energy for the plant during early germination stages. The conversion of large molecular weight compounds to low molecular weight compounds by amylase during starch metabolism increases the osmotic potential of the cellular solution and may impart a growth response through increased osmotic pressure, leading to cell expansion, as well as providing increases in the availability of substrates for metabolism. Lingual lipase is another enzyme present in saliva that is a part of the digestive process (Hamosh and Scow 1973). In animals, lingual lipase catalysis the digestion of lipids initially in the mouth which continues to occur in the stomach (Hamosh and Scow 1973). Lipases are also present in plants, however, they are primarily found in tissues of growing seedlings (Pahoja and Sethar 2002) where they catalyse they hydrolyse triacylglycerols to fatty acids to be converted to sugars that support the growth of seeds during the process of germination (Lin, Yi et al. 1987).

Glycoproteins are proteins which are integral membrane proteins and play a role in cell-to-cell interactions. Glycoproteins are predominantly N-linked and O-linked and are often found in the body as mucins (Gamblin, Scanlan et al. 2009). Glycoproteins have a variety of functions including acting as a structural molecule within collagen, a lubricant/protective agent in mucins, an immunologic molecule in immunoglobulins, and hormones such as thyroid stimulating hormone (Murray, Granner et al. 2006, Maverakis, Kim et al. 2015). In plants glycoproteins are present in plant cell walls and assist in adaptation of a plant to its environment (Selvendran and O'Neill 1982, Josè-Estanyol and Puigdomènech 2000). Mucin is a glycosylated protein, and a macromolecular component of the 'mucus' component of saliva (Voynow and Fischer 2006). Mucus is a mixture of water and a diverse range of 'defensive proteins' that are largely comprised of glycans of both the N-linked and O-linked type (Voynow and Fischer 2006, Dolan and Hansson 2023). Mucin in livestock originates from secretions in submaxillary glands, and its function is to aid in digestion so food can easily travel through the digestive tract (Proust, Baszkin et al. 1984). Whilst there is no obvious mechanism by which mucin can stimulate plant growth, saliva is a crucial line of defence against microbial species due to its richness in antimicrobial compounds. Antimicrobial agents in this instance are natural substances that can kill or inhibit the growth of microorganisms such as bacteria, fungi, or algae (Burnett-Boothroyd and McCarthy 2011). Secretory immunoglobulin A (SlgA) is a known antimicrobial agent produced in large

amounts in animal saliva and stimulates immune protection and preservation of immune homeostasis (Mach and Pahud 1971, Myer, Smith et al. 2015, Fouhse, Smiegielski et al. 2017). Other antimicrobial proteins evident in saliva include lysozyme (Salton 1957), lactoperoxidase (Singh, Smith et al. 2012), and lactoferrin (Sánchez, Calvo et al. 1992). Of the aforementioned proteins, only two are expressed in plants, lysozyme, and peroxidase, which serve the same antimicrobial function in plants. Peroxidases function in many of the physiological processes in plants, such as biosynthesising lignin, and similarly to animals, acting as a defensive agent against pathogens and wounding (Fujiyama, Intapruk et al. 1995, Vicuna 2005). In plants, physiologically, lytic vacuoles achieve a similar outcome to lysosomes in animals, as these vacuoles degrade cellular materials (Hara-Nishimura and Hatsugai 2011) whilst lysosomes digest waste in a cell (Salton 1957). Mucopolysaccharides, or glycosaminoglycans, are comprised of repeating two-sugar units (disaccharides) and are found in bacteria, vertebrates, and invertebrates (DeAngelis 2002, Esko, Kimata et al. 2009). The former, is often found in mucus and fluid in joints. Mucopolysaccharides also vary in their mass, structure, and sulfation, and can be further subdivided depending on their main structures (Sasisekharan, Raman et al. 2006). Their function in animals ultimately depends on which of the four classes the mucopolysaccharide belongs to, for example, cellular wound repair, brain development, pathogen infection, and functions in skin, vessels, and heart valves (Funderburgh 2000, Trowbridge and Gallo 2002, Sugahara, Mikami et al. 2003, Tortora 2013). The mechanism in which glycosaminoglycans function on plant growth, however, has not been described (Yamada, Sugahara et al. 2011).

Ions

Electrolytes include most soluble salts, acids, and bases, and may be either positive or negative ions or minerals that help the body maintain fluid balance and a constant pH (Enderby and Neilson 1981, Ruckebusch, Phaneuf et al. 1991). The primary ions in electrolytical physiology are sodium, potassium, calcium, magnesium, chloride, hydrogen phosphate, and hydrogen carbonate (Enderby and Neilson 1981). Sodium in particular, is the main electrolyte found in the body and is involved in blood pressure control (Enderby and Neilson 1981). Though little research has been undertaken on the extent of plant growth promoting ions in livestock saliva, there are studies on electrolytes (i.e. sodium, chloride, and potassium) in livestock for other areas of research such as patterns during oestrus and pregnancy (Devi, Singh et al. 2016, Mojsym, Wawrzykowski et al. 2022), in calves (Kumar and Singh 1981), and in long term research on electrolyte changes (Grimm, Humann-Ziehank

et al. 2021). Whilst the physiochemical interactions of free ions are likely limited to their influence over osmotic potential (and increasing cellular turgor pressure), induction of signal cascades by increase in ion abundance (such as potassium which affects plant hexokinase activity) is observed in both plant and animal cells (Alberts, Johnson et al. 2002).

Species specific interactions and sampling protocols limit extrapolation of results

Despite the physiochemical properties of saliva providing some support for the notion that it imparts or stimulates a growth response on plants, our ability to make broad scale predictions of growth promotion is limited by several factors. In part this is due to the fact only a handful of animal species have thus far been investigated. When saliva has been included in studies such as metabolomics or proteomics, it has been predominantly studied in species such as hogs, grasshoppers, goats, rabbits, rats, dogs, and humans (Palmer 1916, Hines and McCance 1953, Chauncey, Lionetti et al. 1954, Chauncey 1955, McGeachin and Gleason 1956, Chauncey, Lionetti et al. 1957, Chauncey, Lisanti et al. 1958, Cohen 1962, Chauncey, Henriques et al. 1963, Dyer and Bokhari 1976, Rooke 2003, Turunen, Puurunen et al. 2020) (see Table 1) but not in livestock, or was included in studies when analytic capabilities were limited (Reid and Huffman 1949). Recent research has focused on a variety of other biofluids from cattle, though saliva has seldom been the focus of these studies when included. For example, metabolomic investigations performed more recently in cattle included analysis on milk, ruminal fluid, serum, urine, and faeces (Kim 2021, Zhu, Tang et al. 2021). The chemical constituents of saliva in sheep however have been characterised extensively in sheep (Chauncey, Henriques et al. 1963, Lamy and Mau 2012, Palma-Hidalgo, Belanche et al. 2023) and work by Lamy et al., (2012), and Palma-Hidalgo et al., (2023) focused on proteomics and metabolomics-based research, respectively. A literature review by Goldansaz and colleagues in 2017 surveyed metabolomic studies undertaken between 1930 to 2015 did not include saliva as a biofluid within their search parameters. However, this could be due to protein analysis of animal biofluids being a new area of research for factors such as diseases and hormones (Lamy and Mau 2012). Moreover when bovine saliva has been highlighted in 'omics' based research, there is often little interest expressed in its role as elicitor of growth and the responses of a plant to saliva application, and studies instead focus on what has been expressed by the plant post application, i.e. protein expression (Fan, Cui et al. 2011).

These studies highlight another factor affecting our ability to infer firm conclusions; that is the role that saliva collection may play on the data collected and particularly regarding cross

study comparisons. Of the studies investigating sheep saliva, research by Lamy et al. (2009) had saliva sampled from animals under anaesthetic, with catheters sampling directly from the parotid gland. Contrastingly, research by Palma-Hidaglo et al., (2023) had saliva sampled by swabbing the mouth of sheep and may provide a more accurate representation of the constituents of saliva in the mouths of animals at the time of grazing. Research performed by Reardon et al., (1972) collected saliva from cattle from the mouth, whilst that of Johnson and Bailey (1972) collected saliva directly from the rumen. These variations in collection technique undoubtedly have implications for interpretation. For example, saliva from the rumen may contain detrimental contaminants such as bacterial enzymes (Reardon, Leinweber et al. 1974). Though the former three studies were not focused on an 'omics approach to understanding saliva impacts, it is still critical to highlight the importance of 'omic characterisations of saliva based upon standardised protocols, including that of saliva collection.

Previous studies have also attempted to use 'synthetic' saliva as a substitute, and was a concept initially proposed by Jameson in 1964. These results of studies that have used a synthetic version however, have often suggested that the growth response of plants do not elicit the same response compared to the use of real saliva (Reardon, Leinweber et al. 1974, McNaughton 1985, Lamy, Graça et al. 2010, Huang, Peng et al. 2014, Li, Liu et al. 2014). Animal saliva used in contrast, has been found to have a larger impact on plant growth than any synthetic substrate used, further contributing to the complexity of understanding the impact that individual substrates may have (McNaughton 1985, Li, Liu et al. 2014). Huang et al., (2014) proposed that herbivore saliva is 'unstable' possibly due to the presence of bacteria, which further reiterates the need for characterisation of livestock saliva, due to the potential interactive effects between the different components within saliva. This interactive effect further highlights the overall complexity of salivary composition. The various protein and growth factors present in saliva, regardless on the level of individual presence, may not be encapsulated in a synthetic mixture and therefore, not accurately resemble saliva itself (Cohen 1962, Miles and Lloyd 1967, Kessler and Baldwin 2002). Furthermore, there are likely to be large costs of making a synthetic solution due to the mixture of proteins and growth factors evident.

Applications of this knowledge include the ability to design experimental procedures which can identify growth promotion as separate to other factors such as damage and wound responses. Along with a multi-omic approach to investigate what is in saliva, it is suggested

that studies should be performed to identify what the potential contributors to plant growth are by testing all salivary components individually, to rule out if this effect is likely due to the addition of growth promoters, proteins, and nutrients, or if it likely a wound response by the plant. Additionally, research should be undertaken to determine if a salivary response differs in livestock species (i.e., cattle or sheep) and breeds within the species (e.g., a Jersey or Angus in cattle). Research should also be devoted to increase the range of species of pasture studied both *in situ*, and in field, so an extensive library can be created on the various growth effects that saliva has. By doing so, we will develop a much clearer understanding of how grazing impacts pasture production, including stimulatory responses, in order to understand the extent that plant-animal interactions can be better utilised to maximise the sustainable productivity of grazed systems.

Conclusion:

Despite the extensive amount of existing research, many questions remain regarding the impact that saliva has on pasture production. These range from understanding the contradictions present in the existing literature on the effects that saliva has, all the way through to determining what is present in ruminant saliva both qualitatively and quantitatively. Through investigation of existing literature, out of the 21 defoliation studies found to have utilised saliva and/or various constituents presumed to be in saliva, only 11 studies identified a positive influence of saliva on plant growth, and five studies identified a mixed effect. Evidently, more research is required to understand why certain constituents within livestock saliva might cause growth promotion (or inhibition) post-grazing. More recent analytic techniques such as multi-omic approaches to determine what is in livestock saliva show great promise here. Studies such as those would allow a deeper understanding of the constituents within saliva, and so too, which compound may impact growth responses by plants. With the ability to isolate the active constituents that promote growth in plants, a chemical tool may be identifiable, and utilised to enhance the resilience of pasture production systems. Although the likely impact of saliva overall being of a minute scale when at a paddock level, the influence of saliva on individual plant growth is likely to be a significant contributor to overall pasture production.

Data Availability Statement:

Data sharing is not applicable as no new data were generated or analysed during this study.

Conflicts of Interest:
The authors of this work declare no conflicting interests.

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Supplementary Material:

Table 1: Summary table of papers indicating the various responses of plant species to saliva application, grazing, and manual defoliation. NB: + indicates positive effect, - indicates negative effect.

Author and year of publication	Effect (+, -, NA, none, mixed)	Plant species studied	Saliva (sampled from) or compound	Collected saliva (C), Grazed (G), manual defoliation (MD) not applicable (NA)	Lab (L) or field (F)	Effect seen/Major takeaway
Positive effects (saliva/compounds)						
Dyer and Bokhari, 1976	+	*Bouteloua gracilis* (Blue grama)	Grasshoppers	C	L	• Largest effect displayed by insect grazers is increase in below ground respiration and root exudation. • Grazed by grasshoppers had a faster regrowth rate than that just clipped mechanically.
Dyer 1980	+	Sorghum seedlings	Mouse (submaxillary glands)	C	L	• Increase in shoot response in 3 and 6 days. • Proposed that EGF or EGF like compounds provide the basis that regulate plant productivity.
Frank et al., 2002	+ (saliva effect not explicitly stated)	Mixed; *Agropyron cristatum, Festuca idahoensis, Pseudoroengaria spicata, Poa pratensis,*	*Cervus elaphus* (elk), *Bison bison* (bison), *Antilocapra americana* (pronghorn)	G	F	• NA for saliva (not discussed) • Grazing animals stimulated above aboveground and below ground production by 21% and 35%. • Whole plant production increased by 32% when graziers were on the land. • Root mortality increased by 22%.

		Deschampsia caespitosa, P. compressa				• Predominant effect of grazers was an influence on below ground root growth (7x that of shoot growth). • Aboveground productivity and grazer facilitation was positively related to consumption and grazing intensity, but weakly associated with site condition.
Howe et al., 1982	+	*Lolium perene* (annual ryegrass)	*Sigmodon hispidus* (Hispid cotton rat)	C, G	L	• Regrowth was faster by rat grazing than mechanical clipping. • Assumes that saliva contains an agent that promotes growth when coming into contact with open plant wounds. • Alternatively could be due to specific manner of tissue removal which stimulates growth.
Huang et al., 2014	+	*Leymus chinensis*	Bovine serum albumin (BSA), compound	NA	L	• BSA deposition triggers gene expression which differs to defoliation only. • Established that apoptotic pathway responded to grazing stressors.
Kato et al., 1993	+	*Vigna angularis*	Epidermal growth factor (EGF), compound	NA	L	• EGF promoted adventitious root formation in epicotyl cuttings, presumably from promotion of cell division. • Most effective time periods were between 8 – 16 hours and 16 – 24 hours.
Liu et al., 2012	+	*Leymus chinensis*	Sheep (mouth)	C	L	• Primarily identified that there was a link between mobilisation of carbohydrates post grazing.

						• Animal saliva increased tiller number, buds, and biomass, but no significant effect on height. • Effect also depended on intensity. • Accelerated hydrolysation of fructans and accumulation of glucose and fructose.
Loeser et al., 2004	+	Mixed species pastures including: *Pascopyrum smithii* (western wheatgrass), *Elymus elymoides* (squirreltail) and *Artemisia frigida* (prairie sagewort)	Cattle Grazed and manual defoliation.	G, MD	F	• Aboveground net primary productivity (ANPP) was greater in all species when defoliated. • Single year defoliated subplots had 31% higher average ANPP than non-defoliated plots, and second year defoliated subplots had 27% higher average. • Clipping in plots with ongoing grazing yielded 20% higher ANPP on average than non-defoliated.
Reardon et al., 1972	+	*Sideoats grama*	Cattle Collected	C	L	• Speculated the presence of thiamine and it's influence on pasture growth. • Saw an increase in plant growth response.
Rooke, 2003	+	*Combretum apiculatum*	Goat saliva	C	L	• Clipped shoots had enhanced growth though, unclipped shoots grew more than clips.
Teng et al., 2010	+	*Leymus chinensis*	Sheep saliva Collected	C	L	• Saliva increased leaf weight and elongation. • No difference between in clipping treatments on height growth rate and aboveground biomass.
Positive effects (defoliation only)						

Gao et al., 2008	NA for saliva + for defoliation	*Leymus chinensis*	NA	NA	L	• Significant interactions on water availability and clipping intensity on relative height growth rate and bud number. • Interactions between 'herbivory' and water or nutrients were opposite to each other. • Low tolerance to heavy clipping when there was high nutrients available, than when there were low nutrients available.
Morvan-Bertrand et al., 1999	NA for saliva + for defoliation	*Lolium perenne* (perennial ryegrass)	NA	NA	L	• Plants were severely defoliated, and fructan levels in leaf sheaths and elongating leaf bases strongly influenced the shoot yield during the first 2 days after defoliation. • Fructans from sheaths, but fructans from immature cells may be a substrate used for growth.
Negative or no effects evident (saliva/compounds)						
Detling et al., 1980	None	*Bouteloua gracilis* (Blue grama)	*Bison bison* (Bison)	C	L	• No statistical significance of foliage application on photosynthesis, root respiration, fixed C or regrowth rates. • In this study, unclipped plants invariably outproduced clipped plants following defoliation after 10 days.
Detling and Dyer, 1981	-	*Bouteloua gracilis* (Blue grama)	Grasshoppers (*Brachystola magna*)	C	L	• Found a reduction in tillering and growth (contrasting to that of the Dyer's 1976 paper).

						• Presuming the diseases/chemicals in pest saliva may have caused the reduction.
Gavloski et al., 2000	None	*Brassica napus* and *Salix alba*	Insects (*M. configurata*, *P. xylostella, P. cruciferae*)	G	L	• Saliva effect not explicitly discussed. • Herbivory effect by insect does lead to plant compensation, however, varied. • Related to the different plant species defensive mechanisms, or the particular insect used.
Johnston and Bailey, 1972	-	*Festuca scabrella* and *F. idahoensis*	Cattle (from rumen fistulated cow)	C	L	• After clipping plants were sprayed with freshly collected bovine saliva. • No significant differences in yield of tops or roots in tiller number at any clipping height regardless of saliva.
McNaughton, 1985	None	*Sporbolus icolados* and *S. pyramidalis*	Thiamine, compound	NA	L	• Leaf treatment of thiamine can impact yield and metabolic balances at low applications. • Defoliation rates reduced AG plant biomass to a fourth of the level produced by unclipped plants. • Growth was strongly limited by defoliation. • Promotion of yield and modified chemical balance of plants, reduced N concentrations. • Plant species from heavily grazed grasslands were more sensitive to thiamine application.
Negative and no effects evident (defoliation only)						
Gold and Caldwell, 1990	NA – no saliva used	*Agropyron desertorum, Artemisia tridentata*	NA – no saliva used	MD	F	• Photosynthetic rate increased following clipping regardless of defoliation pattern

	None					• Increases were greater when older leaves were removed, versus younger leaves • Photosynthetic competence varied with foliage age • Any structural differences caused by defoliation will influence regrowth potential.
Harradine and Whalley, 1981	NA - for defoliation	*Aristida ramosa, Danthonia linkii*	NA – no saliva used	MD	L	• Root weight and depth were severely reduced by clipping at weekly or monthly time periods and were sensitive in roots. • Defoliation led to a concentration of root mass between 0 – 10cm (48% for *D.* linkii, 33% for *A. ramosa)*
Capinera and Roltsch, 1980	NA	Wheat seedlings	*Melanoplus sanguinipes* (grasshopper)	MD	L	• NA for saliva (not explicitly discussed) • Focused on damage by grasshoppers to seedlings • Severe defoliation by grasshoppers had lower rate of seedling regrowth than manual clipping. • Chewing insects may be more difficult to simulate than generally acknowledged.
Hodgkinson, et al., 1989	NA - for defoliation	*Cenchrus ciliaris, Themeda triandra*	NA – no saliva used	MD	L	• Frequent close defoliation greatly reduced shoot production for both species. • *T. triandra* was less able to withstand defoliation compared to *C. ciliaris* likely from structural and functional responses of both plants.

						• *C. ciliaris* is likely to have evolved effective structural and functional strategies to cope with heavier grazing.
Jarvis and Macduff, 1989 9	NA – no saliva used None for defoliation	*Lolium perenne, L. multiflorum*	NA	MD	L	• Little effect of N deprivation on the growth rates of recovering defoliated shoots. • Possible mechanism is the roots of N defoliated plants retains high capacity for absorption after resupply/defoliation. • Stressed plants could not assimilate NO_3^- or utilise it.
Lindgren and Moen, 2007	NA – no saliva used - for defoliation.	*Carex bigelowii*	NA – no saliva used.	MD	L	• Clipping at different intensities with different harvest times found that the amount of soluble plant proteins is lowered in wounded plants.
Macduff et al., 2002	NA – no saliva used None for defoliation	*Lolium perenne*	NA – no saliva used	MD	L	• The stay-green mutation in this species has no significant/adverse effect on growth of *L. perenne* during N starvation or during severe defoliation in the short to medium term.
Mackie-Dawson, 1999	NA – no saliva used - for defoliation	*Lolium perenne*	NA – no saliva used	MD	L	• Single defoliation event resulted in reduced tillering, biomass increment, and N uptake after 3 weeks from defoliation.

						• Root growth was reduced by defoliation for all N impacted treatments, and in shoots that were derived from root uptake from 7 – 14 days.
Stroud et al., 1985	-	Western wheatgrass	NA – no saliva used	MD	L	• Simulated continuous grazing caused no major decrease in plant production. • Declined however under 4 uniform clips.
Thornton and Millard, 1996	NA – no saliva used - for defoliation.	*Lolium perenne, Poa trivialis, Festuca rubra, Agrostis castellana*	NA – no saliva used	MD	L	• Clipping reduced biomass of leaf and roots when done at high intensity. • Root growth decreased with increase of severity. • Uptake by roots was more important in supplying N to the shoots of repeatedly defoliated grasses than remobilisation.
	Mixed response to defoliation (with and without saliva)					
Bergman, 2002	Mixed	*Salix capera*	Moose (Alces alces)	C (sedated)	L	• Increased branching on saplings. • No significant effects on other growth characteristics. • Consistent stimulatory effect on branching of sallow saplings, but not on growth traits.
Gullap et al., 2011	Mixed	*D. glomerata* (Orchardgrass) *F. ovina* (Sheep fescue)	Cattle	C	L	• Cut with saliva had greater relative height growth rate (RHGR) than controls. • Saliva applied in orchardgrass increased below ground biomass in sandy based soils but decreased it for sheep fescue in the same.
Li et al., 2014	Mixed	*Leymus chinensis*	Sheep saliva Thiamine	C	L	• No compensatory response in clipped plants.

			Epidermal growth factor (EGF)			• Plants in clipping with saliva had more biomass and buds than those with water or components. • Clipping with salivary components had no stimulatory effects on plant growth, compared to clipping with water. • Herbivore saliva had greater impacts than saliva components. • No additive effect between salivary components on plant growth.
Reardon et al., 1974	Mixed	*Sideoats grama*	Cattle and thiamine	C, G	L	• Highly significant response to height and frequency of clipping. Grazed was taller than the clipped. • Plants responded to additions of thiamine but not saliva.
Reardon and Merrill, 1978	Mixed	*Sideoats grama*	Cattle and thiamine	C	L	• Environment can be a variable impacting response of plants to thiamine and saliva.
Discussions on defoliation studies (with and without saliva)						
De Visser et al., 1997	NA – no saliva used	*Lolium perenne*	NA – no saliva used	NA	L	• Discussed the movements of various carbohydrates in plants pre, and post defoliation.
Fan et al., 2010	NA	Rice seedlings	Cattle Collected	C	L	• NA – this paper focused on the analysis of proteins expressed in rice seedlings after ovine saliva treatment.
Harris, 1978	NA – no saliva mentioned	NA – a review paper on pastures in general	NA – no saliva mentioned	NA	NA – review paper	• One of the early papers that discussed the importance of defoliation on pasture

						regeneration, highlighting the key influencers as frequency, intensity, and timing of defoliation.
Jameson, 1964	NA – no saliva used, review paper	NA – variety of species were mentioned	NA – no saliva used	NA	NA	• Summarised work that has been done on various aspects of harvesting and grazing impacts on plants. • Highlighted that attention should be paid to environmental control, plant parts, and interpretation of physiology involved.
Kessler and Baldwin , 2002	NA – review paper	NA	Insects	NA	NA – review paper	• Review paper that highlighted the advancements of technology to understand elicited effects of insect herbivory, and responses at a molecular level. • Suggested saliva may be an area of interest, and difficulty to emulate saliva.
Matches, 1992	NA – a review paper	NA – review paper	NA – review paper	NA	NA – review paper	• Summarises responses found of plants to grazing from animals. • Grazing intensities may change morphology of the plant • Animals also impact pasture composition and growth (urine/treading) • Animal based defoliation early may assist in characterising plant response long term.
McNaughton, 1976	NA – did not look at saliva effects	*Themeda pennisteum*	Wildebeest	G	F	• NA – study did not focus on the effect of saliva explicitly, or growth response, but did focus on the energy and nutrient flow in the system.

Miles and Lloyd, 1967	NA – study did not look at saliva and growth rates	Sunflower seedlings	Insects	NA	L	• NA for saliva explicitly, but the study identified plant damaged caused by saliva of these insects. • Saliva of aphids converted tryptophan to B-indolyl acetic acid (IAA) and contains various amounts of this plant hormone. • Results indicated that synthesis of IAA occurs naturally during the salivation of these insects.
Parsons et al., 1983	NA – study did not look at saliva and growth rates	*Lolium perenne* (perennial ryegrass)	Sheep	G	F	• Hard grazed had highly efficient photosynthetic activity (77% net activity). • Photosynthesis was substantially less than leniently/lightly grazed sward.

Chapter 3:

Saliva and understanding its impact on *Phalaris aquatica* in rhizoboxes

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AUTHOR ATTRIBUTION STATEMENT FOR THESIS WITH INTENT TO PUBLISH

All author contributions and the role of **Danica Parnell**, in preparation of the following manuscript are listed below:

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Task	***Author contribution***
Conceptualisation	DP, LI, AM
Methodology	DP, LI, AM
Investigation/data collection	DP
Analysis and interpretation of results	DP
Draft manuscript preparation	DP
Reviewing and edits	DP, LI, AM

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the above authorship attribution statement for this chapter is correct.

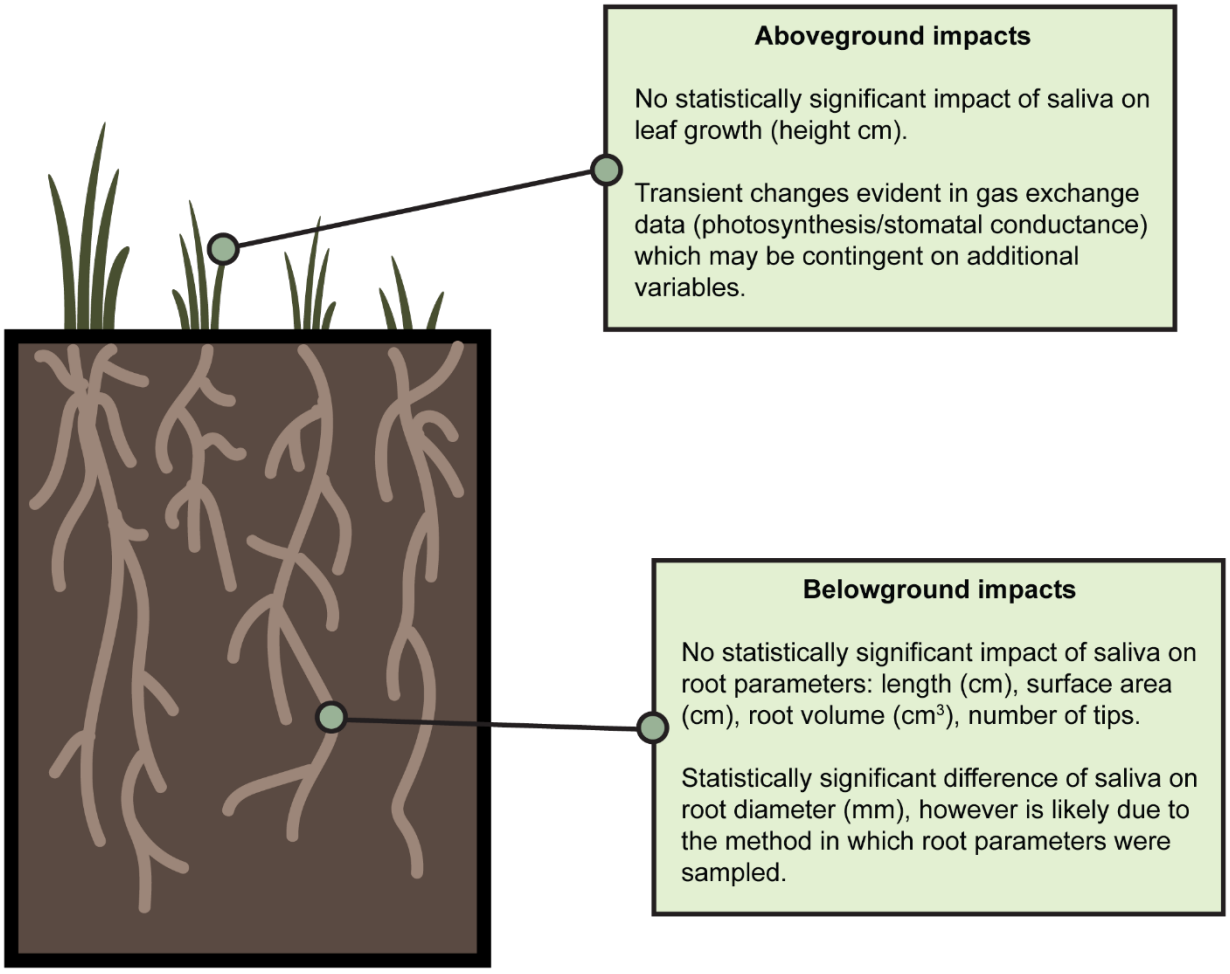
Associate Professor Andrew Merchant

15th December, 2023

Graphical Abstract:

Saliva and understanding its impact on *Phalaris aquatica* in rhizoboxes

Parnell et al., 2023



Understanding plant growth responses to the effects of herbivory is important to optimise grazing management strategies in pasture production systems. Prior studies have revealed a diverse range of mechanisms that may influence plant growth or inhibition in tissues. This study highlighted the complexity of the interactions among saliva application, defoliation, and plant responses. As such, further research is crucial to aid in unravelling the dynamics between grazing ungulates and plants in a range of environmental contexts.

Saliva and understanding its impact on *Phalaris aquatica* in rhizoboxes

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Abstract:

Understanding plant growth responses to the effects of herbivory is important for the optimisation of production systems. Whilst the effect of foliage loss alone is relatively well characterised, studies concurrently using saliva as part of the defoliation process have shown varied responses in both leaf and root growth. Prior studies have revealed that there may be a diverse range of mechanisms which ultimately influence growth promotion or inhibition in plant tissues. This study examined the repeated application of bovine saliva and defoliation events on growth responses in leaves and root attributes, of *Phalaris aquatica,* a commonly grown species across much of Australia's improved pasture grazing systems. Plants were grown for a period of 12 weeks in a glasshouse, with defoliation events beginning after five weeks of establishment. Defoliation and application of saliva, and gas exchange measurements were performed in alternate weeks during the course of the study. There was no significant ($p > 0.01$) impact of saliva and defoliation events on *P. aquatica* observed in both leaves and roots. While no conclusive results were evident for changes to photosynthetic activity and stomatal conductance, variations in contrast to the control measurements suggests a transient influence on leaf physiology, which may be contingent on additional variables (timing, environmental conditions, chemical elicitors, pH). The absence of definitive results indicating a mechanistic mode of action underscores the complexity of the interactions among saliva application, defoliation, and plant responses. Further research is imperative to comprehensively understand how saliva interacts with plants, with consideration of additional factors such as environmental conditions (water, light levels, nutrients). This deeper understanding is crucial for unravelling the intricate dynamics between grazing ungulates and plants in diverse environmental contexts.

Keywords: Rhizobox; plant-animal interactions; cattle saliva; grazing interactions

Introduction:

Growth and associated regrowth rate of pasture is assumed to be influenced by grazing processes of livestock, as they play an integral part within all grassland systems (Loeser et al., 2004). Pasture productivity is additionally dependent on quality and quantity of forage, which is influenced by the individual plant type and associated regrowth rates. From the variability evident in both the plant and animal inputs, the direct effects of grazing on plants are often unclear. Due to this, herbivory and plant productivity has been an active area of research in the last several years (Gullap et al., 2011; Teng et al., 2010). Prior studies report that in simulated defoliation events, the addition of saliva promotes a growth response in plants (Dyer, 1980; Dyer & Bokhari, 1976; Dyer et al., 1982; Howe et al., 1982; Liu et al., 2012; Reardon et al., 1972) whilst in others, there is a negative (Detling et al., 1981; Gavloski & Lamb, 2000; Johnston & Bailey, 1972; Reardon et al., 1974) or mixed response (Bergman, 2002; Gullap et al., 2011; Li et al., 2014).

Physical damage from grazing through biting, ripping, and chemical transfer of saliva are the likely causation of physiological responses in plants. Saliva is known to contain plant growth promoting chemicals, for example epidermal and transforming growth factors (Cohen, 1962; Frazier et al., 1974), and the addition of such may result in an increase in aboveground productivity (Frank et al., 2002), or changes in belowground root development (Amiard et al., 2003; Mackie-Dawson, 1999; Morvan-Bertrand et al., 1999; Thornton & Millard, 1996). Studies attempting to simulate a grazing event *ex-situ* use the process of mechanical clipping (defoliation) to remove leaf material from the plant and commonly place a considerable focus on understanding photosynthetic capacity, nutrient uptake, and root growth, post application.

The method of grazing simulations in glasshouses does not commonly include saliva (Christopher et al., 2004; Clement et al., 1978; Jarvis & Macduff, 1989), and thus ignores the known impacts saliva has on physiology, which have been highlighted by many authors (i.e., Liu et al., 2012; McNaughton, 1985; Teng et al., 2010). Growth responses may be dependent on the intensity of the simulated grazing event, the 'bite' or simulated cutting process, the amount of saliva deposited, collection location of saliva, or the plant growth conditions during the time of study. Prior studies have not quantified the amount of saliva applied after defoliation (Li et al., 2014; Liu et al., 2012) or characterised how 'saliva' solutions are applied to different areas of the plant surface or soil using various methods (Johnston & Bailey, 1972; Reardon et al., 1974). To enable a more direct comparison between *in-situ* and *ex-situ* grazing simulations, the saliva should be applied directly to an area on the plant which had been most recently cut, due to the possibility of increased effectiveness where cell growth is active (Harris, 1974). Furthermore, the method and/or type of saliva collection is likely to influence results. Some studies have collected saliva directly from the mouth (Liu et al., 2012; Reardon et al., 1972) whilst others directly collected from other locations such as the rumen (Johnson and Bailey 1972), or from salivary glands (Lamy & Mau, 2012).

To date, studies aimed at determining the influence of saliva on growth responses have adopted a range of pseudo-quantitative methods. Whilst herbivore grazing may only be a smaller aspect of the bigger picture in livestock grazing, evidence suggests mechanisms of growth promotion/inhibition in plant species can provide additional insight to understanding the variations in pasture productivity. In this study, we aimed to investigate the effects that cattle (*Bos taurus*) saliva on plant physiology. This was done by manually defoliating and treating Phalaris (*Phalaris aquatica* L.) with saliva in a greenhouse study, that emulated grazing. The study was conducted using a rhizobox system to simultaneously investigate both above and below-ground components. Various physiological and growth responses were measured both above- and belowground in response to saliva treatments. We expected to see a positive response in aboveground biomass, based on the positive responses evident in literature, and no response to saliva in below ground biomass measurements as any changes present are likely due to physiological growth unrelated to saliva presence.

Methods

Saliva collection

Saliva collection was approved by the University of Sydney Ethics Committee (approval number: 2022/2113). Cattle were placed in a chute with a head restraint for safe handling for both animal and collector. Approximately 100 mL of saliva was collected from five Holstein-Friesian cows (~20 mL per cow) by 'tickling' the roof of their mouth to induce salivation and containing samples in a falcon tube (see Chapter 4 for further details on livestock, and

samples collected). Once saliva was collected from the animal, cattle were let back into yards prior to being released back to their original paddocks. Samples were kept on ice for transportation back to the lab, before being stored in a – 80 °C freezer. When required, saliva was thawed prior to use. The pH of the saliva was 8.8 prior to the commencement of the greenhouse study.

Experimental conditions and treatment application

Twenty-five rhizoboxes measuring 21cm (W), 5cm (D), 29.7cm (H) (Vienna Scientific Instruments, GmbH, Austria) were set up in a climate-controlled glasshouse for a total duration of 12 weeks, from the 19th of September until the 5th of December 2022. The glasshouse was set to maintain a temperature between 24 and 27 ˚C during the day, and 16 and 18 ˚C at night. Seeds of *Phalaris aquatica* were planted in a peat-based medium and were left to establish for five weeks, until plants reached the four-leaf stage. During this time, plants were fertilised once a fortnight with an all-purpose liquid fertiliser (Nitrogen, 12.4% w/v; Phosphorous, 2.7% w/v, Potassium, 6.2% w/v) before the treatment began, and irrigation occurred four times daily for two minutes. When the trial began, irrigation was reduced to three times daily for two minutes. Rhizoboxes were set up in trays of five, where each tray acted as replicates (Figure 1).

Figure 1. Rhizobox experimental set up in the glasshouse (a) and planned layout (b). Boxes related to saliva application amount (none (box 1), one (box 2), two (box 3), three (box 4), four applications (box 5)), whilst trays related to replicates.

Assessments of above and below ground biomass

During the trial, individual plants were measured weekly to obtain estimates of aboveground height, and belowground root length. Each fortnight, all leaves on plants were cut ('defoliated') down by 50% of their height using Fiskars shears. During fortnightly measurements, boxes not requiring repeated saliva application were cut first, and boxes that required saliva treatment had 400 µL of saliva pipetted onto the blade of the scissors before defoliating the plants. After a box was cut, the scissors were wiped down and cleaned with acetone. Plants were defoliated three times over six weeks.

Each rhizobox (B) was randomly assigned to a tray and represented a different treatment with B1 having no saliva (NO) applied throughout the duration of the experiment, B2 only having saliva applied once (ON), B3 having saliva applied twice (TW), B4 having saliva applied three times (TH), and B5 having saliva applied four times (FO) (Figure 1). It is important to note however that by the time the fourth application was to occur, it was decided that the plants had already reached maturity, based on the root length and number of nodes established (though not recorded), and therefore were not as active in their growth. As such, the experiment ceased before the fourth defoliation and application event occurred, and treatment FO was instead counted as part of TH (i.e., 10 replicates for the TH rather than 5 replicates as per the other treatments). In this study, trays were used as replicates and the box number was being used to identify treatments to consider any potential variation relating to positioning within the glasshouse.

Assessments of photosynthesis and stomatal conductance

During the alternate week when defoliation events weren't occurring, gas exchange measurements were taken using a LI-COR 6400XT photosynthesis system (LI-COR, Lincoln, NE, United States) to determine photosynthetic activity and stomatal conductance of the plants. Chamber conditions were set to approximate the ambient growth conditions. Leaf temperature was set to 22 °C and chamber CO2 regulated to 400 μmol m^{-2} s^{-2}. Relative humidity was maintained at approximately 75%. Light conditions were set at 1000 PAR. Leaves were left to equilibrate under the chamber conditions for 10 minutes, or until an approximate steady state was achieved. Average activity levels for both photosynthetic activity and stomatal conductance are presented in this chapter as relative to the control (NO) treatment to standardise the data to account for inherent variations between samples and sampling conditions.

Data analysis

Images were taken during the measurement period of the below ground root production on a weekly basis and were analysed using WinRHIZO software (Regent Instruments Inc., Canada), to obtain root data values such as root length, surface area, volume, and root tips (Arsenault et al., 1995). Relative growth rate (RGR) per day was calculated for shoot and root values to determine the RGR after a defoliation event occurred, prior to the next defoliation event. The RGR was calculated as follows:

$$\frac{RGR = \left(\frac{(T2 - T1)}{T1}\right)}{d}$$

where T1 is the initial value after a defoliation event, T2 is the final value prior to the next defoliation event, and d is the number of days between the measurements, which in this instance was seven.

The RGR for plant height, root length, root surface area, root volume, root tips, as well as photosynthetic activity and stomatal conductance data were analysed using linear mixed models (Pinheiro et al., 2023) in R 4.0.3 (R-Core-Team, 2020). In these models, the target variable was RGR (shoot and root models), photosynthesis, and stomatal conductance. Variables as predictors included a combination of treatment, week, cut time and final weight (as a covariate), and random variables included tray, and area (for photosynthesis and stomatal conductance models only), were included in initial models and certain variables were removed from the final models throughout the model selection process when not

significant (Table A1). To determine the best model, Akaike Information Criterion (AIC) scores were utilised as the measure of the quality of the response for a given set of data in relation to other models (Akaike, 1974), with the best model having the lowest score. Additionally, *p*-values values were utilised to determine the significance of the effect of treatments (NO, ON, TW, TH), and predicted estimates were obtained through the use of the package 'emmeans' (Lenth, 2023).

Results:

Above ground changes in shoots

Plant height varied among the treatments. Notably, the NO treatment saw a relative reduction in plant height when compared to the other treatments (ON, TW, TH). Treatments with saliva applied saw a general increase in height within the first two weeks (cutting period), before steadily declining after the proceeding cuts for the remainder of the experimental period.

Using linear mixed modelling methods, the model produced for the shoot dataset produced an AIC score of -618.88 (Table A1). Utilising RGR as the target variable in models, it was evident that there was no significant effect ($p > 0.01$) of saliva treatment, or repeated saliva treatment on *P. aquatica* (Figure 2).

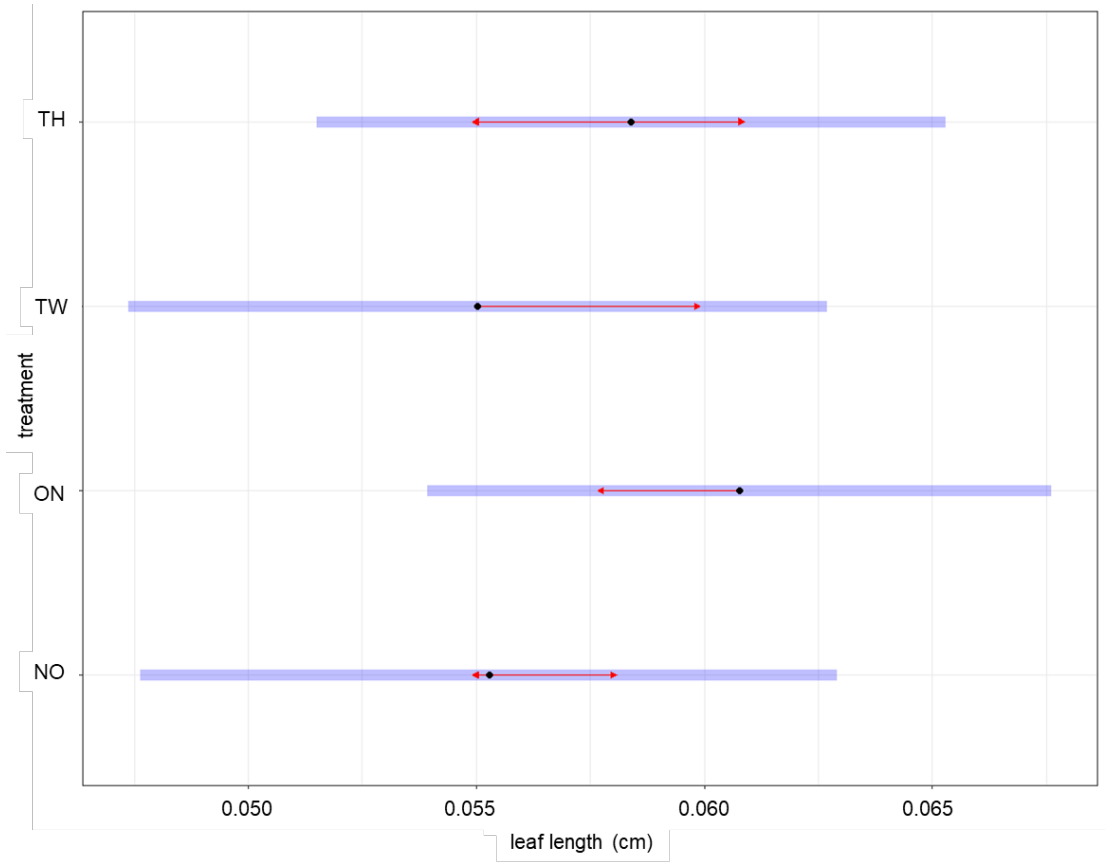

Figure 2. Predicted leaf length and marginal mean comparisons (EMMs) between treatments across the duration of the study. Treatments include one application of saliva (ON), two applications (TW), and three applications (TH). NB: blue bars indicate confidence intervals for the EMMs, red arrows are based on the confidence intervals for the pairwise differences of means. Significant differences are evident when the red arrows don't overlap and are indicated with an asterisk (*).

Below ground changes in roots

The linear mixed model for root length produced an AIC score of -504.49, root area produced a score of -418.65, root volume produced one of -342.11, and number of root tips had an AIC score of -560.87 (Table A1). The impact of saliva on these variables aforementioned were not significant, and there was additionally no significant difference between treatments NO compared to other treatments (ON, TW, TH), or between the saliva treatments (Figure 3). In contrast however, the root diameter model produced an AIC score of -796.79 (Table A1), and the effect of saliva on root diameter was significantly different ($p < 0.01$) for TW and TH applications when compared to NO applications of saliva (Figure 3c).

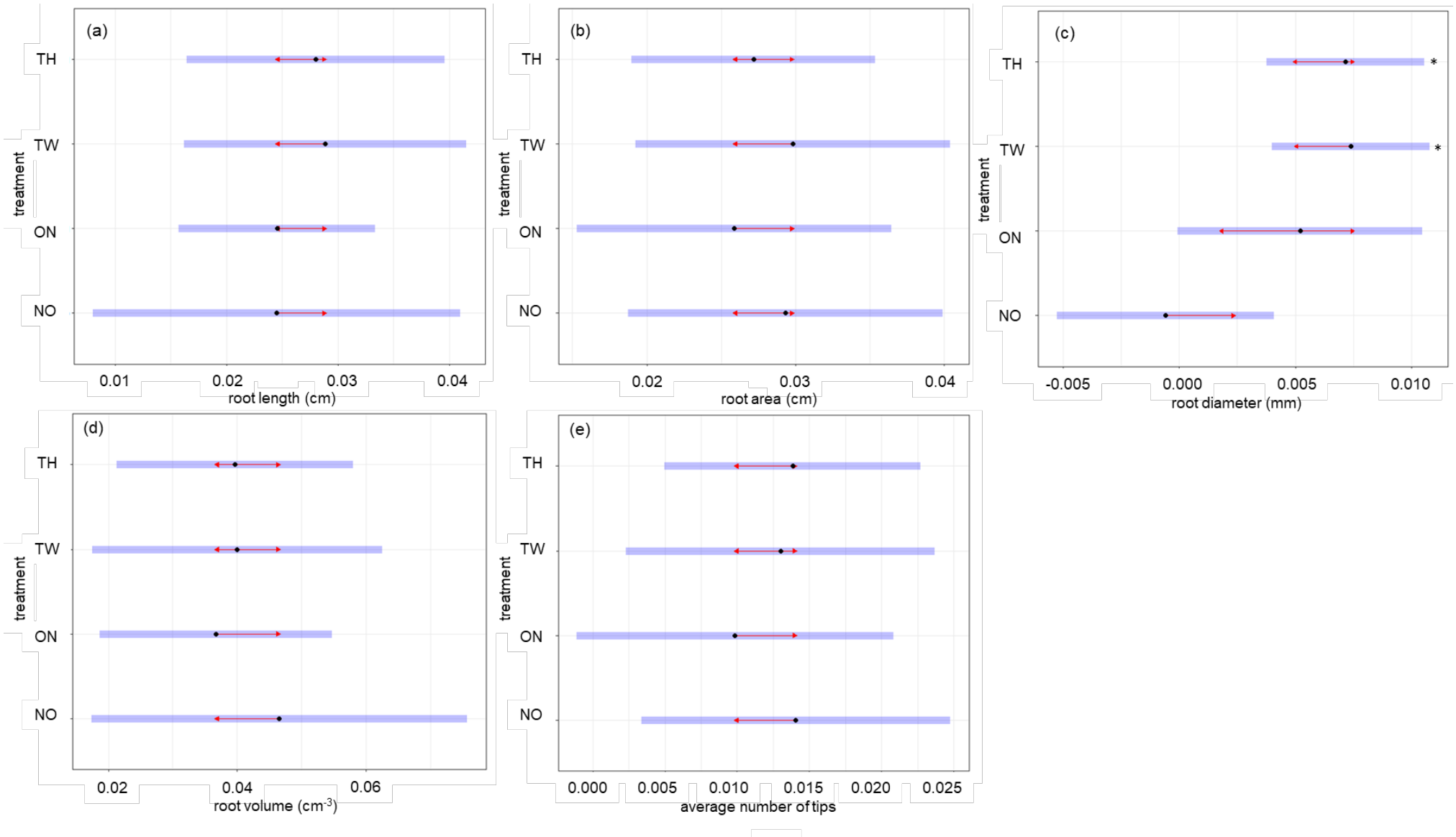

Figure 3. Predicted root parameters and marginal mean comparisons (EMMs) between treatments across the duration of the study. Panels are indicative of (a) length, (b) area, (c) diameter, (d) volume, and (e) number of tips. Treatments include one application of saliva (ON), two applications (TW), and three applications (TH). NB: blue bars indicate confidence intervals for the EMMs, red arrows are based on the confidence intervals for the pairwise differences of means. Significant differences are evident when the red arrows don't overlap and are indicated with an asterisk (*).

Gas exchange

Photosynthetic activity and stomatal conductance were measured at three time points during the study on the 2nd, 14th, and 28th of November 2022. As such the models and data created for both variables used in this study were measured on each of these dates independently. Mean activity levels are presented in Figure 4, as relative to the control (NO) treatment. Mean ranges of photosynthetic activity were 12.48 – 18.87 µmol m^{-2} s^{-2} for treatment NO, 14.97 – 17.98 µmol m^{-2} s^{-2} for ON, 19.37 – 19.39 µmol m^{-2} s^{-2} for TW and was 14.05 µmol m^{-2} s^{-2} for TH (Figure 4). Additionally, stomatal conductance ranges were 0.40 – 1.31 µmol m^{-2} s^{-2} for NO, 0.53 – 1.75 µmol m^{-2} s^{-2} for ON, 1.14 – 1.60 µmol m^{-2} s^{-2} for TW and was 0.70 for TH µmol m^{-2} s^{-2} (Figure 5).

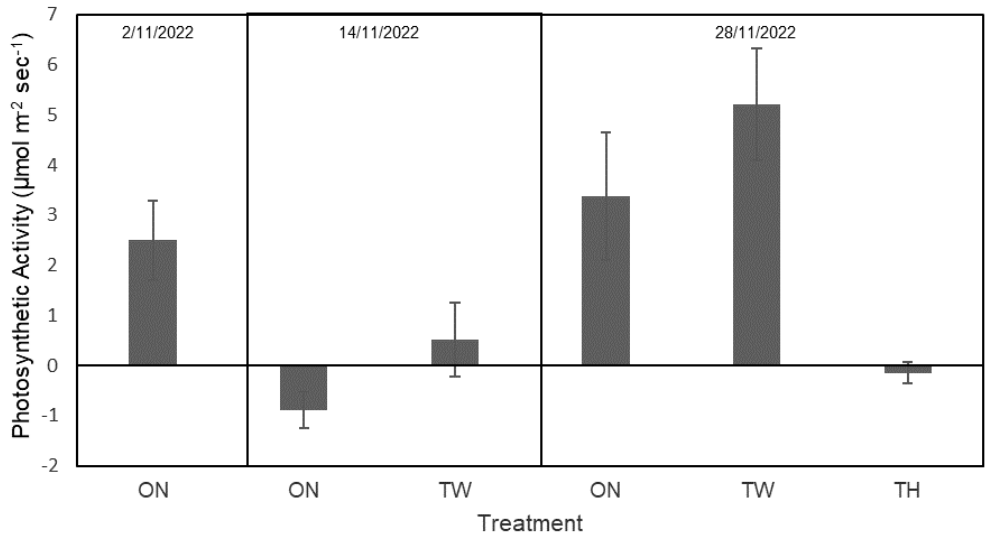

Figure 4. Mean (n = 3 readings per box, 5 replicates per treatment) with standard error for photosynthetic activity, relative to the control (no saliva; NO) for each treatment on the three different sampling dates (2nd, 14th, and 28th November 2022). Treatments include one application of saliva (ON), two applications (TW), and three applications (TH). An asterisk (*) indicates a significant difference from treatment NO.

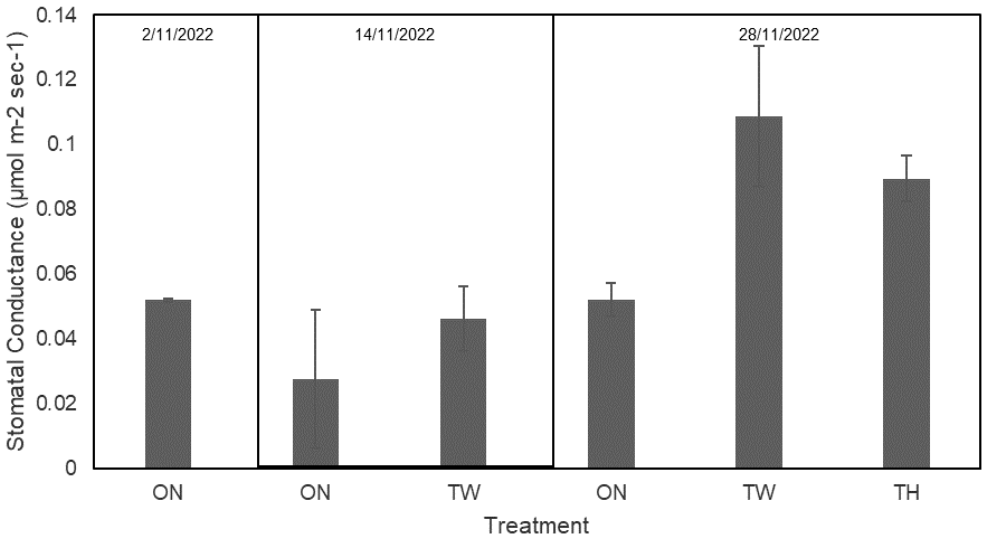

Figure 5. Mean (n = 3 readings per box, 5 replicates per treatment) with standard error for stomatal conductance, relative to the control (no saliva; NO) for each treatment on the three different sampling dates (2nd, 14th, and 28th November 2022). Treatments include one application of saliva (ON), two applications (TW), and three applications (TH). An asterisk (*) indicates a significant difference from treatment NO.

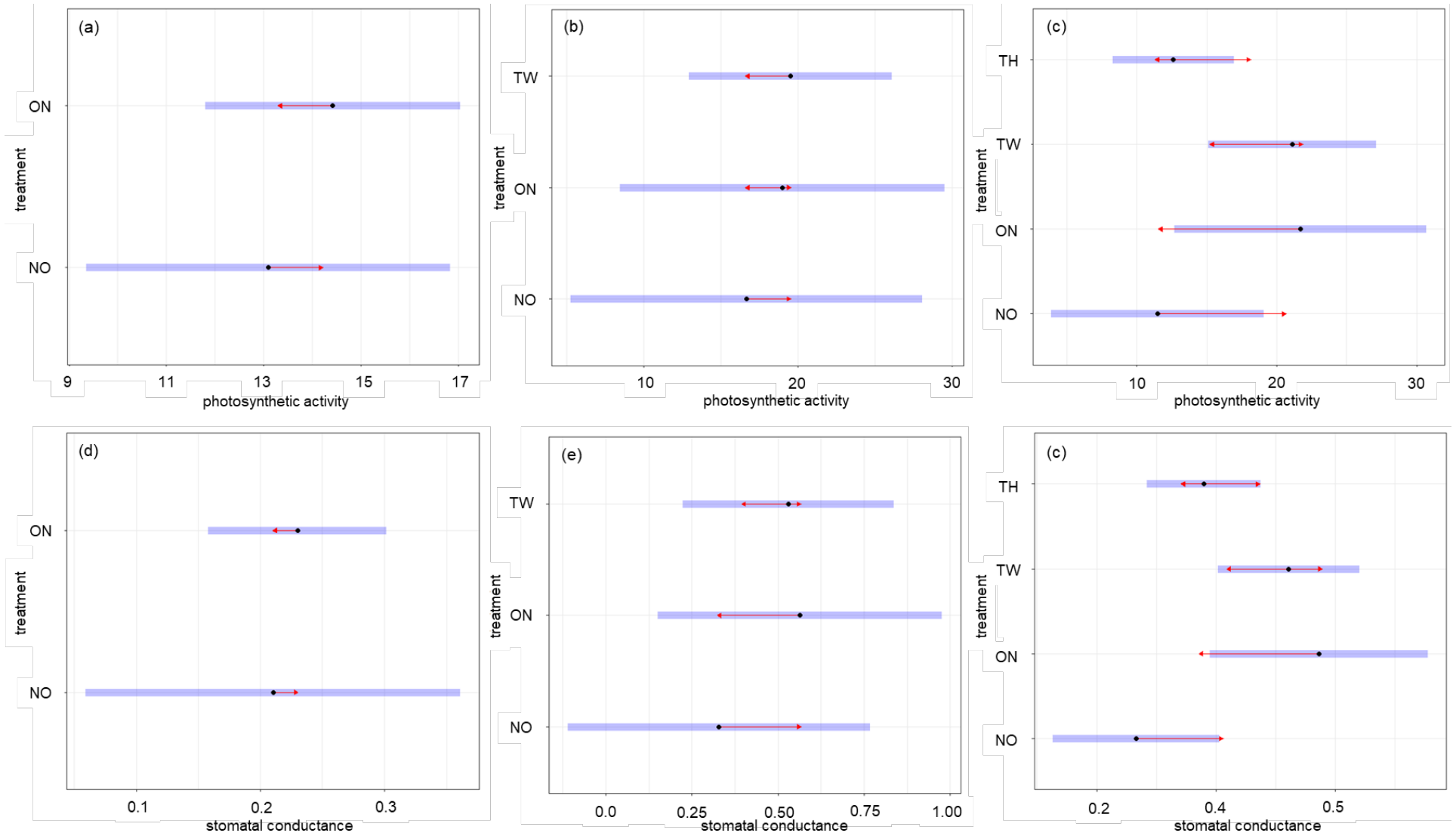

Figure 6. Estimated marginal means (EMMs) comparisons between treatments for photosynthetic activity (a – c), and stomatal conductance (d – f) for sample dates on the 2nd November (a, d), 14th November (b, e), and 28th November (c, f) 2022. Treatments include one application of saliva (ON), two applications (TW), and three applications (TH). NB: blue bars indicate confidence intervals for the EMMs, red arrows are based on the confidence intervals for the pairwise differences of means. Significant differences are evident when the red arrows don't overlap and are indicated with an asterisk (*).

Models produced for photosynthetic activity had an AIC score of 168.53 on the first measurement period (2nd Nov), 125.37 on the second measurement period (14th Nov), and 111.95 on the third measurement period (28th Nov) (Table A1). Those produced for stomatal conductance had AIC scores of -285.08 for the first measurement date, -251.78 for the second measurement period, and finally, produced an AIC of -222.50 on the third and final measurement period (Table A1). Furthermore, the effect of saliva on both photosynthetic activity and stomatal conductance was not significant ($p > 0.01$), nor was there a significant difference between treatments ($p > 0.01$), indicating that there was no added effect with saliva reapplication (Figure 6).

Discussion:

Within the scope of this study, including both the parameters measured and the intensity and duration of our treatments, no clear growth promoting effects were detected in either above or below-ground components of the plant. However, robust changes in short term physiology through gas exchange measurements were detected, indicating an effect of saliva on plant physiological function on a background of environmental variation and ontogenic development. This study represents a quantitative investigation of grazing effects on plant growth and offers useful indications to explain potential growth promoting effects of saliva seen in prior studies. Defoliation changes root to shoot ratios, elicits a wound response, and impacts the allocation of resources such as water, nutrients, and photo assimilates (Rosenthal & Kotanen, 1994). Some hypothesize that saliva may stimulate compensatory growth, and changes in growth rates are evident (McNaughton, 1979, 1985). In this study, we observed variations in root, shoot, and gas exchange responses to defoliation, often with increased response (average growth/activity), when we applied saliva to leaves. However, these differences were not apparent when we standardized using relative growth rates.

Potential saliva-derived elicitors of changes in photosynthetic activity and growth.

Consistent with current literature, influences on plant growth responses from saliva-based defoliation (i.e., (Liu et al., 2012; McNaughton, 1985; Teng et al., 2010) could be related to growth promoting compounds such as thiamine that are likely to be present in animal saliva (Bonner & Greene, 1939; Reardon et al., 1974). The presence of other metabolites in saliva, such as linolenic acid, are known to stimulate defensive secondary metabolites in plants, due to the induction of mechanical damage to the plant, or the presence of a chemical elicitor in saliva (studied in insects) (Taiz et al., 2014), and could additionally be an influencing factor here. For example, it is not entirely clear whether the defensive metabolites are activated due to the mechanical damage caused, or the addition of saliva. However, in plants, 20% of known secondary metabolites are alkaloids, with majority of their function relating to protection, and regulation of plant growth (Nowacki & Waller, 1973; Seneca, 2007a, 2007b; von Linné, 2007). As the saliva utilised in this study had a pH of 8.8, the potential alkalinity evident could be a potential catalyst for the growth responses observed. The addition of alkaline saliva could enact as an inducing agent for enzyme pectin methyl asterase (PME), to begin catalysing pectin in plant cell walls, ultimately altering cell wall integrity allowing cellular expansion. Studies have discussed plant PME activity occurring when there are modifications of the local pH in cell walls (Di Matteo et al., 2005; Giovane et al., 2004). The variability in effect of pH and PME has been further highlighted by Giovane et al., (2004), whereby activity can cause stiffening of cell walls, or may contribute to cell wall loosening. Contrastingly, acidic conditions (existent in the apoplastic environment) generate higher levels of inhibitors of PME and activate other enzymes which may lead to cell wall loosening, facilitating growth, expansion and separation of cells (Bosch & Hepler, 2005;

Wen et al., 1999). However, studies have identified that with higher pH levels, a reactivation of PME occurs, creating wall stiffening and decreases in growth response (Bosch & Hepler, 2005). For example, an increased extracellular pH correlated with reduced root elongation and alteration of cellular morphology in pea plants (Wen et al., 1999). Studies which have discussed manipulation of PME positively influencing physiological processes, such as stem elongation and root development (Giovanetti et al., 2017; Micheli, 2001; Tieman & Handa, 1994; Wen et al., 1999), have predominantly been completed in dicotyledonous plants. Whilst *P. aquatica* is monocotyledonous, the potential influence and interaction of pH and PME on cell wall expansion/growth should be similar, further investigation of this potential mode of action is required.

Regardless of the mechanism that causes growth promotion or inhibition in plants, the presence, and concentrations of metabolites within saliva (i.e., thiamine, linolenic acid) have not previously been quantified in literature until now (see Chapter 4). The quantification of the presence of these specific metabolites would assist in determining if the change in chemical environment on the plant during defoliation events with deposition of saliva is the primary driver of growth response, or if it remains with the mechanical damage applied.

The effect of grazing simulation on above-ground growth.

Despite the positive growth trends evident in the data primarily within the first week of application (Figure 3), there was no statistically significant effect of saliva application on *P. aquatica* relative growth rates suggesting no, or very limited net effect of saliva on shoot growth in the short term (< 3 months). There are several possibilities as to why this could have occurred. The study began when plants reached the four-leaf stage to closely emulate when a grazier is likely to place livestock on pasture, due to plants reaching stem elongation which is beneficial for grazing (Frank, 1996; Watson et al., 2001). As the plants were well developed prior to the commencement of the study, it is likely that the overall decrease in response to fortnightly defoliation could have correlated with the age of the plant. Specifically, the plants may have reached maturation and not have been as actively growing within the latter weeks during the study period. During maturation of plants, cessation of cell growth occurs, causing rigidification within the cell wall and a decrease in growth response (Taiz et al., 2014). Furthermore, younger leaves are known to be more responsive to herbivory, having greater ‘defences’ (secondary metabolite presence, creating increased regrowth) response to defoliation of leaves than mature leaves (Taiz et al., 2014).

Water availability, in addition to factors aforementioned (saliva presence, plant age), can additionally impact the extent of plant growth. Changes in turgor pressure impact the way in which cells enlarge or shrink (Johnson, 1978; Taiz et al., 2014). A defoliation event will reduce the demand for water, due to the reduction of surface area, resulting in plants undergoing expansive growth, re-establishing turgor pressure and rigidity within the remaining leaves (Taiz et al., 2014). Regrowth and regulation of turgor pressure is additionally dependent on several other factors such as species of plant, environmental conditions, and height of defoliation. Studies have shown that there are significant interactions between water availability and intensity of defoliation on relative growth rate of grasses (Gao et al., 2008).

Furthermore, the varied results in relative growth rate may have been influenced by the ontogenic stage that each of the plants were in the rhizoboxes or their location within the growth medium. Seeds were planted in the rhizoboxes to ensure that roots were established

within the box, and to enable growth of the roots along the Perspex for optimal measurements. Nevertheless, plants formed an increasing proportion of roots along the surface of the rhizobox toward the end of the experiment (due to gravitropism). The extent to which this proportion changed was not accounted for within our experimental procedure and may have influenced the rate at which water was taken up from the growth medium.

The effect of grazing on below-ground growth

Previous studies have identified that defoliation events can impact root development, likely due a shift in demand for sucrose from the root sink (Amiard et al., 2003; Mackie-Dawson, 1999; Morvan-Bertrand et al., 1999; Thornton & Millard, 1996). In this study, there was no impact of saliva on the measured root parameters, aside from root diameter. While there was a significant effect of TW and TH applications of saliva compared to NO saliva application on root diameter (Figure 7c), a single application was not significantly different from NO saliva application. However, results here indicated that root diameter increased with repeated saliva application, and may in fact not have a biological explanation. Typically, monocot species such as *P. aquatica* do not have the capacity for secondary growth as it lacks a vascular cambium, where increases in root diameter are biologically explainable (Tomlinson & Zimmermann, 1969). Further, a study performed on mature field grown maize (monocot species), suggest that diameter decreased along most roots, and during elongation, longer roots either maintained or sometimes increased in diameter (Wu et al., 2016).

The observed root diameter results may have instead been impacted by the methodology used for data collection and sampling in WinRHIZO software, rather than underlying physiological factors. Prior studies have highlighted the importance of proper sample preparation and scanning techniques for measuring root length and diameter using WinRHIZO (Bouma et al., 2000). However, it's important to note that these studies typically employ destructive sampling methods, which is not feasible in this study due to the continuous sampling approach. Though the uniqueness of this study (continuous, non-destructive sampling) allowed for more in depth of analysis on root attributes during the plant's active growth period, the mechanism in which defoliation and chemical alteration of the wound site alters root parameters cannot effectively be determined here.

Weekly photographing of the roots within the rhizoboxes may have impacted the accuracy of data collected from WinRHIZO software. Though images were taken as consistently as possible, due to the set up in the glasshouse, there was inherent variability each week with external weather conditions, leading to changes in lighting within the glasshouse. During cloudy days internal UV lights would switch on, and during sampling the colour of the light would reflect a purple hue on the Perspex, leading to a slight change in colour of images. Similarly, during some sampling periods it was quite bright, and Perspex was reflective in the sunlight leading to issues in images. For example, when there were pockets of moisture and air in the rhizoboxes close to the Perspex surface, light reflection created a glaring spot in some pictures. Additionally, plants were grown in was a neutral substrate (cocopeat) to ensure that there was no subsequent influence of fertilisers present in typical potting mixes. Due to the use of cocopeat, the fibres of coconut husks being so stringy inevitably got picked up in certain images in WinRHIZO software as a root. The latter two issues with images and analysis were counteracted for by selecting areas where the predominant root growth was, avoiding picking up the stringy husks for most part, however, at times the moisture pocket could not always be avoided in the analysis.

The effect of saliva on leaf physiology and gas exchange

A range of variables interact in complex manners and influence both photosynthesis and stomatal conductance, including light intensity and temperature as examples (Taiz et al., 2014). Plant photosynthetic capacity and its relationship with defoliation has been investigated previously (Clement et al., 1978), with defoliation being reported to influence photosynthetic capacity, mobilize carbon and nitrogen, and improve source-sink relations (Iqbal et al., 2012). In this study, treatments ON and TH had a negative photosynthetic rate in contrast to NO (Figure 4) after the second, and third defoliation event respectively. During these periods, compensatory photosynthetic activity may not have occurred. Results presented here are similar to that of Detling & Painter (1983), whereby net photosynthetic activity was not significant after defoliation events. Though grasses are known to have increased photosynthetic rates after defoliation, they may be constrained by age, and remaining leaf areas (Rosenthal & Kotanen, 1994), which could explain the non-significant effect seen here. Additionally, other variables such as reallocation of resources to ungrazed leaves during those periods may have influenced potential compensatory photosynthetic rates for treatments ON and TH (Hamerlynck et al., 2016). Previous publications have highlighted that higher leaf blade/sheath ratios and larger numbers of tillers have allowed plant populations to withstand defoliation events (Detling & Painter, 1983; Hamerlynck et al., 2016), likely due to the ability of a plant to reallocate sources to younger leaves and therefore, initiate compensatory photosynthesis. However, due to study limitations, the number of tillers were not recorded within this study prior to or after defoliation events and as such, the effect of saliva on tillering of *P. aquatica* remains unknown.

Increases in net photosynthesis is often concurrent with higher stomatal conductance (Detling & Painter, 1983; Hamerlynck et al., 2016). Within the study, while stomatal conductance was greater for all treatments compared to NO (Figure 5) and similar to prior studies which have found that defoliated grasses had higher stomatal conductance rates (Anderson et al., 2013), there were no significant differences due to saliva application (Figure 4, 5). It is more likely here that stomata opened and changes in photosynthetic activity was a response to the defoliation event itself rather than as the result of saliva application. A possible explanation for this effect lies within the effect of turgor pressure within stomatal guard cells (Yi et al., 2022). An increase in turgor pressure post defoliation event as the plant drastically increases the demand for water, ultimately causing an expansion of the guard cell wall, and so too, widening/opening of the stomatal pore (Taiz et al., 2014; Yi et al., 2022). Another potential explanation to stomatal conductance being higher in saliva-based treatments (ON, TW, TH) compared to NO, could be due to the alkalinity of the saliva, which catalyses cell wall opening, as discussed previously.

Conclusion:

This study investigated the impact of repeated application of saliva and defoliation events on relative growth rates of leaf and root attributes and found no significant effect of the application of saliva and repeated defoliation of *Phalaris aquatica*. Though no conclusive results were identified in regard to photosynthetic activity and stomatal conductance, the evidence suggests that there is a transient impact on leaf physiology and may ultimately be contingent on a range of variables such as defoliation and measurement timing, environmental lighting conditions, chemical elicitors, and pH of saliva. Limitations within the study may have restricted the results obtained, as such authors suggest potential changes to current experimental design for future research. Additionally, research should investigate

the effects of livestock bite patterns and saliva deposition rates, and fresh and frozen/rethawed effects on saliva to further grasp the magnitude in which saliva is the true elicitor of growth.

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Appendix

Model results

Table A1. Final model metrics for each of the datasets, detailing variables included within the model, and associated metrics such as Akaike information criterion (AIC) and Bayesian information criterion (BIC) scores, Log-Likelihood (logLik) values, and the minimum (Min), median, and maximum (Max) residuals.

Dataset	Model	AIC	BIC	logLik	Min	Median	Max
Leaf length (cm)	Relative growth rate length ~ treatment + week*cut time + final weight, random = ~1|tray	-618.68	-521.13	342.34	-2.07	-0.06	2.47
Root length (cm)	Relative growth rate length ~ treatment + week + cut time, random = ~1|tray	-504.49	-412.64	283.25	-2.82	-0.01	2.20
Root area (cm^2)	Relative growth rate area ~ treatment*cut time + week, random = ~1|tray	-418.65	-371.59	225.33	-3.25	-0.07	3.17
Root diameter (mm)	Relative growth rate diameter ~ treatment + week*cut time, random = ~1|tray	-796.79	-702.20	430.40	-3.08	-0.07	2.42
Root volume (cm^3)	Relative growth rate volume ~ treatment*cut time + week + final weight, random = ~1|tray	-324.11	-221.40	197.06	-2.41	-0.06	2.52
Number of root tips	Relative growth rate of tips ~ treatment + week*cut time, random = ~1|tray	-560.87	-466.28	312.44	-2.98	-0.07	2.60
Photosynthetic activity – first measurement	Photosynthesis ~ treatment + final weight, random = ~1|tray/area	168.53	201.55	-65.27	-1.34	0.18	1.21
Photosynthetic activity – second measurement	Photosynthesis ~ treatment + final weight, random = 1|tray/area	125.37	161.36	-41.68	-1.16	0.20	1.14
Photosynthetic activity – third measurement	Photosynthesis ~ treatment + final weight, random = 1|tray/area	111.95	142.48	-35.98	-1.18	0.12	1.14
Stomatal conductance – first measurement	Stomatal conductivity ~ treatment + final weight, random = ~1|tray/area	-285.08	-252.06	161.54	-1.24	0.07	1.33
Stomatal conductance – second measurement	Stomatal conductance ~ treatment + final weight, random = ~1|tray/area	-251.78	-236.36	134.89	-1.67	0.21	1.66
Stomatal conductance – third measurement	Stomatal conductance ~ treatment + final weight, random = ~1|tray/area	-248.28	-231.49	135.14	-1.90	0.07	1.45

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Chapter 4:

Moving the knowledge forward on *Bos taurus* saliva using a metabolomic approach

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AUTHOR ATTRIBUTION STATEMENT FOR THESIS WITH INTENT TO PUBLISH

All author contributions and the role of **Danica Parnell**, in preparation of the following manuscript are listed below:

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Task	***Author contribution***
Conceptualisation	DP, AM
Methodology	DP, AM, ND
Investigation/data collection	DP, ND
Analysis and interpretation of results	DP, AM, MP
Draft manuscript preparation	DP
Reviewing and edits	DP, AM, LI

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the above authorship attribution statement for this chapter is correct.

Associate Professor Andrew Merchant

15th December, 2023

Graphical Abstract:

Moving forward the knoweldge on *Bos taurus* saliva using a metabolomic approach

Parnell et al., 2023

Livestock saliva holds paramount importance not only in the process of grazing, but additionally through health diagnostics. Literature suggests that there is a **bioactive function of saliva on plant growth responses**. This study employed a non-targeted, metabolomic approach to characterise the polar metabolite constituents of cattle saliva, considering different breeds, and utilising two collection methods. Results **identifed 132 metabolites in saliva samples**, and indicated a **large presence** of **nitrogen containing compounds** within the top 20 constituents.

Sampling methods:

Tickling roof of mouth
Sedation

Breeds sampled:

Holstein-Friesian
Angus
Brahman
Charolais

Top 20 metabolites and their functional classes

Amino acids
Alanine
Glycine
Leucine
Valine

Alcohol and aldehydes
Glycerol

Carbohydrates
myo-Inositol
Sucrose

Inorganic acids
Hydroxylamine

Ketone bodies
3-hydroxybutyric acid

Lipids and sterols
Palmitic acid
Stearic acid

Organic compounds
Urea

Organic acids
Benzoic acid
Caproic acid
Glycolic acid
Hydrocinnamic acid
Lactic acid
Pyroglutamic acid
Pelargonic acid

Vitamins
Thiamine

Moving the knowledge forward on *Bos taurus* saliva using a metabolomic approach

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Abstract:

While animal derived biofluid studies have commonly focused on urine, blood, serum, interstitial fluid, faeces, milk, and ruminal fluids in livestock, saliva is one that is often unexplored, despite its potential diagnostic significance. Additionally, saliva holds paramount importance in the process of grazing, which is further reiterated through existing literature that suggests there is a bioactive function of livestock saliva on plant growth responses. This study employs a non-targeted metabolomic approach to characterise the polar metabolite constituents of cattle saliva, considering different breeds, environmental locations, diets, and collection methods. Results identified 132 metabolites present in cattle saliva, with urea being the most prevalent. Additionally, high presence of nitrogen containing compounds was identified, and as such can be suggested to be the main contributor to variations in plant growth responses in existing literature. Correlation analysis performed indicates that subtle differences occur within animals, and non-negative matrix factorisation performed highlights that the amount of metabolites present can be associated with specific saliva profiles. Ultimately, the analysis performed aids in not only understanding the diverse range components present in cattle saliva, but additionally may allow for insights for both animal health diagnostics, and for identifying biologically significant constituents that can influence plant regrowth rates.

Keywords: Livestock; cattle; saliva; metabolomics; sialochemistry; ruminants

Introduction:

Animal derived biofluids have potential to inform advanced diagnosis of animal diseases and nutritional imbalances. Common biofluids derived from animals include urine, blood, interstitial fluid, serum, faeces, and specifically from livestock, milk and ruminal fluid (Kim, 2021; Zhu et al., 2021). However, despite the regular use of biofluids for diagnosis of disease and illness, saliva is rarely used. Saliva has been included in proteomic and metabolomic based studies, albeit predominantly in species such as grasshoppers, rabbits, rats, dogs, and humans (Chauncey, 1955; Chauncey et al., 1963; Cohen, 1962; Dyer & Bokhari, 1976; Hines & McCance, 1953). A review of metabolomic based studies in livestock between 1930 and 2015 did not include saliva as a biofluid search parameter (Goldansaz et al., 2017). Only a handful of studies have attempted to elucidate the constituents of saliva in livestock, often when there were limiting analytical capabilities (Reid & Huffman, 1949).

To date, chemical constituents of saliva in sheep (*Ovis aries*) have been characterised, with more recent research taking a proteomic and metabolomic based approach (Lamy et al., 2009;

Palma-Hidalgo et al., 2023). Research for cattle (*Bos taurus*), has largely focused on identifying plant protein identity and function, post saliva deposition (Fan et al., 2011), rather than comprehensively characterising the diversity of compounds found in this important biofluid. In addition to the generic constituents of saliva in cattle, intra-species variation of saliva characteristics remains unexplored, despite the known significance of influential factors such as stimuli, individual animal, diet, and activity (Kay, 1960).

Saliva, with a pH of generally between 8 – 8.5 for livestock, plays a pivotal role in contributing water and salts to the rumen of livestock (Bailey, 1961). Saliva from the two major glands, the parotid and mandibular glands, produce serous (water based, rich in proteins) and meocrine based (rich in mucopolysaccharides and glycoproteins) solutions, respectively (Nonaka & Wong, 2022). The differing solutions produced by each gland alone further highlights the need for a deepened level of knowledge on the constituents present. The importance of this research is further highlighted as previous investigations indicate the bioactive function of cattle saliva on plant growth. There is however little information about the specific metabolites present or their properties and the potential role they may play on stimulating or inhibiting plant growth (see Chapter 2, Chapter 3 in this thesis). One metabolite found in saliva that is hypothesised to stimulate growth post-grazing is thiamine, an essential metabolite in plants that acts as a critical growth factor in the shoot, aiding in cell functioning (Addicott, 1941; Bonner, 1937; Clark, 1942). Currently, the application of thiamine containing solutions has not conclusively shown that stimulation of plant growth occurs, as only negative or neutral effects have been observed (Li et al., 2014; McNaughton, 1985; Reardon & Merrill, 1978). Such observations are supported by additional studies using thiamine alone which demonstrate the inability to reliably stimulate plant growth compared to the use of real saliva (Cohen, 1962; Huang et al., 2014; Kessler & Baldwin, 2002; Lamy et al., 2010; Li et al., 2014). This suggests other metabolites may be responsible for stimulating plant growth and demonstrates the need for an ‘omic approach to the characterisation of metabolites found in and among saliva obtained from a range of cattle to potentially identify chemical elicitors of plant growth and ascertain the usefulness of saliva as a potential health diagnostic biofluid.

Here we complete a non-targeted, metabolomic approach to characterising the polar (soluble) metabolite constituents of cattle saliva. We contrast samples collected across a number of breeds, collected across different sites/diets. We also compare saliva samples collected from sedated cattle to those samples collected using a “tickling” technique on fully conscious/non-sedated cattle.

Methods:

Saliva collection

Holstein-Friesian cows from May Farm grazing on mixed pastures of native species and kikuyu, and female Charolais, Brahman, and Angus breeds from John Pye Farm grazing on mixed pastures of rye and clover, were used for sampling within this study. Five animals of each breed, and collection type (‘tickled’ and sedation) were sampled.

Saliva was collected from cattle using a method that was approved by the University of Sydney Ethics Committee (approval number: 2022/2113). Cattle were placed in a chute with a head restraint for safe handling for both animal and collector. Approximately 100 mL of saliva was collected from cows (~ 20 mL per cow), by ‘tickling’ the top of their mouths to encourage salivation/’drooling’ and collecting samples in a 50ml Falcon tube. Once saliva

was collected from the animal, cattle were let back into yards prior to being released back to their original paddocks. Saliva was additionally collected from cattle which were sedated, approved by the University of Sydney Ethics committee (approval number: 2021/1881). Cattle were sedated with 100mL of Lignocaine 20 (20mg/mL lignocaine hydrochloride) behind their eye. A single bucket was placed underneath each animal for 10 minutes whilst 'drooling', to collect samples in Falcon tubes for storage. Once collection was completed, cattle were let back into yards for observation, prior to being released to original paddocks.

In both instances, saliva volumes were estimated in Falcon tubes and were kept on ice for transportation back to the lab. The pH of each sample was measured using a Laqua twin pH-mV meter (Horiba Scientific Pte. LTD.), before being stored in a – 80 °C freezer. One sample of each breed and collection method were randomly selected to be sent off for exploratory analysis and are outlined in Table 1 below.

Table 1. Details of samples collected including ID, location collection, breed, collection method, collection time (morning/afternoon), and pH value for samples sent for analysis.

Sample ID	Location	Breed	Diet/Pasture	Age (approx.)	Method	Collected amount (mL)	pH
MFC2	May Farm	Holstein –Friesian	Native spp./Kikuyu mix	5	Tickle (am)	50 mL	8.8
C1	May Farm	Holstein –Friesian	Native spp./Kikuyu mix	5	Sedation (am)	125 mL	9.2
RF	May Farm	Holstein –Friesian	Native spp./Kikuyu mix	5	Tickle (pm)	3.5 mL	8.7
S70	John Pye	Charolais	Rye/clover mix	3	Tickle (am)	36 mL	8.5
S26	John Pye	Brahman	Rye/clover mix	1	Tickle (am)	4.5 mL	8.1
S3	John Pye	Angus	Rye/clover mix	3	Tickle (am)	11.5 mL	8.3

Analysis of saliva

Saliva samples were thawed prior to being sent off for analysis, and were centrifuged to remove unwanted particulate (i.e., hay, hair, dirt) from the solution. Samples remained cryogenically frozen (– 80 °C) during subsequent transportation among different laboratories for analysis.

Sample Extraction

A 25 µL aliquot of cow saliva was transferred into a 1.5 mL microtube. One hundred and seventy-five µL of 6:1 methanol/water (containing 1.3 µM of $^{13}C_5{}^{15}N$ valine and $^{13}C_6$ Sorbitol) was added to each sample. The samples were vortexed and then agitated at 950 rpm and 4°C for 10 min. Seventy-five µL of the supernatant of each sample was pooled to make five pooled biological quality controls (PBQCs). Seventy-five µL of each sample (study samples & PBQCs) were evaporated to dryness using a Christ RVC 2-33 Speed vacuum concentrator in preparation for GC-MS analysis.

Sample derivatisation for gas chromatography analysis

Dried samples for targeted analysis were derivatised online using the Shimadzu AOC6000 autosampler (Shimadzu, Japan). Derivatisation was achieved by the addition of 25 µL methoxyamine hydrochloride (30 'mg/mL in pyridine, Merck) followed by shaking at 37°C for 2h. Samples were then derivatised with 25 µL of *N*,*O-bis* (trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (BSTFA with 1% TMCS, Thermo Scientific)

for 1h at 37°C. The sample was allowed to equilibrate at room temperature for 1 h before 1 µL was injected onto the GC column using a hot needle technique. Split (1:10) injections were performed for each sample.

Mass spectrometry

Samples were analysed using a 2030 Shimadzu gas chromatograph linked to a TQ8050NX triple quadrupole mass spectrometer (Shimadzu, Japan), equipped with an AOC6000 autosampler. Electron ionisation was set at 70eV for fragmentation. The mass spectrometer was tuned according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43). GC-MS was performed on a 30m Agilent DB-5 column with 0.25mm internal diameter column and 1µm film thickness. Injection temperature (inlet) was set at 280°C, MS transfer line at 280°C and the ion source adjusted to 200°C. Helium was used as the carrier gas at a flow rate of 1 mL/min and argon gas was used in the collision cell to generate the MRM product ion. The analysis of TMS samples was performed under the following oven temperature program; 100°C start temperature, hold for 4 minutes, followed by a 10°C min^{-1} oven temperature ramp to 320°C with a following final hold for 11 minutes. Approximately 520 targets were collected using the Shimadzu Smart Metabolite Database, where each target comprised a quantifier MRM along with a qualifier MRM, which covers approximately 350 endogenous metabolites and multiple stable isotopically labelled internal standards. Resultant data was processed using Shimadzu LabSolutions Insight software, where peak integrations were visually validated and manually corrected where required.

Metabolomic data and statistical analysis

The data obtained was analysed using MetaboAnalyst 5.0 (Xia et al., 2009) for analysis and was transformed using a log10 transformation (Figure A1). A heat map cluster analysis using the default parameters (normalised data, auto scaled features, Euclidean distance measure, clustering method ward, t-test and Anova methods) was performed. The transformed data was then further analysed using a non-negative matrix factorisation (NMF) approach to identify patterns evident between composition and the breed from which the saliva was collected, through feature extraction (Lee & Seung, 1999). It is a technique used where in a given matrix (V), NMF aims to identify two matrices, a basis matrix (W) and coefficient matrix (H), such that their product approximates to the original matrix, expressed as:

$$VV \approx WW \times HH$$

V is the original matrix to be factorised, where the elements present represent the relationships or values between two sets of entities. W contains the basis vectors, where the elements present are non-negative, and columns represent a pattern or feature present within the data. Finally, H contains the coefficients which express how much each basis vector (column from W) contributes to each column in V. Factorisation occurs when minimising the difference between the V, and the product matrix WH through optimisation (Lee & Seung, 1999). Both W and H are updated iteratively until a satisfactory approximation is achieved. The analysis was performed using the 'NMF' package (ver. 0.26) (Gaujoux & Seoighe, 2010), within R (R-Core-Team, 2020).

Results:

General observations of fluids

During sampling, visual observations of the saliva collected were made to distinguish any characteristic differences among each of the collected sample types. When the sedation method was utilised, saliva samples were more 'aqueous' in consistency, contrasting greatly to those collected through tickling methods, samples of which were more viscous. Samples collected via the tickling method in the morning additionally were clearer in colour than samples collected in the afternoon, which had a 'greenish' hue, similar to that of ruminal fluid. There were no observational differences between the saliva from each breeds. Interestingly however, the quantity of saliva collected varied between each of the breeds (Table 1). On average, 38.5 mL (± 46.3 mL) were collected from all cattle, with the greatest collection being from the Holstein-Friesians which were sedated (125 mL), and the smallest collection occurring from the same breed, however, were collected using the tickling method in the afternoon (3.5 mL). Upon comparing samples collected using the tickle method in the morning, the Holstein-Friesians still produced the most saliva (50 mL), followed by the Charolais (36 mL), Angus (11.5 mL), then Brahman (4.5 mL). Prior to metabolic analysis, the samples were analysed for their pH content, with the mean pH being 8.6 (± 0.4), sedated Holstein-Friesians having the highest pH (9.2), and the Brahman breed having the lowest pH (8.1), as seen in Table 1 along with sample characteristics.

Top identified metabolites based on peak intensity

The metabolomic analysis of saliva samples resulted in the identification of 132 different metabolites (Table A1). Samples were additionally analysed in a pooled sample to identify the vast range of metabolites present, of which the top 50 metabolites are depicted in Figure 1. The most prevalent metabolite detected in these samples was urea, which on average was 6.5 times greater in magnitude than the next detected metabolite, hydroxylamine (Figure 1a). Aside from urea, the remaining top 10 metabolites identified had a peak intensity count between one and five million (Figure 1a). The remaining 40 metabolites identified in the top 50 had an average peak intensity range between 100 to 600 thousand (Figure 1b), which is 45 to 270 times smaller in abundance than that of urea.

Once the range of metabolites were identified, samples were run individually to understand the range in abundance of each metabolite and variations within the species and collection methods. Urea was the metabolite with the highest peak intensity, with a range that varied between the breeds of 27 to 32 million (Figure 2). The abundance of urea compared to other metabolites ranged from 4.3 to 10.8 times greater than the second most detected metabolite for each of the breeds, with the greatest difference occurring in the sedated Holstein-Friesians, and the smallest difference occurring in the tickled Angus. Interestingly, hydroxylamine was only the next most prominent metabolite detected for sedated and tickled in the afternoon Holstein-Friesians (Figure 3a, e), and the Charolais (Figure 3f). Thiamine was the second most prevalent metabolite instead for the Holstein-Friesians when tickled in the morning (Figure 3b) and the Brahman (Figure 3d), whilst sucrose was the second most detected metabolite for the Angus (Figure 3b).

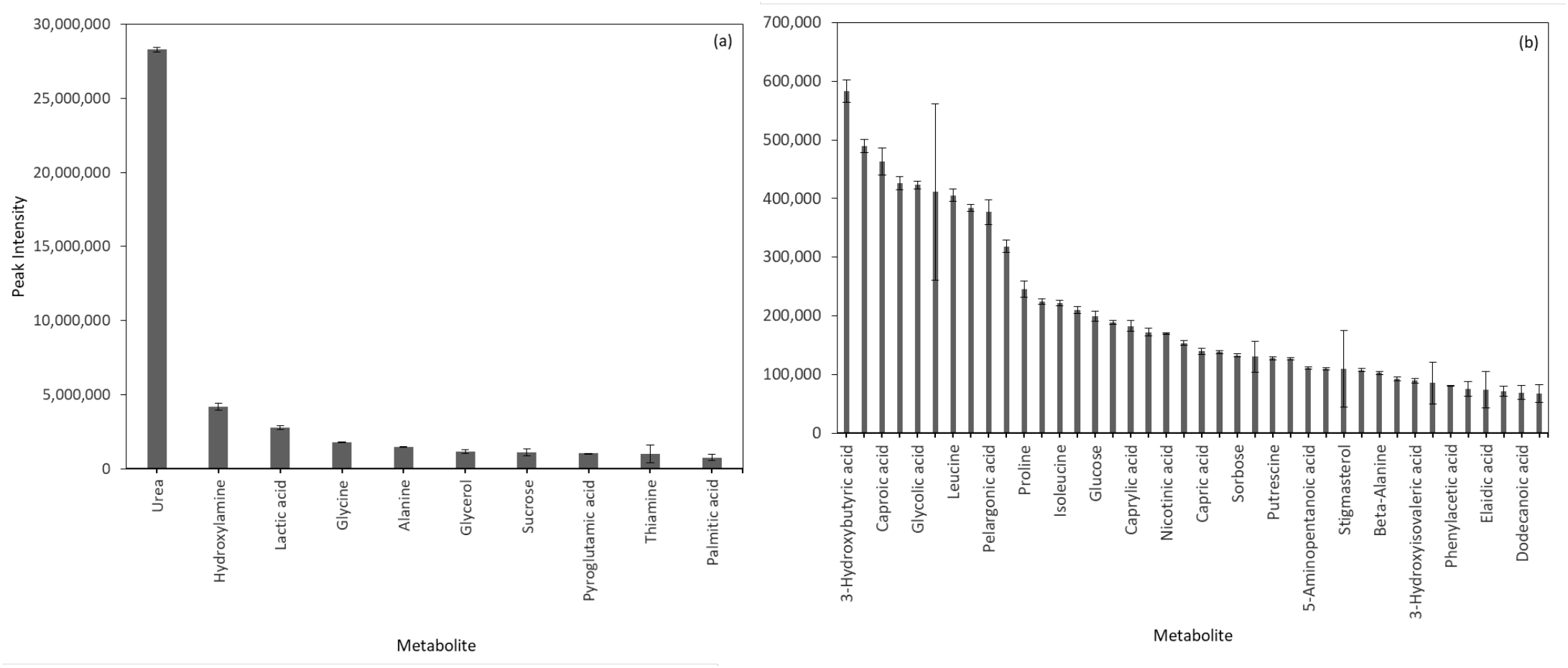

Figure 1. Top 50 detected metabolites for pooled (n = 5) samples of all breeds and collection types, sorted by magnitude. Panel (a) highlights the top 10 metabolites, whilst panel (b) represents the remaining 40 metabolites evident in analysis. Error bars are indicative of the standard error across the pooled samples.

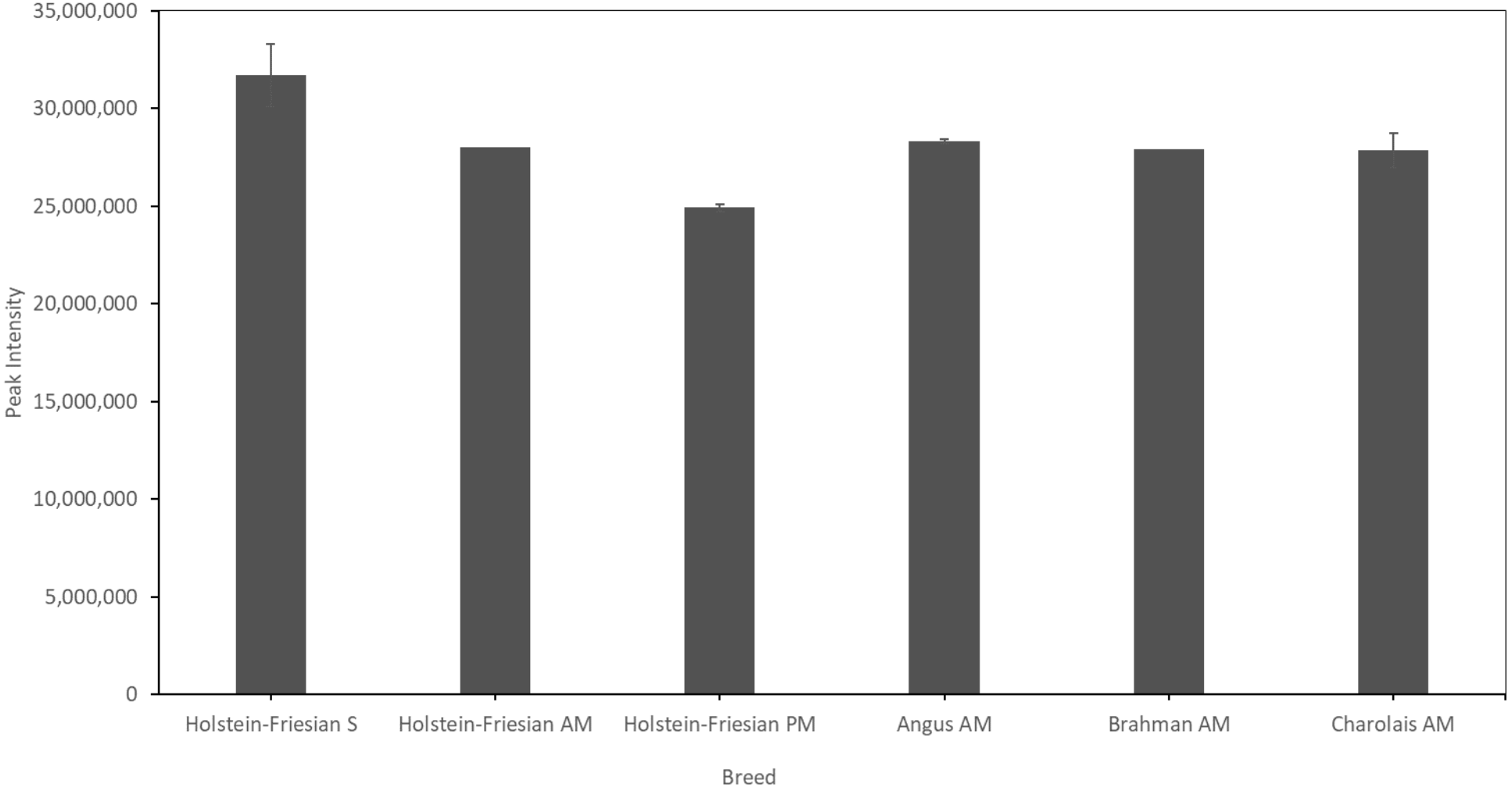

Figure 2. The most prevalent metabolite by magnitude, urea, from dairy cattle (Holstein-Friesian) from May Farm, and beef cattle (Angus, Brahman, Charolais) from John Pye Farm. Nomenclature for collection type: S = sedated, AM = morning collection period, PM = afternoon collection period. Error bars are indicative of the standard deviation of the mean (n = 2) across the individual samples.

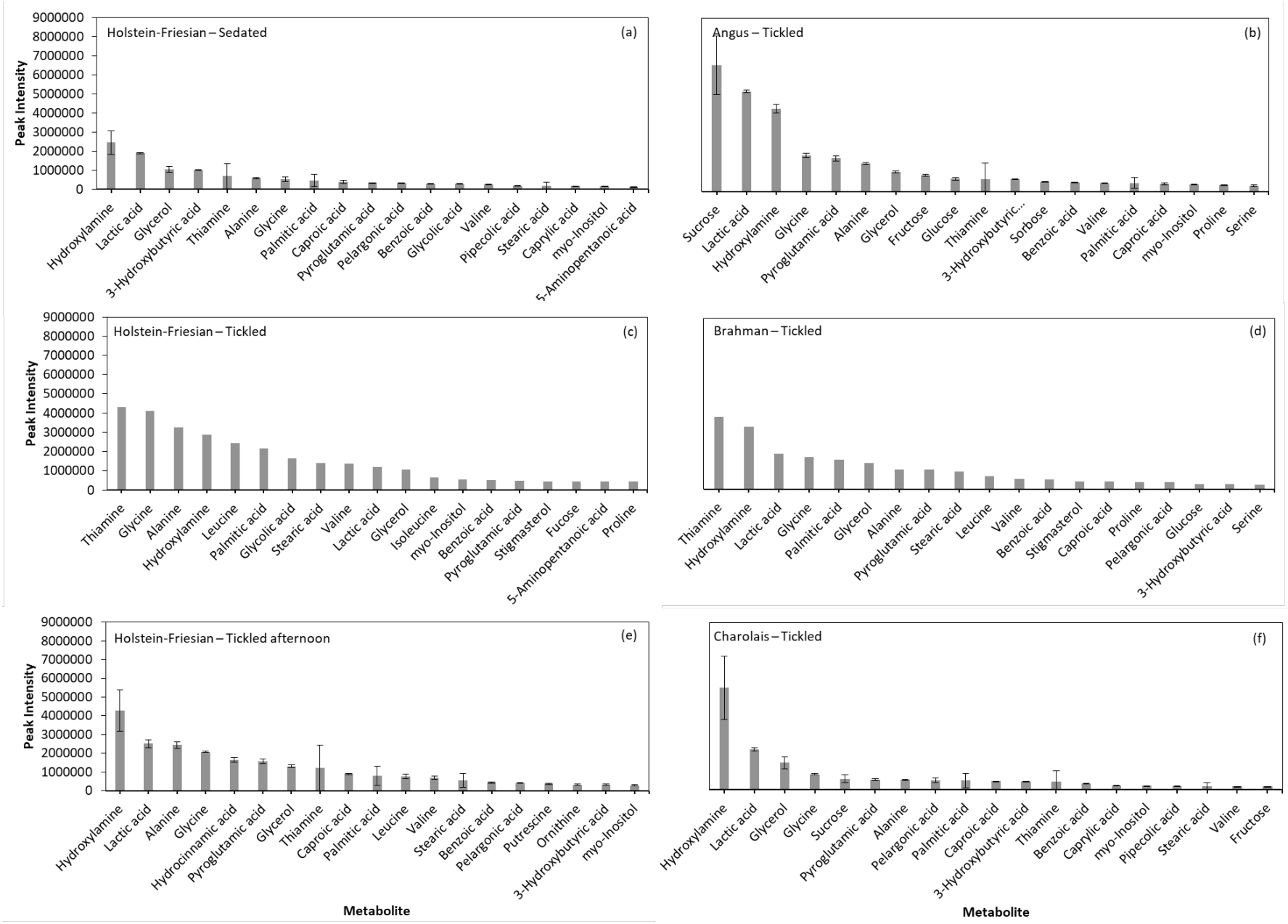

Figure 3. Remaining 19 top detected metabolites by magnitude for saliva samples taken. Urea ranked number one is not included here. Panels (a), (c), and (e) were sampled from dairy cattle (Holstein-Friesian) from May Farm, whilst (b), (d), and (f) are indicative of beef cattle (Angus, Brahman, Charolais, respectively) from John Pye Farm. Panels (b) – (f) utilise a 'tickled' sampling approach, whilst panel (a) utilised a sedation approach to saliva collection. All collections for panels (a) to (d) and (f) took place in the morning, whilst collection for panel (e) occurred in the afternoon. Error bars are indicative of the standard deviation of the mean (n = 2) across the samples.

Major constituents by compound class

We categorized the 132 identified metabolites into nine distinct functional groups to facilitate the discussion of the diverse range of metabolites evident and highlighted the most prevalent metabolite for each group. A table is also presented as supplementary data (Table A1).

Overwhelmingly, urea was the most predominant metabolite regardless of functional grouping (Figure 1; Table A1). Organic compounds are classed as chemical compounds that primarily contain a carbon and hydrogen atom, with the presence of other elements such as oxygen, nitrogen (N), sulphur and phosphorous. In this instance, the most overwhelming organic compound is urea. Alcohols and aldehydes are a subclass of organic compounds which function in various biological processes, such as carbohydrate metabolism. Across the saliva samples four metabolites were classed within this group, with glycerol being the major metabolite in this grouping overall (Table A1). Hydroxylamine was the only inorganic compound identified within the saliva profiles (Table A1). Organic acids are often involved in metabolic pathways, energy production, and acid-base balancing, and form the largest functional group of compounds found in saliva (Table A1). Of the 38 identified, lactic acid had the largest concentration from this functional group across all samples (Figure 1). Amino acids are primarily involved in protein synthesis, essential for numerous biological functions such as enzyme catalysis, structural support, and signalling. From the samples, 27 metabolites were classed as amino acids (Table A1). Glycine was the most prominent metabolite overall from this functional group (Figure 1).

Twenty-one different carbohydrates and their derivatives were identified (Table A1). Carbohydrates and their derivatives are a primary source of energy for living organisms. The metabolite within this group which had the greatest magnitude was disaccharide sucrose (Figure 1). Lipids and sterols are a group of metabolites which are vital for maintaining the integrity of cell membranes, energy storage, and metabolic processes. Of the eight different lipids and sterols classed, palmitic acid had the largest presence of the group in the samples, sitting within the top 10 overall (Figure 1). Within the metabolite profiles of saliva sampled only three vitamins, Vitamin B1 (Thiamine), B3 (Nicotinic acid), and B5 (Pantothenic acid), were identified, with thiamine, having the largest presence (Table A1).

Cluster analysis of samples and metabolites

A correlation analysis was completed identifying relationships between the saliva samples and pooled (control) samples (Figure 4). All the control samples (PBQC), and samples from the Holstein-Friesian breed (MFC, C1, RF1), regardless of collection type were clustered close together, with the highest correlation being evident in the pooled samples (Figure 4). Additionally, this analysis highlights that each of the samples had similar features to each other, except for the Charolais sample (S70_001), which was only moderately correlated to its sub replicate (S70_002) and had low correlation to the remaining beef species (Angus, S3; Brahman, S26), and the rest of the samples (Figure 4). The Holstein-Friesian tickled sample (MFC) had a moderate level of correlation to all samples, except for the Charolais sample (S70_001), which had the lowest correlation evident (0.5; Figure 4).

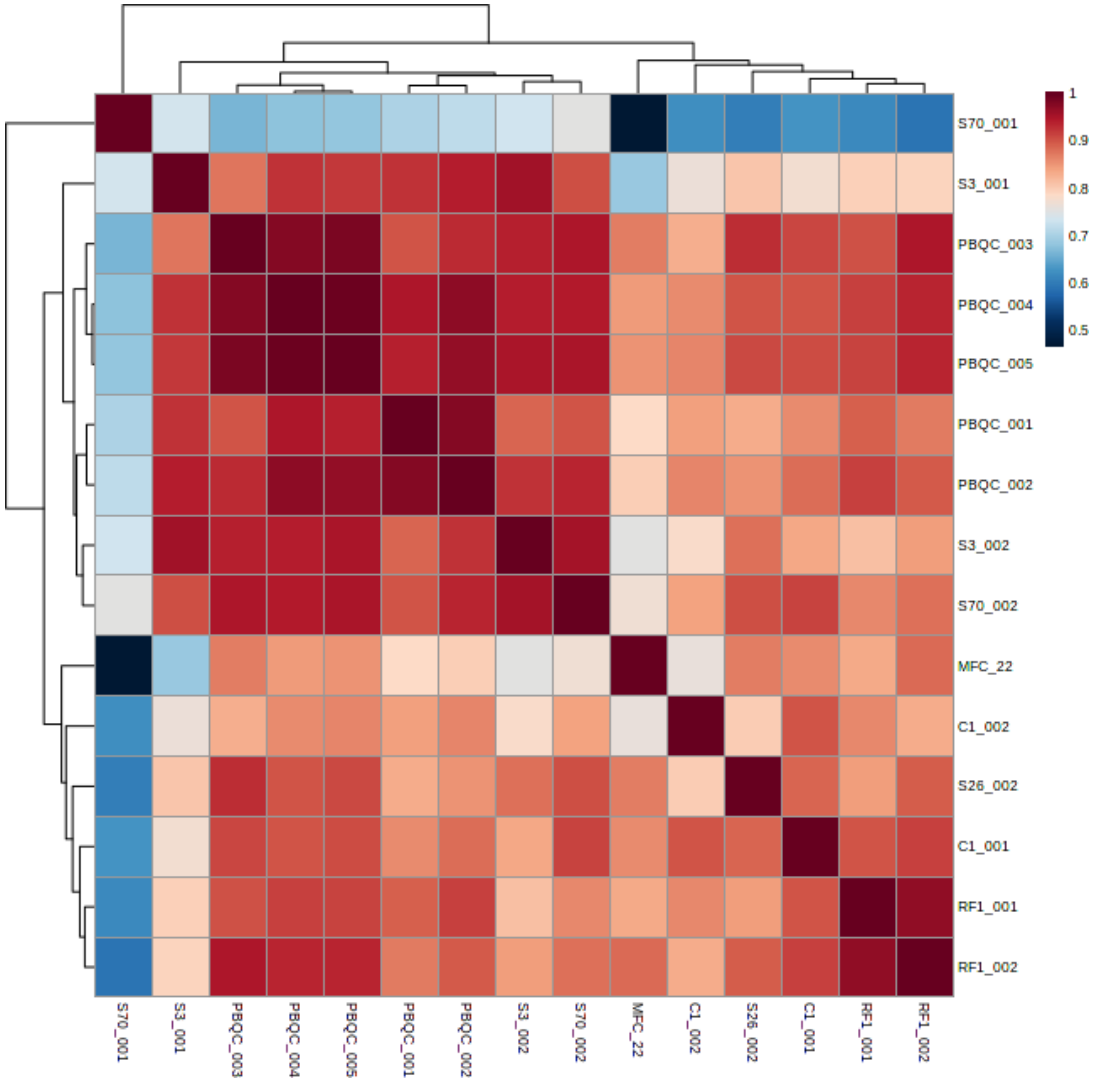

Figure 4. Overall correlation heatmap produced for each of the saliva samples (n = 2) alongside the control (n = 5). Nomenclature for samples are as follows; C1 = Holstein-Friesian sedated, MFC = Holstein-Friesian tickled, PBQC = control pooled samples, RF = Holstein-Friesian tickled afternoon, S26 = Brahman tickled, S3 = Angus tickled, S70 = Charolais tickled.

Saliva was further analysed using NMF to understand trends between the presence of particular metabolites, and the saliva profile that the composition best fitted with. As identified in Figure 5, certain metabolites were clustered together, and had a stronger relationship than others. For example, metabolite number 7, or pyroglutamic acid (Table 2), was present in larger quantities in the beef cattle (S26, S70, S3), than the dairy cattle (MFC, RF1, C1), as seen in basis group 1 (Figure 5). Similarly, a stronger relationship is evident with both metabolite 14, glycolic acid, and metabolite 16, hydrocinnamic acid (Table 2) in basis 2 and can be suggested to be in higher amounts in the dairy cattle than that of the beef cattle (Figure 5). Of note, though urea was previously identified as the most prevalent metabolite across all saliva profiles (Figure 2), it was on average higher in the samples from beef cattle which may further explain the greater relationship identified in the NMF between urea (metabolite 1; Table 2) and basis 1/beef cattle (Figure 5). A larger presence of amino acids was strongly associated with the saliva from the beef cattle, and only C1 (sedated Holstein-Friesian) from the dairy cattle grouping, whilst in contrast, there was a greater presence of sugars and organic acids from RF1 and MFC (Figure 5).

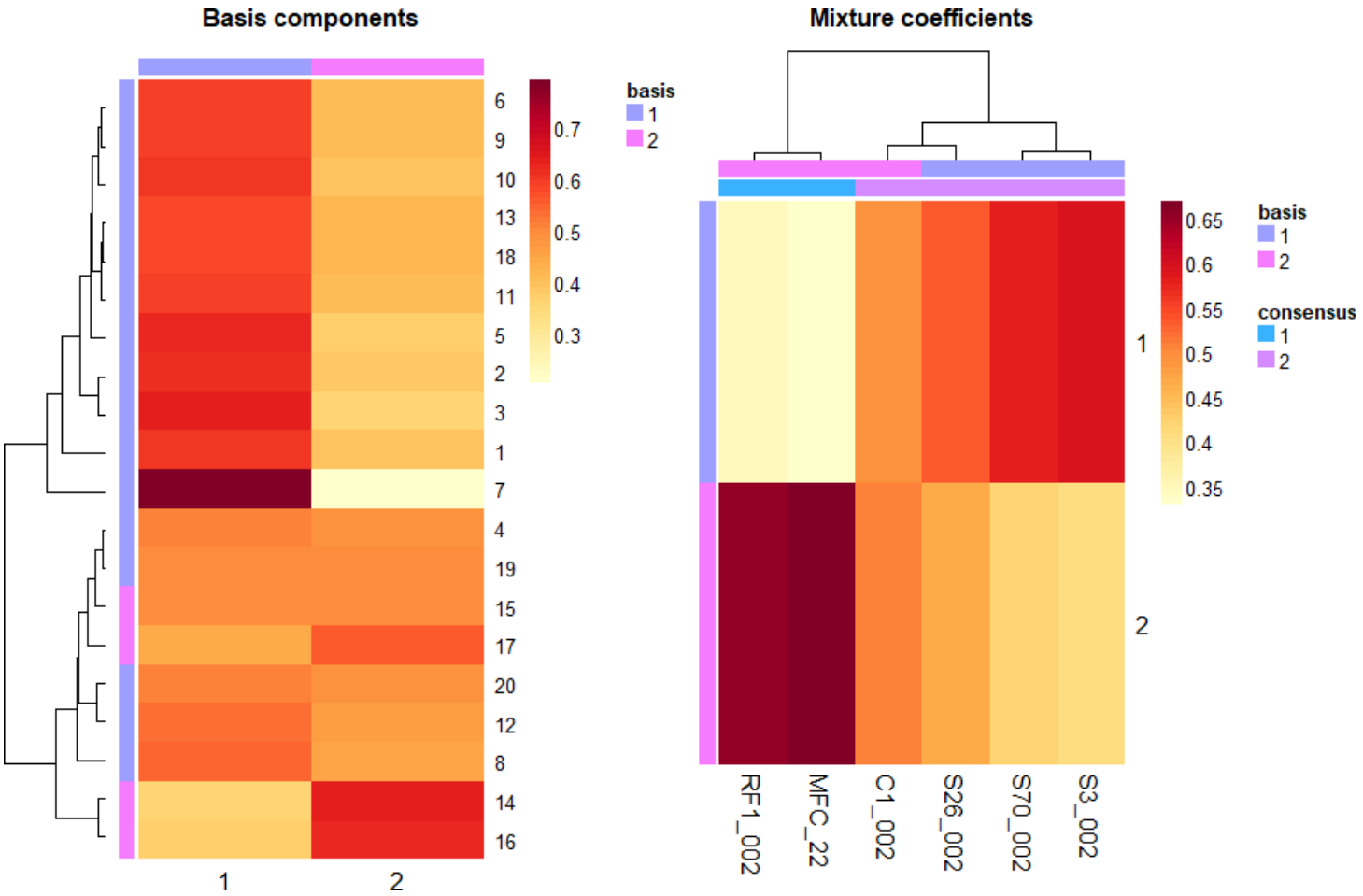

Figure 5. Non-negative matrix factorisation of saliva samples collected for dairy cattle (RF1, MFC, C1) and beef cattle (S26, S70, S3). NB: Nomenclature of metabolites (basis components) are outlined in Table 2.

Table 2. Top 20 metabolites from the non-negative matrix factorisation data, in order of clustering in figure 5.

Rank	***Metabolite***
6	Glycerol
9	Palmitic acid
10	3-hydroxybutyric acid
13	Benzoic acid
18	Stearic acid
11	Valine
5	Alanine
2	Hydroxylamine
3	Lactic acid
1	Urea
7	Pyroglutamic acid
4	Glycine
19	myo-inositol
15	Leucine
17	Pelargonic acid
20	Thiamine
12	Caproic acid
8	Sucrose
14	Glycolic acid
16	Hydrocinnamic acid

Discussion:

Saliva originating from glandular tissues within the mouth of livestock is described as complex in origin (Phillipson & Mangan, 1959), and as such, profiling the composition of saliva is critical for future work in both animal and plant management. Metabolomic profiling of cattle saliva has not been thoroughly explored, though it has previously been analysed in goat and sheep (Palma-Hidalgo et al., 2023). Here, 132 metabolites were identified within six unique saliva profiles across different cattle breeds (Table A1), with the most prevalent metabolite being urea which was between 4.3 – 10.8 times greater than the next most abundant metabolite in each breed (Figure 1, 2, 3).

Correlation analysis highlighted the relationships between different saliva profiles, identifying that all samples analysed were closely clustered together, aside from one replicate of the Charolais saliva sample, which had the lowest correlation amongst all profiles (Figure 4). This finding suggests that there may be subtle differences within pseudo replicates, despite overarching similarities persisting across saliva profiles in each breed. As such, a non-parametric approach to identification of trends within the features of the saliva profile was utilised, whereby NMF analysis highlighted that specific metabolites exhibited stronger associations with certain saliva profiles in contrast to others (Figure 5). The outlined investigations present a coherent framework for understanding the diverse components present across various saliva profiles. Future research could look at pinpointing specific metabolites that may influence plant regrowth rates in defoliation studies or could be utilised in animal health diagnostic studies, based on the exploratory research presented here.

The saliva profile – metabolites, the major constituents, and nitrogen containing compounds

Saliva in this study had a pH from 8.1 to 9.2 (Table 1). Aside from consistency changes described between the samples collected from each breed and method collection type (i.e., tickling, versus sedation) it was evident that in this study, the pH was also influenced by the collection method and time of day during sampling. The pH of saliva has been reported in prior studies as alkaline, within the range of 8.2 and 8.4 (Phillipson & Mangan, 1959; Somers, 1957). The samples collected using sedation methods saw not only an increase in volume collected (125 mL), but additionally saw an increase in pH, at 9.2, compared to the mean pH of 8.6 across all samples. It is a known side effect of cattle to produce an excessive amount of saliva when exposed to alpha-2 antagonists (i.e., xylazine and lignocaine hydrochloride), within 10 – 30 minutes (Brearley et al., 1990; Lin et al., 2022). The increase in salivation rate was expected, however, the increase in salivary pH was not. It is not explicitly clear what effect pH may play within defoliation studies on plants, due to confounding factors. However, as seen with results here, increased salivary pH could be a potential indicator of the use of sedation drugs used in cattle. The alkaline pH of the saliva is likely a natural buffer to maintain favourable condition (Akula et al., 2023; Somers, 1957). Additionally, recent publications have identified high amounts of a pH stabilising enzyme, carbonic anhydrase 6 (Akula et al., 2023) which is known to regulate pH in the mouth of cattle (Asari et al., 2000; Kivelä et al., 1999).

In humans, regulation of saliva pH is achieved by the presence of urea (Zabokova Bilbilova et al., 2012). In this study, regardless of the saliva profile, the organic compound, urea, overwhelmingly had the largest abundance present and was 4.3 to 10.8 times greater than the next detected metabolite. In sheep, positive correlations are evident between blood urea and salivary urea in ruminants during digestion of proteins (Barker, 1970), and a portion of

salivary urea is produced from the parotid duct (Cirio et al., 2000). The overall presence of urea within saliva may be influenced by factors such as protein intake, diet, and dehydration (Khozeimeh et al., 2017) and perhaps recently masticated foodstuffs. Here it can be suggested that these variables, along with others such as secretion rate (Cirio et al., 2000), may have influenced the varying range detected in each sample (250 – 325 million peak intensity; Figure 2). The presence of urea in animals lies with metabolism of proteins and amino acids (Hailemariam et al., 2021; Khozeimeh et al., 2017), and is a main product which supports growth for plants (Matiz et al., 2020). Promotion of plant growth is due to urea being a common source of N. Additional sources of N containing compounds were present within the profiles. Figures 1 and 3 list a high presence of hydroxylamine within cattle saliva, particularly for the sedated and tickled Holstein-Friesians (Figure 3a, e) and Charolais (Figure 3f) samples. It is likely that its presence as one of the top five metabolites is related to the occurrence of urea within the samples, as urea hydrolyses into ammonia within plant cells, ready for uptake (Sigurdarson et al., 2018). Hydroxylamine is an inorganic compound which is produced as part of the first step of nitrification whereby ammonia (NH_3) catalyses into hydroxylamine (NH_2OH)), before being broken down into nitric oxide (NO) (Caranto & Lancaster, 2017). In addition to urea and hydroxylamine, there were six other N containing compounds within the top 20 detected metabolites in the saliva profiles. These six are the amino acids glycine ($C_2H_5NO_2$), alanine ($C_3H_7NO_2$), pyroglutamic acid ($C_5H_7NO_2$), valine ($C_5H_{11}NO_2$), and leucine ($C_6H_{13}NO_2$). The large presence of N containing compounds within saliva (8/20 most prevalent metabolites) could therefore be the likely reason for varying growth responses by plants during defoliation and grazing studies. Prior research has identified that it is possible for plants to absorb nutrients through leaf surfaces (Seshadri, 1980; Thorne, 1954).

From the wide range of compounds categorised into functional groups (Table A1), of note, vitamin B1 (thiamine) was within the top five detected metabolites only for the tickled Holstein-Friesian, and Brahman (Figure 3c, d), and the 9th overall (Figure 1a). Thiamine is required for carbohydrate metabolism, synthesised by rumen microbes within ruminants (Pan et al., 2016), so it is unsurprising that thiamine had a large presence in saliva overall (Figure 1). Additionally, thiamine is the predominant metabolite in livestock saliva that has been hypothesised in literature to stimulate a growth response in grasses (Bonner, 1937; Bonner & Greene, 1939; Reardon et al., 1974). Upon investigating this particular metabolite presence within different breeds and collection methods, it is evident that the concentration of thiamine varies between individual animals (Figure 3), which is likely due to the inherent affect rumen acidity has on thiamine synthesis (Karapinar et al., 2010). Similar to the variations of N containing compounds in saliva, the potential variation of thiamine within species alone may be a confounding factor on the variation of responses seen by prior research in defoliation-based studies. However, any growth promotion observed within plants is most likely due the large N supplies evident within saliva profiles, and rate of foliar uptake of N (Seshadri, 1980; Thorne, 1954).

Development of saliva-based diagnostics

In addition to furthering our understanding of what may influence the physiochemical changes within plants that lead to increased growth rate, the metabolomic investigation detailed here provides an additional avenue for use of saliva as a diagnostic tool in animal health and production. Livestock saliva changes have been more recently investigated using salivary cortisol as a metric in a range of studies to determine the impact of changes to feed,

lameness, and inflammation (Contreras-Aguilar et al., 2019; Contreras-Aguilar et al., 2022; Contreras-Aguilar et al., 2021; Contreras-Aguilar et al., 2020), and using proteomics based methods to understand certain physiology, nutrition, and disease-based traits (Franco-Martínez et al., 2021; Lamy & Mau, 2012). Here, we propose that metabolic analysis of saliva could be a useful diagnostic tool, due to saliva sampling being a low stress, minimally invasive method for sample collection to identify disease and biochemical markers (Džermeikaitė et al., 2023). Metabolic based analysis has been performed in other biofluids (i.e., blood, urine, milk, ruminal fluid) (Kim, 2021; Zhu et al., 2021), however has seldom been used in saliva-based investigations (Goldansaz et al., 2017).

This study has detected the presence of specific metabolites which can be used in health diagnostics. From the metabolite groups identified, ketone bodies are water soluble molecules predominantly evident in animals, produced in the liver, ruminal wall, and lactating mammary glands, often when glucose is not readily available (Laffel, 1999; Sato et al., 2002). Of this particular class, three types of ketone bodies were identified in cattle saliva, with the most prevalent being 3-Hydroxybutyric acid (3-HB A) which was more predominant within the beef than the dairy cattle (Figure 5). This metabolite in particular has been studied previously in cattle, however, has been done so utilising fluids other than saliva (i.e., blood plasma (Li et al., 2022); cerebrospinal fluid (Sato et al., 2002); urine (Knodt et al., 1942)). 3-Hydroxybutyric acid occurrence overall was high (11/132), however varied greatly depending on the breed and collection method of the sample (Table A1; Figure 3). The presence of 3-HB A was not as high for the beef breeds, as it was for the sedated Holstein-Friesian sample (Figure 3). Aside from potential influences of health aspects (i.e., identifying lactation period, overall health) that was not investigated per animal in this study, 3-HB may have been elevated in the sedated sample due to the animals being off pasture for an extended period prior to sedation (Sato et al., 2002). Furthermore, a prior study has identified the presence of lactate in saliva of cattle as an indicator of inflammation due to the presence of mastitis (Contreras-Aguilar et al., 2019). Lactic acid was identified here as the third most frequent metabolite overall (Figure 1) and had a stronger relationship and so too, higher presence in beef cattle in contrast to the dairy cattle (Figure 3, Figure 5). Though the health status of the cattle sampled in this study was not explicitly investigated, this comparison alone highlights the potential use of metabolomics to perform saliva-based health assessments in future studies.

Aside from health issues, potential exists for this approach to be utilised in dietary based assessments. Metabolomic analysis may allow for further insight into the presence of various carbohydrates, such as sugars and associated derivatives (Table A1). Sugars and their derivatives are a primary source of energy for living organisms due to their soluble carbohydrates and are found in the tissues of most plants (Flitsch & Ulijn, 2003). Of the metabolites within the sugar functional group, sucrose had the greatest presence (7/132; Figure 1). Surprisingly, sucrose was only within the top 20 detected metabolites for the Angus (2/20; Figure 3b), and Charolais (5/20; Figure 3f) samples (Figure 5). Once again, though not explicitly investigated here, this could be due to individual animal differences, such as diet-based preferences (i.e., increased/decreased intake of grains vs pasture). Changes in sucrose levels within animals has previously been described as useful to understand diet impacts on the rumen and overall production of dairy cattle (Ravelo et al., 2022).

Cluster analysis suggests that the identity of cattle can be determined by metabolic abundance due to the grouping of relevant replicates, and additionally, successful clustering of replicate samples can occur using only a handful of identified metabolites. This suggests that there is

potential to utilise salivary biomarkers in metabolomic analysis with genomic analysis. Despite the anomaly with Charolais saliva profile (S70_001 and S70_002), the correlation analysis presented high levels of correlation within the Holstein-Friesian samples regardless of collection type (i.e., tickle in the morning, tickle in the afternoon, sedation), and similarly, a correlation was evident with the remaining Angus and Brahman breed samples (Figure 4). The NMF analysis was able to cluster metabolites present and relate these to the particular saliva profiles (Figure 5). Whilst the results presented here are preliminary, inter-sample variation within this set of cattle breeds, diets, locations, and sampling protocols suggest that biologically significant patterns in saliva constituents exist and may be informative for future diagnostic tests of animal health and production.

Further investigations are required to deepen our understanding of the various changes in salivary metabolite profile under numerous scenarios. There are various cofactors that are not considered here which we are not able to assess, due to sampling size limitations. Specifically, additional research would be beneficial to elucidate the impact of factors such as time of day, changes in diet (i.e., pasture-only, total mixed rations, grain-only diets), and underlying health conditions on salivary metabolites. Understandably, enhancing the robustness and reliability of the findings in this study is contingent on increasing the number of replicates for each breed, and collection method. A larger sampling size not only strengthens the statistical power of the analysis but may also allow for a comprehensive understanding of underlying patterns per animal.

Conclusion:

Metabolomic analysis of cow saliva obtained from four cattle breeds differing in diet, location and sampling method has uncovered biologically significant properties and variation among samples that may be useful for future development of diagnostic testing. This ‘omic approach combined with targeted bioinformatics has for the first time uncovered significant constituents, properties and patterns in cattle saliva that likely explain observed patterns in plant growth promotion. Further, major constituents and physiochemical properties of the identified metabolites suggest that with further development, saliva-based bioindicators may serve to provide monitoring and evaluation of cattle production and health.

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Appendix:

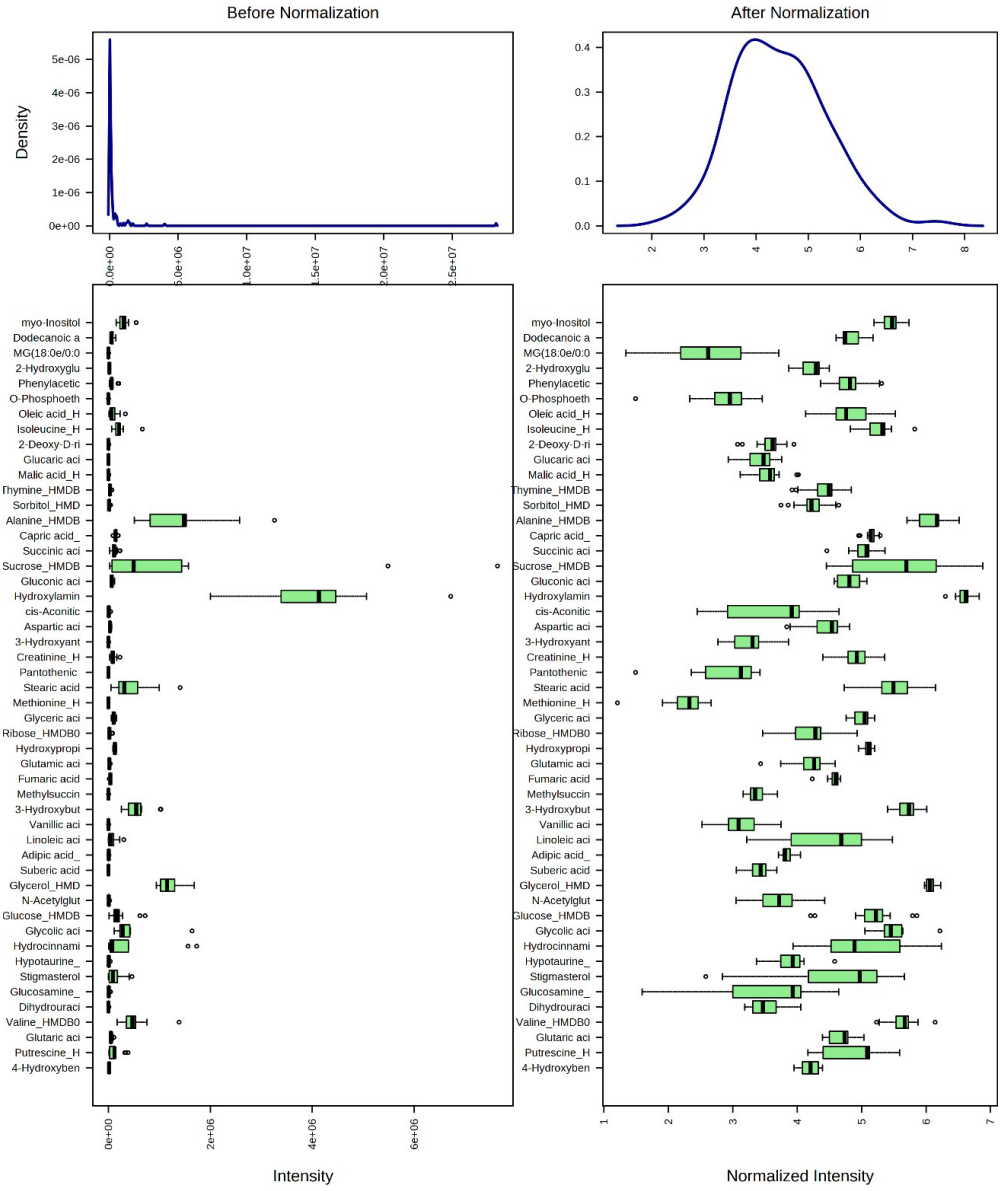

Figure A1. Box plots and kernel density plots before, and after normalisation of all metabolomic data. Box plots show only 50 features due to space constraints, whilst density plots are based on all samples. The methods of analysis were row wise normalisation, and Log10 transformation, produced by MetaboAnalyst 5.0.

Table A1. List of 132 metabolites prevalent across all saliva samples (n = 15), ordered by their functional grouping and associated mean peak intensity (± standard error).

Group	Compound Name	Mean intensity	Rank (1 - 132)
Alcohols and Aldehydes	Glycerol	1200150.73 ± 60609.86	8
	Octadecanol	59245.00 ± 4219.31	53
	Glycerol 3-phosphate	26850.33 ± 1791.51	69
	1-Hexadecanol	11392.93 ± 753.78	92
Amino Acid	Glycine	1702282.47 ± 223517.55	4
	Alanine	1454585.53 ± 203022.87	5
	Leucine	506589.60 ± 149542.05	12
	Valine	503822.33 ± 75803.95	13
	Proline	236818.07 ± 28110.04	21
	Isoleucine	217240.60 ± 37069.40	23
	Serine	188907.53 ± 17992.82	25
	Ornithine	166819.20 ± 26319.43	27
	5-Aminopentanoic acid	105234.73 ± 26998.16	38
	Cysteine	102475.20 ± 13571.94	39
	Phenylalanine	94895.60 ± 23883.70	42
	Beta-Alanine	87898.40 ± 12718.26	43
	Aspartic acid	33532.00 ± 4679.41	62
	Thymine	31464.47 ± 4605.69	65
	Gamma-Aminobutyric acid	29287.80 ± 3328.76	66
	Threonine	28826.47 ± 2174.69	67
	Tyramine	27174.20 ± 6330.06	68
	Tyrosine	26003.87 ± 13024.54	70
	N-Acetylmannosamine	24696.07 ± 10974.81	71
	Glutamic acid	19249.47 ± 2717.91	77
	N-Acetylneuraminic acid	18218.67 ± 8209.80	79
	Homogentisic acid	16216.53 ± 2565.79	84
	Maleic acid	10003.80 ± 906.54	97
	Lysine	7052.13 ± 1863.98	101
	N-Acetylglutamine	6845.20 ± 1673.79	104
	Theanine	4791.40 ± 422.30	112
	Methionine	219.80 ± 33.37	132
Ketone Bodies	3-Hydroxybutyric acid	556771.20 ± 59833.46	11
	Acetoacetic acid	18237.47 ± 3157.74	78
	2-Ketobutyric acid	9316.13 ± 1276.59	99
Lipids and Sterols	Palmitic acid	797015.20 ± 148327.33	10
	Stearic acid	443631.53 ± 103625.65	15
	Stigmasterol	137467.00 ± 40057.54	32
	Oleic acid	98428.93 ± 24916.01	40
	Elaidic acid	86437.73 ± 21570.58	44
	Linoleic acid	79144.13 ± 24402.75	46
	Myristic acid	78471.60 ± 9539.47	47
	Cholesterol	1335.87 ± 268.79	128

Organic Acid	Lactic acid	2706519.60 ± 291088.01	3
	Pyroglutamic acid	1009243.40 ± 124731.25	9
	Caproic acid	493320.47 ± 44667.38	14
	Benzoic acid	420503.80 ± 18386.28	16
	Glycolic acid	381586.33 ± 94619.44	17
	Pelargonic acid	377983.47 ± 20891.37	18
	Hydrocinnamic acid	367112.73 ± 141128.49	19
	Caprylic acid	188522.73 ± 7288.54	26
	Capric acid	142680.00 ± 7353.19	31
	Hydroxypropionic acid	127744.07 ± 5019.85	35
	Succinic acid	119787.80 ± 14263.14	36
	3-Hydroxyisovaleric acid	84939.53 ± 3757.58	45
	Phenylacetic acid	76159.80 ± 13743.43	48
	Dodecanoic acid	74156.60 ± 9201.70	49
	Gluconic acid	70048.13 ± 8179.33	50
	Glutaric acid	56021.93 ± 7468.27	54
	Fumaric acid	38102.93 ± 2035.70	59
	Quinic acid	33447.27 ± 8704.21	63
	Alpha-Hydroxyisobutyric acid	31928.20 ± 2066.59	64
	Citric acid	21432.07 ± 3019.62	74
	Phenyllactic acid	19386.80 ± 7665.40	76
	2-Hydroxyglutaric acid	18159.33 ± 1740.13	80
	4-Hydroxybenzoic acid	16629.13 ± 1338.33	83
	Citramalic acid	11885.87 ± 2212.82	90
	Oxoglutaric acid	10749.60 ± 2921.24	93
	cis-Aconitic acid	10515.40 ± 3675.18	94
	Azelaic acid	7177.80 ± 637.90	100
	Adipic acid	6979.00 ± 405.32	102
	Pyrocatechol	6909.07 ± 401.56	103
	Salicylic acid	6841.47 ± 986.70	105
	Pyruvic acid	6557.53 ± 183.76	106
	Galacturonic acid	5655.00 ± 549.60	110
	Malic acid	4158.07 ± 694.20	114
	Glucaric acid	3101.07 ± 376.45	120
	Suberic acid	2784.80 ± 241.44	121
	Methylsuccinic acid	2597.73 ± 266.51	122
	3-Hydroxyanthranilic acid	2285.47 ± 459.55	124
	3-Methyl-2-oxovaleric acid	1994.20 ± 288.96	125
	Vanillic acid	1839.20 ± 428.58	126
Organic Compounds	Urea	28198293.87 ± 490274.37	1
	Pipecolic acid	154738.80 ± 17115.28	29
	Threonic acid	147228.67 ± 20099.61	30
	Putrescine	128353.93 ± 32061.04	34
	Glyceric acid	105648.13 ± 8646.97	37
	Creatinine	94904.60 ± 13362.47	41
	Uracil	47647.87 ± 3877.06	56

	Ethanolamine	37110.80 ± 3950.91	60
	Dimethylglycine	35527.60 ± 1278.69	61
	Gluconolactone	16002.27 ± 2780.95	86
	2-Aminoheptanedioic acid	14956.20 ± 2225.49	88
	Threitol	13264.33 ± 795.45	89
	Dihydroxyacetone phosphate	11802.40 ± 869.65	91
	Hypotaurine	10185.07 ± 2174.37	96
	Pinitol	6159.87 ± 428.67	108
	Triethanolamine	5793.73 ± 255.14	109
	p-Hydroxyphenylacetic acid	4771.47 ± 1013.38	113
	2-Deoxy-D-ribose	4095.53 ± 507.81	115
	Dihydrouracil	4074.00 ± 755.71	116
	Sedoheptulose	4029.93 ± 682.83	117
	Hydroquinone	3400.27 ± 545.21	118
	Erythritol	3363.93 ± 219.20	119
	Xylitol	2364.67 ± 336.19	123
	N-Acetylserine	1404.87 ± 211.72	127
	MG(18:0e/0:0/0:0)	1135.00 ± 386.51	130
	O-Phosphoethanolamine	1067.40 ± 196.59	131
Other Compounds	Hydroxylamine	4010534.47 ± 286562.86	2
Sugars and Sugar Derivatives	Sucrose	1352165.80 ± 572942.30	6
	myo-Inositol	299427.13 ± 25272.42	20
	Fructose	228053.53 ± 69931.36	22
	Glucose	216169.93 ± 51378.78	24
	Sorbose	131093.13 ± 41956.98	33
	Fucose	67600.13 ± 27835.69	51
	Levoglucosan	65033.80 ± 8015.82	52
	2-Deoxyglucose	49617.40 ± 7563.02	55
	Lactitol	41247.73 ± 7525.94	57
	Trehalose	39315.73 ± 7477.89	58
	Xylulose	24555.20 ± 3302.23	72
	Ribose	24081.33 ± 6106.52	73
	Sorbitol	20092.67 ± 3009.19	75
	Mannitol	18081.93 ± 3457.27	81
	Galactitol	17147.87 ± 2657.20	82
	Ribitol	16138.40 ± 3009.94	85
	Arabitol	15618.73 ± 3285.68	87
	Glucosamine	10449.87 ± 3362.13	95
	Tagatose	9892.20 ± 3288.82	98
	Arabinose	6471.87 ± 1062.47	107
	Lyxose	4863.47 ± 647.50	111
Vitamin	Thiamine	1269649.80 ± 371921.90	7
	Nicotinic acid	156525.13 ± 24004.37	28
	Pantothenic acid	1278.67 ± 225.48	129

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Chapter 5:

Understanding sheep baa-haviour: Investigating the relationship between pasture and animal grazing patterns

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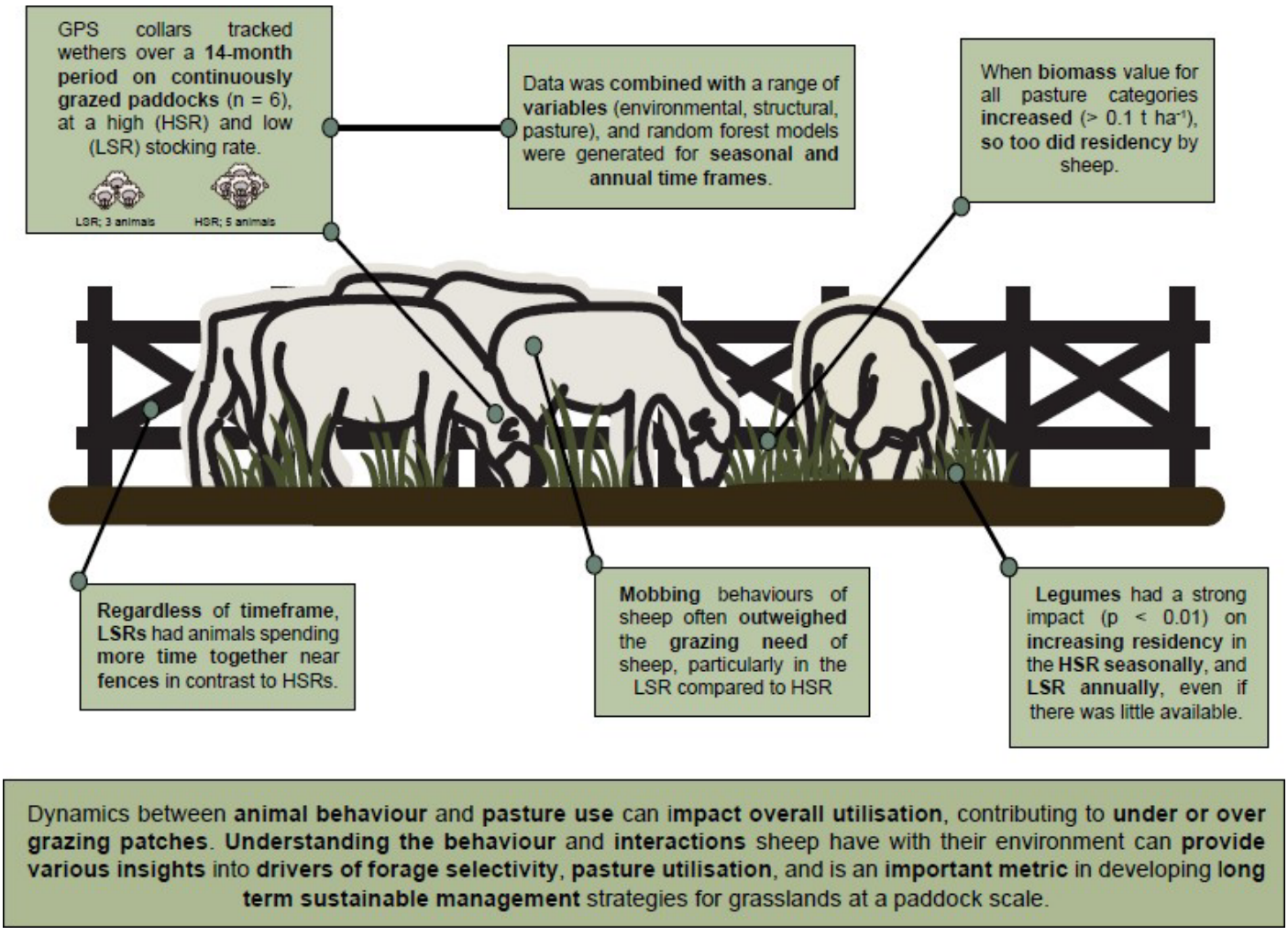

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RESEARCH ARTICLE

Grassland Research

Understanding sheep baa-haviour: Investigating the relationship between pasture and animal grazing patterns

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Abstract

Background: Grasslands are the primary source of feed for grazing livestock, and as such, knowledge on how to best manage livestock and grasslands, through the use of spatiotemporal modelling, will assist in the long-term management of a valuable ecosystem resource.

Methods: This study was conducted over 14 months between March and April 2017 in Orange, NSW, Australia. The study evaluated sheep behaviour in relation to the presence of pasture species, environment and paddock structures, using random forest modelling, to predict sheep location under continuous high (HSR, 13 DSE ha^{-1}) and low (LSR, 7 DSE ha^{-1}) stocking rates.

Results: In the LSR, significant drivers included water, shade and fence lines ($p < 0.01$). In the HSR, only fence lines and available biomass were found to be significant ($p < 0.01$). The presence of green legumes in both stocking rates often increased residency by sheep. Animals spent more time together in the LSR, suggesting that social behaviour played a larger role than pasture quantity and quality in driving grazing behaviours.

Conclusions: Understanding how pasture type can influence grazing behaviours and also how animal behaviour affects pasture performance and utilisation is important in developing long-term sustainable management strategies on a paddock scale.

KEYWORDS

behaviour, GPS, grazing interactions, livestock grazing, pasture production, sheep, wethers

INTRODUCTION

The dynamics between animal and pasture attributes can impact pasture utilisation and can contribute to under- or overgrazing. Grasslands are the primary and most economical source of livestock feed and a potential carbon sink (Boval & Dixon, 2012; O'Mara, 2012). Therefore, a thorough understanding of the determinants of grazing intake as well as the associated dynamics between both animals and pasture is beneficial for pastoralists (Baumont et al., 2004). The implementation of management strategies on free-grazing ruminants such as sheep is, however, strongly influenced by a variety of social and environmental factors that can influence their behaviours and, presumably, their grazing patterns. The ability to match the locations of grazed areas to identified plant species and biomass values has the potential to further our knowledge of paddock utilisation gaps in livestock enterprises (Ingram et al., 2016; D. B. Taylor et al., 2011; Thomas et al., 2008). Understanding the behaviour and interactions that sheep have with their environment can provide insights into the various drivers of sheep location, forage selectivity and pasture utilisation.

Previous studies have identified that sheep will often show social attachments and social organisation, that is, the need for social interactions will influence sheep grazing patterns, including both diet and feed preferences (Dumont & Boissy, 2000; Michelena et al., 2009; Squires, 1976). For example, in a large grazing mob, sheep may ignore higher-quality feed available in the paddock for various reasons, such as fear of separation and predation (Dumont & Boissy, 2000). This may lead to areas of overgrazing, reducing the overall pasture productivity

compared to relatively more evenly grazed pastures. The presence of a diverse mix of pasture species can often result in having feed available with contrasting quality, quantity and nutrient concentration, which can lead to variations in grazing preferences and the spatial distribution of a flock (Parsons et al., 1994). Besides grazing preferences and behaviour, pasture use can be influenced by the location of resources, such as shelter, water and fence lines, and distances from other resources (Ingram et al., 2016; D. B. Taylor et al., 2011). Alternatively, regrazing heavily grazed areas due to higher pasture quality (e.g., new regrowth) can lead to other feedback processes, commonly referred to as patch grazing.

Drivers of short-term foraging decisions may differ from those made over the medium or long term (Dumont et al., 1995). For example, grazing preferences for a pasture species may only be evident in a particular month or season. This could be due to the preference for a particular species that only grows within certain seasons, pasture being more palatable during certain plant growth stages (i.e., higher nutritive value and palatability during early growth stages) or due to the impact that underlying soil or landscape properties (wetter, greater soil fertility, aspect, etc.) have on vegetation. Studies that focus on grazing over a short duration may not be useful in detecting behaviours that may change over the course of seasonal cycles and identifying differences in behaviours of animals. These seasonal behavioural changes will likely be influenced by the ability to feed on reproductive patches of pasture, depending on the time of year of the study (Dumont et al., 1995). As such, one can conclude that long-term grazing trials are likely to be highly beneficial for determining foraging decisions in pastures as they allow behaviours to be investigated across multiple time scales. Few papers have been published that have focused on the various drivers of sheep behaviour using a modelling perspective. Additionally, few studies have highlighted grazing behaviour trends and their relationships to mixed pasture species in detail on continuously grazed paddocks over a long term.

The aim of this study was to assess drivers of sheep location by investigating the spatiotemporal characteristics of sheep behaviour with respect to pasture biomass, quantity and species. It is hypothesised that forage quality and presence of functional biomass groups (i.e., introduced pastures both living and dead, weeds and legumes) will be the primary driving factors behind the animal location, with social behaviours (i.e., mobbing) a secondary factor. The influence of structures such as shelter and water troughs will not be as significant as the presence of a pasture type.

MATERIALS AND METHODS

Site description

The study was undertaken between March 2017 and April 2018 at the Orange Agricultural Institute (OAI) (922 m ASL 33.324 250° S, 149.089 472° E), a long-term grazing trial undertaken by the NSW Department of Primary Industries (DPI). This grazing trial started in 2012 to compare the performance of short- and long-term rotational grazing paddocks against a long-term continuous grazing paddock (Badgery et al., 2017). Climate and rainfall data have been collated at the OAI by the Bureau of Meteorology (BOM site 063294) located approximately 0.5 km from the grazing site. Long-term average annual rainfall was 916 mm, with average minimum temperatures of 7.2°C and average maximums of 18.2°C (Australian Bureau of Meteorology, 2022). The average annual rainfall was lower than long-term averages, with 631 mm in 2017 and 620 mm in 2018. The average minimum and maximums were 5.7°C and 19.9°C in 2017 and 7.5°C and 19.7°C in 2018 (Australian Bureau of Meteorology, 2022).

A period of drought occurred during the mid to late portion of the study, with dramatically reduced rainfall between May and September in 2017 when compared to the long-term average (Figure 1). The severity of the drought ranged from nondrought during the start of the trial (March 2017), intense drought during Spring (September–November 2017) to a drought through Summer (December 2017–May 2018) until the end of the trial.

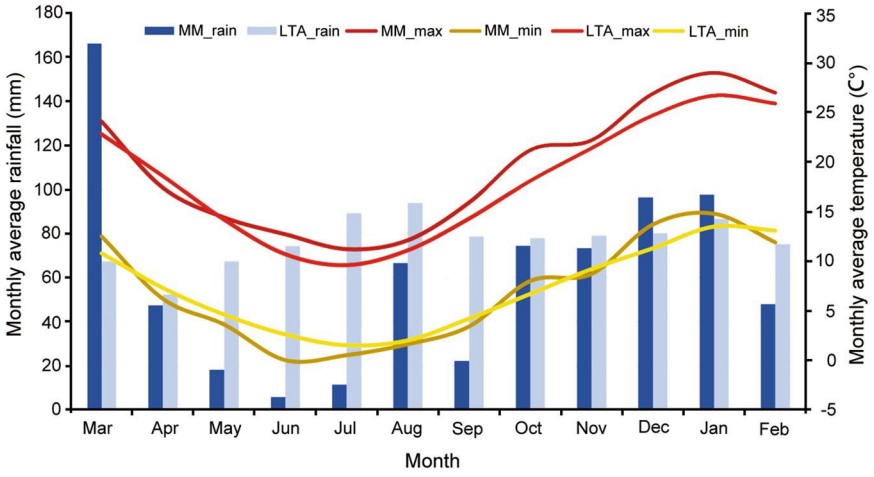

FIGURE 1 Comparison of long-term average temperature and rainfall data (1976–2022), and monthly average temperature and rainfall data (2017–2018) for the Orange Agricultural Institute study site (Australian Bureau of Meteorology, 2022). LTA, long-term average; MM, mean monthly.

The site in which the study took place included replicated paddocks that were either assigned to a high stocking rate (HSR; High 1, High 2 and High 3) stocked at 13 DSE ha^{-1} (five sheep) or a low stocking rate (LSR; Low 1, Low 2 and Low 3) treatment at 7 DSE ha^{-1} (three sheep). All six of the replicate paddocks were approximately 0.5 ha in size. The paddocks were dominated by sown species, phalaris (*Phalaris aquatica* L.), tall fescue (*Festuca arundinacea* Shreb.) and cocksfoot (*Dactylis glomerata* L.). Clover species such as white clover (*Trifolium repens* L.), vetch (*Vicia sativa* L.) and sub clover (*Trifolium subterraneum* L.) became more prevalent from May to June 2017. Other species of grasses, legumes and weeds evident in the paddocks included Wallaby grass (*Austrodanthonia caespitosa*), a variety of medics, catsear (*Hypochaeris radicata*), spear thistle (*Cirsium vulgare*) and silver grass (*Vulpia myuros* (L.) C.C.Gmel.). Using pasture data derived from BOTANAL measurements (see following) across all paddocks for the duration of the study, there was approximately 0.19 t ha^{-1} brown and 0.8 t ha^{-1} green sown grass, 0.2 t ha^{-1} brown and green legumes, 0.1 t ha^{1} brown and green weeds and 0.5 t ha^{-1} and 0.3 t ha^{-1} of brown and green grass, respectively.

Each paddock contained a continually filling water trough, a shaded area and a hand feeding trough, and H1 had a small walkover weighing (WoW) station (~ 5 m^2) (Figure 2). H3 and L3 had shading from trees within the paddock, and from a large tree on the perimeter, as well as the presence of a small rock outcrop. All resources including the WoW, watering, feeding and shading areas were geolocated using a global positioning system (GPS) unit (Garmin etrex 30, Garmin Ltd.).

Pasture measurements

The BOTANAL method (Jones & Hargreaves, 1979) was used for pasture composition surveys across 90 fixed locations, with 15 locations per treatment replicate. At each location, pasture was ranked using a pair of 0.25 m^2 quadrats for green or dead dry matter (DM) tonnes per hectare. Pasture composition was estimated visually with each plant species and then allocated into one of eight different green or dead functional groups: legumes, sown grasses and grasses (which included natives and annuals) and weeds and litter (loose green and brown biomass). Pasture was assessed at the initiation of the trial (March 14, 2017) and was reassessed 10 times at each of the permanently marked locations over the duration of the current trial (April 27, 2018), and due to the nature of survey, usually took a few days to complete.

Due to the number of pasture surveys undertaken over the course of the trial and the need to attempt to interpolate data for those dates when surveys were not conducted (often many months apart), machine learning methods were used to develop predictions of DM for available biomass (I. Kardailsky, personal communication, August 29, 2019). This model was based on ~ 24 000 independent data points (spanning ~ 8 years of observations) with > 50 predictors derived from available Sentinel or Landsat 8 imagery and daily weather information for the site and incorporated use of the 'xgboost' (V1.6.2; Chen & Guestrin, 2016) package in R (I. Kardailsky, personal communication, August 29, 2019). The DM of the functional groups was estimated at Sentinel spatiotemporal resolution, that is, at a 10 m^2 scale on average every 5 days. While the larger model used both Landsat 8 and Sentinel 2 data, the DM predictions used for this study were based entirely on Sentinel 2 (10 m^2 pixel).

For the purposes of this study, we used a subset of this larger functional groups model that covered the 14 months for which this study was undertaken (Figure 3). After the removal of satellite images that were either partially or fully obscured by clouds, we were left with 78 days when Sentinel 2 images were available for biomass predictions.

Pasture biomass was generally higher in the LSR for legumes and weeds but lower than the HSR for grass and sown grasses (Figure 3). Normalised difference vegetation index (NDVI) values, which were used as a

FIGURE 2 Map of Orange Agricultural Institute paddocks and selected features with allocated treatment and replication number. Blue rectangles represent water locations, while white hashed rectangles indicate shaded areas.

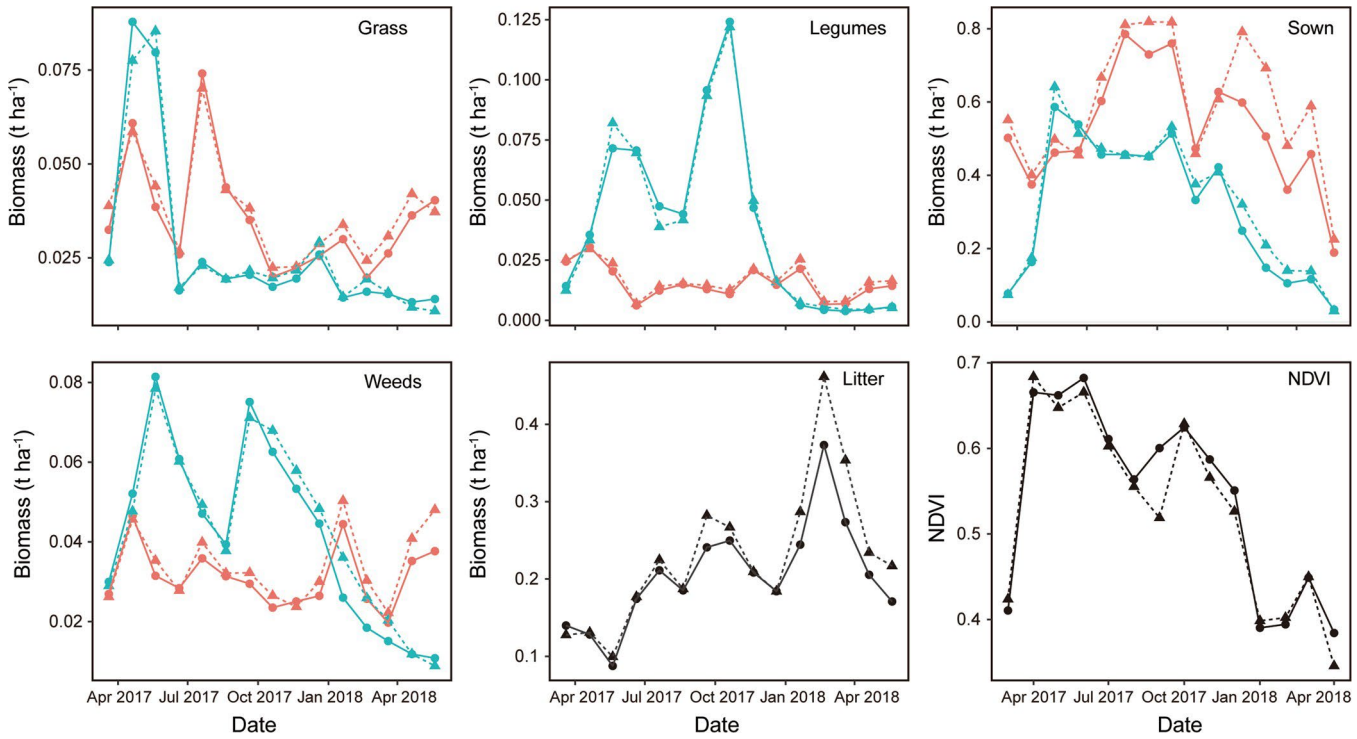

FIGURE 3 Monthly biomass (t ha^{-1}) predictions and normalised difference vegetation index (NDVI) values in paddocks for both high ($n = 3$) and low ($n = 3$) stocking rate (SR) treatments for grass (top left), legumes (top middle), sown grasses (top right), weeds (bottom left), litter (bottom middle) and the NDVI (bottom right). high SR, solid circles with solid lines; low SR, solid triangles with dashed line; brown biomass, red-coloured icons; green biomass, green-coloured icons.

proxy for pasture quality, and litter biomass were quite similar between the stocking rates (Figure 3e,f). Grass (~ 0.1 t ha^{-1}) and weed (~ 0.08 t ha^{-1}) biomasses were the highest during autumn, sown grass (~ 0.8 t ha^{-1}) during winter and legumes (0.125 t ha^{-1}) during the spring of 2017 (Figure 3).

Livestock and maintenance

For the duration of the trial, twenty-four 4-year-old Merino wethers were sourced from a DPI flock (approved by The University of Sydney Ethics Committee [2016/1098] and the DPI Ethics Committee [ORA 11/14/014]). Fifteen wethers were selected for the HSR treatment, before being divided evenly into the three replicate paddocks. Similarly, nine animals were randomly selected and split evenly into three replicate paddocks for the low LSR treatment. Wether weights averaged 73.2 ± 6.6 kg at the start of the study and 74.8 ± 7.7 kg at the end of the study and had an average body condition score of three across both treatments. The animals were estimated to have a DSE feed requirement of 1.1 DSE. Both treatments were continuously grazed for the duration of the study. Standard animal husbandry procedures were undertaken over the course of the study including being regularly weighed (between April 2017 and April 2018), drenched (in June, November 2017 and May 2018), crutched and wigged (June 2017) and shorn (December 2017) as part of regular welfare maintenance. Due to the drought that occurred during the study and subsequent poor pasture growth, wethers were fed between 250 and 900 g of supplementary feed (barley) on a total of 43 days across the study (predominantly in 2018) when pasture growth was insufficient for maintaining body weights.

GPS collar configuration, fitting and data

The UNETracker II (Trotter et al., 2009) animal tracking collars were used in this trial. Collars were randomly placed onto two sheep in each of the LSR replicate paddocks and three sheep in each of the three HSR paddocks. The weight of each collar (~ 440 g) relative to the weight of the sheep was less than 1% of the total body weight and was therefore assumed to have had minimal impact on sheep behaviour (Hulbert et al., 1998; Manning et al., 2017). Collars were configured to take one measurement (fix) approximately every 15 min from early March 2017 until the end of April 2018, when the collars were removed from the animals at the conclusion of the study. Collars were removed from animals for 3–7 days in June and December 2017 to change batteries and download data. GPS data from each collar were downloaded and the coordinate points for each fix were converted from degrees, minutes, seconds (DMS) into Universal Transverse Mercator (UTM Zone 55S) and all times were transformed into Australian Eastern Standard Time. There was no adjustment to account for daylight saving periods within the trial. Finally, as the objective of this study was to determine relationships between biomass and animal behaviour based on biomass, the GPS data were filtered to remove those days when there were no satellite estimates of

functional group biomass available, leaving a total of 78 days of observations. Of the 78 selected days, 2 days coincided with supplementary feeding and, as such, were removed from the analysis as it was evident that had impacted pasture grazing behaviours (data not shown). All analyses hereafter refer only to these data and the remaining 11.5 months of data were not used in any ongoing analysis.

Grazing windows

Data analysis was undertaken for each of the treatments (LSR, HSR) separately. As well as determining what drove sheep behaviour over the course of 24 h ('all hours'), 'annual' and 'seasonal' data sets were created to split the data for analysis. 'Annual' contained all the data from the entire 14-month period over which the study was undertaken, while 'seasonal' periods aggregated data into summer (December–February), autumn (March–May), winter (June–August) and spring (September–November) periods. Data were looked at both annually and seasonally to discern the trends between seasonal variations in climate and pasture supply.

As no data were available to determine 'actual' grazing behaviour over the day (i.e., observational data), diurnal data were used to estimate grazing windows by the sheep. Previous research has found that there are select times in which animal grazing is prominent. The most frequent hours of movement for grazing are typically the 3 h before and after both sunrise and sunset times (Cox, 2013; Hulbert et al., 1998). These periods are often referred to as diurnal grazing patterns, and often include 'camping' behaviour in the middle of the day (Ferreira et al., 2013; Lynch, 1971; Squires, 1976). For the purpose of this paper, the term 'grazing periods' is used to describe those diurnal periods of time during the day when sheep were most likely to be grazing. In addition, 'annual' and 'seasonal' data sets were also created for analysis for these grazing windows.

Statistical analysis

Random forest modelling is a highly endorsed tool in remote sensing and spatial mapping studies to predict and account for the impact of a range of variables in relation to another (Allen et al., 2013; Wiesmeier et al., 2011). The random forest models internally separate ~ 67% of the data for training and ~ 33% for validation and form an 'out of bag' (OOB) subset, similar to a cross-validation (Akter et al., 2021; Naghibi & Pourghasemi, 2015). In this paper, the largest-size data set was in the HSR annual model, with n = ~ 19 019 for calibration and n = ~ 9797 for validation, and the smallest data set belonged to the LSR spring model with n = ~ 2419 for calibration and n = ~ 1246 for validation. Mean square error (MSE) was used as an estimate of the relative importance of each variable used in the model, where variables with the lowest MSE indicated high importance. Additionally, OOB R^2 values were used to evaluate the prediction error of the random forest models produced and ranged from the overall lowest produced value in the LSR Spring model (R^2 = 0.017) to the highest in the HSR Winter model (R^2 = 0.469). While importance values are the main way in which each of the factors is ordered, relative to their importance in contributing to the target variable, the livestock residency index (LRI; a count of the number of times animals spent in a given pixel per hour), not all importance values necessarily significantly contributed to prediction. To determine the significance of each of the predictors, random forest models were permuted 50 times and their contribution to the final model was determined. In the following results section, only those factors with importance values that significantly ($p < 0.01$) contributed to the target predictor will be discussed. Partial dependence plots (PDPs) are part of the model output and are a visualisation tool to depict the effect of the selected response variable (LRI) on the predictor variables (12 other variables). Analyses were undertaken in R 4.0.3 (R Core Team, 2020) using 'Ranger' (V0.12.1; Wright et al., 2020) for the random forest modelling, and the 'pdp' (V0.7.0; Greenwell, 2018) package was used to visualise the results.

A total of 12 variables were used in the random forest analysis: near distance to water and shade (WS), near distance to fence lines (F), NDVI, minimum temperatures (MiT), maximum temperatures (MaT), rainfall (R), green grass (GG), green legumes (GL), green sown (GS), green weeds (GW) and litter (L). While total green biomass (TG), total green pasture (green biomass not including green weeds; TGP), total brown biomass (TBB) and total biomass (TB) were also considered to be included in the random forest model, due to high multicollinearity (determined using the 'car' R package; V3.0-12; Fox et al., 2021) between these 'total' variables and the other variables, we decided to remove TB, TGP and TG from the modelling process. TBB was retained as an indicator of the role that brown pasture plays in grazing.

The objective of the model was to understand what factors (social, environmental or plant functional group) were best for predicting where sheep will be in a paddock as well as to determine the greatest influential factor(s) on sheep location. Sheep location was based on each animal's LRI (calculated for each treatment replicate), and treatment replicate LRI was used as the target variable for all models, while the 12 variables mentioned previously were used as predictors in all random forest models.

RESULTS

Annual

All hours (24-h periods)

For the HSR across all hours and on an annual basis, OOB had an R^2 of 0.407 and an MSE value of 0.019. Of the 12 variables included in the final model to predict LRI, ten were significant: WS, F, MaT, MiT, NDVI, GG, GS, GW, L and TBB, with the latter five variables having a positive influence on LRI ($p < 0.01$; Table 1). Of the ten, the top three variables ranked by importance

TABLE 1 Importance values from random forest modelling for all hours and grazing hours data, both annually and seasonally (summer, autumn, winter and spring)

		Annual		Summer		Autumn		Winter		Spring	
Variable	Treatment	All hours	Grazing hours	All hours	Grazing hours	All hours	Grazing hours	All hours	Grazing hours	All hours	Grazing hours
Near distance to water and shade	HSR	0.007* (−)	0.006* (−)	0.004* (+)	0.004* (−)	0.006* (+)	0.004* (−)	0.007* (−)	0.005* (+)	0.005* (−)	0.003* (−)
	LSR	0.009*,b (−)	0.009*,b (+)	0.009*,b (+)	0.006*,c (−)	0.012*,c (−)	0.011*,c (−)	0.005*,a (−)	0.005*,a (−)	0.007*,a (−)	0.007*,a (−)
Near distance to fences	HSR	0.009* (−)	0.008* (−)	0.007* (−)	0.006* (−)	0.008* (−)	0.008* (−)	0.010*,3 (−)	0.009*,2 (−)	0.005* (−)	0.005* (−)
	LSR	0.005* (−)	0.004* (+)	0.004* (−)	0.002 (−)	0.004 (−)	0.004 (−)	0.004*,b (−)	0.004*,b (−)	0.005* (−)	0.003* (−)
Maximum temperature	HSR	0.007* (−)	0.007* (−)	0.003* (−)	0.004* (−)	0.006* (+)	0.004 (−)	0.005* (−)	0.004* (−)	0.002* (−)	0.002* (−)
	LSR	0.008* (+)	0.006* (+)	0.002 (+)	0.002 (+)	0.005 (+)	0.004* (+)	0.001 (−)	0.001 (−)	0.001 (+)	0.002 (+)
Minimum temperature	HSR	0.006* (−)	0.005* (−)	0.003* (−)	0.003* (−)	0.010* (−)	0.006 (+)	0.005* (−)	0.003* (−)	0.003* (−)	0.003* (−)
	LSR	0.007* (−)	0.006 (+)	0.003 (+)	0.003 (+)	0.007* (+)	0.005 (+)	0.001 (−)	0.001 (−)	0.002 (+)	0.002 (+)
Rainfall	HSR	0.001 (−)	0.001 (−)	0.001 (−)	0.001 (−)	0.001 (−)	0.001 (−)	0.002* (−)	0.001 (−)	0.001 (−)	0.001 (−)
	LSR	0.001 (−)	0.000 (+)	0.001 (+)	0.001 (+)	0.000 (+)	0.000 (+)	0.000 (−)	0.000 (−)	0.001 (−)	0.001 (−)
NDVI	HSR	0.013*,1 (−)	0.010*,1 (+)	0.010*,1 (−)	0.010*,1 (−)	0.015*,2 (−)	0.012*,2 (−)	0.013*,2 (−)	0.009*,3 (−)	0.008*,3 (−)	0.008*,2 (+)
	LSR	0.009c (−)	0.007 (+)	0.006 (−)	0.005 (−)	0.012b (−)	0.008 (−)	0.004 (+)	0.003 (+)	0.006*,b (+)	0.005*,b (+)
Green grass	HSR	0.008* (+)	0.007* (+)	0.007* (+)	0.007*,2 (+)	0.011* (−)	0.011* (−)	0.007* (−)	0.006* (−)	0.005* (+)	0.005* (+)
	LSR	0.005 (−)	0.004 (+)	0.005 (−)	0.005 (−)	0.007 (−)	0.007 (−)	0.003* (+)	0.002 (+)	0.004 (+)	0.003* (+)
Green legumes	HSR	0.009 (+)	0.008 (+)	0.008*,3 (+)	0.006* (+)	0.011* (−)	0.009 (+)	0.009* (−)	0.008* (−)	0.007* (+)	0.006* (+)
	LSR	0.015*,a (−)	0.013*,a (+)	0.006 (−)	0.006 (−)	0.015a (−)	0.011a (−)	0.004 (−)	0.003 (−)	0.005c (+)	0.005c (+)
Green sown grass	HSR	0.011*,2 (+)	0.009*,2 (+)	0.007* (+)	0.005* (+)	0.010 (+)	0.011* (+)	0.014*,1 (+)	0.010*,1 (+)	0.010*,1 (+)	0.008*,1 (+)
	LSR	0.007 (−)	0.006 (+)	0.005 (−)	0.005 (−)	0.010 (−)	0.008 (−)	0.003 (+)	0.003 (+)	0.004 (+)	0.004 (+)
Green weeds	HSR	0.010*,3 (+)	0.009*,3 (+)	0.008*,2 (+)	0.007*,3 (+)	0.013*,3 (+)	0.014*,1 (+)	0.009* (+)	0.007* (−)	0.009*,2 (+)	0.008*,3 (+)
	LSR	0.008 (−)	0.008c (+)	0.007* (−)	0.006*,b (−)	0.011 (−)	0.010c (−)	0.004*,c (−)	0.003c (−)	0.004 (+)	0.004* (+)
Litter	HSR	0.009* (+)	0.007* (−)	0.008* (+)	0.006* (+)	0.015*,2 (+)	0.011*,3 (+)	0.006* (−)	0.006* (−)	0.006* (+)	0.006* (−)
	LSR	0.006* (+)	0.005* (+)	0.010*,a (+)	0.010*,a (+)	0.011* (+)	0.009* (+)	0.002 (−)	0.002 (−)	0.003 (−)	0.004 (−)
Total brown biomass	HSR	0.007* (+)	0.006* (+)	0.007* (+)	0.005* (+)	0.006* (+)	0.007* (+)	0.008* (−)	0.006* (−)	0.007* (+)	0.006* (+)
	LSR	0.007* (+)	0.005 (+)	0.007*,c (+)	0.006* (+)	0.011* (+)	0.008* (+)	0.002* (+)	0.003 (+)	0.004 (−)	0.003 (+)

Note: Cells with an asterisk (*) indicate a significant p value (<0.01), and superscript values (1, 2 and 3 represent HSR; a, b and c represent LSR) indicate the top three important variables. Brackets indicate whether the variable was positively (+) or negatively (−) correlated with LRI, based on a simple correlation matrix (data not shown).

Abbreviations: HSR, high stocking rate; LRI, livestock residency index; LSR, low stocking rate; NDVI, normalised difference vegetation index.

were NDVI, GS and GW (Table 1). Common significant trends during this period suggest that animals spent more time further away from water and shade locations (40–60 m away), but closer to fence lines (0–10 m) in the HSR (Figure 4a,b). General pasture trends suggested that when more than 0.1 t ha^{-1} of each pasture variable (GG, GS, GW, L and TBB) was present, this had a much greater influence on animal location (Figure 5f–j). While NDVI was both significant and ranked as the most important variable in the model, its importance was limited to when NDVI values were between 0.3 and 0.5 or > 0.8 (Figure 4e). Temperature also played an important role in that when temperatures were between −5°C and 5°C, wethers spent more time together, whereas at higher temperatures, there was a decrease in grouping behaviour (Figure 5c,d).

In contrast to the HSR, the LSR had OOB R^2 of 0.356 and an MSE value of 0.022. Seven variables were significant: WS, F, MaT, MiT, GL, L and TBB, but only three (MaT, L and TBB) had a positive influence on LRI (Table 1). In general, higher values of residency occurred while animals were close to both fences and water and shade locations (0–10 m), but also when animals were between 60 and 80 m away from water and shade points (Figure 4k,l). Similar to the HSR, pasture trends for GL, L and TBB suggested that as biomass increased above 0.1 t ha^{-1}, LRI also increased (Figure 4o–q). As for HSR, temperature was also important, with MaT of increasing importance of LRI when temperatures were > 25°C, while MiT had a greater influence on LRI when temperatures were > ~ 8°C (Figure 5m,n).

Grazing hours

Models were produced to look at trends that occurred within hours of expected grazing. For the HSR, the model produced an OOB R^2 value of 0.404 and an MSE of 0.018. Of the 12 factors included in the model, 10 were of significance (WS, F, MaT, MiT, NDVI, GG, GS, GW, L and TBB; Table 1). Of these significant variables, five had a positive influence on LRI (NDVI, GG, GS, GW and TBB) and five had a negative influence on LRI (WS, F, MaT, MiT and L), with the top three important variables being NDVI, GS and GW (Table 1). Common trends within this period indicated that wethers spent more time further away from water and shade locations (> 50 m), as well as spending time within the first 10 m from a fence line

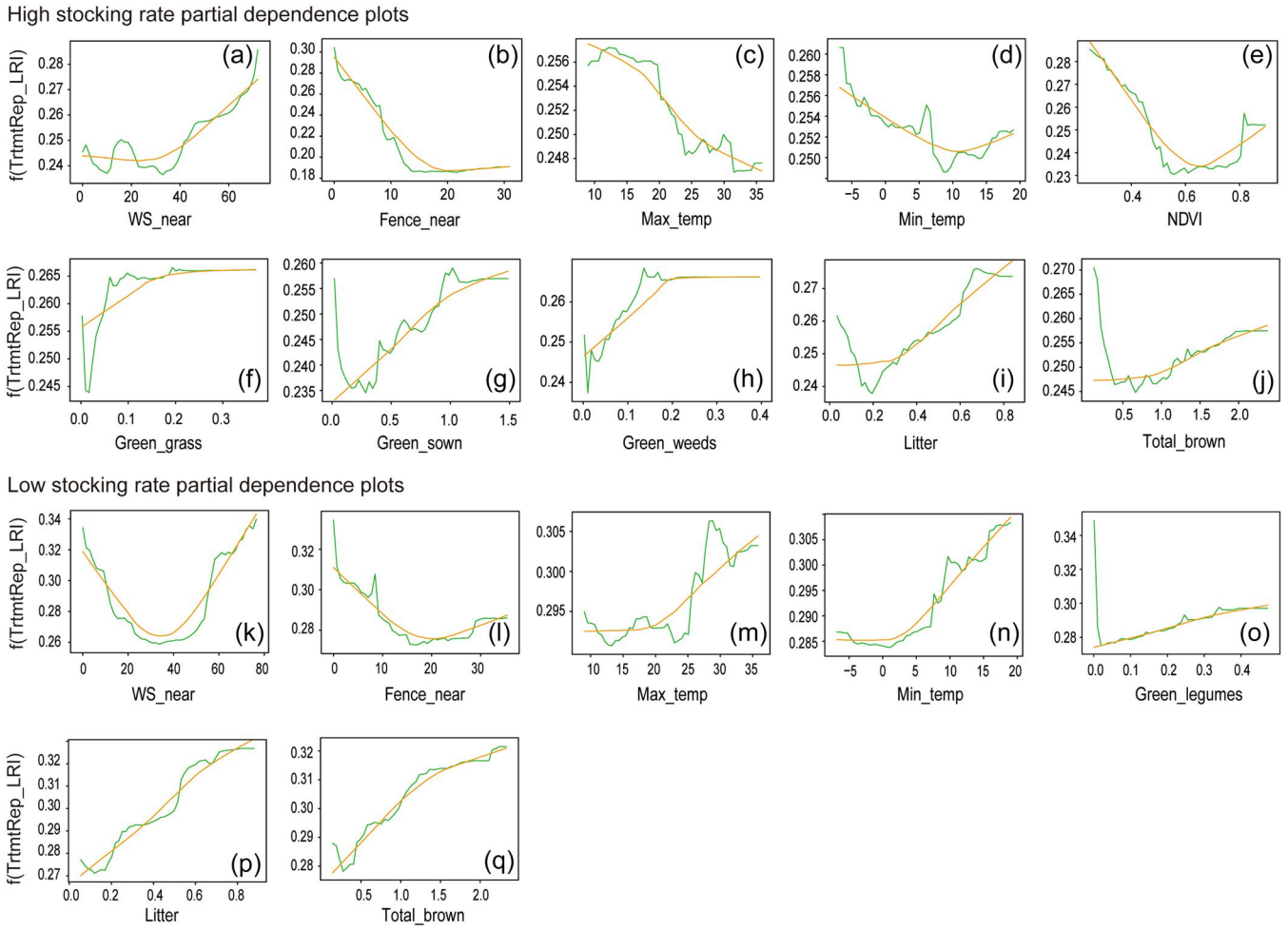

FIGURE 4 Partial dependence plots for significant factors ($p < 0.01$) in annual random forest models produced for the (a)–(j) HSR and (k)–(q) LSR, for 24-h periods. Green lines in each figure represent the data, while the light orange line depicts the trendline. Values on the *x*-axis reflect the variables chosen in the model, while the *y*-axis represents the predicted livestock residency for the treatment. HSR, high stocking rate; LRI, livestock residency index; LSR, low stocking rate; NDVI, normalised difference vegetation index; WS, water and shade.

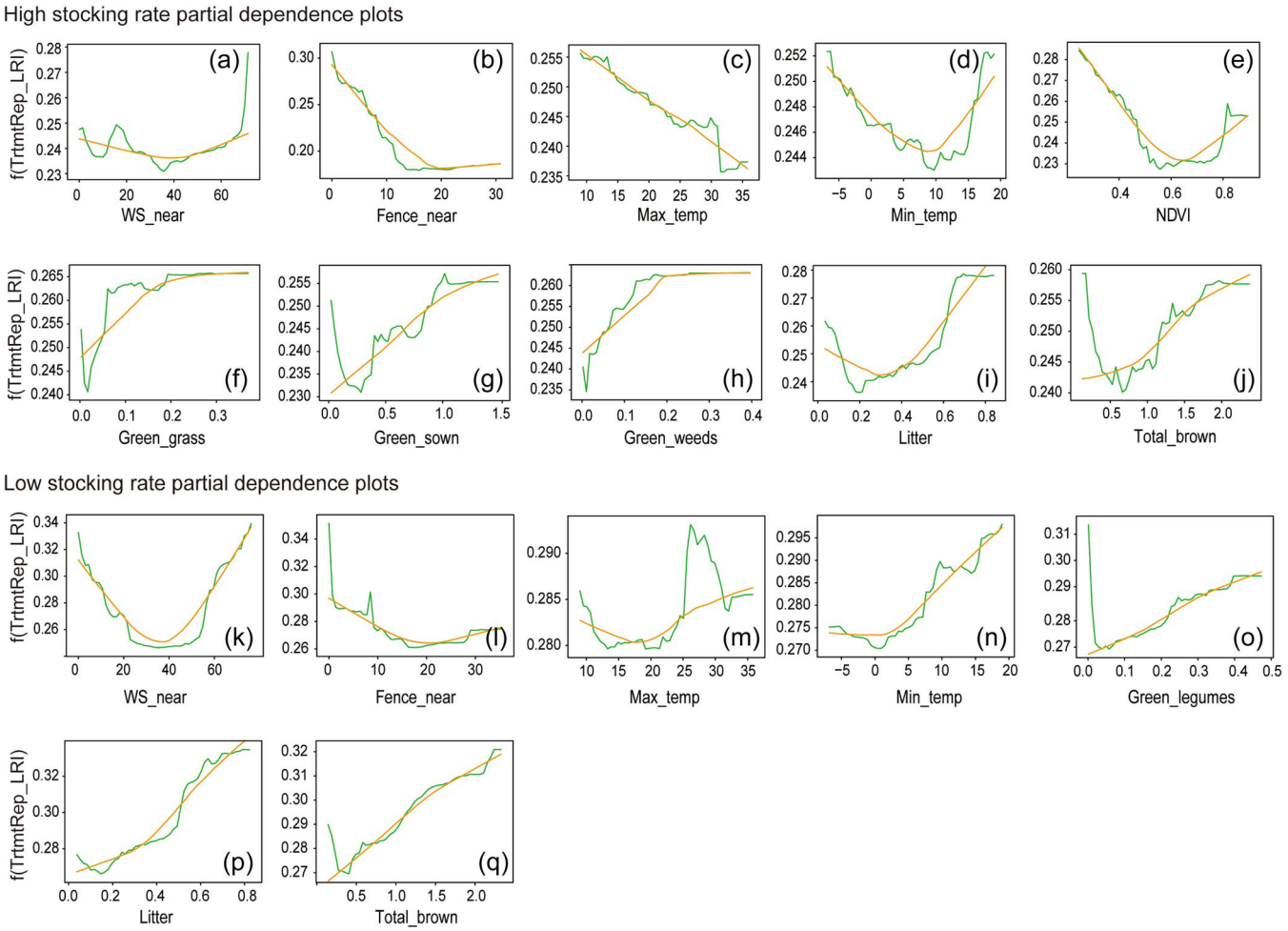

FIGURE 5 Partial dependence plots for significant factors ($p < 0.01$) in annual random forest models produced for the (a) – (j) HSR, panels and (k) – (q) LSR, for grazing hours only. Green lines in each figure represent the data, while the light orange line depicts the trendline. Values on the *x*-axis reflect the variables chosen in the model, while the *y*-axis represents the predicted livestock residency for the treatment. HSR, high stocking rate; LRI, livestock residency index; LSR, low stocking rate; WS, water and shade.

(Figure 5a,b). Similar to its 24-h period model, pasture trends suggested that there was higher LRI when available biomass was > 0.1 t ha^{-1} for GG and GW, > 0.5 t ha^{-1} for GS and L and > 1.5 t ha^{-1} for TBB (Figure 5f–j). As found previously, NDVI was a significant determinant of LRI at both lower (0.3) and higher (0.8) values (Figure 5e), and likewise, temperature was also an important driver of LRI (Figure 5c,d).

For the LSR, models produced an OOB R^2 value of 0.388 and an MSE value of 0.018. Five factors were of significance (WS, F, MaT, GL and L), all with a positive influence on LRI, with GL and WS being the top two most important variables (Table 1). Although not statistically significant, GW was the third top important variable (Table 1). During grazing hours, sheep spent much of their time at distances of 40–60 m away from water and shade locations. Similar to the 24-h model, higher residency was observed near fence lines (0–10 m) (Figure 5k,l). It was generally observed that when there was > 0.1 t ha^{-1} GL biomass available, there would be higher residency and this held true for TBB (> 1 t ha^{-1}) and L (> 0.4 t ha^{-1}) (Figure 5o–q). Temperature-based trends were similar to the 24-h period models for the LSR (Figure 5m,n).

Seasonal

All hours (24-h periods)

Models were further split into seasons to understand if there would be a seasonal effect to grazing patterns across the study years. Model performance varied across each of the seasons and for each of the treatments, ranging from an OOB R^2 value of 0.386 to 0.475 for the HSR, and 0.298 to 0.381 for the LSR. An OOB MSE range of 0.017–0.021 was produced for the HSR and 0.015–0.027 for the LSR. All HSR models had NDVI as one of the top three important variables regardless of the season, closely followed by GW, GS or GL (Table 1). Near distance to F was in the top three important variables during winter. Interestingly, however, NDVI was only in the top during autumn and spring for the LSR, and WS was in the top regardless of the season (Table 1). GL was the next most prevalent variable for the LSR in terms of importance.

Near distance to WS was significant for all seasons, regardless of treatment, but had a positive influence during summer and autumn for the HSR, as well as during summer and winter for the LSR (Table 1). Distance to F was always significant, except for the LSR

during autumn (Table 1). For the HSR, F was always negatively correlated with LRI, while for the LSR, when it was significant, it was only a positive influence on LRI during winter. Weather-based variables (MaT, MiT and R) had a varying influence on both treatments. Minimum temperature was the only variable that was a significant predictor on LRI and was a positive influence on LRI for the LSR during autumn, suggesting that seasonal models account for greater variability than annual models (Table 1). In contrast, weather had more of an impact on animals in the HSR instead, with R being significant and having a negative influence on LRI during winter. Similarly, MiT was significant in all seasons and consistently had a negative influence on LRI; similarly, MaT was significant, but was only a positive influence on LRI during autumn and spring (Table 1). Pasture variables such as GW and GS consistently had a positive influence on LRI when they were significant for the HSR (Table 1). Across all seasons, both GG and GL were significant; however, there were positive relationships between them and LRI in summer and spring, but with a negative effect on LRI during autumn and winter (Table 1). Importance values for both L and TBB were also significant regardless of the season and only had a negative influence during winter for the HSR (Table 1). In contrast, pasture variables such as GL and GS, which might reasonably be expected to be positively linked to LRI, were not often significant for the LSR, despite often having high importance values (Table 1). Both TBB and L were significant, positive influences on LRI only during summer and autumn, and GW was significant during summer (negative influence) and winter (positive influence). Additionally, for the LSR, NDVI was a significant positive influence during spring and GG was a significant negative influence during winter (Table 1).

PDPs for structural features (WS and F) were similar across all seasons and treatments, with livestock in the HSR predominantly spending more time away from WS, around 40 m or more away, and within the first 10 m of a fence line (Supporting Information: Figures S1–S4), and LSR spending more time within 5 m of F, but 60 m away from WS. During winter, livestock in the HSR spent time within 10 m of both WS and F, while animals in the LSR spent more time between the first 10 m for F and WS, although additionally, some LSR animals spent > 60 m away from WS during this season (Supporting Information: Figures S1–S4). Weather variables were only significant for the LSR during autumn, in which PDPs produced saw higher residency when temperatures were higher (10–15°C), rather than lower as seen in previous PDPs when weather was not significant (Supporting Information: Figures S1–S4). The HSR, in contrast, saw more grouping together when MaT was between 10°C and 15°C and a decrease in grouping when temperatures were higher and increased mobbing (i.e., increased LRI) when MiT was < 5°C during summer and autumn and < 0°C during winter and spring (Supporting Information: Figures S1–S4).

For the most part, as biomass values increased, so too did animal LRI regardless of the biomass amount available in either treatment (Supporting Information: Figures S1–S4). Similar trends for LSR to their annual counterparts for GW, L and TBB were also observed across all seasons (where significant). During winter, the LSR also saw an increase in residency when there was more GG present (Supporting Information: Figure S3o). NDVI was only significant during spring for the LSR and it was found that high LRI values were evident when NDVI was > 0.6 (Supporting Information: Figure S4n). The trends shown in the PDP for NDVI in the HSR varied with the seasons, with high residency often observed when NDVI was low (0.3) and low residency observed when values were > 0.4, except for in spring, when LRI was the highest for the HSR when NDVI was 0.4–0.5 and 0.8 (Supporting Information: Figure S4e). Generally, in the HSR, as variables such as GG, GL, GW, L and TBB increased, so did residency across the seasons, predominantly during summer and spring (Supporting Information: Figures S1–S4). However, at certain times of the year, there were specific variables that had a higher influence on LRI than others. The presence of GL during autumn was most influential when there was either almost nothing available, or more than 0.2 t ha^{-1}, L when there was > 0.6 t ha^{-1} and TBB when there was only 0–0.2 t ha^{-1}, suggesting that for this season, low levels of brown biomass were driving grazing (Supporting Information: Figure S3g,j,k). Interestingly, GG in autumn and GW in autumn and winter were highly influential on LRI regardless of how much was available (Supporting Information: Figures S3 and S4).

Grazing hours

Models were also produced for both HSR and LSR treatments seasonally for the aforementioned grazing hours (3 h before and after both sunrise and sunset). Models produced a range of OOB R^2 values ranging from 0.376 to 0.469 for the HSR and from 0.326 to 0.398 for the LSR. The MSE values produced for each stocking rate had a range from 0.015 to 0.021 for the HSR and from 0.013 to 0.023 for the LSR. Similar to the 24-h period models produced seasonally, NDVI was within the top three most important variables each season for the HSR, but only during spring in the LSR (Table 1). Green weeds and GS were among the most important variables for the HSR, while L and F were also important during autumn and winter (Table 1). WS was one of the top three most important variables for the LSR across all seasons, followed by GW in all seasons, except for spring, and GL in autumn and spring.

Structural features of significance were significant for all seasons and for both the HSR and the LSR (Table 1). Distance from WS showed a positive relationship with LRI for the HSR in all seasons, except for spring, and F was a positive influence on LRI only during summer and autumn. In contrast, WS was a negative influence on LRI for all seasons in the LSR, and F was a negative influence when it was significant (winter and spring) in the LSR (Table 1). Weather-based variables such as MaT were only significant in the LSR during autumn, when it was positively associated with LRI (Table 1). Both MaT and MiT had a significant positive influence on LRI for the HSR for summer and a negative influence for winter and

spring (Table 1). Pasture variables of significance during grazing hours for the LSR were often only TBB and L during summer and autumn, and often had a positive influence on LRI (Table 1). GW was another significant variable for the LSR during both summer and spring; however, it was a negative influence on LRI during summer and a positive influence during spring. There was a large difference in the role of NDVI as a predictor of LRI between treatments as it was a positive influence across all seasons for the HSR and only negative during winter, whereas NDVI was not a significant predictor for LSR, except in winter, when it was negatively correlated. All other pasture variables (GG, GL, GS, GW, L and TBB) were often negative influences on LRI in the HSR, except for spring, when all were significant positive influences on LRI, and L was negative (Table 1).

Structural feature (WS) showed similar trends to those previously produced. High LRI was often found when animals were between 0 and 10 m from F, 0 and 10 m away from and > 40 m away from WS, regardless of the season for the HSR. During winter, there was variable residency along WS for the HSR, with LRI being the highest at 0, 20 and 30 m away from them (Supporting Information: Figure S7a). Trends were similar for the LSR; however, rather than > 40 m away, high residency was also seen when animals were > 60 m away (Supporting Information: Figures S5–S8). Weather variables varied across the seasons; however, for the LSR, it was only significant during autumn and evidently, the highest residency in areas was often when it was 25°C (Supporting Information: Figure S6j). Temperature-based PDPs for the HSR, however, had similar trends to their 24-h period counterparts, whereby high residency for MaT was often when temperatures were lower, and when MiT was at its lowest, there was highest residency (Supporting Information: Figure S5–S8). When pasture-based variables were of significance in both treatments, trends were similar to their 24-h model counterparts, where there was an increase in residency when there was also an increase in biomass (> 0.05 t ha^{-1}), as well as when NDVI was between 0.2 and 0.4 (Supporting Information: Figure S5–S8). It is noteworthy, however, that during winter, GL and GG in the HSR saw a decrease in LRI as biomass values were at 0.1 and 0.04 t ha^{-1}, before slowly increasing again (Supporting Information: Figure S7f,g).

DISCUSSION

Drivers of sheep behaviour

Social-driven behaviours and implications

Although pasture greenness or the legume functional group was often significant in predicting where animals were located, in both treatments and during different seasons, there were other clear drivers of grazing behaviours shown in this study than just pasture. It was evident that regardless of the stocking rate, infrastructures such as water, shade and fence lines were frequently found to be important in predicting where animals were located regardless of whether the predictions were based on seasonal or annual, 24-h periods or grazing periods. There was clearly a very strong effect of F on sheep location. Although this was likely driven by the small paddock sizes (0.5 ha) used in this trial (and thus sheep were never further than 10 m away from a fence), it is reasonable to assume that mob behaviour was also a large driving factor, particularly for the LSR treatment. It was common for sheep location to be predominantly driven by proximity to areas where other sheep were present. Areas where LRI was greatest were often seen in the corners of the paddocks where they came into close contact with one or more mobs of sheep present in other treatments. This often occured when a LSR paddock boundary was shared with a HSR paddock, and the grouping effect was not likely to be associated with grazing (Figure 2). This was likely a 'camping' area for sheep of both paddocks. Previous studies have described camping location of an individual to be areas where they have spent majority of their time, often with other animals, usually within high points of paddocks (Bowens, 1971; Gillingham & During, 1973; Grubb & Jewell, 1974; Mottershead et al., 1982; J. A. Taylor et al., 1984). However, camping behaviour has also been referred to as areas where sheep either cease grazing (Arnold & Dudzinski, 1978) or an area where sheep graze in certain periods during the day (J. A. Taylor et al., 1987), often where shade is located (D. B. Taylor et al., 2011). With the latter, the likelihood of this camping behaviour occurring is reduced in this study, due to the small paddock sizes. It was evident that the inherent social factor dominates over all other factors determining camping location.

Although mobbing effects were still apparent in the HSR with a strong presence around fence lines, water and shade locations, both annually and seasonally, they were not as prevalent as they were for animals in the LSR. On an annual basis, animals in the LSR over a 24-h period would be observed within 5 m of a fence (Figure 4), while for the HSR, distance to fence lines was only significant ($p < 0.01$) if they were right at the fence line. Comparatively, during only grazing hours, animals in the LSR were still much more likely to be found within 5 m of fence lines, whereas the HSR were generally located at greater distances (up to 10 m) away from fences (Figure 5). This suggests that annually, during expected grazing hours, animals in the HSR were searching for food at times, while animals in the LSR often stayed near fence lines, regardless of the time (grazing hours vs. all hours). Although dusk is considered the most important and is one of the longest periods for forage consumption, it is believed to have direct influences on animal behavioural patterns, such as selectivity of pasture (Burritt et al., 2005; Gregorini, 2012). Based on previous studies, sheep have been identified to have been grazing on forage, which will sustain them overnight (Gregorini, 2012). During the grazing periods, sheep in both stocking rates were often found in areas of pasture where legumes were present. This is unsurprising due to the known preferences that sheep have for legumes due to greater palatability, crude protein and metabolisable energy (Parsons & Dumont, 2003). Although not explicitly investigated during this study, it is suggested that future studies compare the potential

differences in drivers of grazing behaviours across dawn and dusk periods, separately, as this trend may also highlight key differences between grazing and camping behaviours.

Previous studies have found that mob behaviour will significantly affect areas grazed and is an influential factor that needs to be considered regarding the underlying reasons that determine where sheep are found (Dumont & Boissy, 2000; Michelena et al., 2009; Squires, 1976). This relationship was consistently found across both treatments and all seasons but was more apparent in the LSR treatment. While it is likely that mob behaviour was the predominant driving force behind sheep location in general, overlain on this is the influence of individual animal behaviour. The unique behaviours shown by individual sheep and their willingness to move away ('boldness') or stay within ('shyness') a mob mentality mindset are key to understanding the differences that could have occurred between the two stocking rates, as highlighted in previous studies (Dumont & Boissy, 2000; Michelena et al., 2009; Squires, 1976).

Pasture-driven behaviours and implications

While mob behaviour played a significant role in determining sheep location, pasture attributes also played a key role. Annual models predicted that animals in the HSR would spend time in areas with large amounts of green grass, while those in the LSR spent more time in areas with greater amounts green legumes. This suggests that a commonly known behaviour, satiety theory, is occurring here with green legumes. This is where animals show a preference for legumes compared to other grasses and will adjust their daily grazing substantially to respond to various dietary choices and nutritional needs (Chapman et al., 2007; Curll & Wilkins, 1982; Parsons & Dumont, 2003). However, it is noteworthy that importance values for green legumes were generally greater and more spatially diverse in the LSR annually and during autumn and spring, but only in summer for the HSR (Table 1). Despite the overall amount of legumes on offer in comparison to other available feed (Figure 3), both treatments saw a significant decline in the level of importance that it had when there were little to none on offer.

Additionally, there is a considerable amount of selectivity by grazed animals here, due to the variation amongst the species of grass present across the study year. As can be seen in Figure 3, sown grasses, followed by litter were the most available in both treatments. Litter may have only been significant for most models as high residency in certain areas will increase the amount of litter present in other locations (Figures 4 and 5). Bimodal patch states could explain this trend, with animals preferring to graze short- or medium-length grass (Gibb et al., 1997), instead of moving to areas where more grass growth is apparent (i.e., opposing areas of the paddock), and as such, increasing the amount of litter present in other areas. With the high importance and significance of litter for most models (Table 1) and at various times of the year, total brown biomass, it is evident that patch grazing can influence mobbing behaviours, as discussed previously.

Preference for actively growing grasses is a known driver in grazing, due to the sensory experience of the feed and improved nutritional quality (Baumont et al., 2004; Edwards et al., 1994). In this study, NDVI was used as a proxy for pasture quality and was identified as a factor of significance both annually and seasonally in the HSR, but only significant in spring for the LSR (Table 1). Regardless of the period (annual or seasonal) or time (all hours or grazing hours), NDVI was either the top or within the top three most important variables annually for both treatments, as well as in each season for the HSR, but only autumn and spring for the LSR (Table 1). This suggests that regardless of whether it was a significant variable or not, it was still important within models to predict animal residency in areas. Both annually and seasonally, and during 24-h periods and grazing periods, NDVI was associated with high residency when there were values of 0.3–0.4, but were also seen when values were 0.6–0.7 (Figures 4e and 5e). This suggests that even though animals were often found in areas where NDVI was 0.3–0.4, if values were higher in other areas, they would often move to select higher-quality feed (Thomas et al., 2021). For example, there was slightly higher residency by animals when there was an NDVI value of 0.6–0.7 during grazing hours in contrast to models looking at 24-h periods, so it is therefore assumed that animals here were actively seeking pasture with higher quality/greenness during these hours. It is important to note that sheep may not have had access to pasture with high NDVI values frequently, as they may not have been available for prolonged periods of time.

Overall implications for pasture management

It is evident that the grazing trends by wethers at this location may have had a huge effect on pasture growth across the paddocks, aside from the usual selectivity that sheep show on mixed pastures as mentioned previously (Baumont et al., 2004; Chapman et al., 2007; Curll & Wilkins, 1982; Edwards et al., 1994; Gibb et al., 1997; Parsons & Dumont, 2003). The grouped behaviour shown predominantly by the LSR stocking rate, although largely influenced by the low stocking rate, created reduced usage of the remainder of the paddock. Long term, this mobbing behaviour can have an impact on the overall production and productivity of pasture due to constant overgrazing, which can lead to and was reflected in the commonly observed positive correlation in this study, between LRI and increasing amounts of brown biomass and litter. This can imply that where sheep were spending substantial amounts of time, a general decrease in productivity and fowling of the pasture were caused due to the increased presence of the animals (i.e., damage from impact trampling) (Morris & Reich, 2013). This in turn may also have an impact over time on pasture sustainability and overall soil health over the long term (Condron et al., 2014; McCarthy et al., 2016; Sharp et al., 2012).

Limitations of the study and future directions

There were limitations to the study, as apparent with research. Paddock size and the stocking rates of the high and low treatments were limitations to understanding large grazing herds, despite them being appropriate for the seasonal conditions at the time. The authors suggest that this study be investigated at a larger scale to remove the inherent impact that infrastructure and mobbing behaviour have on results. An intense drought period coincided with 4 months of the study, and the drought for the remaining 7 months may have had an impact on the overall pasture production and, as a consequence, the drivers of animal location during this time. Although temperature and rainfall variables were included in models to understand the impact they have on animal grazing and location behaviours, and not on pasture, it is important to note that a large rainfall event before the commencement of this study would have increased the level of 'greenness' on NDVI and so too, available pasture (Figures 1 and 3).

CONCLUSIONS

The prevalence of fence lines, water troughs and shade significantly influenced expected grazing behaviours by animals, regardless of the time (expected grazing hours vs. 24 h in a day), or period of study (annually vs. seasonally) and stocking rates. In this study, mobbing behaviours appeared to far outweigh the grazing needs of a sheep at a scale as small as this study. As such, the inherently strong social behaviours shown by wethers seem to be primary drivers of behaviour as indicated by their distance to other sheep in neighbouring paddocks and, as such, our hypothesis that pasture attributes would be the primary driver of sheep location was not found to be true. Despite this, it was evident that the presence of legumes had a strong impact on residency by animals, even if legumes were either present for a short period or in low amounts. Generally, as biomass values increased > 0.1 t ha^{-1}, so too did residency by wethers. Future studies investigating grazing behaviours shown by animals should utilise larger paddocks and also larger flocks to determine if trends are similar on a larger scale. Understanding how pasture type can influence grazing behaviours and also how animal behaviour affects pasture performance is important in developing long-term sustainable management strategies on a paddock scale.

AUTHOR CONTRIBUTIONS

Danica Parnell: Formal analysis; investigation; methodology; resources; writing – original draft, writing – review and editing. Igor Kardailsky: Data curation; software; writing – review and editing. Jacob Parnell: Resources; software; writing – review and editing. Warwick Brabazon Badgery: Conceptualization; resources; supervision; writing – review and editing. Lachlan Ingram: Data curation; formal analysis; funding acquisition; investigation; methodology; resources; supervision; writing – review and editing.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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Chapter 6:
Exploring 'wether' grazing patterns differed in native or introduced pastures in the Monaro region of Australia

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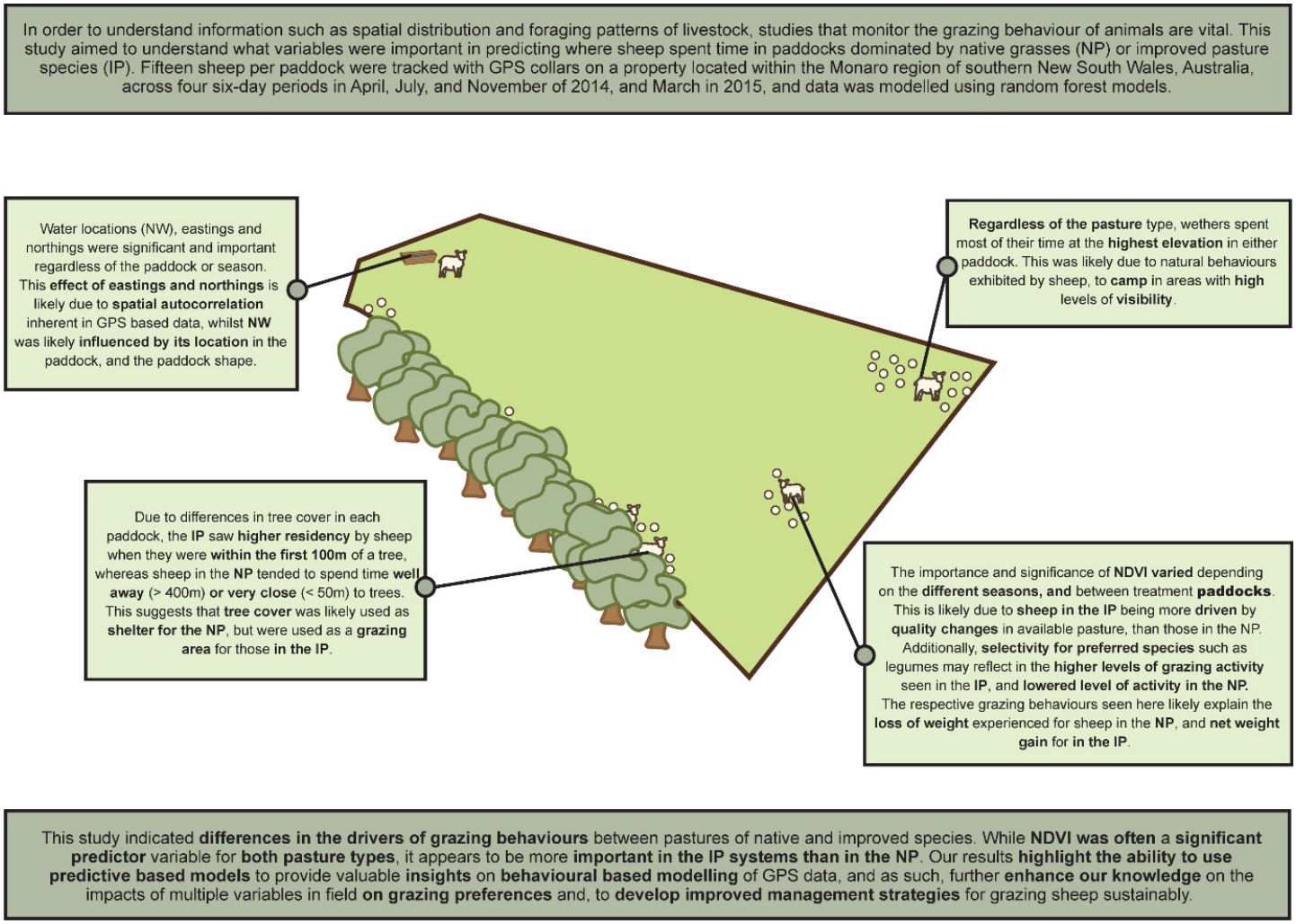

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Article

Exploring 'Wether' Grazing Patterns Differed in Native or Introduced Pastures in the Monaro Region of Australia

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Simple Summary: Understanding how and why livestock choose to graze on pasture can provide valuable information to farmers, such as overall pasture use, foraging patterns, and behavior trends. This information may be used to further assist farmers in making management decisions on how to increase production. This study specifically aimed to understand what the important drivers of grazing were, such as landscape attributes (pasture quality, distance to trees, distance to fences, distance to water) and climatic variables (rainfall, temperature), in paddocks comprising either native or improved/introduced grass species, in the Monaro region of SE Australia. Pasture quality was a highly important and a significant variable, along with temperature and elevation for the improved pasture paddock, in contrast to the native paddock, where in addition to elevation and temperature, near distance to trees was more important in determining sheep location. Results from research such as this can provide valuable insights into various grazing behaviors of sheep and also assist in shaping the sustainable management of sheep.

Abstract: Monitoring livestock allows insights to graziers on valuable information such as spatial distribution, foraging patterns, and animal behavior, which can significantly improve the management of livestock for optimal production. This study aimed to understand what potential variables are significant for predicting where sheep spent the most time in native (NP) and improved (IP) paddocks. Wethers (castrated male sheep) were tracked using Global Positioning System (GPS) collars on 15 sheep in the IP and 15 in the NP, respectively, on a property located in the Monaro region of Southern New South Wales, Australia. Trials were performed over four six-day periods in April, July, and November of 2014 and March in 2015. Data were analyzed to understand various trends that may have occurred during different seasons, using random forest models (RFMs). Of the factors investigated, Normalized Difference Vegetation Index (NDVI) was significant ($p < 0.01$) and highly important for wethers in the IP, but not the NP, suggesting that quality of pasture was key for wethers in the IP. Elevation, temperature, and near distance to trees were important and significant for predicting residency of wethers in the IP, as well as the NP. The result of this study highlights the ability of predictive models to provide insights on behavior-based modelling of GPS data and further enhance current knowledge about location-based choices of sheep on paddocks.

Keywords: grazing behavior; sheep; pasture management; small ruminant management

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1. Introduction

With advancements in tracking technology for livestock, extensive animal production systems have the ability to compile uninterrupted data over a range of environmental conditions and allow us to better understand both foraging patterns/behaviors and spatial distribution of animals [1]. Combining this technology simultaneously with pasture-based data, insights can be gleaned that can provide a better understanding of paddock utilization and behavioral interactions [2–4]. Access to these technologies is more readily available

and can reduce the need for observational data, which are quite intensive in nature due to the demand on labor and range of hours required [5–8]. As such, detailed behavioral studies with this technology can assist in further refining the way sheep are managed, with increased knowledge being available on grazing distribution patterns and the influence of various behavioral attributes. For example, social interactions are known to have a large influence on how sheep graze [9]. Sheep may choose to graze non-preferred pasture species to stay close together [9–11]. When in larger herd sizes, sheep have been reported to form subgroups of up to five or six sheep [12] or to leave the main flock if they are followed by other animals [10,13]. Furthermore, there are reported individual personalities which sheep can exhibit that will overall influence grazing choices (i.e., choosing poorer forage and revisiting areas versus breaking off from the herd for better-quality forage) [13].

Aside from the aforementioned livestock factors, environmental factors are also known to influence grazing behaviors, such as time of day and climate. Sheep have been reported to graze just prior to daybreak, often camp during the middle of the day, then graze once again in the late afternoon [12,14,15]. This grazing pattern is also often referred to as diurnal grazing behavior [9]. The grazing around the dusk period that occurs often has a longer observed grazing window. It has been hypothesized that the reason for this is that sheep consume forage, which is higher in palatability to fill the rumen for longer overnight [16–18]. Weather variables such as temperature may also have an impact on grazing behaviors. Ewes were reported to visit watering points and have reduced grazing times when temperatures were higher, whereas on cooler days, they grazed more frequently [19]. Wethers in contrast were found to spend more time grouped together at lower temperatures and were less grouped when temperatures reached above 25 °C on high stocking rates [9]. Pasture availability is another known variable which influences grazing patterns exhibited. Sheep may selectively graze areas in paddocks purely based on the forage availability, with preference for species such as legumes, until that particular feed is either restricted or limited [3,12,17,18].

Pasture systems in Australia are composed of native, improved, or mixed pasture systems, with native pastures being more predominant than the others [20]. Though native pastures have the ability to provide reliable feed during all seasons due to their hardiness and ability to thrive when water and nutrients are limited, improved pastures offer higher quality and quantity of feed [21,22]. Improved pastures have higher levels of productivity and better responses to fertilization, which largely contrasts with lower-production native pastures found on poorer-quality soils that often receive lower rates of fertilizer [21,22]. Native grasslands often contain a higher proportion of C4 (summer active) grasses, which are usually of lower quality and produce less biomass than the C3 (spring active) grasses that are more commonly found in improved pastures. These C3 grasses have maximal growth rates in spring and potentially a smaller amount of growth in autumn and late spring; however, they can also be largely impacted by climate and weather. Therefore, though improved pastures are the preferred pasture option due to the larger quantity and greater quality of forage they provide, they must be effectively managed in these cultivated lands for livestock to reap the benefits.

The overall objective of this study was to determine which variables were best for predicting sheep location in paddocks of native and improved pastures in the Monaro region using global positioning system (GPS) data. In addition, both observational data and accelerometer data were used for additional information to the GPS data to predict grazing periods throughout the day. Our hypothesis is that animals here will seek out areas with higher Normalized Difference Vegetation Index (NDVI) values regardless of the pasture type (evident through higher residency and importance value). It is also assumed that wethers may spend more time grazing in the native pastures than the improved pastures due to the quality disparity between both pasture types. Pasture quality may therein play a larger role in grazing behavior than environmental and social behaviors. With a better understanding on how these interactions occur, this research could not only aid in better

management of both sheep and pasture in the Monaro region of Australia, but also improve current knowledge about prediction-based modelling for grazing using GPS data.

2. Materials and Methods

This research was performed on a Merino wool-based enterprise, 'Coolringdon' (36.28° S, 148.98° E), in the Monaro region of southern NSW. Research was conducted over four six-day periods in April (T1; autumn), July (T2; winter), and November (T3; spring) of 2014 and March (T4; summer/autumn) of 2015, with the aim to encapsulate behavior across all seasons. The Monaro region is often characterized by low ambient temperatures, high wind chill, and severe frosts, all which are common from April to September. Annually, the region receives on average 540 mm of rain, with a summer dominant pattern in 2014 [23]. Prior to the initiation of this study, there was a long summer drought, which was followed by heavier-than-usual rainfall during the study (Figure 1). It is important to highlight, however, that due to this unusual pattern of rainfall in 2014, pasture growth was much greater over the duration of the study than what may be normally expected.

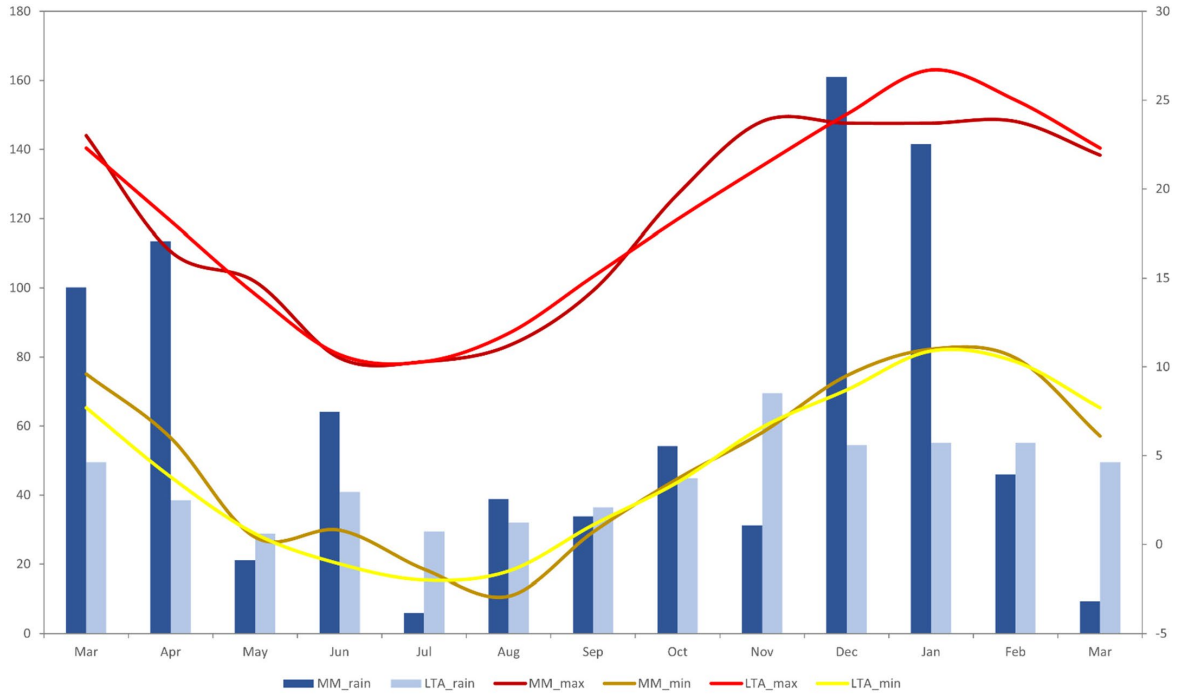

Figure 1. Comparison of long-term average temperature and rainfall data (1976–2023) and monthly average temperature and rainfall data (2014–2015) for the Coolringdon site over the period of the study [23]. MM = mean monthly; LTA = long-term average.

Two paddocks were chosen that were within close proximity (<100 m) of each other (Figure 2) and had soil profiles that matched their pasture composition. The two main soil profiles of this area are basalt and granite originated from basalt and granite, with basalt-derived soils being more productive and used for improved pastures and the granite-derived soils being less productive and used for native pastures [22]. The Monaro region of Australia is known to have one of the largest native pasture systems across all of New South Wales (NSW), at 70% [22], and as such, native grasslands are often an important component of sheep grazing operations in the area. The "native" paddock (NP) (21.3 ha^{-1}) was mainly composed of *Hordeum leporinum, Poa* spp., and *Stipa* spp. This paddock had a smattering of *Eucalyptus* spp. trees in the northern end and had an elevation of 984 m above sea level (asl) in the north and 935 m in the southern end and had a water trough situated in the southwestern corner (Figure 2). The "improved" paddock (IP) (26.6 ha^{-1}) was composed of *Festuca* spp., *Phalaris aquatica*, and *Trifolum subterraneum*. Unlike NP, the IP had relatively sparse shade coverage due to the lack of trees present overall, had an

elevation range of 932–949 m, and had a water trough located in the northwestern area of the paddock (Figure 2). The IP in particular, however, had a row of *Pinus radiata* which often provided protection from prevailing winds. In both paddocks, the positions of trees and water troughs were logged using a handheld GPS unit (Garmin etrex 30, Garmin Ltd., Olathe, KS, USA).

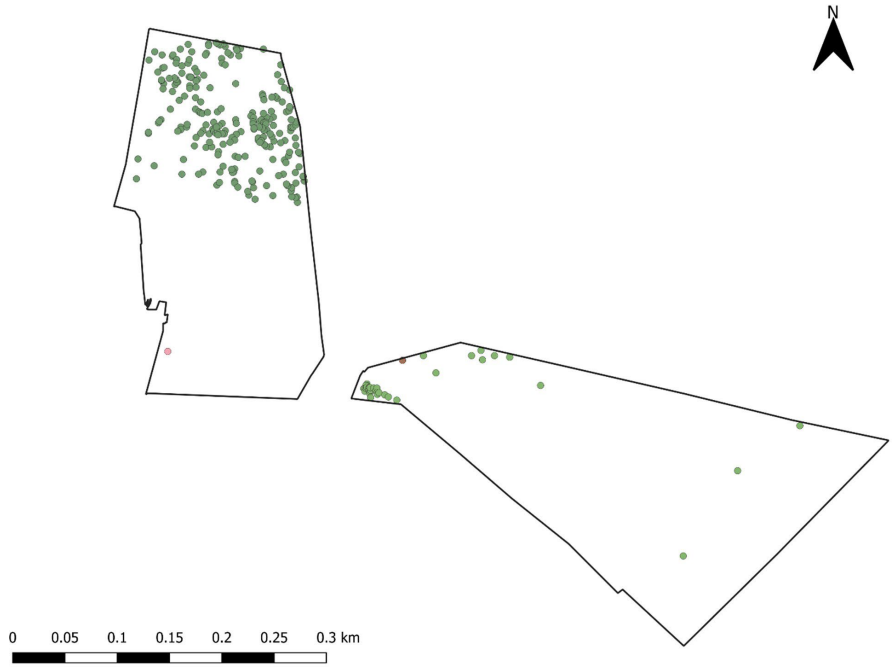

Figure 2. Map of native and improved paddocks used for the duration of the study and selected features. Red circles = water troughs; Green circles = GPS-logged tree locations.

It is important to note that during T1, T2, and T4, it was not possible to perform a botanical survey, as grasses were within a vegetative state and therefore, there was an inability to identify all pasture species in both paddocks. Paddocks were sampled for their normalized difference vegetation index (NDVI) 1–2 days prior to the initiation of each trial, on approximately 40 m transects across each paddock. NDVI within the study is utilized as a proxy for forage quality and quantity across the landscape [24], as the data obtained from the collection are collected at a relatively fine scale. The NDVI values of each paddock were measured using a vehicle-mounted ACS-470 CropCircle System (Holland Scientific, Lincoln, NE, USA) using the red band wavelength (670 nm) and the near-infrared band wavelength (760 nm) at a rate of 20 Hz, with GPS coordinates continuously recorded alongside it using a Trimble 250 EziGuide (Trimble Navigation Ltd., Westminster, CO, USA). All data were stored on a data logger before being processed using Variogram Estimation and Spatial Prediction with Error (VESPER) software [25] to produce an interpolated 5 × 5 m gridded NDVI map, which was then imported into ArcGIS (10.5 ESRI, Redlands, CA, USA) for analysis (Figure 3). The average NDVI for each paddock was relatively consistent with values of 0.273 ± 0.025 for T1 (Figure 3a), 0.217 ± 0.036 for T2 (Figure 3b), 0.235 ± 0.035 for T3 (Figure 3c), and 0.222 ± 0.015 for T4 (Figure 3d).

Wethers were chosen from the commercial flock of 'Coolringdon', and for the course of the trials, they were maintained as a separate mob from other sheep present on the property and were subject to the same standard farming operations (drenching, shearing, etc.). Fifty wethers approximately three years old were utilized for the study as approved by the University of Sydney University Animal Ethics Committee (2014/570). Of the 50 wethers chosen, 25 were randomly allocated into the IP, and the remaining 25 were allocated to the NP. Wethers were rotated between the two paddocks for each trial, for example, wethers in NP for T1 were assigned to the IP for T2, then back to the NP for T3, and so on. Of the 25 sheep in each paddock, GPS collars were randomly allocated to 15 sheep in each

paddock to record their locations ('fixes') over a minute at 0, 15, 30, 45, and 60 s, before going into sleep mode for 4 min. The remaining 10 animals acted as buffer sheep. Logging of GPS data occurred from the day before each trial started to around lunchtime of the final day of the trial. Once the collars were removed from the sheep on the 7th day, the data were cleaned to remove any fixes that took longer than 16 s to log, as it was not an accurate representation of location. Data were imported into ArcGIS and any points which were outside paddock boundaries were also removed.

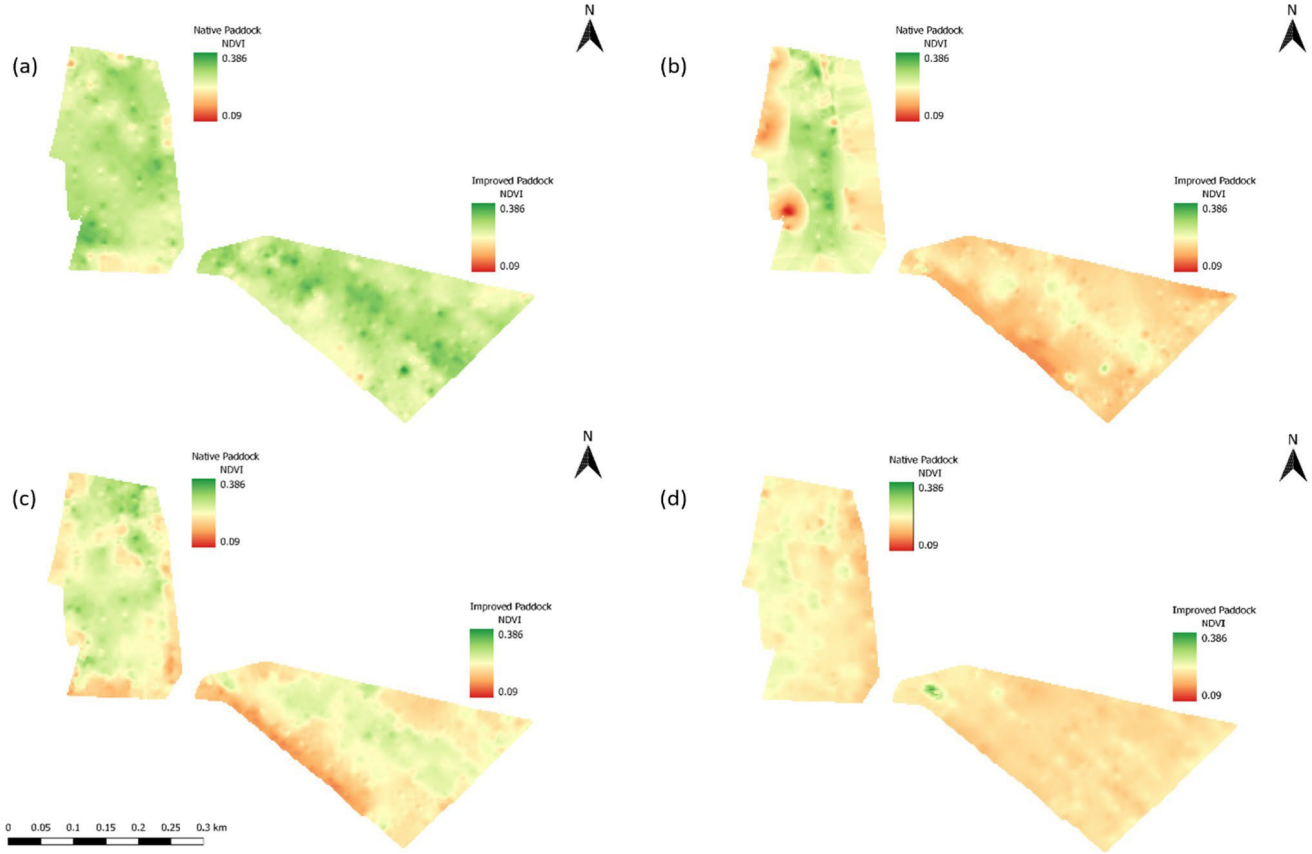

Figure 3. Normalized Difference Vegetation Index (NDVI) maps of native and improved paddocks during (**a**) Trial 1 (23–28 April 2014), (**b**) Trial 2 (9–14 July 2014), (**c**) Trial 3 (11–16 November 2014), and (**d**) Trial 4 (27 March–1 April 2015).

Over the course of the four trials, various sheep behaviors were visually observed to understand how often various traits occurred. At the same time that GPS collars were placed on the randomly selected 15 sheep in each paddock, a numbered, colored vest was also placed on the wethers to identify the individuals. The traits recorded were standing (S), standing grazing, walking grazing, standing walking grazing, lying (L), walking, running, and drinking. Due to the low levels of recordings, walking and running were combined into 'moving' (M); standing grazing, walking grazing, and standing walking grazing were combined into 'grazing' (G); and as only a total of four observations of drinking was recorded across all the trials, it was removed from the analysis. Measurements started around dawn and finished at dusk, occurring every two hours, lasting for six hours across the day. Starting on the hour, the observed behaviors of each sheep were recorded until all sheep had a recorded observation, using a scan sampling technique [26,27]. At 10 min past the hour, the process was repeated and occurred every 10 min. In total, observations were recorded in 10 min blocks over an hour, until all 15 bibbed wethers had recorded observations. As there was only one observer throughout the trials for consistency of observations, sheep on the NP were observed on days one, three, and five, whilst sheep on the IP were observed on days two, four, and six.

IceTags are a three-axis accelerometer that was attached to the leg of a sheep and collected count data of steps and lying bouts to ultimately provide a count of the time spent lying and standing over one-minute intervals. IceTags were placed on the legs of seven randomly

selected wethers per paddock during the collar- and cloak-fitting process prior to the start of the trials. As a smaller number of IceTags were available than GPS collars (14 IceTag accelerometers, 30 GPS collars) and there was a loss of units due to battery failure, seven sheep per paddock were fitted with the unit in T1 and six sheep per paddock in T2.

Speed was calculated from the GPS data using both the date and time stamp to obtain a 'seconds' (time) value and a distance between the recorded coordinates (easting and northings) for each "fix" of the animal at the time. Distance between fixes (x) is a measure of the difference between two points and was calculated as follows:

$$x = \sqrt{\frac{(aa-a)^2}{(bb-b)^2}} \quad (1)$$

whereby aa is the second eastings value, a is the first eastings value, bb is the second northings value, and b is the first northings value. Once the distance (m) by fixes is obtained, speed (m/s) was calculated by dividing the distance by the seconds.

Data were analyzed for each of the trials separately to compare the various trends that may have occurred within each season. The objectives of each model were to understand what variables were best for determining where sheep were located in a paddock (IP, NP) across each season (T1, T2, T3, and T4). Sheep location was based on a livestock residency index (LRI), a count of the number of times an animal spent in a given pixel (5 × 5 m) per hour, calculated for each individual animal and each treatment [4,27,28]. The LRI was calculated for each treatment (n = 15 collared animals; treatment replicate LRI) and was the target variable for all models generated.

Random forest modelling (RFM) was used to explain and predict sheep location and has well-established means to explore spatially mapped data in agriculture and conservation studies [29–32]. During the RFM process, data are internally separated into a validation (~33%) and training (~66%) to form an out-of-bag (OOB) subset. Details of how RFMs process data have been outlined previously [33,34]. Mean squared error (MSE) and R^2 values taken from these OOB subsets were utilized as an indication of overall performance of each model, importance values were used to identify the scale and relative importance of each variable in the model prediction, and partial dependence plots (PDPs) were used as a visualization tool depicting the effect of the selected predictor variable (LRI) on the response variable.

The data prepared for RFMs included near distance to various features such as trees (NTs), water troughs (NWs), and fence lines (NFs), as well as elevation (EL), aspect (AS) and the normalized difference vegetation index (NDVI) value as a measure of pasture quality [35]. Additionally, weather data were included to understand the importance of climatic variables such as rainfall (mm) and cumulative rainfall (mm), temperature (°C), apparent temperature (°C), dew point, relative humidity (%), wind direction, wind speed (km/h and m/s), wind gust (km/h), and wind pressure (QNH, MSL), sourced from the Cooma Airport weather station [23]. Data for each animal (A) were also included to identify if there were any significant influences by individual animal behavior [9]. Due to high collinearity between the climatic data, variables with high variance inflation factor (VIF) values over five were removed, leaving rainfall (R) and temperature (T). Sheep wind chill (WC) was calculated based on calculations determined by Weeks et al. [36]. Additionally, eastings (EA) and northings (NO) were also included in the models to attempt to reduce the potential for biased random forest models and account for any potential spatial autocorrelation in the GPS data. Data for each individual animal were also included in the models to determine if an individual animal's behavior was a significant factor on grazing choices [9]. In total, 12 variables were included as predictors in the final IP and NP models. Data were analyzed using packages 'ranger' (V0.12.1), 'pdp' (V0.7.0), 'car' (V3.0-12) [37–39] using R 4.03 [40].

Models were produced for 'all hours', which aimed to identify important variables for predicting where animals would be located across a diurnal cycle, and models were also produced for 'grazing hours' (GH), which were used to identify key drivers of why

sheep were in a given location during times when grazing was observed. Using the aforementioned visual observations of grazing behavior, the GH model only included the times in which grazing observations were recorded. In this paper, the smallest-sized dataset belonged to the GH model for T4 NP, with n = 9742 for calibration and n = 4799 for validation, and the largest dataset belonged to the 'all hours' (AH) model for T3 IP, with n = 70,368 for calibration and n = 34,659 for validation.

3. Results

3.1. Movement Data

The predominant behavior observed across all seasons was grazing (45% for IP, 41% for NP), except for in T3 where the dominant behavior for IP was lying (38%) followed by grazing (29%), which contrasts from the NP, where lying and standing were equally proportionate (32%) (data not shown). From these observations, the grazing activity counts were taken, and plots were produced for each trial and treatment (Figure 4). There was variation with grazing counts for T1–T3 in both paddocks across each hour, but in contrast, there was consistency across the hours for T4 in the IP and NP (except during the fourth hour) (Figure 4).

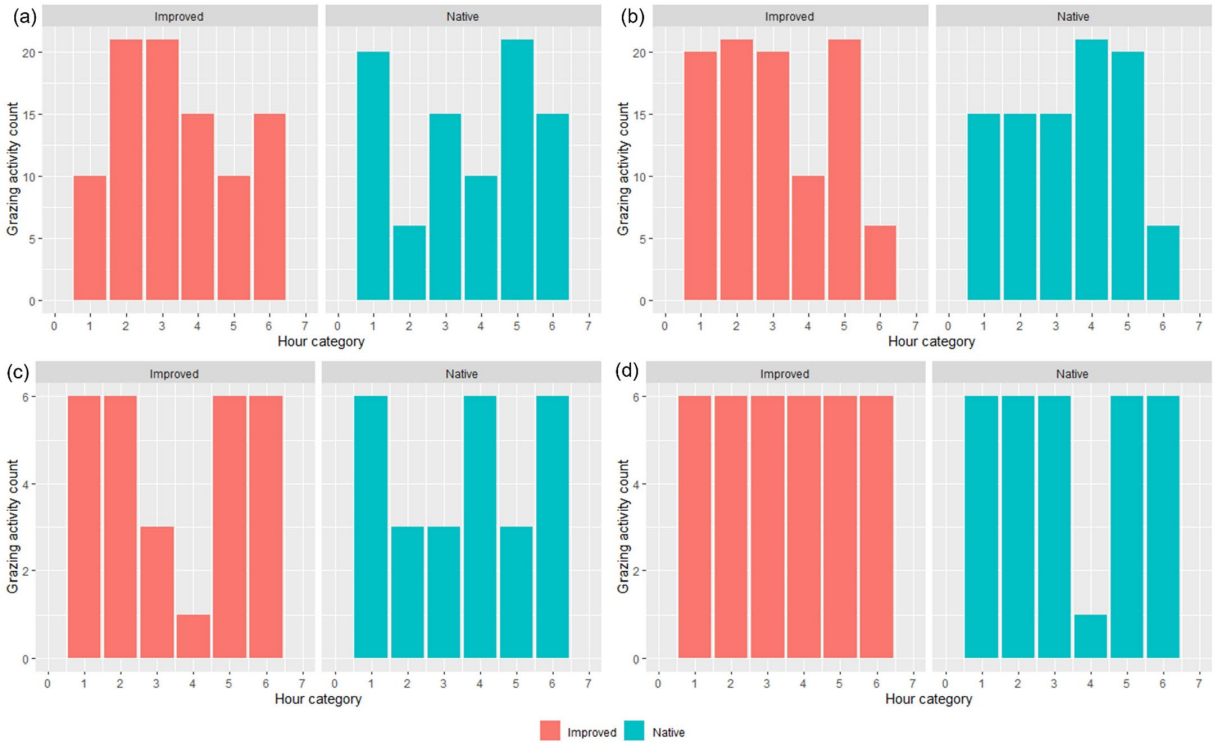

Figure 4. Average (n = 15 bibbed sheep) grazing activity counts for both native and improved paddocks during (**a**) Trial 1, (**b**) Trial 2, (**c**) Trial 3, and (**d**) Trial 4, per hour. NB: due to the slightly different start times across the trials (i.e., 7:30 a.m. vs. 6:00 a.m. start; effects of daylight savings), hour is presented in categorical notion whereby '1' was the first hour of recording, '2' was the second, and so on.

After calculating the average speed (m/s) of wethers across the different seasons, distinct patterns were evident for each trial and per treatment basis (Figure 5). Sheep in the IP consistently spent more time walking across the course of the day, in contrast to the NP, which had more uniform 'patterns' of higher speeds/activity during the morning and in the afternoons (Figure 5). Both treatments spent greater amounts of time grazing throughout the day during T2, as well as the NP in T1 (Figure 5a,b). Speeds were often greater than 0.15 m/s in the mornings and afternoons for both paddocks, except for in T2, where wethers in the IP spent more time walking at a faster pace than 0.15 m/s regardless of the time day.

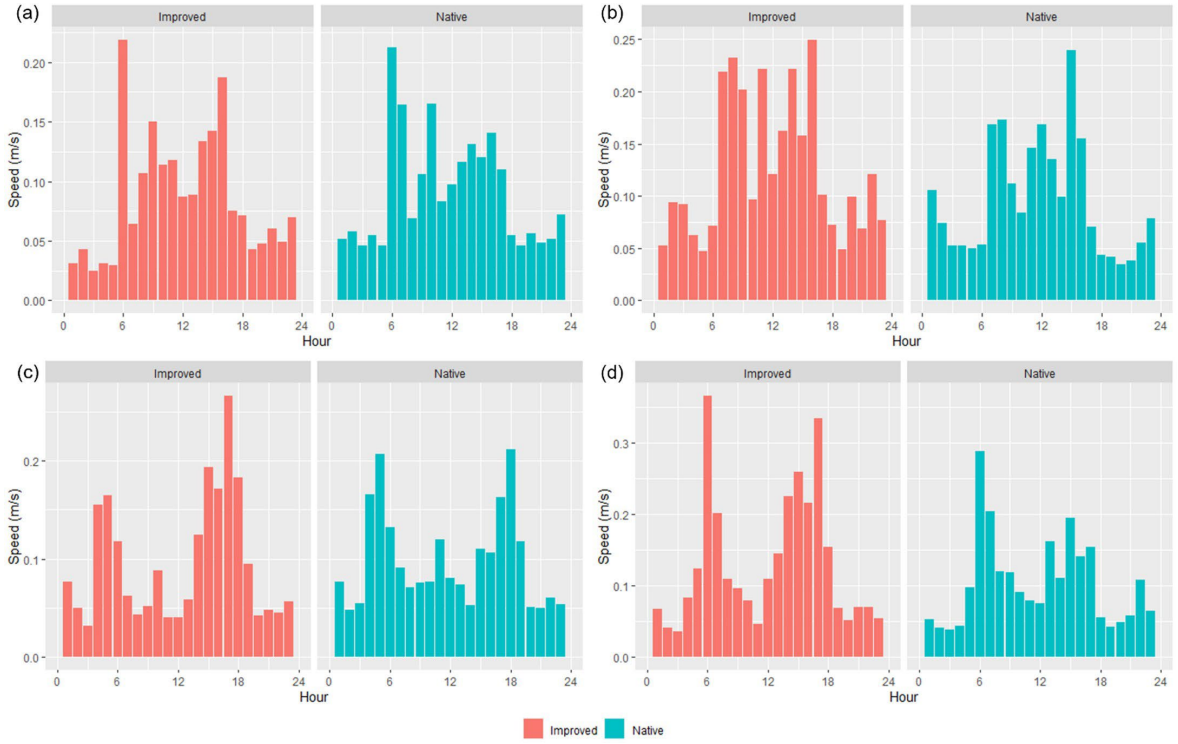

Figure 5. Calculated total average (n = 15 collared sheep) speed (m/s) of sheep using GPS collars, for both native and improved paddocks during (**a**) Trial 1, (**b**) Trial 2, (**c**) Trial 3, and (**d**) Trial 4.

IceTag accelerometer data showed an inverse relationship between step count and lying-bout counts (Figure 6). During the early hours of the morning and the hours in the late afternoon when step count decreased, lying count increased for both treatments (Figure 6). Across both treatments and trials, there seemed to be a trend roughly around the first few hours of daylight (~0600 to 0900) and the last few hours in the afternoon before nightfall (~1500 to 1700) where the most steps were taken by wethers (Figure 6a,b). Furthermore, fewer steps were taken overall across the day by the wethers in the NP, in contrast to those in the IP, and this is more apparent in T1 than T2 (Figure 6a,b).

3.2. *Random Forest Models*

Models were created for each trial to gain an understanding of the various seasonal effects that occurred across the duration of the study. The best performing model overall was T1 IP grazing hours (0.98 R^2; 2.0×10^{-6} MSE), and the lowest performing model was T3 NP all hours (0.94 R^2; 3.0×10^{-6} MSE) (Table 1). Across all of the trials, the significance ($p < 0.01$) and importance values varied for each of the variables included, however, distinct similarities were also evident. As the main intention is to identify variables which may drive grazing behaviors, the next section will predominantly focus on the results from the GH models, and where relevant, include a brief discussion of the AH models. General trends for all of the models produced often saw A, R, and NF as the three least important variables, regardless of the treatment or trial (Table 2). Similarly, EA, NO, and NW were commonly ranked as the top three important variables, except for certain periods where they were ranked between fourth and seventh (Table 2) and appeared to be influenced by the season more in the IP than NP.

In addition, residency (LRI) maps were created to visually identify the predominant locations where sheep were spending time during each of the trials (Figure 7). Evidently, for each trial, there were areas of the paddocks that were completely unused, predominantly in the NP compared to the IP (Figure 7). Wethers in the NP clearly congregated within the northernmost part of the paddock, where there were large amounts of tree cover (Figure 2) and there was the highest elevation across all trials. In contrast, LRI varied, as during each

of the different trials for the IP, as in T1 and T4, wethers mainly resided on the southeastern area of the paddock (Figure 7a,d), whilst in T2, wethers resided closer to the tree line in the northwestern area of the paddock (Figure 7b). Interestingly, wethers had consistent residency levels across the entire paddock in IP for T3 (Figure 7c).

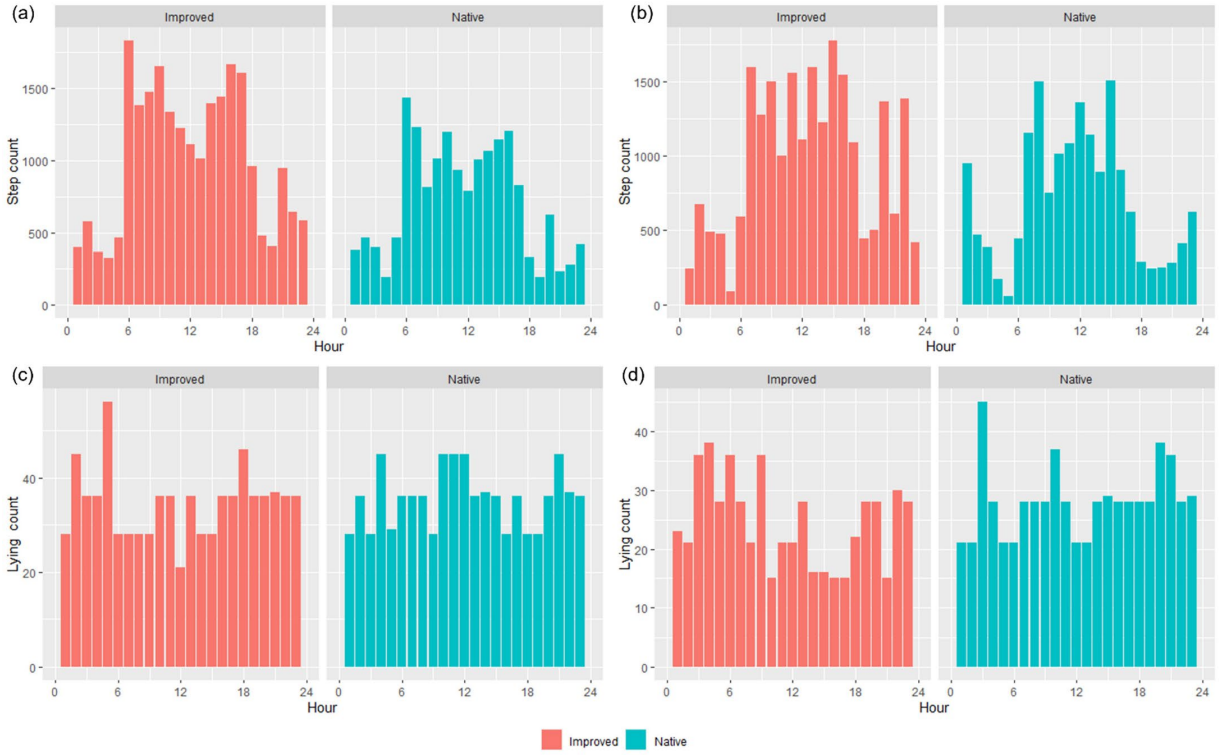

Figure 6. IceTag data for the average step count (**a**,**b**) and average lying count (**c**,**d**) per hour for Trial 1 (**a**,**c**) and Trial 2 (**b**,**d**) in both the improved and native paddocks.

Table 1. Model performance of OOB R^2 and MSE values for the models produced for each trial, treatment, and data type—all hours (AH) and grazing hours (GH).

	Trial 1				**Trial 2**			
	Improved		Native		Improved		Native	
	AH	GH	AH	GH	AH	GH	SH	GH
OOB R^2	0.978	0.982	0.949	0.957	0.958	0.952	0.944	0.956
MSE	2.0×10^{-6}	2.0×10^{-6}	3.0×10^{-6}	3.0×10^{-6}	3.0×10^{-6}	3.0×10^{-6}	3.0×10^{-6}	4.0×10^{-6}
	Trial 3				**Trial 4**			
	Improved		Native		Improved		Native	
	AH	GH	AH	GH	AH	GH	Ah	GH
OOB R^2	0.981	0.977	0.941	0.943	0.976	0.975	0.949	0.950
MSE	2.0×10^{-6}	2.0×10^{-6}	3.0×10^{-6}	3.0×10^{-6}	2.0×10^{-6}	2.0×10^{-6}	3.0×10^{-6}	3.0×10^{-6}

Table 2. Rankings for variables in models produced based on importance value for each trial, treatment, and data type—all hours (AH), and grazing hours (GH).

	Trial 1				**Trial 2**				**Trial 3**				**Trial 4**			
	Improved		**Native**		**Improved**		**Native**		**Improved**		**Native**		**Improved**		**Native**	
Variable	**AH**	**GH**	**AH**	**GH**	**AH**	**GH**	**AH**	**GH**	**AH**	**GH**	**AH**	**GH**	**AH**	**GH**	**AH**	**GH**
Animal (A)	11th	10th	12th	11th	10th *	10th *	12th	12th	11th	12th	12th *	12th	10th *	10th *	11th	12th
Eastings (EA)	4th	4th	1st *	1st *	3rd	1st	1st	2nd	1st *	1st *	2nd *	2nd *	2nd *	2nd *	2nd *	1st *

Table 2. *Cont.*

	Trial 1				Trial 2				Trial 3				Trial 4			
	Improved		Native		Improved		Native		Improved		Native		Improved		Native	
Variable	**AH**	**GH**	**AH**	**GH**	**AH**	**GH**	**AH**	**GH**	**AH**	**GH**	**AH**	**GH**	**AH**	**GH**	**AH**	**GH**
Northings (NO)	1st	1st *	3rd	2nd *	1st	4th	3rd	5th	2nd *	4th	3rd	3rd *	1st *	1st *	1st *	3rd *
Near distance to trees (NT)	8th	6th *	7th	6th *	7th	7th	7th	7th	5th *	8th *	6th *	5th *	3rd *	8th	7th	5th *
Near distance to water (NW)	6th	7th	2nd	3rd *	4th	3rd	2nd	4th	3rd	2nd *	1st *	1st *	4th	3rd	3rd	2nd *
Near distance to fences (NF)	12th	11th	10th	10th	12th	12th	11th	11th	12th	11th	11th	11th	12th	12th	10th	11th
NDVI	2nd *	2nd *	8th *	7th *	8th	8th	8th	8th	4th *	3rd *	8th	8th *	6th *	4th *	8th	8th *
Aspect (AS)	9th	9th	9th	8th *	11th	11th	9th	9th	9th	9th	9th	9th *	9th	9th	9th *	9th *
Elevation (EL)	5th	5th	5th	4th *	6th	6th	5th	3rd	6th	7th	4th	4th *	7th	5th	5th	4th *
Temperature (T)	3rd *	3rd *	4th *	5th *	2nd *	2nd *	6th *	6th *	7th *	6th *	5th *	6th *	5th *	6th *	4th *	6th *
Rainfall (R)	10th *	12th	11th *	12th *	9th *	9th *	10th *	10th *	10th *	10th *	10th *	10th *	11th	11th *	12th	10th *
Wind Chill (WC)	7th *	8th	6th *	9th *	5th *	5th *	4th *	1st *	8th *	5th *	7th *	7th	8th *	7th *	6th *	7th

* Indicates significance ($p < 0.01$).

3.2.1. Improved Paddocks

The IP models produced for grazing hours for each of the trials produced an OOB R^2 range from 0.95 (T2) to 0.98 (T1), with MSE values between $2.0 \times 10^{-}$ (T3) and 3.0×10^{-6} (T2) (Table 1). Interestingly, based on importance value, NDVI was the second highest ranked variable for the IP, regardless of hours included in the model, for T1 only, and only became important again in T3 during GH (Table 2). NDVI was also significant for all trials except for T2. Weather variables such as T changed in their relative importance depending on the season; T was ranked third and second only in T1 and T2 for both GH and WC, which, for the IP, was only ranked highly during T2 for both AH and GH and T3 for only GH (Table 2). Each of the variables and their rankings, as aforementioned, were also significant ($p < 0.01$). The landscape variable AS was not significant or as important compared to other variables for the IP. However, in contrast, EL was ranked fifth during GH for T1 and T4, despite its statistical insignificance (Table 2). NT was only moderately important, often ranking between sixth and eighth during GH; however, it was placed as the third and fifth most important variable for T4 and T3 AH (Table 2). Significance only occurred during T1 and T3 GH and T3 and 4 AH. The landscape variable NW was within the top three important variables for all trials except for T1 and was only significant for GH in T3 (Table 2). Furthermore, EA was often ranked highly for all trials but was only significant in both AH and GH for T3 and T4. In contrast, NO was ranked first in T1, T4, both AH and GH, in T2 AH, and was significant for GH in T1 and T4, but only T2 and T4 AH (Table 2).

Partial dependence plots produced showed the general trends between each of the variables and residency by wethers. For GH in all trials, as NDVI values increased, the LRI by wethers had decreased, often occurring when NDVI reached 0.2, except for in T1 where this was when NDVI reached 0.25 (Figure A1). General trends for both EA and NO saw that as they both increased, so too did residency, often suggesting that wethers in the IP spent time around the northwestern corner of the paddock (Figures 2 and A1). Landscape variable EL saw a similar trend where regardless of the trial, as EL increased to 930 m ASL, so too did LRI (Figure A1). Interestingly, AS had the highest residency when south-facing (between 150° and 200°) for all trials except for T2, where LRI was greatest when AS was western-facing (between 250° and 300°) (Figure A1). Residency was highest for NF when wethers were between 0 and 2 m from a fence line, regardless of the trial. Contrastingly, NT saw variations in residency depending on the trial, as for T1, residency was highest when wethers were more than 100 m from trees but was higher when animals were within the first 100 m from a tree for T2–T4 (Figure A1). In addition, NW was another variable which fluctuated with LRI depending on the trial, as within T2 and T3, LRI was greatest when animals were 600 m

or more away from water troughs; however, in T4, predicted residency was greatest when wethers were within 50 m or at least 550 m away from a water trough (Figure A1). As expected, all weather variables changed predicted LRI depending on the trial/season. As seen in WC, higher residency was evident when WC was lower (500–700) for T3 and T4; however, in T1 and T2, the predicted LRI was higher as WC was elevated (800 for T1, 750 and 900 for T2). This could have been correlated with the effects of T, as residency was often higher at lower temperatures during T3 and T4 but was higher as T increased (15 °C or greater for T1 and 5 °C for T2) for T1 and T2 (Figure A1). Trends between plots created for GH were similar for AH.

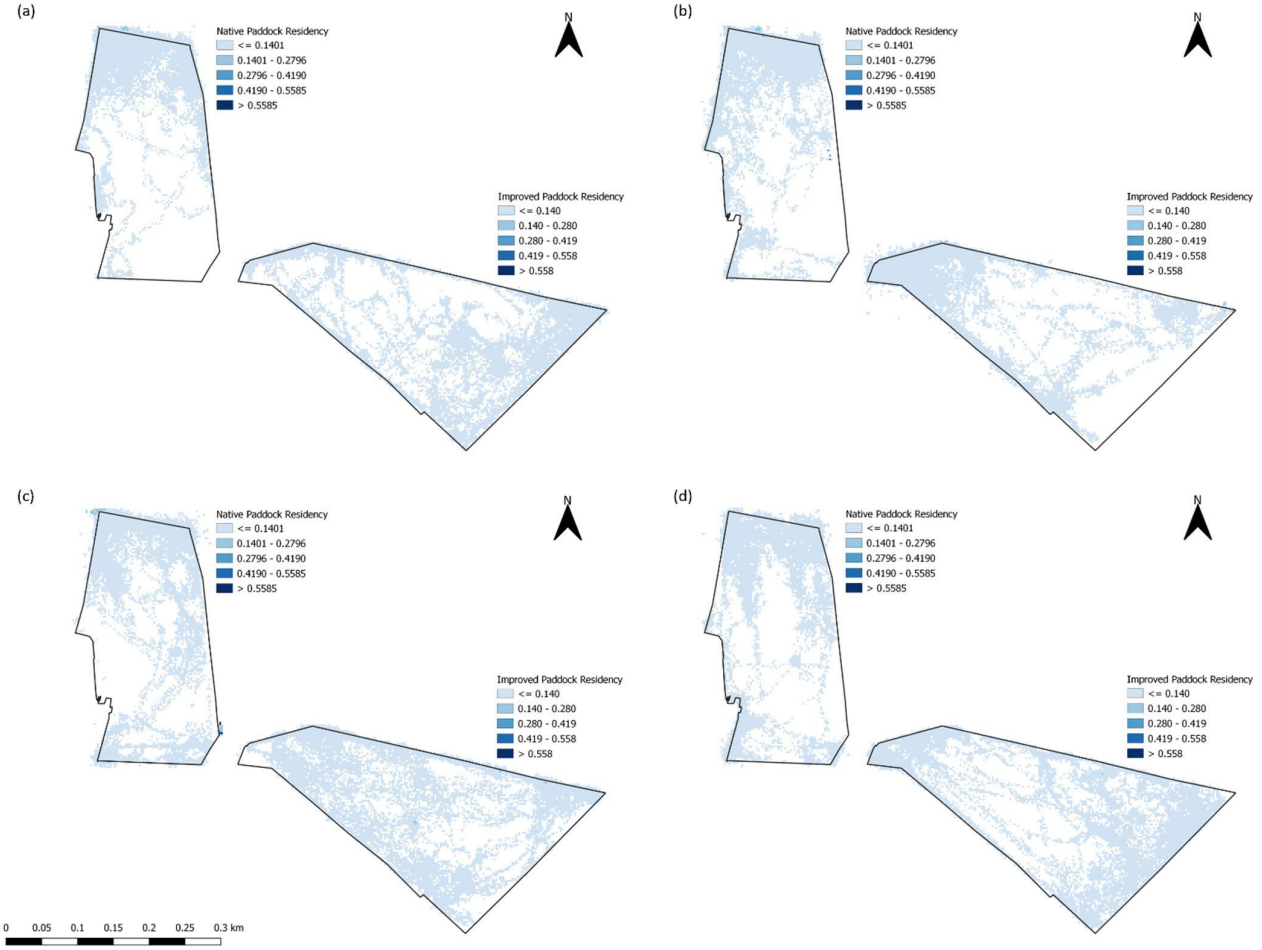

Figure 7. Livestock residency index (LRI) values in the improved and native paddocks for (**a**) Trial 1, (**b**) Trial 2, (**c**) Trial 3, and (**d**) Trial 4.

3.2.2. Native Paddocks

Models created for the NP produced an OOB R^2 range of 0.94 (T3) to 0.96 (T1) and MSE values of 3.0×10^{-6} and 4.0×10^{-6} (Table 1). The landscape variable EL was one of the higher-ranked variables across all trials, being significant for all except T2 (Table 2). In contrast, while the importance value for AS often ranked quite low across all trials, nonetheless they were generally significant for the GH models, except for T2, and all trials for AH (Table 2). Contrastingly, NT was ranked moderately, being significant for GH in all seasons except for T2 and only T3 AH (Table 2). Weather variables were significant for all trials during GH, with T being ranked moderately high, R being ranked quite low, and WC being ranked moderately high, except for in T2 where it was ranked first for GH or fourth for AH (Table 2). Of interest, NDVI was not a highly important variable to wethers in the NP as much as it was for those in the IP (Table 2). NDVI was, however, significant for all trials except T2 for GH and was only significant in T1 for AH in the NP (Table 2).

Across all trials, sheep were generally located either well away (>400 m) from trees, at often the southern end of the paddock where pasture quality was higher (Figures 3 and A2),

or were relatively close to trees (<50 m), regardless of AH or GH, likely indicating that trees were often providing shelter to the sheep from factors such as sun, rain, and wind (Figure A2). In contrast to NT, NF had higher LRI values only when animals were between 0 and 4 m away from fences, regardless of the season (Figure A2). Similarly, partial dependence plots (PDPs) for NW across all trials had low LRI until wethers were at least 600 m away from a water trough (Figure A2). Due to sheep spending the majority of their time at the northern and higher end (>960 m ASL) of the paddock, EL was evidently an important driver of sheep location. Similarly, as the paddock was southern-facing, AS had higher residency in T1 and T2 when sheep were southeasterly (between 100° and 200°) and western-facing (above 200°) in T3 and T4 (Figure A2). The level of residency for NDVI varied across the trials; however, the greatest LRI often occurred when NDVI values were around the 0.2–0.3 mark, regardless of higher NDVI values available (Figure A2). The weather variable WC saw greater LRI as WC increased for T1–T3, but lower residency as WC increased in T4. Interestingly, residency dramatically increased for WC in T2 (0.05, compared to 0.02 in T1, 3, and 4) when wind chill was 900 or greater (Figure A2). Rainfall also decreased residency in wethers as R increased, except for in T2 where LRI was also high when there was 3 or more mm of R (Figure A2). Residency by wethers also varied depending on T and generally saw a decrease in residency as T increased for T1 and 3, but there was greater grouping as T increased for T2 and 4 (Figure A2). Additionally, trends for NO saw higher LRI values when NO was greater in all trials and suggested a relatively even distribution of LRI for EA in T1, and high residency occurring at either the most western or eastern point (lower or higher value) of the paddock for T2–4 (Figure A2). The forecasted residency that occurred between AH and GH was quite similar, except for in T2 where residency within the first 100 m from a tree was greater than further away, and higher residency was also evident at lower NDVI values (0.10–0.20).

3.3. Weight Data

Weight data collected over the course of the study show that sheep in the IP increased in their liveweights, whilst sheep in the NP lost weight across the duration of the study. There was no effect of trial or trial x treatment on net weight changes; however, there was a pure treatment effect with an average of 1 kg difference between IP and NP. Wethers in the IP gained ~0.2 kg per trial, whilst those in the NP lost ~0.8 kg.

4. Discussion

4.1. Movement Behavior Data

All of the 'movement'-based data evidently had similar trends to each other and provided a sound basis for estimating when grazing was likely to occur. The behavioral observations presented in Figure 4 had similar trends with calculated speed data (Figure 5) and the IceTag data presented for T1 and T2 (Figure 6), as there were distinct peaks for T1–T3 for both treatments, both in the morning and the early afternoon. Evidently, trends in T4 were likely apparent that as more pasture became available, sheep were grazing during each observation period. However, this inconsistency could also be attributed to a change in observers that occurred for T4. As has been highlighted in existing literature, a change in observers is likely to cause inconsistencies between results, attributed to human-based errors [41]. Visual monitoring and observation of animals in research is often of a labor-intensive nature, and as such there is a demand for alternative methods of observing animals [41]. Aside from this, sheep are likely to be easily startled during observation periods if the observer makes sudden movements, impacting how accurate an observation period may be. The ability to monitor the behavior of certain livestock without requiring the labor-intensive nature of visual observations would therefore be useful for research. Furthermore, the IceTag data presented showed trends consistent with the both the calculated GPS speeds and the visual observation data. The lack of data due to malfunction, however, inhibited the depth of analysis able to be performed in this study. Accelerometer-based data in research are useful for research, identifying various behaviors

such as grazing, resting, walking, and ruminating in livestock, and offer the ability to continuously monitor behavior remotely [5,42,43]. When used in terms of generating a 'step count' or level of acceleration, it is clear that though not as granular, GPS speeds can also produce a similar level of detail when accelerometers are absent. As represented in Figure 5, sheep in the IP spent more time walking across the day, in contrast to those in the NP, which had more distinct movement speeds in the morning and the afternoon across all trials; this was also reflected in their residency locations (Figure 7). The higher greenness of feed on offer in the NP consistently across the trials, compared to the IP (Figure 3), likely suggests that sheep in this paddock were spending less time grazing. Evidently, in T1 and T2 alone, sheep in the NP had higher counts of lying down than those in the IP (Figure 6c,d). Additionally, the decrease in the level of grazing activity for T3 in the IP (Figures 4c and 5c) but increase in total pasture use (Figure 7c) could be due to having an increase in pasture quality in contrast to the prior trial/season, which inevitably led to a decrease in walking events to find quality pasture. This may be reflected with the known adaptivity animals have, where the level of foraging behavior changes depending on the available quantity and nutritive value of vegetation present [44].

4.2. Random Forest Models

Data from random forest models produced suggested that NDVI varied in its importance and significance for determining where sheep spent time in the paddock, across different seasons. Across the different trials, pasture quality, as identified through NDVI, was more variable for the IP than NP (Figure 3). Sheep in the IP evidently may be driven more by quality changes in the paddock than the sheep in the NP. Sheep are known to feed more selectively than other livestock and often do so to compensate for lower rumen residence time [14,45]. Whilst it is not possible to know exactly what was being consumed, pasture in the IP had clover species present (i.e., *Trifolum subterraneum*), which may have heavily influenced the selectivity by sheep when in the IP, due to the inherent preference known by livestock when grazing on clover species, for their ease to graze and high levels of sugars [44,46–49]. In contrast, the lack of such valued pasture species in the NP and lower levels of grazing activity across most trials for NP may further reiterate the narrative of sheep in IP spending more time looking for better-quality feed than what was often available (Figures 3–5 and 7). The activity level differences between the paddocks may have attributed to the changes in liveweight seen, for example, wethers in the NP lost weight across the duration of the study. In addition to this, weight loss along with lower grazing activity could have also correlated with seasonal forage variation. Biomass production through pasture surveys was not explicitly measured in this study; through NDVI, it was evident that the production of 'green' forage was high in T1, decreased during the winter (T2) and increased again during T3 during the spring period (Figure 3a–c). Though these aforementioned green forage trends should correlate with weight changes [50], this was not the case for the study. As sheep in the IP were actively grazing on more nutritionally viable pasture, the lessened availability of such pasture in the NP impacted diet preferences of the grazing sheep, resulting in the consumption of less desirable pasture [14]. Evidently, sheep liveweight here was greater on the IP over the NP, consistent with previous research [51,52].

As previously discussed, EA and NO were ranked in the top three variables regardless of the treatment or trial period. This effect is likely due to the temporal correlation between GPS points [53]. Similarly, distance is a variable which can also be impacted by spatial autocorrelation between GPS points [53]. It has been stated in prior literature, however, that the position of water locations in the paddock can have an impact on the utilization, grazing, and overall distribution of livestock [3,12,19]. With the results evident, it is likely that NW was only significant and important in this study due to the paddock shape and the placement of the troughs over inherent spatial autocorrelation (Figures 2 and 7). The literature has also suggested that in a larger pasture, livestock may head to water less often, spending more time away from it [54], which was a trend evident for both paddocks during all trials, as wethers spent most time at least 550 m away from the trough at the other end

of the paddock (Figures A1 and A2). A change was evident in T4, when residency was also high when wethers were within 50 m of water troughs, a likely event due to the warmer weather experienced in T4. The assumption here is that an increase in temperature led to an increase in water intake by sheep [55]. Warmer or cooler temperatures, high winds, and rainfall inherently also influenced location-based grazing for animals in this study. Primarily occurring during T2, weather variables R, WC, and T were ranked significantly higher for both paddocks than the rest of the trials. It is presumed that this is a large driving influence of grouping and residency changes in this trial in particular, as poor weather (high wind chill and rainfall) has been reported to impact heat loss from animals, influencing their location and utilization of shelters such as trees, shrubs, or structures within a landscape [56]. This was reflected in the trends evident in the residency by wethers within the paddock as during T2, wethers were often located near tree points which may have provided a form of shelter in both paddocks (Figure 7b). Additionally, any differences between LRI and the three weather variables during AH and GH could likely be attributed to the times which were included and excluded. For example, there is a lowered effect of weather variables on AH due to the larger range of hours included in contrast to the GH models, which saw higher influences of weather variables on LRI due to the smaller range of hours included (Figures A1 and A2).

Landscape variables such as AS and EL in these models reflected that wethers here often preferred to spend more time in areas more northeast in the IP, whilst in the NP, animals spent the most time in the northern end (regardless of eastings), commonly in the areas where EL was the highest (Figure 7). This influence of topography has been reported in prior research, identifying the need of animals to be in areas of good visibility [57–59] and in areas where sun was located. It is presumed here that AS may have been further influenced by T during the trials. Furthermore, the differences in the presence of trees and shading between the two paddocks were substantial and may therefore have a large influence on grazing-based choices (Figure 7). As aforementioned, the NP had large amounts of tree cover in the northern end of the paddock, whilst the IP had a minimal line in the western border of the paddock (Figure 2). The presence of trees is known to influence pasture production on both native and introduced pastures [52,60,61], as well as the behavior of livestock [61]. Greater yields are evident when trees are not present; however, trees provide higher-quality pasture due to the access to nutrients trees inherently bring from soil nutrient dynamics [62,63]. Additionally, dry matter digestibility was found to be higher in pasture under tree cover [61,62]. Wethers in the IP were likely grazing closer to the areas where the trees were during all trials, due to more vegetation (as identified in higher NDVI values) present in the lower-lying areas, away from their camping location and closer to the tree line (Figures 3 and A1). In contrast to this, wethers in the NP were found spending time away from tree lines (Figure A2) during T1 to T3. The comparison between grazing preferences in the two paddocks, aside from obvious tree cover differences, could have been attributed to the pasture itself. Despite nutrient concentration being higher in pastures under tree canopy, shade influences the quality and level of biomass more significantly in native pastures over those sown with introduced species [52,60]. Lastly, NF models and PDPs produced suggested that residency was greatest when closest to fence lines; however, it was not significant or important at all, regardless of the trial or the treatment. This effect is likely due to the camping locations predominantly being at the highest point of each paddock being directly next to a fence line (Figure 7).

4.3. Implications of Research and Future Directions

This research has highlighted that there are different drivers of grazing for sheep, depending on the pasture type (native vs. introduced) of the paddock, mainly the importance of NDVI for sheep in the IP over NP. As seen in the trends presented here, wethers on NP were often not walking as far as wethers in IP, which were presumably spending time searching for higher-quality pasture. Despite there being greater NDVI values in the NP than in the IP, the presence of valuable pasture species (i.e., clover) in areas of the

IP as aforementioned is of note here. As indicated in the literature, the incorporation of more nutritional feed resources, such as improved pasture areas, is beneficial for covering nutritional requirements and maintaining sustainable production systems [44,64–67]. As native vegetation often has lower nutritive value and given the inherent grazing patterns observed in wethers in the IP spending more time grazing to find preferred plant species, it is suggested that graziers provide areas of these improved species amongst native pastures to meet the requirements of animals and provide herbage with higher digestibility and quality to assist in the maintenance of liveweights across seasons [52,67,68].

Recent research has often not addressed potential issues of spatial autocorrelation that can be inherent in GPS data, such as the work of Homburger et al. (2014), Gou et al. (2019), Brennan et al. (2020), and Pearson et al. (2021) [69–72]. In this study, we included EA and NO within the models as an attempt to determine the significance and importance of 'space' against data in a given point in time. While random forest models that explicitly consider the spatial autocorrelation of data are available (e.g., spatialRF), due to the size of the distance matrices that are produced for each pair of points, computing power can be a major limitation. In this study, as the size of the datasets used was quite large (~605,000 data points in total for AH, ~150,000 data points in total for GH), we were unable to implement spatial RFMs due to a lack of computing power. It is therefore suggested that future studies that focus on modelling GPS data of similar size to this utilize spatial RFM on high-performance computers to be able to sufficiently address this concern. Furthermore, it is suggested that additional research be undertaken to directly compare the various movement-based data with each other, to determine if modelling GPS behavior data is sufficient. This step is critical, as this type of research has the ability to allow for less labor-intensive monitoring of animals during tracking.

5. Conclusions

With the data presented here, it is evident that there are different drivers of grazing behaviors on pastures of native and improved species. As discussed previously, NDVI is often a significant variable for both paddocks; however, it can be suggested that the quality of pasture was more important for wethers in paddocks of improved species than it was for wethers on native species. The results discussed in this study highlight the ability to use predictive models such as RFMs to provide valuable insights on behavior-based modelling of GPS data. With data such as these, there is greater ability to further enhance current knowledge on the impacts of multiple variables on grazing preferences to assist in developing management strategies to improve the sustainable grazing of sheep within Australia.

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Appendix A

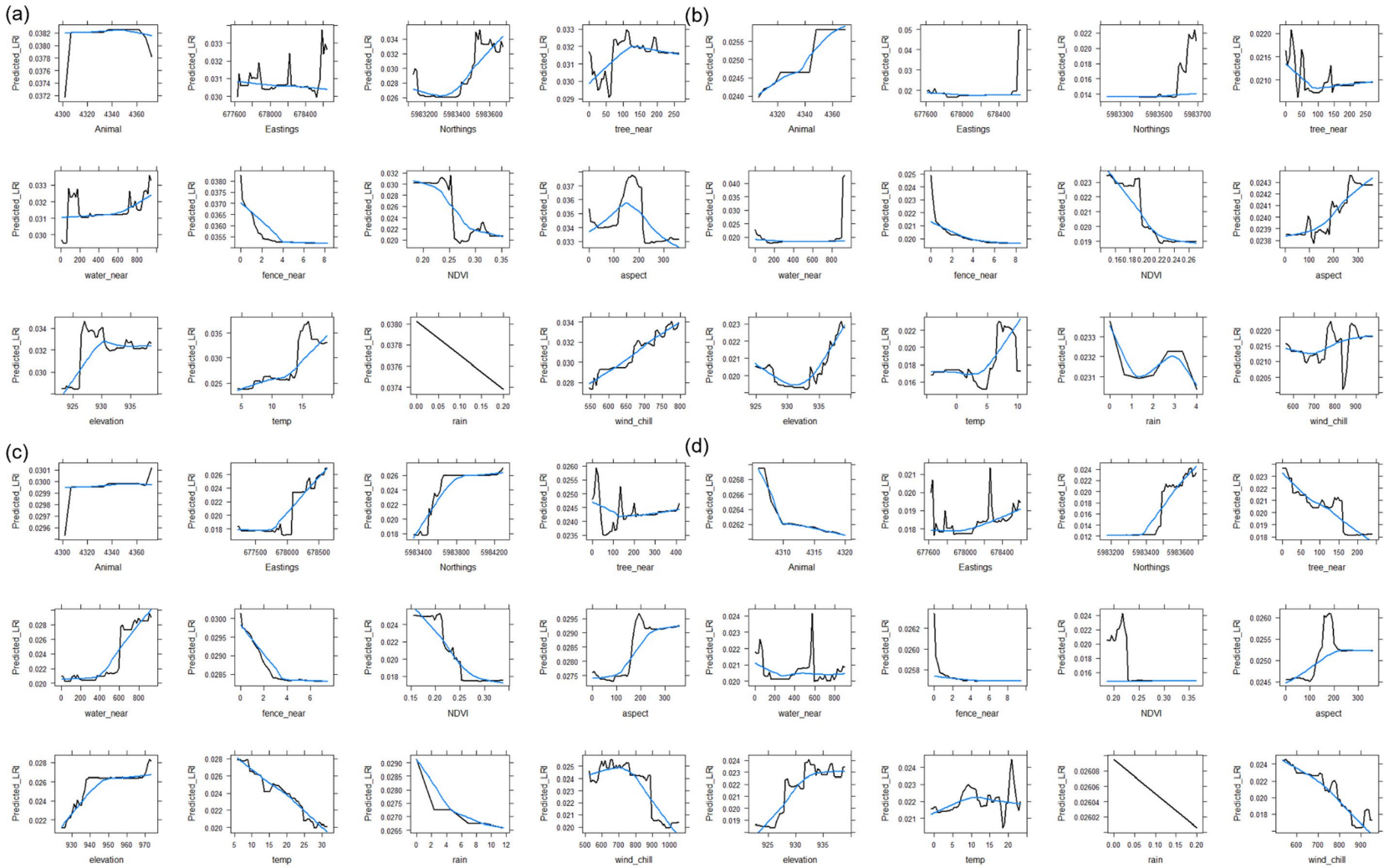

Figure A1. Partial dependence plots produced for the improved paddock (IP) for (**a**) Trial 1, (**b**) Trial 2, (**c**) Trial 3, (**d**) Trial 4. Black lines within the figure represent the data, whilst blue lines represent the trend line.

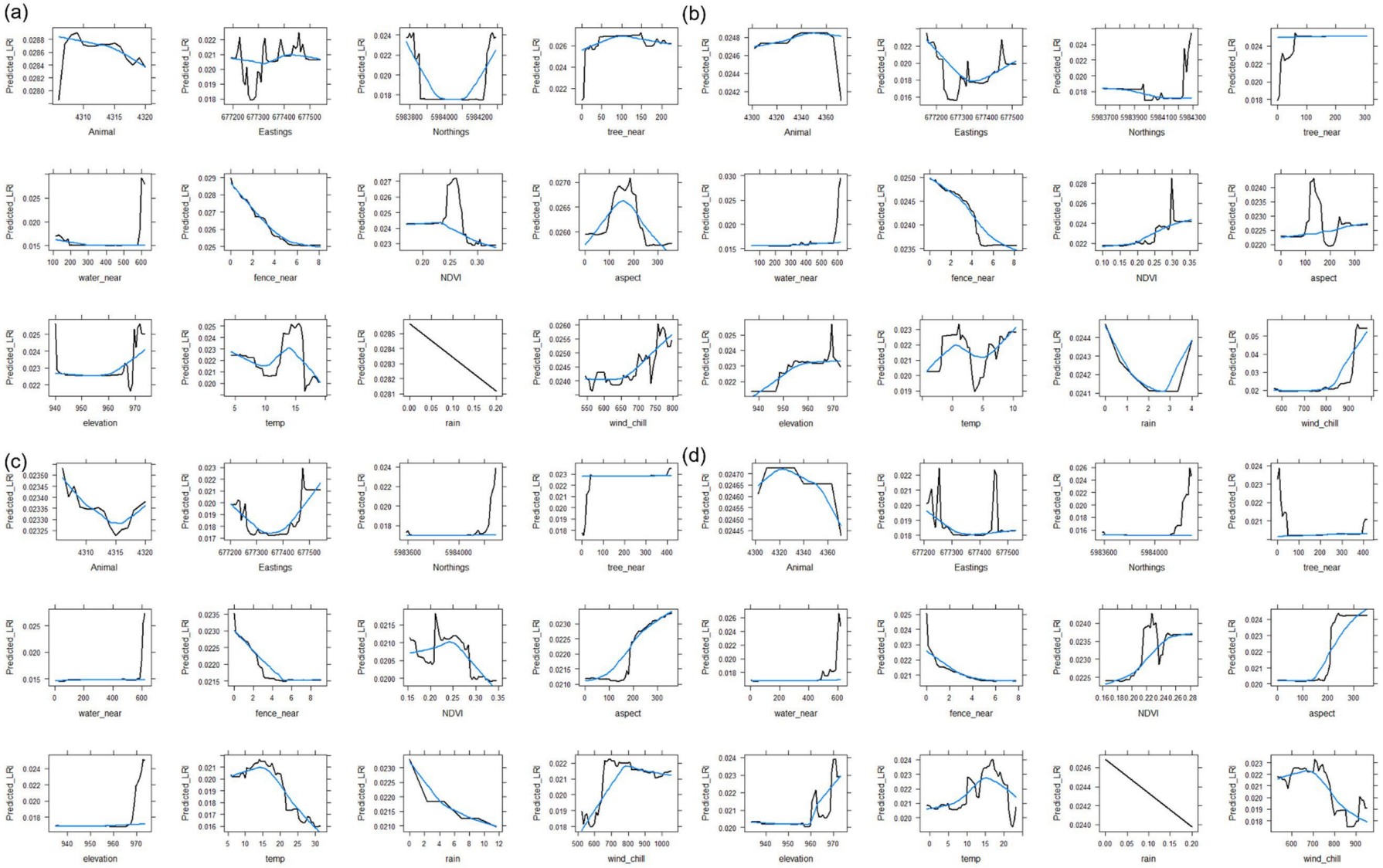

Figure A2. Partial dependence plots produced for the native paddock (NP) for (**a**) Trial 1, (**b**) Trial 2, (**c**) Trial 3, (**d**) Trial 4. Black lines within the figure represent the data, whilst blue lines represent the trend line.

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Chapter 7:
General discussion and future directions

The overarching theme discussed throughout this thesis alludes to the strong need to investigate the associated dynamics and interactions between both biotic and abiotic components in a pastoral ecosystem, to enable the creation of better management strategies for pastoralists in the long and short term. Navigating the intricacies of livestock grazing systems entails working with a significant degree of unpredictability. To ensure the sustained viability of the agricultural industry over the long term, it is imperative to implement effective management strategies which are aimed at enhancing productivity and fostering environmental sustainability. Existing challenges within the industry include the accelerating viability in climate conditions, and the increases in demand for food-based products to support an additional 2 billion people by 2050.

The chapters within this thesis collectively weave a narrative that intricately explores the management of grazing livestock. Our investigations span into two interconnected dimensions of the agroecological landscape – encompassing animals and plants. In the context of this thesis, these approaches highlight the multifaceted relationships between plants and their physiological responses to grazing, and the movement of grazing animals across landscapes. Originally this thesis aimed to delve into three key areas, encompassing investigating grazing impacts with a focus on plant roots and soil productivity ex-situ, the impact of grazing on soil and plant productivity in-situ, and investigating the influence of grazing management on soil organic carbon stocks over a long-term period (Figure 1). Here, the focus was originally directed towards examining the changes in soil organic carbon dynamics during grazing and identify trends in management decisions which could improve sequestration rates. In the context of Australian agriculture, carbon sequestration strategies on farm are of paramount importance for contributing to the mitigation of climate change impacts and management of soil health. However, as a result of the challenges of extensive lockdown periods imposing considerable limitations on fieldwork (see impact statement), a strategic modification of the research focus became imperative.

The revised approach shifted the focus from carbon dynamics during grazing to an intricate examination of plant and animal interactions during grazing periods (Figure 2). This included an exploration of various factors influencing animal grazing-based choices through use of predictive modelling (Chapter 5, 6), delving into the physiological response of a common Australian improved pasture species to defoliation events with livestock saliva (Chapter 3), and a broad scoped examination of the primary metabolic constituents in cattle saliva (Chapter 4).

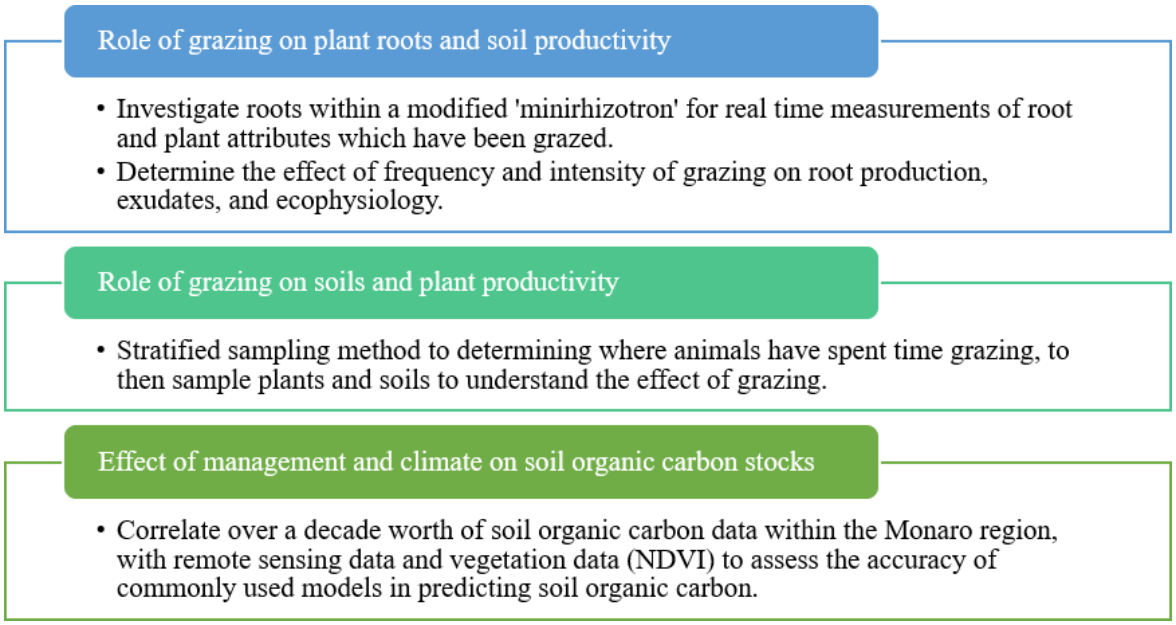

Figure 1. Initial three research areas proposed for this thesis.

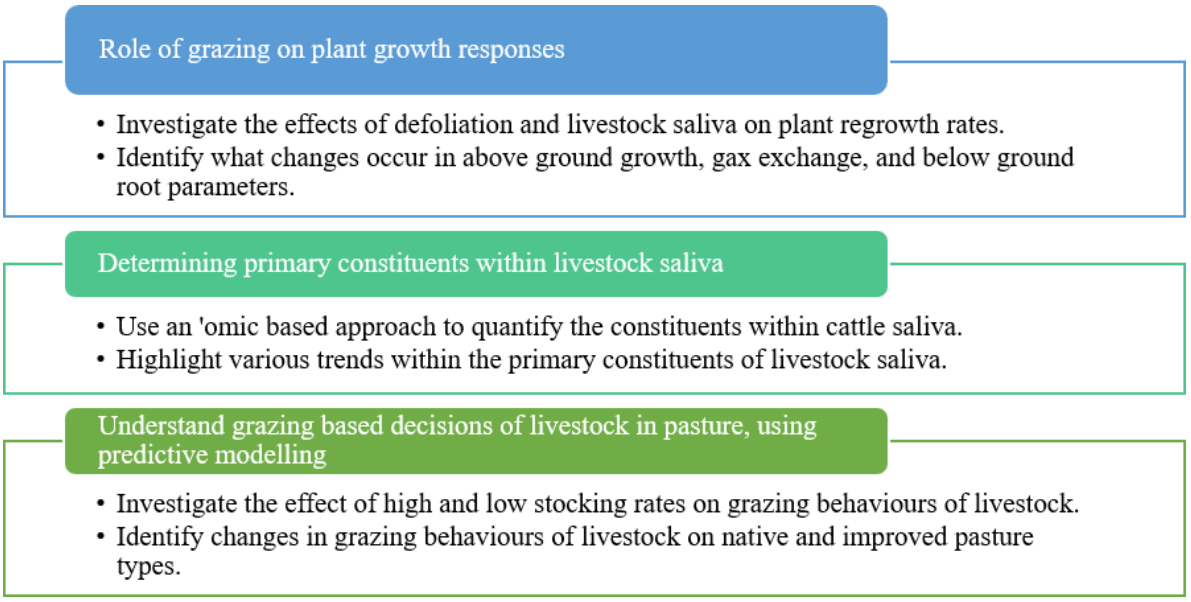

Figure 2. Final research areas investigated within this thesis

Despite the challenges imposed by the lockdowns, the trajectory of this thesis has steadfastly adhered to its foundational goal – to conduct livestock production research through an agroecological lens, and to provide insights towards essential, effective, and sustainable management practices. Figure 3 summarises the bottom-up approach undertaken, outlining the various aims and outcomes of the review and experimental chapters within this thesis.

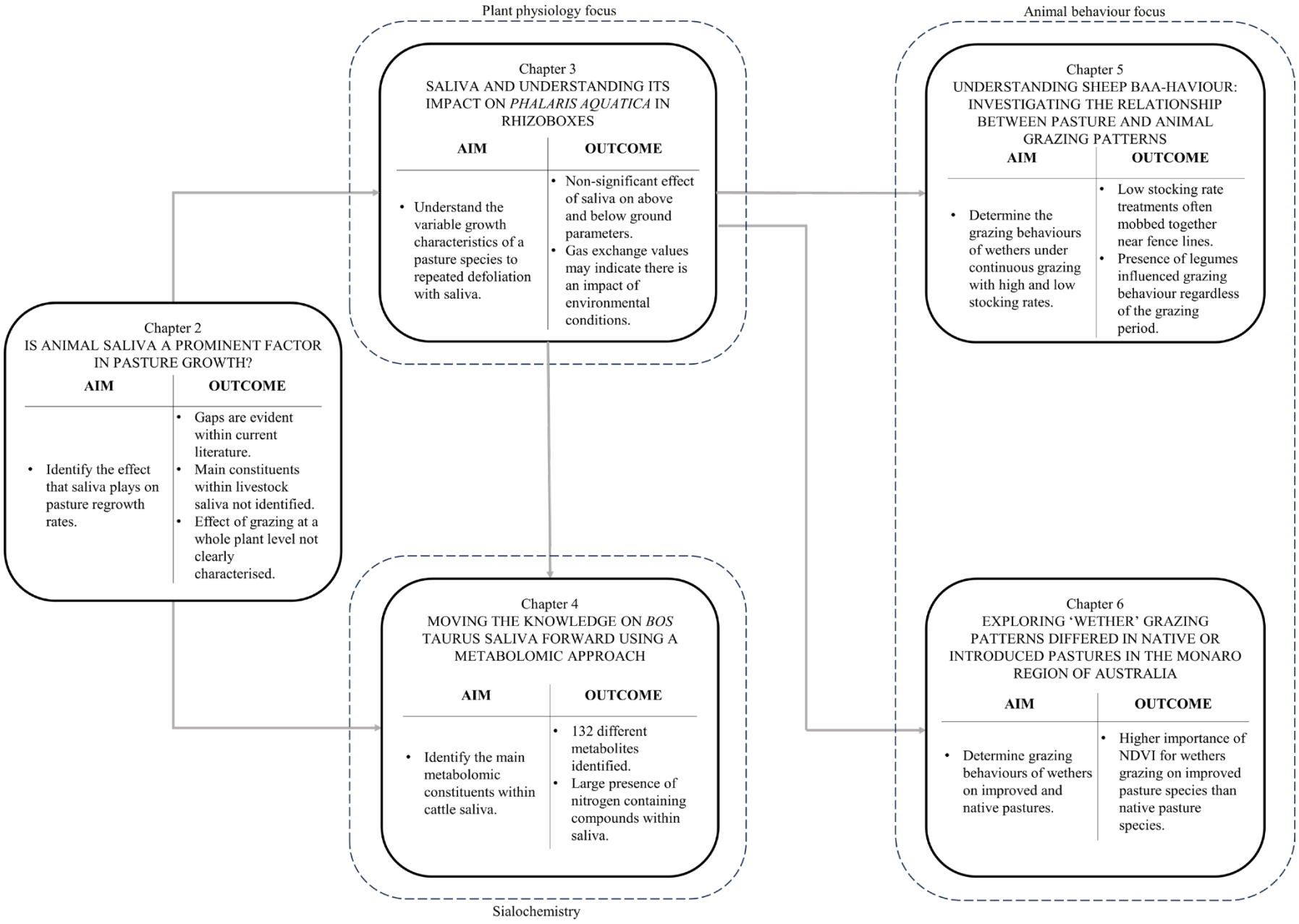

Figure 3. Summary plot of aims and outcomes for each chapter of this thesis.

Saliva and plant physiology

It is clear from the work undertaken in Chapter 2, the existence of gaps in research into the plant-animal interactions during the grazing process, and the potential impact on plants. Though we acknowledge the broad spectrum of factors that can influence grazing, Chapter 2 advocates for a more focused examination of the specific influence that livestock saliva has on plants. Despite the seemingly small role of saliva deposition during the grazing process in the broader context of grazing, the review here highlights the significance of understanding mechanisms behind growth promotion and inhibition, in the context of saliva and associated pasture physiology.

Chapter 2 identifies a lack of explicit understanding of the effect of saliva deposition during defoliation events, particularly in cattle grazing systems. The absence of studies on the constituents of saliva further accentuates the need for further research. The review refrains from undertaking a meta-analysis approach, choosing instead to concentrate on the pool of current literature which incorporates saliva application during simulated grazing events. Notably, many studies on grazing effects have omitted the use of livestock saliva, despite evidence existing in literature to suggest a positive response, prompting the review to emphasise the importance of exploring the specific impacts that saliva. Whilst mechanical defoliation without saliva is important in understanding physical responses in plants, the decision to exclude depth within the review in this area was done to highlight the specific impacts of saliva application on plant physiological responses due to allometric relationships.

The absence of an explicit list of saliva constituents represents an additional knowledge gap. Multiple studies have investigated various proteins using proteomics-based approaches in sheep and goats; however, cattle have not been studied. Of the studies undertaken in a range of animals that have identified what is in saliva, few have gone on to perform a further exploratory analysis to quantify the number of constituents within saliva and potential variations within the same species. Further, recent studies that have utilised saliva during defoliation ex-situ, have only done so using sheep saliva. While a multitude of studies have performed defoliation-based research in the field through mowing pasture, however, this particular method may not be reflective of an actual grazing event and can only be used as an approximation. For example, animals are known to graze in non-uniform patterns, as cattle for example use their tongue to rip and tear plants, whilst sheep bite. Future research proposed in this area is described later in this chapter.

Chapter 3 sought to identify some of these research gaps and delve into the impact of *Bos taurus* (cattle) saliva on the growth of *Phalaris aquatica*, using mechanical defoliation methods within a controlled glasshouse environment. The intended scope of Chapter 3 aimed at exploring changes in roots following a grazing event, with a specific emphasis on resource allocations and alterations to soil productivity (Figure 1), however the modifications imposed on this chapter resulted in the removal of the additional focus of soil productivity, and resource allocation (i.e., carbohydrate movement, root exudates), within the plant, narrowing the overall investigation to look at growth responses exhibited by the plant (Figure 2). Chapter 3 additionally intended to compare the impact of simulated grazing between three key pasture types to Australia: one native species (*Themeda triandra*), one improved species (*Phalaris aquatica*), and one legume species (*Medicago sativa*). The absence of certain constraints would have facilitated a more holistic understanding of the impact on individual species prevalent in Australian pastures and may have allowed for more targeted grazing

management strategies to be developed in future. For example, if a particular species exhibited suboptimal responses to mechanical defoliation events, identifying positive changes could have informed more effective targeted grazing practices for long-term animal management on specific pasture types. The study performed as a result, concentrated on the effect of repeated defoliation and saliva application events, striving to closely replicate a continuous grazing scenario on one pasture species.

Utilising rhizoboxes for continuous monitoring of root growth, presented an approach rarely undertaken in prior research. Unlike conventional destructive sampling methods often employed to assess changes in below ground biomass, the use of rhizoboxes allowed for weekly measurements of root parameters (i.e., diameter, volume, surface area). While the study did not yield statistically significant effects of saliva and repeated defoliation on physiological growth parameters at the time, potential refinements to the methodology could enhance the robustness of future results. For example, adopting a method of scanning the roots each week may provide finer granularity compared to the photographic method undertaken here. Furthermore, insights from photosynthetic activity and stomatal conductance suggest a transient impact on leaf physiology, which may be contingent on various factors such as time of data collection, chemical elicitors, and the pH of saliva. These nuances underscore the complexity of interactions between plant growth response and a chemical elicitor, such as saliva, and further emphasize the need for meticulous consideration of a range of variables in future investigations.

In conjunction with the absence of evidence in Chapter 2, and the quest of this thesis to unravel the details behind the growth promotion or inhibition effects that have been reported in previous research, an analysis of collected saliva became was completed. Chapter 4 achieves this, aiming to characterise the diverse constituents of saliva, and shed light on the potential elicitors of increases in plant growth rates. Beyond its significance in understanding plant physiological responses, the research presented provides a basis for the use of metabolomic analysis of saliva for additional health-based diagnostics. Further, Chapter 2 identified a critical gap in existing literature whereby studies have not investigated the range of ions, proteins, and metabolites present in cattle saliva. Chapter 4 advocates for an unbiased approach to characterising the constituents in saliva through metabolomic methods.

Chapter 2 additionally draws attention to the variations in saliva collection methods for defoliation studies, emphasising the need for standardised approaches. For example, while certain studies employed sponges to collect samples from sheep mouths (Li et al., 2014; Palma-Hidalgo et al., 2023; Reardon et al., 1972), others directly extracted saliva from major salivary glands (Lamy & Mau, 2012). The method adopted for the majority of samples in Chapter 4 involved inducing a salivary response through tickling the roof of the cattle's mouth. This method as such allowed for more a more accurate representation of the saliva within the animal's mouth at the time of grazing. Additionally, opportunistic sampling allowed for the comparison of saliva collected during sedation due to the over production of saliva occurring in response to the sedative. The subsequent results identified 132 unique metabolic compounds within the saliva profile of sampled cattle. Notably, a substantial presence of nitrogen, primarily in the form of urea, stood out, surpassing the next metabolite in magnitude by 6.5 times, and exceeding the remaining metabolites by 45 – 270 times. Intriguingly, the presumed main elicitor of plant growth (see Chapter 2), thiamine, did not exhibit the same quantitative dominance across all species. This leads to the conclusion that positive growth responses that have been observed in prior defoliation studies may in fact, be attributed to increased nitrogen content in applied saliva. The chapter additionally highlights

the importance of considering species and diet variations as critical variables in future defoliation-based studies.

Grazing behaviour choices by livestock

In the broader context of the grazing landscape, a myriad of factors necessitates an exploration of influences that encapsulate real world grazing scenarios. Chapters 5 and 6 address this challenge by investigating the various drivers of grazing behaviour by sheep under two different scenarios. These two chapters take a concept from the initial proposal of the thesis (Figure 1), to investigate where animals have spent time grazing. The modified research work (Figure 2) resulted in use of data which was previously collected in 2017 (Chapter 5), and 2014 (Chapter 6).

Chapter 5 investigates the grazing behaviours of wethers in continuously grazed paddocks, employing random forest modelling methods to discern specific drivers influencing their choices, during expected a 'grazing hours' window, and the full day, across the year. In comparing different stocking rates, variations in grazing patterns between low and high stocking rates were revealed. The results uncovered mobbing behaviours in low stocking rate scenarios, leading to uneven pasture utilisation that overall impacted sward growth, resulting in areas of underused and over grazed pasture. This grazing behaviour has long term implications, significant for overall pasture production and productivity, with potential repercussions overall for sustainability and soil health. The study additionally reaffirmed that animals tend to visit specific grazing areas, irrespective of the time or season, potentially influencing pasture sustainability and overall soil health in the long run. Further, the strong social behaviours observed in wethers emerged as a primary driver of grazing preference, evident in their proximity to other sheep in neighbouring paddocks.

The data analysis approach in Chapter 5 underscores the efficacy that in a small-scale scenario, using models to understand and predict forage use by animals offers valuable insights for enhanced management strategies. For instance, the study revealed that the presence of legumes significantly influenced residency, even if legumes were present for a short period or in low quantities. This information proves instrumental in planning sown pastures, especially in areas favoured by animals due to other abiotic factors such as shade, water troughs, or topography. While pasture availability and type, aside from presence of legumes, did not necessarily impact grazing behaviours in this study, this chapter also acknowledges that the results may be influenced by the small paddocks and mob sizes used.

The results collected in Chapter 5 highlighted the imperative to extend predictive modelling to look at sheep grazing behaviours on a large scale, incorporating more realistic flock and paddock sizes. This was achieved in Chapter 6, with the ability to remove the inherent impact that infrastructure and mobbing may have on results, due to the nature of the experimental design. Chapter 6 explored the influence of pasture type (native vs. introduced species) on grazing behaviour, using larger mob sizes (25 wethers). This research identified distinct drivers of grazing behaviours contingent upon pasture type, indicating that the pasture present can shape the patterns of grazing sheep. Whilst data on specific species of pasture present was not available, NDVI (normalised difference vegetation index) served as a proxy to indicate pasture quality. The resultant models revealed that NDVI did play a crucial role for sheep in improved pastures, compared to those on native pastures.
Further, the models inferred that wethers in the native pastures were often not walking as far

as those in the improved pastures, whom presumably were spending more time searching for higher quality pasture, suggesting that animals undertake different grazing strategies between pasture types.

Overall, a common observation between the models in Chapters 5 and 6 is the pivotal role of pasture quality, weather indicated by NDVI values or the presence of a high-quality pasture species such as legumes, as a significant driver of grazing behaviour in wethers. The absence of such quality pasture may therefore lead animals to spend extended periods of time within a common area, rather than actively searching for preferred pasture types. While random forest modelling provides valuable insights into a less labour- intensive method of behaviour research, the study emphasised the need to address spatial autocorrelation issues inherent in GPS data. As a result, inclusion of eastings and northings measurements were included in the models to understand the importance of 'space' against the data in a given point in time. Computational constraints were identified as a major challenge when attempting spatial random forest modelling, prompting considerations for high-performance computing environments to enhance the robustness of modelling efforts.

Understanding the intricate relationship between pasture type and grazing behaviours, and reciprocally, how animal behaviour influences pasture performance, is paramount for formulating sustainable management strategies at a paddock scale, for our future. The insights evident in Chapters 5 and 6 pave the way for advancement in knowledge regarding the multifaceted impact of variables on grazing preference. Based on the trends identified in both chapters, potential strategies could be implemented in future, such as introducing areas of improved species within targeted areas of native pastures, to meet the nutritional requirements of animals, thereby enhancing the sustainability of livestock grazing practices in Australia.

Future recommendations

The research within this thesis leads to three prospective avenues for further exploration. These areas encompass an in-depth examination of constituents of cattle saliva, advancing the understanding of plant physiological responses to defoliation, and the continued application of modelling approaches to predict grazing behaviours in livestock.

There are many facets in which saliva-based research could delve into. Building on the findings from Chapter 4, research should be furthered into the specific functions of each metabolite identified, in both plants and animals. This knowledge will allow for a deepened understanding into which metabolites present may have originated from the plant, in comparison to those which have originated from the livestock grazing. A comparative metabolomic analysis of the plants in the pasture where animals have grazed could be conducted. Research in various 'omics studies has been an up-and-coming practical approach for understanding the composition of the metabolite pool within plants (Patel et al., 2021), and as such, could be presented in conjunction with future metabolomic analysis of saliva. Furthermore, the research presented in Chapter 4 would benefit from a more comprehensive analysis on the background factors affecting the animals, such as health status, diet, and environmental variations. This would help ascertain whether the metabolite profile is significantly impacted by external changes or remains relatively consistent. A broader quantitative analysis of saliva collected using similar methods from various grazing ungulates (e.g., sheep, goats, horses) could additionally be pursued to identify potential variations in

nitrogen content present in saliva. Furthermore, studies should also be conducted to quantify the amount of saliva deposited during a grazing event, as the current knowledge on this aspect is limited, or not well documented. Studies should also be performed to investigate bite patterns and the variations from animals which may change saliva deposition, and so too, associated regrowth rates of plants. Understanding variations in saliva residue on plants left after grazing bites, which may differ amongst species, would contribute greatly to address the gaps discussed in Chapter 2.

Advancing the research in plant physiological responses to defoliation involves conducting more studies in a glasshouse, before drawing comparisons with field trials. As mentioned previously, the initial idea for Chapter 3 included examining the responses of native plant species to Australia to saliva and defoliation events – and which may in part explain the poor responses of many native grass species to grazing. Identifying how these plants respond may prove beneficial for farmers as native grasses are an integral part of many farms and native pasture species are adapted to the Australian conditions, therefore reducing labour and input costs, compared to improved species.

Similar to the initial proposed research plan (Figure 1), changes in carbon and nutrient dynamics (through shoots, roots, and soil) during grazing events with saliva should additionally be studied, along with differing defoliation rates and saliva application rates. Though these two studies in particular are common with current grazing research, these current studies often do not consider the part that saliva as a chemical elicitor may play. Further, a side-by-side study could be performed to determine if the effects are the same when the same saliva type is used, or if there are differences within species. This type of study would allow for deeper insights into pasture growth trends for mixed livestock enterprises and would coincide with the research discussed on 'omics exploration of various species saliva. Finally, research performed in the glasshouse should be transposed into the field in order to allow for greater accuracy to real scenarios. This could include research similar to the initially proposed field trial (Figure 1), whereby animals are tracked on the pasture, and samples are taken directly from where animals have spent time grazing. Environmental conditions may alter the results significantly in the field in contrast to the set up within a glasshouse.

Alongside the saliva and pasture-based research, studies on animal grazing behaviours should also be continued. Underlined in Chapters 5 and 6, additional research needs to be performed to contrast different movement-based data types with each other (i.e., visual/traditional observation-based measurements, GPS tracking, accelerometer data, Brownian bridge models), to determine if modelling and creating predictions around GPS based behaviour data, is sufficient. This step ensures that the data used for modelling can encapsulate a holistic picture of an animal's grazing event, and so too, potentially allow for less labour-intensive monitoring of animals during studies. Furthering the GPS tracking of other animals and using similar predictive efforts (i.e., random forest modelling) would be beneficial to identify if these models will work successfully for other livestock species. Overall, investigating the efficacy of GPS-based behaviour data for modelling purposes can lead to more efficient and precise predictions in research, as it is clear that the spatial distribution of livestock ultimately impacts use and productivity of the pasture. The research is needed to improve predictive modelling capacity, and potentially enable the use of the data on farm to allow pastoralists to have a more targeted approach to grazing management.

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