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for the Environment

Integration of simulated and true herbivory with an emphasis on rapidly deployed anti-herbivore silicon defences

By

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Hawkesbury Institute for the Environment

Western Sydney University

October 2021

Declaration of Authenticity

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

Acknowledgements

My principal PhD supervisor, Scott Johnson deserves the greatest thanks for his guidance and support throughout my PhD. Whether it be experimental design, manuscript writing, or even just menial administrative tasks, I am deeply grateful for the time and invaluable insights Scott provided. I would like to thank my secondary supervisors, Sue Hartley and Chris Cazzonelli, for their excellent guidance and sage advice, especially regarding manuscript writing. Shinichi Nakagawa also deserves immense thanks for his time, effort, and critical assistance with Chapter 6 of this thesis. I would also like to recognise the 'Silicon Crew', with special thanks to Ximena Cibils-Stewart and Casey Hall for their collaboration and support throughout my three years at HIE. I would also like to thank Meena Mikhael and Tim Mann for their collaboration, technical support, and expertise.

I must thank Anthony D'Amato, Karl Fetter, Steve Keller, and Ann Hagerman, all of whom played a key role in my early scientific development and catalysed my interest in ecological research as an undergraduate student.

Furthermore, I would be remiss if I did not thank the following WhatsApp and Instagram groups (and humans therein) for providing invaluable moral support and entertainment value: Nautical Phasmids, 'I'm sorry, mate', Lads that lunch, Pinging and Ponging + NRL, Smelly herbs for Yaz, and woque bois only. I would especially like to emphasise the importance of the support and companionship provided by Claire Allison. Also, my desk-mates, Bruce and Edith (pigeons that nest on my balcony), as well as Heathcliff and Elizabeth Bennett (beloved stick insects), provided excellent company during the extensive work-from-home periods due to COVID-19.

My wonderful family (Ma, Gran, D, Kim, Mike, Shay, Blue) deserve the utmost gratitude for their unyielding love and support, not only during my PhD (from 10,000 miles away), but throughout my development. Finally, I would like to dedicate this thesis to my loving mother (Ma), Kristine Waterman, who has done so much to ensure I am able to live a life of joy, wonder and fulfilment. The amount of gratitude and recognition she deserves is incalculable and I will owe, at least in part, any success for the remainder of my life to her.

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Abstract

Plants and herbivorous insects have been locked in an evolutionary arms race for over 300 million years. As a result, plants have evolved a plethora of defences against herbivory, many of which are triggered by herbivore-associated stimuli, including mechanical stimulation (e.g., vibrations from herbivore movement), tissue damage (wounding), chemical elicitation, and transmission of microbes (including pathogens). Understanding how these stimuli affect plant defences is confounded by the fact that herbivores introduce uncontrolled bias stemming from variation in feeding patterns, intensity of damage, and the introduction of biotic and abiotic signals in a non-standardised way. Simulated herbivory is often incorporated into studies to uncouple the relative impacts of herbivore-associated stimuli, glean mechanistic details regarding plant defences, and for standardisation purposes.

Some plants, namely grasses, have evolved the ability to uptake Si from the soil and accumulate it throughout their aboveground tissues. The role of Si in plant ecology is complex, as it has proven beneficial for plants in the context of growth, reproduction, and mitigation of diverse environmental stressors. But perhaps one of the most apparent advantages of Si accumulation is its strong anti-herbivory quality. In grasses specifically, it has been suggested that Si plays a critical role in their ability to combat herbivore attack. Although Si is well known to mitigate the negative impacts of herbivory, there are many knowledge gaps regarding the temporal scales of induced Si-based resistance and the mechanisms behind Si accumulation and deposition. Using both simulated and authentic herbivory techniques, this work identifies the extent that Si is integrated into wider plant defence machinery, how rapidly Si defences can be effectively deployed, and how quickly plants develop resistance to herbivores once Si is supplied.

Chapter one provides the necessary background and context for the work conducted in chapters 2–6.

Chapter two of this thesis synthesises studies that incorporate simulated herbivory, highlights the application of simulated herbivory in experiments, and identifies how simulated herbivory might be used to address research questions that are unanswerable when using herbivores.

Chapters three, four and five were conducted using the model grass and Si-hyperaccumulator, *Brachypodium distachyon*. In chapters 3 and 5, the global insect pest, the cotton bollworm (*Helicoverpa armigera*) was used as a model herbivore.

Chapter three investigates the role of specific herbivore stimuli, oral secretions (OS) and their microbial constituents, in activating wound responses. Crude OS from *H. armigera* was shown to activate greater levels of senescence around wounds in *B. distachyon* leaves compared to OS with reduced microbial abundance and mechanical damage alone. Nonetheless, plant wound closure was greater when treated with *H. armigera* OS regardless of the microbial component. This highlights the importance of herbivore-specific signals for the activation of an important defence response to both herbivores and microbes.

Chapters four and five focus on the short-term dynamics of Si-based defences, integration of Si with alternative defence responses, and whether plants with only brief exposure to Si can successfully defend themselves against herbivores. Chapter four investigates how rapidly Si accumulation is induced in response to simulated herbivory. In *B. distachyon*, within 6 hr of simulated herbivory treatment plants increased foliar Si accumulation by 20%. This increase

was tightly correlated with increased jasmonic acid concentrations and suppressed foliar salicylic acid levels. Additionally, the effects of Si on further biochemical defences (e.g., phenolics) were dependent on whether plants were treated with simulated herbivory. Chapter five expands on this finding by determining how short-term Si exposure and rapid Si accumulation impact herbivore feeding and performance. Within 72 hr of exposure to Si, plants were as resistant to herbivory as plants exposed to Si for over 34 days, despite having considerably lower levels of Si in their tissues, likely due to the rapid filling of Si cells (phytoliths) on the leaf surface. These findings provide novel insights regarding the temporal dynamics of Si-based plant defences and highlight that Si-based resistance to herbivory can be achieved in plants much more rapidly than previously envisaged, perhaps underpinned by their integration with phytohormonal (jasmonic acid and salicylic acid) signalling pathways.

Chapter six investigates how responses induced by simulated herbivory compare to those induced by live herbivores through a meta-analysis of 110 peer-reviewed studies, covering 56 plant species and 5 arthropod orders, that measured biochemical defence responses induced by both simulated and true herbivory. This chapter contains meta-analysis on both the mean response and the variability of responses, as variation in responses is emerging as a major structuring force in plant–herbivore interactions. Comparability between simulated and authentic herbivory was shown to vary with methodology, herbivore taxa and treatment duration. Simulated herbivory generally induces responses conservatively compared to true herbivory, however, can accurately mimic herbivores when responses are measured soon after plants are treated and when multiple herbivore-associated stimuli are incorporated. Further, this chapter identifies the key factors which are important for accurate simulation of herbivory, potential knowledge gaps and room for further exploration in the literature, as well as methodologies that should be used or avoided given research objectives.

Collectively, this PhD research highlights the importance of herbivore-specific signals in shaping plant defence responses and integrates simulated and true herbivory to yield a robust mechanistic understanding of the temporal scale at which Si-based defences, which are critical for resistance to herbivory, are deployed in a model grass. These findings could have implications for the way Si is utilised in agricultural systems and provide novel insights regarding potential evolutionary strategies evolved in grasses to utilise Si as an inducible defence in an analogous way to inducible specialised metabolites. Collectively these works provide novel evidence for the specified role of Si as an anti-herbivore defence in grasses and systematically identify the role of simulated herbivory in ecological research.

Preface

This thesis contains solely original research conducted by myself with guidance from my supervisors, Scott N. Johnson (primary supervisor), Christopher I. Cazzonelli and Susan E. Hartley (University of Sheffield), as well as Shinichi Nakagawa from the University of New South Wales Sydney. I conceptualised the research together with my supervisory panel. I have conducted the collection, analyses, interpretation, and presentation of the data contained within this thesis. I have written this thesis and all publications therein with guidance and assistance from my supervisory panel and additional collaborators.

This thesis is a standard thesis and not a thesis by publication. However, it consists of a review chapter and four stand-alone experimental chapters that have been written in a format appropriate for specific peer-reviewed journals. Chapters 2–5 have been published in peer-reviewed journals and chapter 6 is to be submitted for publication in a peer-reviewed journal. Chapters are written either as published in their respective journals or as intended to be printed upon acceptance, and thus some repetition in terms of information and methodology will be present. Although chapters 2–6 are multi-authored works, I am responsible for the large majority of the work contained therein (Table 0-1):

Table 0-1. Percentage contribution made by Jamie M. Waterman to each published (or formatted to be published) chapter within this thesis

Chapter	Journal	Ideas	Writing	Collection	Analysis	Data presentation
2	*Trends. Ecol. Evol.*	75%	First draft: 100% *Co-authors helped revise* **Total: 80%**	N/A	N/A	100%
3	*Ecol. Entomol.*	80%	First draft: 95% • *Co-author wrote small section of methods* *Co-authors helped revise* **Total: 85%**	75%	100%	100%
4	*Funct. Ecol.*	85%	First draft: 95% • *Co-author wrote small section of methods* *Co-authors helped revise* **Total: 80%**	85%	100%	100%
5	*Ecology*	85%	First draft: 95% • *Co-author wrote small section of methods* *Co-authors helped revise* **Total: 85%**	80%	100%	100%
6	**In Preparation**	80%	First draft: 90% • *Co-author wrote small section of methods* *Co-authors helped revise* **Total: 85%**	100%	85%	100%

1   Chapter 1: General introduction

1.1   Plant-herbivore interactions

Plants and insect herbivores have been evolving in the presence of one another for several hundred million years (Hartley and Jones 1997). Over half a century ago the notion was put forward that plants and insect herbivores had likely experienced coevolution, whereby plants evolved complex defence mechanisms to which herbivores developed counter adaptations, ultimately resulting in an 'arms race' between the two (Ehrlich and Raven 1964). Plants and insect herbivores comprise roughly 50% of multicellular terrestrial organisms, and thus plant–herbivore interactions play a substantial role in shaping natural ecosystems (Hartley and Jones 1997, Nentwig and Vaes-Petignat 2014). Insect herbivores also have dramatic impacts on the agricultural industry and are responsible for billions of dollars in crop losses annually, making understanding the mechanisms of plant–herbivore interactions, and subsequent development of mitigation strategies, exceedingly important for ensuring agricultural sustainability (Oerke 2005, Deutsch et al. 2018). One factor that makes addressing these mechanisms a challenge is the wide diversity in feeding strategies employed by insects, ranging from those that severely damage plant tissues (such as lepidopteran chewing herbivores) to those who do minimal damage to tissue by inserting very narrow feeding parts between cells to feed on fluids from plant vascular tissue (e.g., Hemiptera) (Leitner et al. 2005). Although both feeding strategies can have deleterious impacts on plants, the subsequent responses from plants vary greatly (Walling 2000). Additionally, even within feeding guilds (i.e., closely related herbivores with similar feeding strategies) there are many adaptations, such as host specificity, that contribute to nuances in feeding patterns, plant responses, tolerance, and resistance (Cates 1980, Ali and Agrawal 2012).

As a result of substantial herbivore pressures during more than 300 million years of coevolution, plants have evolved a plethora of biochemical and physical defence mechanisms to combat a multitude of attacking herbivores (Levin 1973, Turley et al. 2013, Kessler and Kalske 2018, Defossez et al. 2021). Determining the mechanistic bases of the intricacies of plant–herbivore interactions and mechanisms through which plant defences impact herbivores is essential not only from an eco-evolutionary perspective, but also to inform effective pest-management strategies in agricultural systems, which ultimately has major impacts on high-priority global issues such as food security (Gregory et al. 2009).

1.2 Plant defences in context

When plants are exposed to insect herbivores, they are inundated with multiple signals at once, commonly referred to as herbivore- and damage-associated molecular patterns (HAMPs and DAMPs, respectively) (Mithöfer and Boland 2008, Hou et al. 2019). In many chewing herbivores, chemical signals contained within their regurgitant, or oral secretions (OS), and saliva can have substantial impacts on plant defence responses beyond what would be induced by wounding alone (Tian et al. 2012, Sobhy et al. 2017, Li et al. 2019). Making matters more complicated, insects form associations with microbes that expose plants to microbe-associated molecular patterns (MAMPs) that are also known to modify plant defence responses (Newman et al. 2013, Schausberger 2018). For example, Colorado potato beetle OS contain bacterial symbionts that supress anti-herbivore defence responses in tomato plants (Chung et al. 2013). Upon removal of microbes from OS, Chung et al. found defence responses to be markedly higher than OS with the natural microbial community intact. In contrast, chewing herbivore-associated (*Helicoverpa zea*) microbes have also been shown to strengthen the anti-herbivore response, highlighting the complexity of these multi-trophic interactions (Wang et al. 2017). Considering that, under herbivore attack, plants are exposed

to signals of generic wounding as well as herbivore- and microbe-derived signals, it can be challenging to identify the precise source responsible for defence response induction (details covered in Chapter 2). For example, some responses, such as cell senescence, might be induced during herbivory, but considering the importance of senescence in resistance to biotrophic microbial pathogens, is also likely induced by herbivore-associated microbes (Guo and Gan 2012, Häffner et al. 2015). Therefore, to determine a precise mechanistic basis for responses such as cell senescence, it is essential to identify the relative weight of microbial and herbivore signals.

Upon perception of HAMPs, within seconds, early signalling events such as plasma transmembrane depolarisation, increases in cellular Ca^+ concentrations and production of reactive oxygen species such as hydrogen peroxide occur, catalysing a cascade of defence responses, primarily regulated by the phytohormone, jasmonic acid (JA) (Maffei et al. 2004, Howe and Jander 2008, Erb et al. 2012, Erb and Reymond 2019). Further downstream in these metabolic pathways, often occurring within hours, days or even weeks (Lu et al. 2014, Wang et al. 2014, Erb et al. 2015), many plants biosynthesise specialised metabolites and proteins that can have deleterious effects on herbivores either due to toxicity or feeding deterrence (Kessler and Kalske 2018, Erb and Reymond 2019). Some plants rely heavily on highly specified defence metabolites that target herbivores to defend themselves, such as nicotine in tobacco and glucosinolates in *Brassica* spp. (Steppuhn et al. 2004, Ahuja et al. 2010). Other plants, including plants in the family Poaceae (grasses), may be less equipped with diverse metabolites (Moore and Johnson 2017, Defossez et al. 2021) and rely more heavily on alternative means of defence against herbivory such as (i) formation of symbioses with microorganisms that produce their own anti-herbivore metabolites in exchange for plant nutrients (Bastías et al. 2021), (ii) physical and structural defences including hairs, thorns,

spines and trichomes on the surface of tissues (War et al. 2012, Kariyat et al. 2017, Moore and Johnson 2017, Hall et al. 2020a), and (iii) the ability to uptake metals and metalloids from the soil that enhance resistance to herbivory (Davis and Boyd 2000, Plaza et al. 2015, Hall et al. 2019).

1.3 Silicon (Si) in plants

Silicon (Si) is the second most abundant element in the earth's crust and is thus found in soil systems throughout the world (Epstein 1994). This considered, most terrestrial plants have evolved in the presence of Si, however Si is only available for plant uptake in the form of orthosilicic acid ($Si(OH)_4$), which only makes up a small fraction of total soil Si (Liang et al. 2015). Despite constant exposure to Si throughout terrestrial plant evolution, the degree to which Si is incorporated into plant tissues varies drastically across taxa, from < 0.01% to > 10% dry weight (Epstein 1994). Plants uptake Si through both active (requiring ATP) and passive (no energy input) transport through a series of channel-type influx (Lsi1, Lsi6; passive) and efflux (Lsi2; active) proteins (Ma and Yamaji 2015). The influx transport proteins facilitate the translocation of Si from the soil environment into plant root systems and out of the xylem into tissues (Ma et al. 2006, Yamaji et al. 2008), whereas the efflux proteins transport Si from the root tissue into the xylem, where the Si then moves through the transpiration stream to aboveground tissues (Ma et al. 2007).

In plants, Si is deposited in cell walls, tissue surface structures (trichomes, macro-hairs, etc.), and as discrete silica structures such as phytoliths (Si cells) (Hartley et al. 2015, Kumar et al. 2017b). Additional proteins have been identified for Si deposition (solid silica formation in plant tissues), however the mechanisms behind this process are much less understood (Kumar et al. 2020). While Si is not considered an essential element for plants (Ma 2004), Si uptake

and accumulation are increasingly being recognised as an effective and highly important strategy for certain plants to mitigate the impacts of a multitude of biotic and abiotic stressors, including herbivores, pathogens, nutrient deficiency or toxicity, and extreme climatic conditions (Cooke and Leishman 2016, Leroy et al. 2019, Vandegeer et al. 2021).

1.4 Si as an anti-herbivore defence

In the context of herbivory specifically, the mechanisms underpinning Si-mediated resistance remain contentious (Reynolds et al. 2009, Coskun et al. 2019, Hall et al. 2019). Perhaps the most prevalent hypothesis is that Si deposits in plant tissues act as abrasive structures that can wear down herbivore mouthparts (Massey and Hartley 2009), interfere with the digestibility of plant tissues (Massey and Hartley 2006, Massey and Hartley 2009, Andama et al. 2020), and make tissues more rigid and more difficult to physically crush (Clissold 2007, Hunt et al. 2008). Although the direct impacts of Si as a physical defence are clear, the mechanisms behind Si accumulation and how Si integrates with wider defence machinery (i.e., how Si impacts defence signals and *vice versa*) remain elusive (Coskun et al. 2019, Hall et al. 2019). Recent evidence suggests that Si accumulation, like biosynthesis of certain anti-herbivore metabolites, is induced by herbivory and integrated with the phytohormone and master defence response regulator, jasmonic acid (JA) (Erb et al. 2012, Ye et al. 2013, Hall et al. 2020b, Johnson et al. 2021). This has also been demonstrated on the molecular level; activation of the Si transporters Lsi1, Lsi2 and Lsi6 has been demonstrated in response to herbivore signals (Ye et al. 2013). Further, when JA synthesis (*allene oxide synthase*; *AOS*) and perception (*coronatine insensitive 1*; *COR1*) genes are silenced, the expression level of Si transport genes, and overall capacity for plants to accumulate Si, is substantially diminished (Ye et al. 2013). Nevertheless, less is known about the ways Si interacts with other defence hormones such as salicylic acid (SA), which regulates different pathways than JA such as

those targeted at fluid-feeding insects (Erb et al. 2012, Thaler et al. 2012). Considering successful defence responses can be starkly different between chewing insects and fluid-feeders, it has been demonstrated that SA and JA can behave antagonistically, whereby one might supress downstream responses controlled by the other (Thaler et al. 2012, Phuong et al. 2020). This antagonism has been shown to have consequences on a plant's ability to defend against various herbivore taxa, depending on whether SA- or JA-regulated defences are beneficial (Soler et al. 2012, Ali and Agrawal 2014), however this may not always be the case and may be taxon-specific (Thaler et al. 2012, Fabisch et al. 2019). How Si fits into this system might have important implications for overall defence capabilities of plants (i.e., against a diversity of attacking insects) in a natural setting.

Additionally, as Si and C share many atomic properties (both have 4 valence electrons, can bind to oxygen forming polymers, etc), there is a well-known trade-off between Si and C, likely resulting from the substitution of C with Si during periods of low atmospheric CO_2 in the Miocene, particularly apparent in grasses that rely heavily on Si-based defences (Kürschner et al. 2008, Cooke and Leishman 2011a, Strömberg et al. 2016, Biru et al. 2020). Additionally, Si is considered to be a metabolically 'cheap' alternative to carbon-based structural and defence compounds such as lignin and other phenolics (Raven 1983, Głazowska et al. 2018a), and thus in plants there is often a negative relationship between Si and C in general, as well as between Si and specific C-based defences such as phenolics (Cooke and Leishman 2012, Frew et al. 2016, Klotzbücher et al. 2018). The energetic costs of incorporating lignin, for example, into plant tissues is 27 times higher than Si (per unit mass), so supplementation with Si could reduce the energy investment required to strengthen plant cell walls (Raven 1983, de Tombeur et al. 2021b).

It has been purported that greater incorporation of Si into plant tissue leads to greater benefits for the plant (Ma 2004), although recent evidence suggests that the beneficial effects of Si can be realised at relatively low concentrations, even in low-Si accumulating species (Fauteux et al. 2006, Putra et al. 2020, Wang et al. 2020, Acevedo et al. 2021).

In addition to understanding the threshold for effective resistance to herbivory in terms of overall amount of a defence, of critical importance to the success of a defence response is the rate at which it can be effectively deployed as a means of combatting herbivory; anti-herbivore defence signalling begins seconds after signals are perceived, however when a particular defence is deployed can be anywhere from a few seconds to a few weeks (Karban and Myers 1989). Many responses are substantially induced within a short period of time and maintained for the period of stress, however once stress signals dissipate, levels of defence responses return to baseline (Schmelz et al. 2009, Erb et al. 2015). This is perhaps an adaptation to the dilemma of the 'growth-defence trade-off hypothesis', whereby, in order to maintain high levels of chemical defences, plants must use resources that would otherwise be allocated towards reproduction and growth (Züst and Agrawal 2017). Considering Si deposition is irreversible (Epstein 1994), the impacts of Si accumulation might be more long lasting than other inducible forms of defence. Nevertheless, while the impacts of gradual accumulation (over weeks and months) on herbivore resistance are clear (Epstein 1994, Reynolds et al. 2012), few studies have investigated the dynamics of induced Si accumulation and deposition over short-term temporal scales and how this impacts chewing herbivores (these knowledge gaps are addressed and filled in chapters 4 and 5 of this thesis).

1.5 Integration of simulated herbivory into ecological studies

Understanding the mechanisms associated with defence against herbivory in an experimental setting can be complicated by numerous factors, and perhaps the most prevalent are biases associated with herbivore feeding patterns and behaviour (Caldwell et al. 2016, Robin et al. 2017). For example, herbivores may feed preferentially on one plant over another if plants are part of different experimental treatments, or due to within-plant variation or differences in herbivore behaviour, which makes standardisation of herbivory a challenge when using live herbivores (Gherlenda et al. 2016, Arce et al. 2021). A clear example of this is when experiments utilise Si supplementation as a treatment, as many herbivores feed preferentially on plants with minimal amounts of Si (Ryalls et al. 2017, Islam et al. 2021). Additionally, there are many discrete signals associated with herbivores, including mechanical stimulation, tissue damage, chemical elicitation, and transmission of microbes (Turlings et al. 1993, Schmelz et al. 2009, Tian et al. 2012, Chung et al. 2013, Toyota et al. 2018, Kollasch et al. 2020). Each of these signals can be perceived by the plant, however to ensure that the correct response is deployed, plants may rely on multiple signals at once to identify the attacker (Wu and Baldwin 2009). When insects feed on plant tissues, plants are exposed to many signals in an unstandardised way, making it a serious challenge to discern which specific signals are responsible for inducing a given defence response (Li et al. 2019). As highlighted in greater detail in chapter 2 of this thesis, using simulated herbivory, whereby specific herbivore stimuli are introduced to plants in a standardised and controlled fashion, it is possible to disentangle these stimuli and apply them individually or in desired combinations to develop a precise mechanistic understanding the defence machinery (Turlings et al. 1993, Schmelz et al. 2009, Tian et al. 2012, Chung et al. 2013, Toyota et al. 2018, Kollasch et al. 2020). Nevertheless, there are many scenarios, particularly in ecological research, where simulated herbivory might not be appropriate. For example, simulated herbivory is a known tool for

standardisation across treatments (and thus reduction of variation), however variation in defence responses has been put forward as an important factor for plant resistance to herbivory (Pearse et al. 2018). When and how simulated herbivory might be considered an appropriate substitute for true herbivory or simply as a tool to complement true herbivory assays remains under-studied (details of this are covered extensively in chapters 2 and 6 of this thesis).

1.6   Model system: Plant and herbivore

Brachypodium distachyon (L.) P. Beauv. is a model grass (Poaceae) species that is closely related to many important small-grain crop species such as rice, maize and barley, and is well known to accumulate large amounts of Si (Głazowska et al. 2018a). Additionally, *B. distachyon* has many advantages for experimental use including a short lifecycle, small size at maturity, ease of cultivation, and a small genome in comparison to other grass species. This makes *B. distachyon* an advantageous plant for research purposes and consistency across studies in comparison to larger, longer-lived, and more challenging to grow crop grasses (Opanowicz et al. 2008).

Helicoverpa armigera (Noctuidae: Lepidoptera) is an agricultural pest that is distributed throughout South America, Asia, Europe, Africa and Australasia (Anderson et al. 2018). This species is highly polyphagous and is known to feed on over 180 host plants from over 45 families (Tay et al. 2013). As such it is responsible for approximately $5 billion USD in crop losses annually (Joußen et al. 2012). Further, *H. armigera* is highly resistant to pesticides, making it a serious challenge to control using conventional techniques, and therefore developing alternative methods of controlling this pest is of critical importance (Jones et al. 2019).

1.7 Thesis aims

The overall aim of this thesis was to integrate simulated and true herbivory techniques to better understand the mechanisms underpinning plant defences using laboratory- and glasshouse-based approaches. Uncoupling plant defence mechanisms is essential not only for understanding the bases of coevolution between plants and insect herbivores but also to develop sustainable agricultural practices.

Si-based defences in important cereal crop species have recently been identified as a potential solution to crop losses and are considered of critical importance for resistance to herbivory in such species. A major objective of this thesis was to utilise both simulated and true herbivory techniques to identify some of the mechanisms behind Si defences in a cereal grass model (*Brachypodium distachyon)* and how they impact a major agricultural pest (*Helicoverpa armigera*). Specifically, this thesis fills a knowledge gap regarding the extent to which Si uptake and accumulation integrate with multiple herbivory-associated phytohormones and biochemical defences. Additionally, Si is generally considered as a defence that builds up gradually over time (Reynolds et al. 2012, Ryalls et al. 2017), however the temporal scale of the deployment in plants of Si-based defences is less well understood. This thesis further aims to identify the temporal scales that herbivore-induced Si accumulation occurs and how quickly inductions in Si accumulation and deposition become effective as a defence against herbivores.

The other major objective of this thesis was to understand which simulated herbivory techniques are most effective given research objectives. This thesis identifies that the wounding response is induced to a greater extent in the presence of herbivore specific signals

as opposed to unspecified wound signals. Additionally, the comparability of simulated herbivory to true herbivory across the literature is synthesised to develop a greater understanding of the contributing factors behind herbivore-induced defences. The main aims of this thesis are depicted in Fig 1-1:
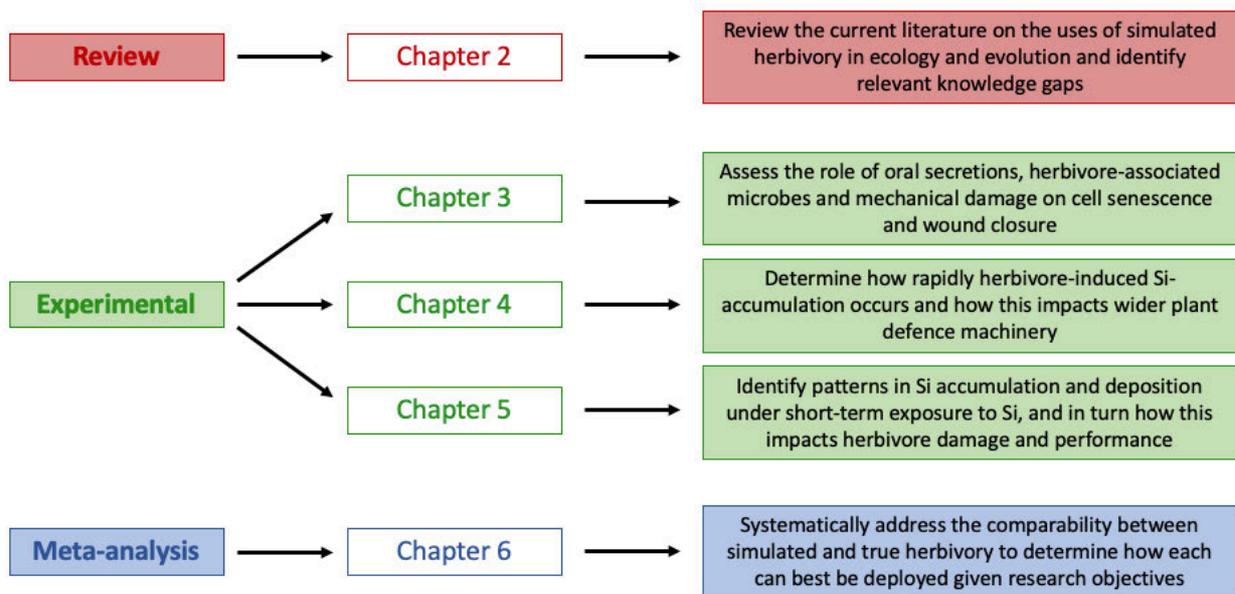
Figure 1-1. Primary objectives of each thesis chapter. Experimental chapters (3–5) were conducted using plants grown in a glasshouse environment under controlled conditions and laboratory techniques. Chapter 6 is a meta-analysis addressing some of the knowledge gaps identified in the review chapter (2).

Further, the general hypotheses of the research contained in this thesis are:

1. Herbivore and microbial signals impact plant wounding response beyond generic wounding signals alone;

2. Si accumulation is rapidly induced by JA signals, similar to the way specialised metabolites might be induced. As Si is integrated into phytohormonal signalling, Si will suppress signals antagonistic to JA such as salicylic acid;

3. The trade-off between Si- and C-based defences is contingent upon whether plants are under stress;

4. The levels of Si accumulated over short periods of time are sufficient to confer adequate resistance to herbivory and this resistance is underpinned by the deposition of specific leaf surface Si structures;

5. The overall trends across the literature will support findings from this thesis that simulated herbivory techniques are most effective for comparison to true herbivory in terms of mean and variation of response when multiple herbivore-associated stimuli and the temporal nature of responses and treatments are considered.

1.8   Thesis overview

This thesis is organised into several chapters with discrete objectives. The introductory chapter covers, generally, plant–herbivore interactions, simulated herbivory, and Si-based defences, and sets the context for the work conducted throughout this thesis.

Chapter 2 reviews the utility and application of simulated herbivory as a tool to understand the mechanisms of plant defence. This review identifies the many complexities associated with understanding plant–herbivore interactions on a mechanistic level. It then highlights how simulated herbivory addresses these complexities and can be used to complement true herbivory to elucidate previously unanswered research questions. This review entitled 'Simulated Herbivory: The Key to Disentangling Plant Defence Responses' (Jamie M. Waterman, Christopher I. Cazzonelli, Susan E. Hartley and Scott N. Johnson) was published in *Trends in Ecology & Evolution*, **34**: 447–458, on 17 April 2019.

Chapter 3 investigates how the wounding response (senescence and wound closure) is differentially impacted by three herbivore associated stimuli: mechanical wounding, oral secretions, and herbivore-associated microbes. This research entitled 'Microbes in

Helicoverpa armigera oral secretions contribute to increased senescence around plant wounds' (Jamie M. Waterman, Timothy J. Mann, Christopher I. Cazzonelli, Susan E. Hartley and Scott N. Johnson) was published in *Ecological Entomology*, **45**: 1224–1229, on 11 May 2020.

Chapter 4 investigates the temporal scale of simulated herbivory-induced Si accumulation and how this response integrates with phytohormone signals and C-based anti-herbivore defences. This research entitled 'Short-term resistance that persists: Rapidly induced silicon anti-herbivore defence affects carbon-based plant defences' (Jamie M. Waterman, Casey R. Hall, Meena Mikhael, Christopher I. Cazzonelli, Susan E. Hartley and Scott N. Johnson) was published in *Functional Ecology*, **35**: 82–92, on 16 October 2020.

Chapter 5 investigates how brief plant exposure to Si impacts defence against chewing insect herbivory. Specifically, this research investigates Si deposition patterns and the mechanisms behind associated reductions in herbivore fitness and performance. This research entitled 'Short-term exposure to silicon rapidly enhances plant resistance to herbivory' (Jamie M. Waterman, Ximena Cibils-Stewart, Christopher I. Cazzonelli, Susan E. Hartley and Scott N. Johnson) was published in *Ecology*, doi: 10.1002/ecy.3438, on 17 June 2021.

Chapter 6 is a meta-analysis that compares simulated and true herbivory as means of inducing biochemical plant defence responses. This research identifies the important factors that affect said comparability, such as timing, technique, taxa, and type of defence. Further it provides potential solutions for issues pertaining to the use of simulated and true herbivory and identifies scenarios in which each might be most effective. This research entitled 'Meta-analysis shows that simulated herbivory can imitate short-term plant defences induced by real

herbivory' (Jamie M. Waterman, Shinichi Nakagawa, and Scott N. Johnson) is formatted for

Nature Plants and will be submitted for publication in October 2021.

2 Chapter 2: Simulated herbivory: the key to disentangling plant defence responses

2.1 Abstract

Plants are subjected to a multitude of stimuli during insect herbivory, resulting in a complex and cumulative defence response. Breaking down the components of herbivory into specific stimuli and identifying the mechanisms of defence associated with them has thus far been challenging. Advances in our understanding of responses to inconspicuous stimuli, such as those induced by microbial symbionts in herbivore secretions and mechanical stimulation caused by insects, have shed light on the intricacies of herbivory. Here we provide a synthesis of the interacting impacts of herbivory on plants and the consequential complexities associated with uncoupling defence responses. We propose that simulated herbivory should be used to complement true herbivory in order to decipher the mechanisms of insect herbivore-induced plant defence responses.

2.2 Plant Defences Vary Depending on the Nature of Herbivory

Around a quarter of multicellular organisms on the planet are thought to be insect herbivores that have been locked in an evolutionary arms race with plants for over 300 million years (Hartley and Jones 1997). The plant defence mechanisms driving this battle have been the subject of intense study and debate (Stamp 2003). Insects are typically grouped into two broad categories: chewing insects (e.g., Orthoptera, Coleoptera, and Lepidoptera) and piercing and sucking insects (e.g., Hemiptera) (Bonaventure 2012). During **true herbivory** (see Glossary), chewing insects physically lacerate plant tissue as they feed, whereas piercing

and sucking insects (e.g. phloem-feeders) typically cause minimal cellular rupture (Leitner et al. 2005). However, chewing insects such as leafcutter ants can cause relatively less tissue damage due to their razor-like mouthparts (i.e. the surface area of damage might be lower) (Kost et al. 2011). It is suggested that defence against phloem-feeders typically involves responses similar to those elicited by microbial pathogens, including programmed cell death, a metabolic process that occurs without wounding recognition (Broekgaarden et al. 2011, Hogenhout and Bos 2011). Nevertheless, following penetration and rupture of sieve elements by phloem-feeders, defence responses can be induced (Salvador-Recatalà et al. 2014). Differences in herbivore feeding habits result in variable perception of attack, which can lead to large differences in defence responses (Bos and Hogenhout 2011, Reese et al. 2016).

2.3   Multiple Stimuli Trigger Plant Defences During Herbivory

There are multiple stimuli associated with insect herbivores that are each (independently) known to affect responses in plants. Collectively, these stimuli generate the observed responses of plants to insect herbivory (Fig 2-1). **Wounding** and **mechanical stimulation** induce defence responses in plants (Appel and Cocroft 2014, Blue et al. 2015, Toyota et al. 2018), and plants can recognise self-derived cellular components (e.g. cell wall fragments, glucose, electrolytes, etc.) released in response to tissue damage (Heil et al. 2012). Even unwounded plants activate metabolite signalling processes such as employing defensive hormones including jasmonic acid (JA) following repetitive touch or mechanical stimulation (Chehab et al. 2012, Cazzonelli et al. 2014) (Fig 2-1). Similarly, plant defence responses can be altered by sound vibrations; foliar glucosinolate concentration was shown to increase with higher vibration amplitudes from insect chewing (Appel and Cocroft 2014). Further, in rice (*Oryza sativa*), aldolase (a glycolytic enzyme) mRNA expression was significantly

upregulated at sound frequencies of 125 and 250 Hz, but was downregulated at 50 Hz, indicating that responses to sound might be frequency specific (Jeong et al. 2008).

The complexity of defence response becomes greater upon exposure to chemical **elicitors** and **effectors** classified as herbivore-associated molecular patterns (HAMPs) (Fig 2-1) (Halitschke and Baldwin 2005, Major and Constabel 2006, Peiffer and Felton 2009). All else being equal, plant defences can be suppressed (Musser et al. 2002, Will et al. 2007) or increased in response to said compounds (Turlings et al. 1993, Halitschke et al. 2001, Schmelz et al. 2009, Tian et al. 2012, Wang et al. 2017). In some instances, responses that weren't previously detectable can be realised in the presence of HAMPs (Reymond et al. 2004, Schmelz et al. 2009). Considering chewing insects harbour microbes in their **saliva**, digestive tract, and exoskeleton, certain responses may be solely microbe-induced and thus independent of insect-derived compounds, mechanical stimulation, and wounding. It has therefore proven difficult to uncouple whether the observed defence responses are derived from the insect, associated microbes, or both (Fig 2-1). For example, bacterial symbionts in the **oral secretions (OS)** of both Colorado potato beetle (*Leptinotarsa decemlineata*) and corn earworm (*Helicoverpa zea*) can decrease JA-responsive defences, including polyphenol oxidase activity, relative to OS with lesser amounts of bacteria (Chung et al. 2013, Wang et al. 2017). Similarly, numerous defence response-associated genes in maize (*Zea mays*) were suppressed to a greater extent by western corn rootworm (*Diabrotica virgifera virgifera*) treated with *Wolbachia* sp. than untreated individuals (Barr et al. 2010). It is clear that a multitude of stimuli are responsible for the consequential responses to herbivory, and it is

critical to consider each when investigating the underlying mechanisms associated with

plant–herbivore interactions.
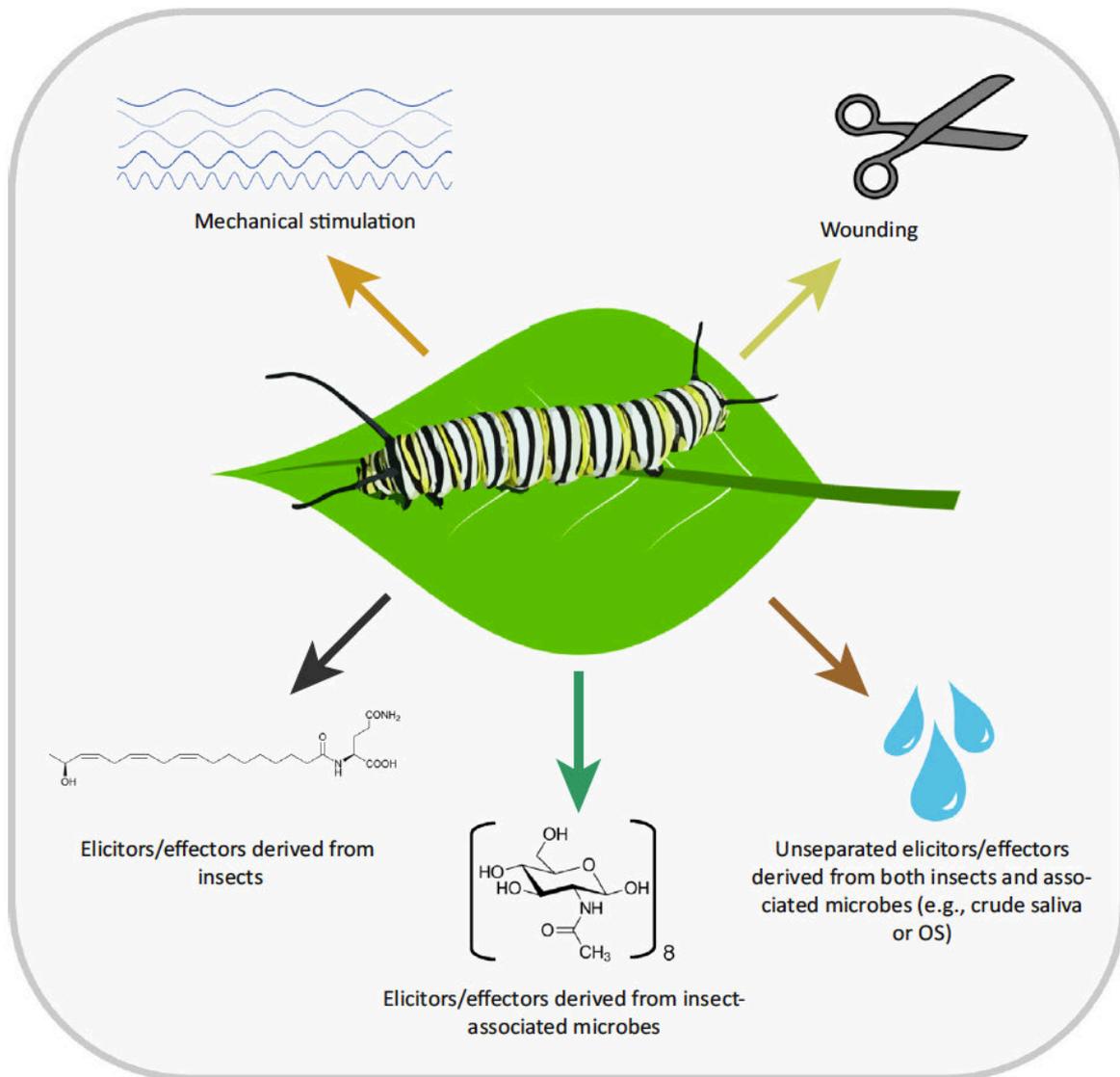
Figure 2-1. Independent stimuli known to elicit a plant response during chewing insect herbivory. The simplest break down of the various defence-inducing stimuli is into physical disturbance and chemical elicitation. Physical disturbance can be further broken down into wounding and mechanical stimulation (i.e., physical movement and/or vibrations), and chemical elicitation can be broken into compounds derived from microbes associated with insects or from the insects themselves.

2.4 The Chemical Machinery of Plant Defences

When a plant perceives herbivore attack various complex signal cascades (e.g. electrical and

chemical signalling pathways) are activated both locally and systemically, resulting in the

activation of defence responses, including the accumulation of reactive oxygen species

(ROS), Ca^+, defence hormones, and volatile organic compounds (VOCs), that contribute to the plant's ability to mitigate the effects of the imposed stress (Rejeb et al. 2014, Choi et al. 2017, Toyota et al. 2018). The major plant hormones known to influence the defence response are JA, salicylic acid (SA), and ethylene (ET) (Wu and Baldwin 2010). It has been shown that JA and SA can exhibit an antagonistic relationship, that is, JA signalling can suppress the SA pathway and *vice versa* (Pieterse et al. 2012). Many microbes induce SA-responsive defences whereas chewing herbivores often stimulate JA-responsive pathways (Reymond and Farmer 1998, Pieterse et al. 2012). In systems in which a plant's JA- and SA-responsive defences interact, microbial symbionts can give herbivores an advantage by inducing the SA pathway and concurrently suppressing JA-dependent defence responses. Although this antagonism has been demonstrated in many plant species, whether or not there is a ubiquitous genetic basis for crosstalk between JA and SA remains contentious (Thaler et al. 2012).

Furthermore, elicitors can trigger a defence response in one species, but have a minimal or differing effect on the same pathway in another (Musser et al. 2002, Schmelz et al. 2009). Even within the same plant family, elicitors can have variable effects on the induction of defence responses. For example, inceptin, a short proteolytic fragment of chloroplastic ATP synthase found in the saliva of fall armyworm (*Spodoptera frugiperda*), upregulated the production of JA, SA, ET, and total VOCs in cowpea (*Vigna unguiculata*), but had a much lesser influence on the same hormones in soybean (*Glycine max*) (Schmelz et al. 2006, Schmelz et al. 2009). In both lima bean (*Phaseolus lunatus*) and cabbage (*Brassica oleracea*), β-glucosidases found in the OS of the large white (*Pieris brassicae*) triggered the emission of VOCs known to act as **indirect defence**s against herbivory by attracting wasps known to parasitise insect herbivores (Mattiacci et al. 1995, Felton and Tumlinson 2008,

Aljbory and Chen 2018). Also in *P. lunatus*, the accumulation of ROS, which affects defence signalling in plants and can result in direct oxidative injury to insects, was greater in leaves that had been fed on by Egyptian cotton leafworm (*Spodoptera littoralis*) than those simply damaged mechanically (Maffei et al. 2006). Specifically, the enzyme glucose oxidase and the fatty acid-amino conjugate N-linolenoyl-L-glutamine (both found in lepidopteran OS) have been shown to promote a significant increase in ROS concentrations within leaf tissue shortly after damage is inflicted (Kerchev et al. 2012, Tian et al. 2012, Block et al. 2018). The fatty acid-amino conjugate volicitin (*N*-(17-hydroxylinolenoyl)-L-glutamine) is found in the OS of lepidopteran larvae and is responsible for the induction of multiple plant VOCs. Additionally, volicitin can stimulate increased activity of both hormone-induced and wound-induced protein kinases (Wu et al. 2007, Kant et al. 2015). Further, caeliferins (disulphooxy fatty acids named due to their presence in the OS of Orthopteran insects in the suborder Caelifera) induce similar defence responses in multiple plant species (Alborn et al. 2007, Schmelz et al. 2009, Kant et al. 2015). In contrast, glucose oxidase in *H. zea* saliva can inhibit the synthesis and functionality of nicotine in tobacco (*Nicotiana attenuata*) and thus decrease resistance (Musser et al. 2002, Steppuhn et al. 2004). Insect-derived molecules can also suppress indirect defences, as it has been shown that a silkworm (*Bombyx mori*) specific enzyme (BmFHD) suppressed the production of leaf VOCs in mulberry (*Morus alba*) (Takai et al. 2018). In order to realise the nature of the complexities associated with insect feeding, development of techniques that enable the uncoupling of the mechanisms that drive the responses observed in plants is critical.

2.5 Simulated Herbivory: A Change in Emphasis

It has been almost 30 years since Baldwin (1990) published the seminal review on the value of using mechanical simulations in ecological research. Baldwin's paper identified

advantages of **simulated herbivory** (see Glossary), including spatial and temporal precision in the application of damage, the ability to standardise damage without the confounding effects of inherent differences in herbivore feeding behaviour, and control over the introduction of material from foreign and unidentified organisms (e.g., pathogens). Shortcomings outlined by Baldwin included differences between simulated herbivory as applied by experimentalists and damage caused by true herbivory (e.g., type and age of tissue damaged, inability to accurately mimic certain feeding guilds, and the geometry of feeding patterns). Moreover, simulated herbivory usually failed to replicate environmental changes associated with true herbivory (e.g., enhanced CO_2 microenvironments due to herbivore respiration).

In the past two decades, the differences between simulated and true herbivory have been reviewed in several articles and book chapters (Tiffin and Inouye 2000, Hjältén 2008, Lehtilä and Boalt 2008). The main purpose of these reviews was to describe the fidelity of simulated herbivory as a proxy for herbivory in nature, and how the two differ in terms of their induction of plant defence responses. The rationale for simulating herbivory in experiments has thus far been either for pragmatic reasons (i.e., not having to include herbivorous organisms in experiments) or for standardisation of treatments. Expanding beyond these prior rationales, we suggest that simulated herbivory has an additional and novel benefit: it is an essential tool for separating how plants perceive and distinguish the various factors associated with insect feeding, including mechanical stimulation, wounding, and introduction of foreign compounds.

Plant defences are highly complex, partly due to the fact that both microbes and insects have strongly influenced the evolution of physiological and chemical plant traits (Futuyma and

Agrawal 2009, Gilbert and Parker 2016). By determining plant responses to specific

components of herbivory, it might be possible to identify the evolutionary rationale for a

given response; In contrast, when live insects are used the exact cause of a response is

difficult to determine, as individual stimuli are more difficult to tease apart. Additionally,

knowledge of whether a specific response is caused by insect- or microbe-derived

compounds can provide insights on how to better manage pests and pathogens. It is clear that

identifying novel mechanisms of defence responses to the various components of herbivory is

useful across disciplines, whether by providing coevolutionary insights, or by directing

sustainable pest mitigation strategies. As of now, unravelling the individual effects of these

interconnected stimuli remains elusive and is thus a subject ripe for synthesis.

Our focus for this synthesis is simulations of chewing-insect herbivory. Although the role of

piercing and sucking insects in plant defence induction has been well-documented (Will et al.

2007, Sharma et al. 2014, Will 2016), methods of simulated herbivory aimed at mimicking

feeding habits of phloem-feeders are, to our knowledge, absent in the literature. This is

presumably due to difficulty replicating proboscis movement, timing of probing, and

injection of saliva directly into the phloem (Garzo et al. 2018).

2.6 Advantages of Simulated Herbivory

2.6.1 Advantage 1: Specified Elicitors and Stimuli Minimises Bias

The dynamics of defences induced by herbivory are clearly complicated and can be species

specific. Using simulated herbivory, it is possible to determine the potential influence of one

single stimulus or a customised combination of stimuli on plant defences during insect

feeding (Fig 2-2). Responses found in studies that apply specific herbivore-associated stimuli

can be conflated if live insects are used, and therefore studies applying a single stimulus and

combinations of stimuli reveal a complexity hidden by true herbivory. Several techniques have been devised in attempts to accurately elicit responses to insect herbivory beyond mechanical wounding, and they typically have two major phases: (i) collection and/or purification of insect-associated compounds and (ii) application of herbivore- and pathogen-associated biomolecules (often coupled with wounding) (Box 2-1).

During bouts of feeding insects secrete variable amounts of OS and saliva. For example, Peiffer and Felton (2009) found that insects can secrete anywhere from 0 to 6 nl of OS in 10 min of feeding. Considering this high variability, it is impossible to ensure that all plants are being treated with the same amount of associated compounds using true herbivory. Chemical, biochemical, and molecular analyses require high-fidelity and consistent treatments, which can be hard to achieve using unpredictable live specimens. Only with artificial herbivory is it possible to run identical treatments and change only one of the variables associated with herbivory. In one method described by Tian et al. (Tian et al. 2012), plants had holes punched in the same part of the leaf, and phosphate buffer was applied to the resulting wounds. In one treatment, plants were given buffer spiked with a constant volume of *H. zea* saliva. Therefore, any differences in plant response between treatments could be more accurately compared, as the amount of saliva and extent of physical wounds were identical across individuals and treatments respectively. Considering it is well known that herbivores can harbour microbes in their saliva and OS, herbivory simulations using isolated elicitors might be particularly useful in experiments that seek to determine the effects of insect-derived and microbe-derived compounds separately.
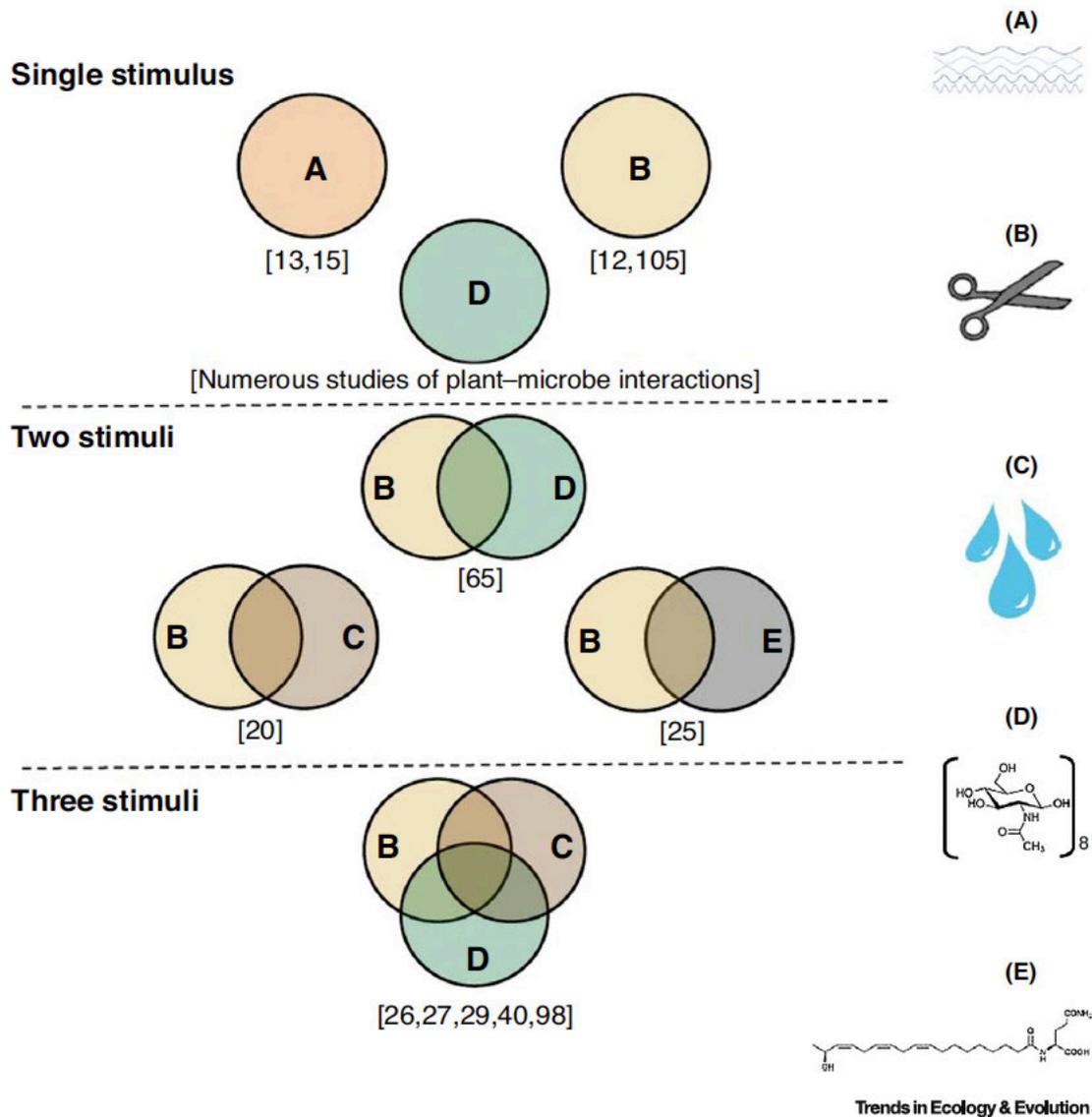
Figure 2-2. Possibilities of simulated herbivory not afforded using true herbivory. Plant defence is known to vary between stimuli, and simulated herbivory allows for customised treatments not afforded by true herbivory (see Figure 2-1). Several studies (numbers shown in brackets correspond to reference number) have used an individual stimulus, two stimuli, or three stimuli to elucidate which components of herbivory were responsible for the observed responses. In all studies listed, regardless of the number of stimuli tested, each was also introduced to the plant independently in order to compare results to the collective response of all the stimuli investigated. Each Venn diagram shows the combination of stimuli used by a given study/studies: (A) mechanical stimulation, (B) wounding, (C) unseparated elicitors/effectors derived from both insects and microbes, (D) elicitors/effectors derived from microbes, and (E), elicitors/effectors derived from insects. Each stimulus' respective icon from Figure 2-1 corresponds to the letter directly above it. The studies referenced are not exhaustive, however to our knowledge no additional combinations directly pertinent to herbivory exist in the literature. For reference: 12 = Toyota et al. (2018); 13 = Appel and Cocroft (2014); 15 = Chehab et al. (2012); 20 = Major and Constabel (2006); 25 = Schmelz et al. (2009); 26 = Tian et al. (2012); 27 = Wang et al. (2017); 29 = Chung et al. (2013); 40 = Mattiacci et al. (1995); 65 = Chassot et al. (2008); 98 = Shinya et al. (2016); 105 = Reymond et al. (2000).

2.6.2   Advantage 2: Eliminates the Effects of Tissue Quality

It has been well documented that insects feed differentially based on the physical and chemical attributes of plant material (Caldwell et al. 2016, Ennis et al. 2017, Robin et al. 2017, Ryalls et al. 2017), and therefore another major challenge associated with the use of true herbivory is the differential feeding patterns likely to be observed between treatments. Ryalls et al. (2017) showed that high concentrations of foliar silicon reduced herbivore feeding compared to leaves with lower amounts of silicon. Robin et al. (2017) found diamondback moth (*Plutella xylostella*) larvae to feed preferentially on *B. oleracea* plants based on foliar glucosinolate profiles; therefore, the size, density, and location of wounding was inconsistent between individual plants and genotypes. Plant phenology also plays a role in determining the extent of herbivory. In *Eucalyptus* spp. the total leaf-area of insect damage was far greater on young leaves compared to mature leaves (~25% vs. < 5% respectively) (Gherlenda et al. 2016). It is also well known that variation in the intensity of herbivory can alter plant metabolism (Hamilton and Frank 2001, Bardgett and Wardle 2003). For example, in *Arabidopsis thaliana*, resistance to grey mould (*Botrytis cinerea*) colonisation was increased based on the intensity of damage (Chassot et al. 2008).

In addition, genetic mutants with particular defence-related genes silenced can be useful in both simulated and true herbivory studies, and have been used with multiple plant species, including *A. thaliana*, *O. sativa*, *N. attenuata*, and tomato (*Lycopersicon esculentum*) (Kachroo et al. 2004, Sánchez-Hernández et al. 2006, Meldau et al. 2012, Ye et al. 2013, Bonifacio et al. 2016). These genotypes can facilitate the uncoupling of defence mechanisms, as changes in resistance in the absence of possible modes of defence allow for validation or repudiation of hypothesised mechanisms of herbivory-induced defences. Ye et al. (2013) showed that the increase in biomass of rice leaf folder (*Cnaphalocrocis medinalis*) was

significantly greater in individuals that fed on *O. sativa* with the expression of allene oxide synthase silenced compared to wild-type plants. This in mind, it could be expected that the extent of damage between *O. sativa* genotypes might have varied due to differing feeding preferences. Therefore, variation in response might be influenced by differences in the quality of damage in addition to differing defence capabilities. Simulated herbivory solves this problem; despite genetic variation, the quality of damage is identical between individuals and treatments.

2.6.3   Advantage 3: Timing of Damage and Measurements

Localisation and intensity of damage are also of importance when measuring defence responses at the transcriptome, proteome, and metabolome level. Gene expression can vary in a single plant between the immediate area damaged and areas further away (León et al. 2001, Koo 2017). Furthermore, over time, mechanically damaged *A. thaliana* increased both apoplastic glutamate and cytosolic Ca^+ concentrations in tissue adjacent to the immediate site of damage (Toyota et al. 2018). In response to herbivory plants transmit systemic signals to distant tissues in order to upregulate defences in preparation for imminent attack, which can further complicate the decision to measure responses in a given tissue locale (Choi et al. 2017); even systemic signalling molecules such as proteins, mRNAs, and large metabolites can be transported at rates of several hundred micrometres per second (Turnbull and Lopez-Cobollo 2013, Choi et al. 2017). Root herbivory, for example, can influence the quality of above ground tissue and *vice versa* (Erb et al. 2011, Johnson et al. 2012), and therefore if one wanted to measure, say, a response in the foliar tissue of a plant to damage undergone in the roots, an understanding of the timing of systemic responses is necessary.

When using live insects, localisation of damage typically requires control over the range in mobility of live insects without interfering with their feeding habits. Mechanisms such as clip

cages can confine insects, but these cages have been shown to influence plant growth, which can interfere with the allocation of resources to defence responses (Moore et al. 2003, Hjältén 2008). Deciding on the location of the clip cages also presents challenges, as herbivory patterns are often significantly different across, for example, varying leaf phenology (Gherlenda et al. 2016). Additionally, the precise timing of feeding can vary considerably between insects over the course of the treatment. Therefore, with true herbivory, measurements of defence responses can differ solely due to inconsistencies in the time at which the wounds were inflicted; although the timing of damage will vary, the timing of harvest will be the same.

2.7   Can We Mimic Herbivore Feeding in Time and Space?

A major concern associated with most simulation techniques in ecological studies is that they fail to account for the fact that plants can discriminate between continuous damage and a single wounding event (Mithöfer et al. 2005). Herbivores feed on plant material over time, whereas the majority of simulation experiments impose damage in one single application (Mithöfer et al. 2005, Hilker and Meiners 2010), despite the suggestion that the spatial and temporal extent of mechanical damage can alter plant defence responses. Responses can also vary due to differences in the quality of damage and uncontrolled stimuli introduced by the insects but omitted in simulations. Considering the inherent dissimilarity between true and artificial herbivory, experiments that use simulations might fail to elicit a response that would be shown with true herbivory, or elicit an unauthentic response. For example, Massey et al. (2007) showed that repeated wounding events in two grass species increased silicon uptake relative to a single wound application, and that damaging tissues with scissors failed to elicit the same response as tissue damaged by desert locust (*Schistocerca gregaria*). In addition, stem-boring insects typically prove harmful to plants; however other insects such as leaf

defoliators have more variable effects on the intensity of both primary metabolic processes (e.g. photosynthesis) and secondary defence responses depending on the amount of tissue removed (Welter 1989, Hjalten et al. 1993, Peterson et al. 1998, Delaney and Higley 2006, Stephens and Westoby 2015). It is well known that plant defences and insect feeding patterns can also vary due to circadian rhythm (Goodspeed et al. 2012); therefore the time of day herbivory simulations occur should be standardised to known circadian patterns of the specific plant–insect system being simulated.

Knowledge of the quality of damage typically inflicted by a given herbivore can yield a more accurate representation of how a plant might respond to herbivory in a 'natural' setting; simulations can then be selected accordingly to induce a similar response. Bricchi et al. (2010) showed that continuous damage with the MecWorm, a robot designed to spatially and temporally replicate the physical nature of various forms of insect damage (Table 2-1), elicited a response in *P. lunatus* VOC emissions more similar to that induced by herbivores than a single entry of damage. Bricchi et al. also showed that only in the presence of OS did ion fluxes closely mimic those induced by true herbivory, regardless if the damage was continuous or not. Similarly, in *B. oleracea*, continuous damage has been shown to induce a response in the production of parasitoid-attracting VOCs more similar to true herbivory damage than final damage or a single-entry and immediate deployment of damage (Connor et al. 2007).

Technical advancements such as MecWorm simulate herbivory with some success, but there are still knowledge gaps that must be addressed. A better understanding of MecWorm's effectiveness across multiple systems might help to identify potential modifications that will increase its utility. Refinement of damage to better resemble true herbivory is imperative,

especially considering that even gentle touch (e.g., bending leaves several times without causing wounds) can activate Ca^+, ROS, and hormone signalling pathways, as well as associated gene expression within minutes of stimulus perception (Chehab et al. 2012, Benikhlef et al. 2013). Defence responses can be sensitive and highly variable, so keeping conditions as similar as possible between individual plants is imperative.

2.8 Herbivore Measurements Are Important

Perhaps the biggest issue with herbivory simulations is the most obvious one: they operate in the absence of real insects. Particularly in ecological studies, recording the effects of plant defence on herbivore performance (e.g. biomass, frass production, fecundity, etc.) is required to provide information regarding the nutritive qualities of the plant tissue and the resulting ecological outcomes (Felton et al. 1992, Kant et al. 2015, Ryalls et al. 2017); measuring defence responses is one thing, knowing if they are of consequence to insects is another. Managing the impacts of herbivory, however, depends on uncoupling the chemical and physiological responses of plants to various types of attack; there are still many gaps in our understanding of the variation in response between herbivores, microbes, and wounding. We propose that many of these knowledge gaps can be best addressed using simulated herbivory, primarily because controlled experiments that clearly distinguish between the effects of each stimulus can be carried out.

2.9 Concluding Remarks and Future Directions

Given the impacts of insect herbivory on ecosystem function, agriculture, and the well-being of the global population (Oerke 2005, Nentwig and Vaes-Petignat 2014, Bradshaw et al. 2016, Deutsch et al. 2018), improving our understanding of plant–herbivore interactions is vital across numerous ecological disciplines. This ranges from crop protection against pests

(e.g., food security), weed biological control, herbivore invasiveness, plant competition, and even conservation of beneficial herbivores (see Outstanding Questions). Detailed comparisons between various forms of tissue damage, that better characterise observed variation in responses to plant antagonists, may be best accomplished by herbivore simulations. Consideration of known plant responses to specific herbivores might ensure that simulations most accurately reflect the nature of the interaction, as it has been well established that stimuli that cause change in the metabolism of one plant species can have drastically different effects in another. Development and increased accessibility of technologies such as MecWorm, that facilitate sophisticated mechanical wounding combined with exogenous biomolecules, will enable the uncoupling of elicitor-specific responses from those of wounding alone. Finally, investigations into mimicking sounds associated with herbivory have almost been completely overlooked by ecologists, yet the evidence is now strong to show that specific sound qualities can impart a plant defence response. Incorporating these concepts into artificial techniques will only increase the accuracy of herbivore simulations and make it possible, for the first time, to mechanistically break down the variation in plant defence responses between chemical signals, wounding, and mechanical stimulation during herbivore attack.

Table 2-1. Various types of artificial wounding used in current literature to mimic chewing damage by herbivores

Tissue Damage	Type of Wound	Major Concern(s)	Sources
Razor blade	Clean lesion, number of wounds can be manipulated	Clean lesion, unlike most chewing herbivore damage, low surface area of leaf damaged, often single entry (non-continuous)	(Schmelz et al. 2001, Schmelz et al. 2009)
Lamina forceps	Crush desired percentage of leaf	No tissue removed, often single entry (non-continuous)	(Reymond et al. 2000)
Tracing wheel	Run over the surface of tissue and make small puncture wound	No tissue removed, often single entry (non-continuous)	(Halitschke et al. 2001, Shinya et al. 2016)
Hole puncher	Remove disks of tissue from desired location	Often single entry (non-continuous)	(Tian et al. 2012)
Syringe	Make puncture wounds in leaf tissue	No tissue removed, often single entry (non-continuous)	(Chassot et al. 2008)
MecWorm	Set parameters to remove desired amount of tissue over specified amount of time	Not widely available	(Mithöfer et al. 2005, Bricchi et al. 2010)

2.10 Outstanding Questions

- Researchers usually aim to replicate the total amount of damage inflicted by an herbivore during a bout of feeding. The signalling events that result from this, however, are likely to vary between damage induced suddenly and damage inflicted continuously (i.e., over time). How can we reproducibly optimise the timing of herbivore simulations?

- Can we accurately simulate herbivory for non-chewing herbivores (e.g., phloem feeders)? This is a major knowledge gap given that this feeding guild contains many

detrimental global pests and keystone organisms that have mutualisms with other taxa.

- How will environmental change affect insect feeding behaviour? Elevated atmospheric CO_2, for example, often results in metabolic changes within the plant and thus indirectly in compensatory feeding and increased damage. How does this relate to individual and collective defence responses?

- Gene editing techniques (e.g., CRISPR-Cas9) and viral vectors provide cutting edge technologies to control gene expression systemically and untangle plant defence responses. How will the utilisation of these technologies facilitate a greater understanding of the molecular mechanisms associated with plants, microbes, and insects during herbivory?

- Can we breed plants to be more resistant when we have a limited understanding of their defence responses to different components of herbivory? If, for example, the use of simulated herbivory can disentangle the responses to wounding and herbivore-associated microbes, and shows that one contributes a disproportionally larger induction of defence mechanisms or reduction in yield, that information can be used for informing both ecological management and sustainable agriculture.

2.11 Glossary:

Effector: A protein derived from an herbivore or microbe that negatively interferes with plant metabolism (Bos and Hogenhout 2011, Hogenhout and Bos 2011).

Elicitor: A molecule derived from an herbivore, microbe, or the plant itself that stimulates (elicits) a response in the plant (Musser et al. 2002).

Indirect defence: A volatile organic compound (VOC) emitted by plants that attract predators and parasitoids of herbivores (Aljbory and Chen 2018).

Mechanical stimulation: Stimulation caused by physical movement or vibrations without wounding tissue (Cazzonelli et al. 2014).

Oral secretions (OS): A combination of bodily fluids derived from both the herbivore gut (regurgitant) and salivary glands (saliva) and secreted from the mouth during feeding (Peiffer and Felton 2009).

Saliva: Secretions derived solely from salivary glands.

Simulated herbivory: Artificial damage techniques meant to replicate herbivore feeding in the absence of a live herbivore.

True herbivory: Feeding on plant tissue by live insects

Wounding: Mechanical stimulation that causes tissue damage. Encompassing tissue laceration and removal (e.g. defoliation).

2.12 Box 2-1. Simulated herbivory techniques

Saliva collection:

Saliva is secreted during feeding across feeding guilds, whereas OS is secreted less regularly (Peiffer and Felton 2009, Chuang et al. 2014, Mugford et al. 2016). After chilling insects on ice, saliva can be collected from the salivary glands using a pipette tip and applied to wounds (Tian et al. 2012).

Ablation:

To compare insect herbivory both in the presence and absence of insect saliva, ablation of the salivary glands, and thus prevention of salivation, is employed (Musser et al. 2002, Peiffer and Felton 2005, 2009, Takai et al. 2018). This method is unique in that it uses true herbivory for both treatments and controls. It has been shown that spinneret ablation does not interfere with feeding habits and therefore consistency between treatments should be expected (Musser et al. 2002). The ventral eversible gland (VEG) also produces secretions known to elicit a defence response, and can be ablated (Zebelo and Maffei 2012).

Oral secretions and gut contents:

Most OS collection techniques involve agitating the mouthparts of insects after feeding and collecting the regurgitant (Shinya et al. 2016). The volume of OS able to be collected from a given insect is larger than saliva alone (Tian et al. 2012). Contents of the alimentary tracts have also been applied directly to plant tissue (Yoshinaga et al. 2014). Insects might not secrete all of these extracted compounds when they feed, and even secreted compounds are produced in highly variable volumes (Peiffer and Felton 2009). The resulting extract will contain compounds found within the salivary gland, but not necessarily released in saliva, unless appropriate purification techniques are used.

Purified elicitors:

Glucose oxidase (GOX) is a major constituent of the proteome of lepidopteran saliva, and applying GOX to wounds is often compared against solely mechanical damage in order to elucidate defence responses specific to the introduction of a single HAMP. Results have thus far indicated variability in defence responses (Eichenseer et al. 1999, Peiffer and Felton 2005, 2009, Celorio-Mancera et al. 2011, Tian et al. 2012, Shinya et al. 2016). Other known elicitors such as inceptin, fatty acid-amino conjugates, and caeliferins have also been isolated and applied to plant tissue (Kessler and Baldwin 2002, Schmelz et al. 2006, Alborn et al. 2007, Schmelz et al. 2009, Wu and Baldwin 2009, Aljbory and Chen 2018).

Mechanical damage of tissue:

Some of the most commonly used mechanical damage techniques are: cutting and/or scratching of the leaf with a razor blade (Schmelz et al. 2001, Schmelz et al. 2009), crushing the leaf tissue with apical lamina forceps (Reymond et al. 2000), puncturing the leaf with a tracing wheel (Halitschke et al. 2001, Shinya et al. 2016), punching holes in the leaf (Tian et al. 2012), puncturing the leaf with a syringe (Chassot et al. 2008), and in few instances the use of a custom-engineered machine designed to simulate the spatial and temporal patterns of insect herbivory as closely as possible (Mithöfer et al. 2005, Bricchi et al. 2010) (Table 1).

2.13 Acknowledgements

3 Chapter 3: Microbes in *Helicoverpa armigera* oral secretions contribute to increased senescence around plant wounds

3.1 Abstract:

1. Plants have long been exposed to insect herbivore attack. Crucial to the plant's ability to defend itself is its ability to identify specific signals associated with attacking insects. Distinctive chemical cues, such as those associated with chewing insect oral secretions (OS), activate targeted defence responses to chewing insect herbivores.

2. Herbivore-associated cues can be complicated by the fact that many herbivores form associations with microbes that produce their own specific signals which may induce alternative defence processes.

3. Here we report that OS of the global pest, the cotton bollworm (*Helicoverpa armigera*), induce senescence around wounds in *Brachypodium distachyon* leaves. Crude OS activate greater levels of senescence than OS with reduced microbial abundance or mechanical wounding alone. Nonetheless, plants closed mechanical wounds more rapidly when treated with *H. armigera* OS regardless of the microbial component.

4. This study concludes that *H. armigera* OS can activate senescence and wound closure in plant tissues and that microbes within OS have an important role in shaping plant–herbivore interactions through additional increases in senescence.

3.2 Introduction:

Plants come under attack from a wide range of natural enemies, some of the most significant being insect herbivores (consuming over 20% of annual net primary productivity) (Agrawal 2011). In order to differentiate various stressors, plants have evolved the ability to initiate targeted defence responses against herbivores following recognition of specific chemical signals (Waterman et al. 2019). For example, many lepidopteran larvae release oral secretions (OS) containing components from their gut and salivary glands during feeding (Peiffer and Felton 2009). The presence of OS modifies how plants respond to insect attack beyond, for example, mechanical wounding alone (Musser et al. 2002, Chung et al. 2013, Wang et al. 2017). Further, microbial associations formed with insects play a pivotal role in plant–herbivore interactions (Douglas 1998, Pieterse and Dicke 2007). For example, microbes derived from the insect gut are present in OS and can modify plant defences against herbivory; however the extent of microbial involvement in plant–herbivore dynamics has not been fully explored (Waterman et al. 2019). Senescence around sites of infection or wounding is an important mechanism by which plants prevent the spread of biotrophic pathogens, as it establishes a physical barrier between infected and healthy cell types and can activate various defence pathways (Glazebrook 2005, Iakimova and Woltering 2018). The extent of senescence is often used to determine the severity of biotrophic pathogen infection, however senescence is seldom considered in experiments involving insect herbivory, even though such a response can be activated by insect-associated signal pathways (Devadas and Raina 2002, Häffner et al. 2015). In order to better understand how plants defend themselves against insects it is important to understand exactly which insect-associated stimuli plants are responding to (Waterman et al. 2019). Additionally, wound closure of areas damaged by insects can also inhibit herbivory through the production of feeding deterrents such as

callose, lignin and other phenolic compounds, which simultaneously provide a barrier against microbes (Cui et al. 2013, Iakimova and Woltering 2018).

In order to address the nuances associated with signals of herbivory and their role in activating senescence in plant tissues, we isolated OS from the global herbivore pest *Helicoverpa armigera* and tested the impacts of its OS on senescence and wound closure in the model cereal grass *Brachypodium distachyon* (Poaceae). Considering senescence is often a response to microbial infection, we significantly reduced the abundance of microbes in *H. armigera* OS and compared the effects on senescence around wounds to OS with normal levels of microbial abundance. Further, we investigated how the closure of wounds was affected by *H. armigera* OS and their microbial components.

3.3   Methods:

3.3.1   *Plant growth and insect rearing*

Brachypodium distachyon (Bd21-3) seeds were obtained from the French National Institute for Agricultural Research (INRA, Versailles, France). Seeds were sterilised in 1% bleach and 0.1% Triton X-100 and stratified in wet perlite for 7 days. Plants were then transferred to a naturally lit glasshouse (22/18° C day/night on a 14L:10D cycle) for germination. After 9 days, 48 seedlings were transferred to black 50 mL centrifuge tubes containing 45 mL of nutrient solution for 21 days (see SI Appendix 1).

Helicoverpa armigera larvae (originally fed on an artificial diet and laboratory reared) were obtained as first instars from Commonwealth Scientific and Industrial Research Organisation (CSIRO, Narrabri, Australia; see SI Appendix 1). Larvae were maintained on artificial diet until 14 days prior to OS experiments, at which point they were fed *B. distachyon* leaves for

14 days. Larvae were reared in 30 mL plastic containers containing 1% agar to maintain leaf moisture and incubated at 25°C.

3.3.2   Antibiotic treatment

Brachypodium distachyon leaves were each treated with 150 μL of either an antibiotic mixture (AB) containing three antibacterial and two antifungal agents (modified from Chung et al. (2013); see SI Appendix 1) or water. Leaves were then air dried in a chemical fume hood. For 4 days, each larva was given two *B. distachyon* leaves per day containing either AB or water. Larvae that consumed over 50% of the leaves over the 4-day period were used for this study. In a laminar flow hood, OS were collected from fourth–fifth instar larvae by gently squeezing their abdomen and probing their mouths with a gel-loading pipette tip. OS from AB fed caterpillars had significantly reduced microbial abundance and are therefore referred to as OS minus microbes (OS-M). OS from caterpillars not fed on AB-treated leaves had normal levels of microbial abundance and are thus referred to as OS plus microbes (OS+M). OS from 7 larvae within each AB treatment (OS-M and OS+M) were separately pooled. In order to determine the effectiveness of the AB treatment, 1/100 dilutions of each OS pool were plated onto 1x LB media and incubated at 28°C for 36 hours. Microbial colony forming units were counted to determine the abundance of microbes within the OS. Water was also plated as a control to ensure that there was no contamination of materials from non-OS microbes. Relative growth rate of larvae was measured to determine the influence of diet on *H. armigera* performance (see SI Appendix 1).

3.3.3   Plant wounding

One attached leaf (third-most recent fully developed) per plant was pierced with a hypodermic needle (0.5 mm x 25 mm) approximately 30 cm from the leaf tip and 1 μl of

either 50% OS+M, 50% OS-M, 50% AB mixture or water was immediately added to each wound.

3.3.4 Confocal microscopy

After 3 days, wounded leaves were detached and senescence was measured with a Leica SP5 confocal microscope (Leica, Wetzlar, Germany) using a 10x water immersion objective. Samples were excited with 405 and 633 nm lasers and emission was captured with channels at 475 – 520 nm and 665 – 695 nm for fluorescent phenolic compounds (e.g. lignin) and chlorophyll autofluorescence respectively (Fig 3-1a-h) (Talamond et al. 2015). Images were collected for a z-stack between the adaxial and abaxial surface of each leaf using a step-size of 20 µm.

3.3.5 Analysis of Images

The area of senescence and openings were measured in ImageJ (National Institutes of Health, USA; Version 1.52). Images from each z-stack were combined and pixels were summed into a single image. Senescence was classified as the total area of tissue where a distinct loss in chlorophyll autofluorescence was observed, excluding the wound opening (Fig 3-1i-l). Loss of chlorophyll is a common proxy for measuring senescence in the foliar tissue of plants (van Doorn and Woltering 2004, Vergeiner et al. 2013, Iakimova and Woltering 2018, Kinoshita and Betsuyaku 2018).

3.3.6 Statistical Analysis

Effectiveness of the antibiotic feeding assay was determined with a Welch two sample t-test. Wound and opening size were analysed using one-way ANOVAs followed by Tukey's HSD

tests. A linear regression was performed to determine the correlation between wound and opening size. All analyses were performed in the programming environment R version 3.5.3 (R Core Team 2020).
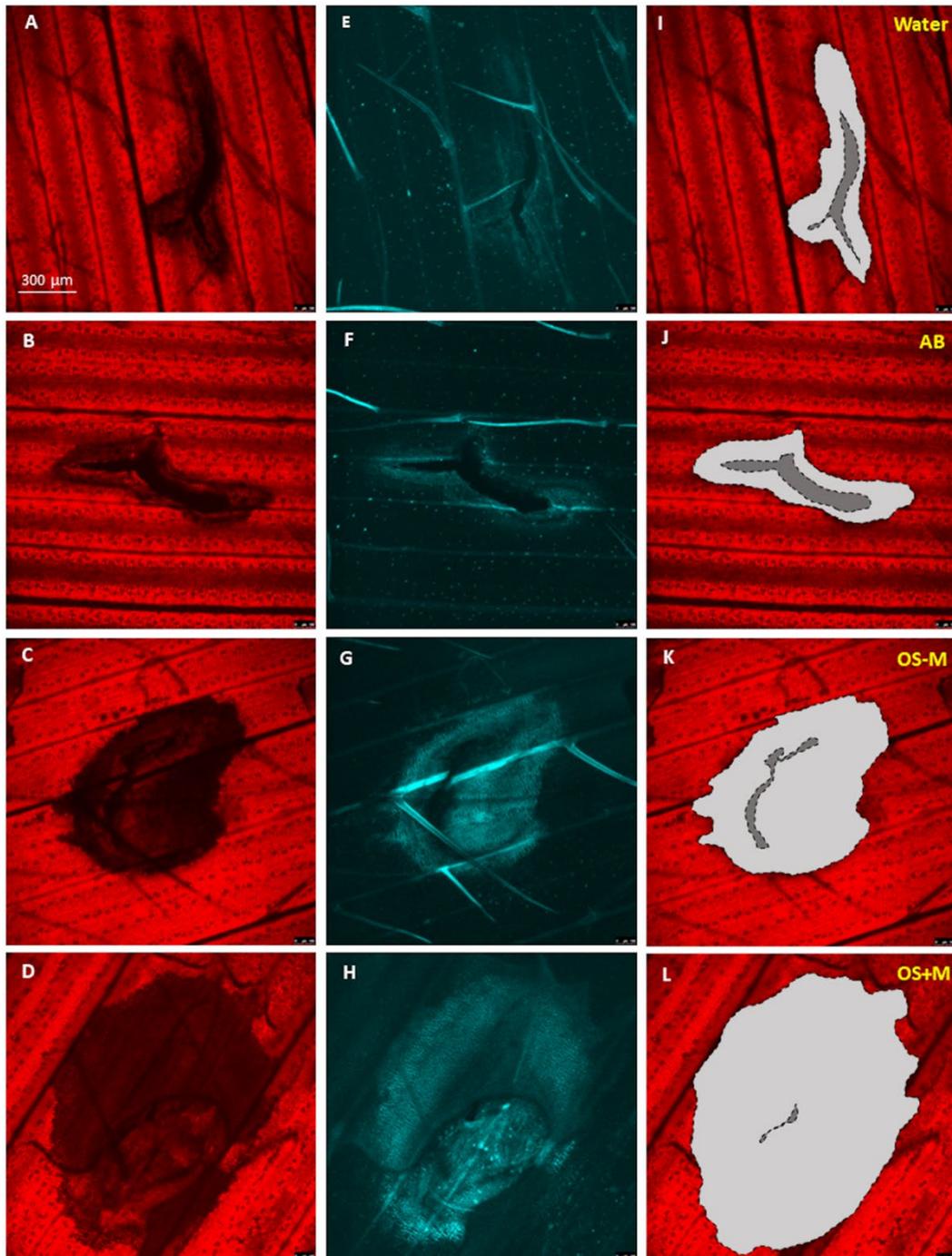
Figure 3-1. The effects of microbes in *Helicoverpa armigera* oral secretions (OS) on senescence (i.e. loss of chlorophyll autofluorescence; A-D), accumulation of cyan-autofluorescent compounds (E-H) and the areas of senescence (light grey) and openings (dark grey) (I-L). For (A-D) autofluorescence was captured at 665-695 nm and for (E-H) 475-520 nm. Image size = 1.55 X 1.55 cm. Abbreviations: OS+M = ½ dilution OS with normal levels of microbial abundance, OS-M = ½ dilution OS with significantly reduced microbial abundance from AB-fed larvae, AB = ½ dilution of the antibiotic mixture fed to larvae.

3.4   Results and Discussion:

Antibiotic (AB) treatment was largely successful in reducing microbes in *Helicoverpa armigera* OS. OS from larvae fed on antibiotics (OS-M) showed a *ca.* 88% reduction in microbial colony formation compared to OS from insects not fed antibiotics (OS+M; Fig S3-1; $t_{(2)}$ = -39.55, $P < 0.001$). Wounds treated with OS+M were *ca.* 30 % larger than wounds treated with OS-M, suggesting that microbe-derived signals substantially increased senescence (Fig 2-2a; $F_{(3,44)}$ = 24.04, $P < 0.0001$). Although OS-M treated leaves showed less senescence than those treated with OS+M, they were still larger than both control (i.e. OS-free) treatments, which might be due to the incomplete removal of microbes and/or components or properties of OS unrelated to the presence of microbes (Fig 3-2a; Fig S1). In the closely related wheat (*Triticum aestivum*, var. Coolah), we found that sterile filtration (0.22 μm) of OS yielded similar results to OS-M (Fig S3-2; see SI Appendix 1). Sterile filtration allows for all dissolved compounds and particles smaller than 0.22 μm (e.g. chemical elicitors in OS) to remain in OS filtrate while ensuring it is sterile (Fig S3-3). These data support the role of microbes in increasing senescence, however also highlight that microbes alone might not be responsible for increased senescence around wounds, and that non-microbial components of OS also activate this response. The AB mixture did not directly influence wound or opening size considering there were no differences between control treatments (Fig 3-2a,b). Additionally, there were no effects of AB treatment on *H. armigera* relative growth rate (Fig S3-4), which supports the notion that antibiotics have minimal influence on lepidopteran physiological processes (Hammer et al. 2017). We found both OS-M and OS+M treatments increased wound closure compared to both control treatments (Fig 3-2b; $F_{(3, 44)}$ = 8.78, $P < 0.001$). Senescent tissues also showed an accumulation of cyan-fluorescent compounds, many of which (e.g. lignin and other phenolics) are induced during wound closure and prevent microbial spread between tissues (Fig 3-1e-h) (Cui et al. 2013).

These compounds are also known to decrease the palatability of plant tissues to lepidopteran larvae, which may ultimately increase plant resistance to herbivory (Moreira et al. 2017). Therefore, plants might rely on microbial signals to a lesser extent to activate wound sealing, as associated increases in phenolics may directly deter chewing herbivory. Mechanical wounds without OS did not close to the same extent, most likely because the plant perceived no further threat of herbivory or microbial presence. Interestingly, we did observe an inverse relationship between the area of senescence and openings, suggesting a linkage between senescence and wound closure, whereby smaller openings were associated with larger areas of senescence (Fig 3-2c). This is to say that increased senescence might signal a greater necessity to invest resources into wound closure due to heightened perception of either herbivore or microbial presence. Although we did not find a significant difference in wound closure between OS-M and OS+M, in light of the relationship observed between area of senescence and opening size, microbes might affect plant resistance through the heightened induction of senescence and thus increased production of anti-herbivore metabolites such as phenolics (Fig 3-2c). Additionally, it is unlikely larger openings in control treatments were the result of continued leaf expansion considering openings not treated with OS, although not significant at a 95% confidence interval ($t_{(17)} = 1.65$, $P = 0.12$), decreased in size between 2 and 14 days (Fig S3-5) and measurements were taken on fully expanded leaves.

Our findings indicate that senescence is in fact a response associated with *H. armigera* OS and is heightened by microbial signals. Nevertheless, it remains unclear how senescence modifies plant defence capabilities against insects. It is our hope that this study will serve as framework for further studies investigating effects of senescence on herbivory dynamics, as well as characterisation of the diversity of microbes contained within *H. armigera* OS and

their potential influence on plant defences including senescence and the production of anti-herbivore metabolites.
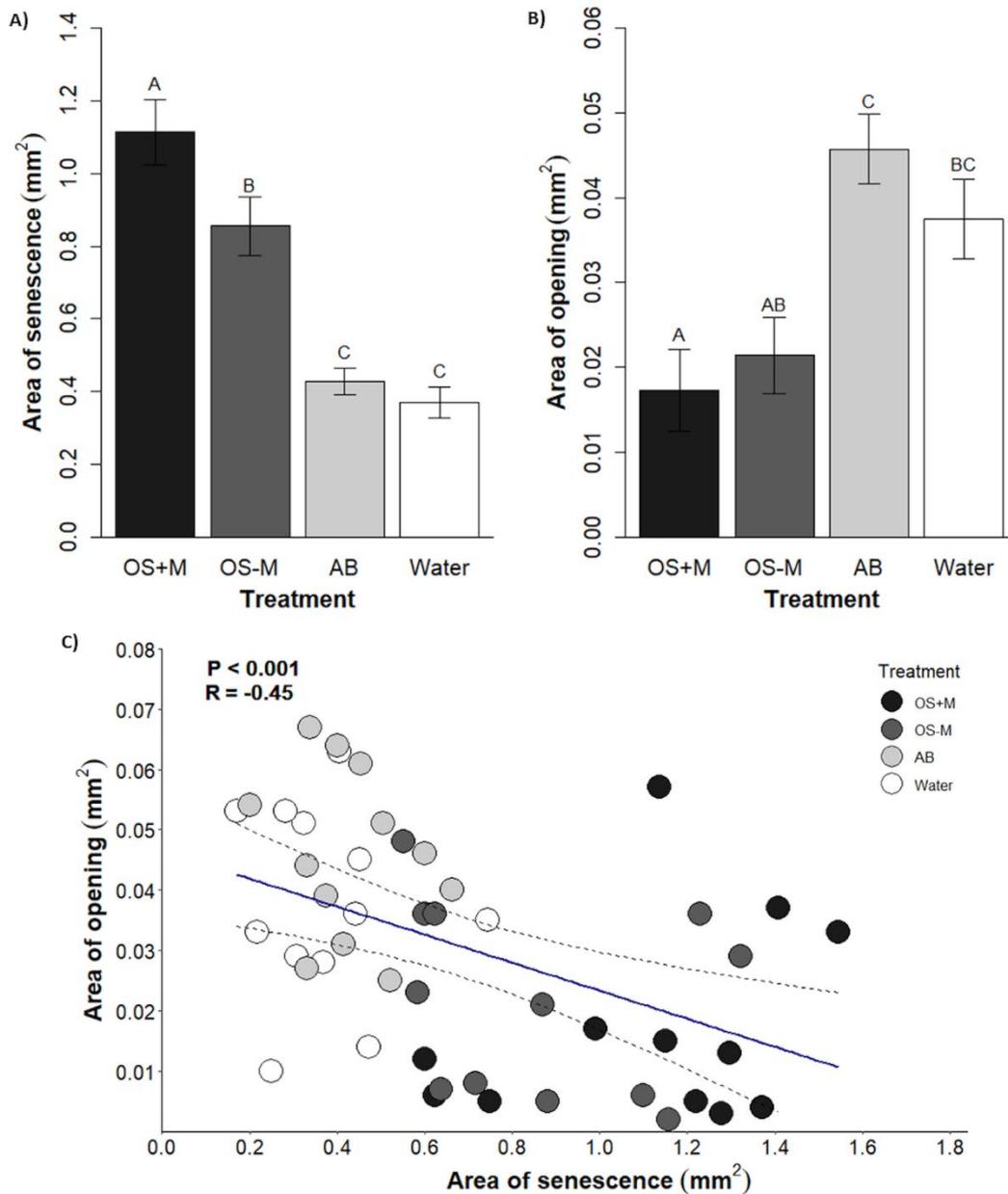
Figure 3-2. The effects of microbes in *Helicoverpa armigera* oral secretions (OS) on (A) area of senescence, (B) area of the opening created by the needle and (C) the correlation between the area of senescence and opening. Abbreviations: OS+M = ½ dilution OS with normal levels of microbial abundance, OS-M = ½ dilution OS with significantly reduced microbial abundance from AB-fed larvae, AB = ½ dilution of the antibiotic mixture fed to larvae. For (A) and (B), values are mean ± SE (n = 12). Letters above each bar indicate significant differences between treatments (ANOVA, $P < 0.05$ followed by an HSD test). One-way ANOVA showed that the effects of treatment on area of senescence and openings were significant ($F_{(3, 44)} = 24.04, P < 0.0001; F_{(3, 44)} = 8.78, P < 0.001$, respectively). For (C) the solid blue line represents linear regression through all data points and the dashed lines represent 95% confidence intervals.

3.5 Acknowledgements

4 Chapter 4: Short-term resistance that persists: Rapidly induced silicon anti-herbivore defence affects carbon-based plant defences

4.1 Abstract:

1. Silicon (Si) is known to alleviate diverse biotic and abiotic stresses including insect herbivory. Si accumulation in plants, notably the Poaceae, can be induced through stimulation of the jasmonic acid (JA) pathway (associated with chewing herbivores). Nevertheless, the temporal dynamics of Si accumulation as a defence response and its consequential effects on carbon-based defences (e.g. phenolics), particularly in the short-term, remain unclear.

2. The model grass *Brachypodium distachyon* was grown in a hydroponic solution where half the plants were supplemented with 2 mM potassium silicate and half had no Si supplied. Plants were treated with methyl jasmonate (MeJA) as a form of standardised simulated herbivory. We measured Si accumulation, the phytohormones JA and salicylic acid (SA), and carbon-based defences over 24 hours to determine the temporal dynamics of Si accumulation and the interplay between Si, simulated herbivory and plant defence machinery.

3. MeJA-induced Si accumulation occurred as early as 6 hr after treatment via increased JA concentrations. Si supplementation decreased SA concentrations, which could have implications on additional downstream defences. We show a trade-off between Si and phenolics in untreated plants, but this relationship was weakened upon MeJA treatment. Further, this trade-off did not apply to the phenolic precursor compound, phenylalanine.

4. We provide evidence for rapidly induced Si accumulation associated with herbivory, and that increased Si accumulation impacts on phytohormones and carbon-based defences over a

24-hr period. Additionally, herbivory modifies the relationship between Si- and carbon-based defences. Thus, in addition to its well-documented role as a long-term defence against herbivores, we demonstrate that, over short-term temporal scales, Si accumulation responds to herbivore signals and impacts on plant defence machinery.

Keywords: herbivory, jasmonic acid, phenolics, phytohormones, plant defence, silicon, simulated herbivory

4.2 Introduction:

Among the most prolific biotic stressors faced by plants are insect herbivores, which have dramatic impacts on ecosystem function, agriculture, and global human welfare (Oerke 2005, Bradshaw et al. 2016, Deutsch et al. 2018). Therefore, it has become increasingly important to understand the mechanisms through which plants are able to defend against insect attack in order to develop optimal mitigation strategies. Over the hundreds of millions of years that plants and insects have coevolved, plants have mounted a plethora of defences against insect herbivores (Hartley and Jones 1997). Many of these defences are specialised metabolites, but there has been increasing interest in the role of silicon (Si) in mediating resistance to insect herbivory (Massey et al. 2007, Ye et al. 2013, Johnson and Hartley 2018). Certain plants, particularly in Poaceae, have evolved the ability to actively uptake Si and deposit it in their tissues as silicon dioxide (SiO_2), with some plants able to accumulate up to 10% Si dry weight (Ma 2004, Hodson et al. 2005). The mechanisms by which Si provides a physical defence against biotic stressors (Massey et al. 2007, Ryalls et al. 2017, Głazowska et al. 2018b, Hall et al. 2020a) and modulates the chemical defence responses to these stressors (Ye et al. 2013, Hall et al. 2019, Hall et al. 2020b) have been the subject of a number of studies over the past two decades (Coskun et al. 2019).

In the context of anti-herbivore defence, several mechanisms have been identified to explain the role of Si in plant resistance to herbivory. One notable hypothesis is that physical defences such as SiO_2 deposits (phytoliths) interfere with herbivore digestion, wear down herbivore mouthparts and increase leaf toughness (Hunt et al. 2008, Massey and Hartley 2009, Johnson et al. 2019). It has also been shown that Si uptake and accumulation can be induced as a response to herbivory (Ye et al. 2013, Hall et al. 2019, Hall et al. 2020b). Defence responses to herbivory are often assigned to one of two groups: short- and long-term defences. Short-term defences are induced during a given herbivore attack, and long term defences are employed following herbivore attack to combat future herbivory (Karban and Myers 1989). In order to better understand the role of Si in mediating plant resistance to herbivory it is important to determine how quickly plants are able to accumulate ecologically relevant amounts of Si. Unlike many inducible defences, Si is considered to be chemically inert in plant systems, and once it is deposited it is unable to be remobilised making its effects on herbivory relatively long-lasting (Hodson 1990, Cooke and Leishman 2011a, Reynolds et al. 2012, Ruffino et al. 2018). Additionally, several studies have shown trade-offs between Si and carbon-based defences such as phenolics, which are known to play an important role in plant resistance to herbivory (Baldwin and Schultz 1983, Cooke and Leishman 2012, Frew et al. 2016, Johnson and Hartley 2018, Klotzbücher et al. 2018, Quigley et al. 2020). To our knowledge, no studies have investigated the effects of herbivore-induced Si accumulation on phenolics or phenolic precursor compounds such as phenylalanine (Bernards and Båstrup-Spohr 2008). Further, how herbivory modifies the relationship between Si- and carbon-based defences such as phenolics is also unknown. Furthermore, the timing of Si accumulation might impact on the effectiveness of Si-based defences against insect herbivores in the short-term, as it is well known that the extent of

insect feeding is based on, and can be constrained by, physical and chemical attributes of plant tissues, including foliar Si (Caldwell et al. 2016, Ryalls et al. 2017).

Jasmonic acid (JA) is a phytohormone known to regulate chemical defence responses to chewing herbivores and other biotic stressors (Erb et al. 2012). Although several studies have investigated the interplay between JA and Si, results tend to vary (Ye et al. 2013, Kim et al. 2014, Hall et al. 2019, Hall et al. 2020b). JA concentrations can change dramatically in relatively short periods of time, and therefore discrepancies between studies could be due to the temporal dynamics of Si accumulation and phytohormone signals (Schmelz et al. 2009, Ye et al. 2013). Additionally, few studies have investigated the effects of Si on other important phytohormones such as salicylic acid (SA), which is known to play a critical role in defence against piercing and sucking insects (e.g. aphids) (Erb et al. 2012). In light of the proposed effects of Si on the JA pathway, the relationship between Si and SA may be particularly interesting, as JA and SA not only regulate downstream defence pathways associated with different biotic stressors, but in some cases have been shown to antagonistically regulate one another (Thaler et al. 2012).

We investigated the interplay between Si accumulation, phytohormone concentration and carbon-based defences in response to methyl jasmonate (MeJA) treatment in the model grass species *Brachypodium distachyon* (Poaceae) over a 24-hr period to determine the timing of herbivore-induced Si accumulation and the consequential impacts on additional anti-herbivore defences. We used MeJA treatment as a standardised form of simulated herbivory (Ye et al. 2013, Waterman et al. 2019, Hall et al. 2020b). MeJA application is a well-known way to mimic herbivore damage without wounding by directly activating the JA pathway (Tamogami et al. 2008). It also ensures that each plant receives an identical amount of

'damage' and eliminates any effect of differences in feeding habits across plants and treatments that might be present when using live herbivores (Waterman et al. 2019). We hypothesised that: (a) Si accumulation is induced by MeJA application over time, which is associated with increased JA concentration and (b) Si supplemented plants maintain higher JA concentrations once the effects of MeJA subside. Considering JA and SA often regulate divergent defence responses, (c) Si and MeJA treatment decrease SA concentrations. Further, (d) in untreated plants (i.e. no MeJA) Si decreases total phenolics, however this relationship is weakened under activation of the JA pathway by MeJA treatment. Finally, (e) Si does not decrease concentrations of phenolic precursors such as phenylalanine.

4.3   Materials and Methods:

4.3.1   Plant growth and treatments

Brachypodium distachyon (L.) P. Beauv. seeds obtained from the French National Institute for Agricultural Research (INRA, Versailles, FR) were sterilised in 1% bleach (NaOCl) and stratified in perlite and 1/4 strength nutrient solution (see below) for 7 days. After stratification, plants were transferred to a glasshouse (22/18° C day/night) for germination. After 12 days, 80 seedlings were transferred to black polypropylene 50 mL LightSafe centrifuge tubes (Sigma-Aldrich) containing 40 mL nutrient solution supplemented with 2 mM potassium silicate (K_2SiO_3; +Si). For plants not supplemented with Si (-Si), 80 seedlings were transferred to tubes containing 40 mL nutrient solution and KCl to balance the K atoms between treatments. The nutrient solution used was a modified version (2x strength) of that used in Hall et al. (2020b). Both -Si and +Si nutrient solutions were adjusted to pH 5.5 using HCl. After 20 days, half of both -Si and +Si plants were treated evenly, over the entire aboveground portion of the plant, with 1 mM methyl jasmonate (MeJA) in 0.05 M sodium phosphate buffer pH 8.0 0.01% Tween-20 as per Ye et al. (2013) (-Si + MeJA and +Si +

MeJA, respectively). Control plants were treated with the same buffer and Tween-20 concentrations without MeJA.

4.3.2 Leaf tissue

The second and third fully developed leaves were removed from the stem, flash-frozen on liquid nitrogen and stored fresh at -80°C and ground to a fine powder. The remaining leaves on each plant were separated from the stem, flash frozen on liquid nitrogen and freeze dried. All freeze-dried leaves from a single plant were combined and ground to a fine powder. Fresh ground tissue was used for enzymatic assays and freeze-dried ground tissue was used for all other analyses.

4.3.3 Si quantification

Approximately 50 mg of ground freeze dried leaf material was analysed to measure Si concentrations 1, 4, 6, and 24 hr after MeJA treatment ($n = 6$–7) using an X-ray fluorescence spectrometer (Epsilon 3^x; PANalytical) following the procedure of Reidinger et al. (2012). Analysis was calibrated using citrus plant material (NCS ZC73018 Citrus leaves, China National Institute for Iron and Steel) of known Si concentrations. All -Si and -Si + MeJA plants had Si concentrations below 0.3 %.

4.3.4 Phytohormone quantification

Jasmonic acid (JA) and salicylic acid (SA) were analysed in 4–5 samples, selected at random, for all treatment combinations at 1, 6, 12 and 24 hr after MeJA treatment. JA and SA were extracted using the Bligh-Dyer method to remove interfering compounds (Bligh and Dyer 1959). Approximately 25 mg of ground freeze dried leaf material was extracted with 220 μL

of 70% methanol spiked with deuterated JA (d5-JA) and SA (d4-SA) as internal standards to yield a final concentration of 100 ppb. Samples were mixed for 30 min at 4º C in a rotator mixer, 180 μL of chloroform was added and samples vortexed for 30 s. This was repeated with another 180 μL of chloroform and then 200 μL of water was added. Samples were then centrifuged at 3,381 x g for 10 min at room temperature. The upper aqueous methanol layer was transferred to a clean 1.5 mL microtube and passed through a 0.22 μm polytetrafluoroethylene filter. The extracts were analysed by UPLC/ESI-MS/MS using an Acquity UHPLC coupled to a Xevo triple quadrupole mass spectrometer (Waters Corporation). The extracts (5 μL each) were injected into a 2.1 mm x 50 mm x 1.7 μm, C18 reverse phase column. The mobile phase was composed of (A) water (B) and acetonitrile both containing 0.1% (v/v) formic acid at a constant flow rate of 0.6 ml/min. Elution was performed as a linear gradient: 80% A at 0 min; 50% A at 2 min; 0% A at 2.1 min. JA and SA were detected by ESI-MS/MS operating in negative ion mode. JA and SA identification was determined by the fragmentation pattern in comparison with authentic JA and SA standards. Quantification was based on a calibration curve of the standards and adjusted for sample recovery based on the internal standards. d5-JA and d4-SA, the internal standards, were purchased from CDN Isotopes. HPLC grade methanol, chloroform, and JA and SA calibration standards were sourced from Sigma-Aldrich.

4.3.5   *Phenylalanine quantification*

Phenylalanine was quantified in 5–7 samples, selected at random, from all treatment combinations at 1, 6 and 24 hr. Soluble phenylalanine was extracted from approximately 50 mg of ground, freeze dried foliar tissue with 350 μL of 50% ethanol and simultaneously heated and vortexed at 50º C/850 rpm for 20 min. Samples were then centrifuged at 21,130 x g for 5 min and the supernatant was collected. The supernatant was then filtered through 0.22

μm nylon filter to remove any remaining suspended solids. Phenylalanine was quantified by reverse-phase high-performance liquid chromatography (HPLC) using an Agilent 1260 Infinity HPLC system equipped with an Agilent Poroshell 120 EC-C18 column (4.6 x 150 mm, 2.7 μm). Using a flow rate of 0.6 ml/min and an injection volume of 7 μL, analyte peaks were detected with a Corona charged aerosol detector (CAD; Corona CAD veo; Thermo Fisher Scientific Inc.) and eluted using two mobile phases (Solvent A: 0.4% heptafluorobutyric acid and 0.02% trifluoroacetic acid (TFA) in distilled water, Solvent B: 0.1% TFA in acetonitrile (Johnson et al. 2020c). The elution gradient was 0–6.86 min, hold 100% A; 6.86–7.7 min, 100–88% A; 7.7–16.38 min, 88–85% A; 16.38–29.4 min, 85–40% A; 29.4–33.2 min, 40-0% A; 33.2–35 min, hold 0% A; 35–42 min, 0-100% A. Phenylalanine standards were used to calibrate the analysis. HPLC mobile phases and the calibration standard were sourced from Sigma-Aldrich.

4.3.6   Total phenolics quantification

Total phenolics were quantified in 3–5 samples selected at random from each treatment combination at 1, 6 and 24 hr. Approximately 10 mg of foliar tissue was extracted twice with 70% acetone (v:v). For the first extraction, 150 μL of 70% acetone was added to 10 mg of foliar tissue and mixed for 30 min at 4° C. The supernatant was removed and 70 μL was added to a clean 1.5 mL microtube. An additional 100 μL of 70% acetone was added to the pellet and was mixed for 1 hour at 4° C. Then 70 μL of the second supernatant were combined with the first. The combined extracts were measured in technical triplicate on a CLARIOstar High Performance Monochromator multimode microplate reader (BMG labtech) using the Prussian blue assay (Graham 1992) modified for a 96-well microplate. Quantification was based on a standard curve of gallic acid (Sigma-Aldrich).

4.3.7   Polyphenol oxidase assay

Polyphenol oxidase (PPO) activity was measured in 6–8 samples from each treatment combination at all time points. In order to remove phenolic compounds, foliar tissue from the second and third fully expanded leaves was combined with 45 mg of polyvinylpolypyrrolidone (PVPP). PPO was extracted in 800 μL of extraction buffer pH 8.3 (6.5 g/L TRIS, 1.5 g/L citric acid monohydrate, 1 g/L cysteine hydrochloride, 1 g/L ascorbic acid, 10 g/L PEG-8000, 110 ml/L glycerol). Then 30 μL of extract was combined with 170 μL 8 mM L-3,4-dihydroxyphenylalanine in 0.1 M sodium phosphate buffer pH 7.0. Using a CLARIOstar High Performance Monochromator multimode microplate reader (BMG labtech), change in absorbance at 490 nm was recorded. Total protein concentration of each extract was determined using modified methods outlined by Bradford (1976). Each sample was run in technical triplicate. Considering the number of samples (6–8 per treatment combination over four time points), individuals were analysed across multiple different batches. Due to variability in measurement there was an effect of batch on PPO activity. Considering individuals were randomly assigned to batches, each batch was standardised to the batch containing the highest measured value.

4.3.8   Statistical analyses

Comparison of Si accumulation was determined using Welch's t-tests. Linear regressions were performed to determine correlation between JA/SA concentration and Si accumulation, Si accumulation and total phenolics, and total phenolics and phenylalanine concentration. Differences between JA concentrations across treatment combinations 1 hr after MeJA treatment were determined using a two-way ANOVA (type = II) on square root-transformed values. Differences in JA concentrations 6, 12 and 24 hr after MeJA treatment did not meet the assumptions of normality, and therefore a non-parametric Scheirer-Ray-Hare test was

used to determine differences between treatments on square root-transformed values. Across time points, SA concentration, phenylalanine concentration, total phenolics, and PPO activity were analysed using two-way ANOVAs (type = II) to determine differences across treatments. Overall differences in total phenolics across treatments was also analysed using a two-way factorial ANOVA. All statistical analyses were conducted in R version 3.6.2 (R Core Team 2020). ANOVAs were run using the R package 'car' (Fox and Weisberg 2019). Both JA 1 hr after MeJA treatment and SA 24 hr after MeJA treatment were analysed using heteroscedasticity-consistent standard errors, obtained by using White-adjusted ANOVAs (White 1980).

4.4 Results:

4.4.1 Effects of methyl jasmonate (MeJA) on silicon (Si) accumulation over time

After 6 hr, plants treated with methyl jasmonate (MeJA) showed an increase in Si accumulation, from 1.519% to 1.823% (+20% in relative terms) compared to controls (Fig 4-1A; Table 4-1). This increase persisted up to 24 hr after treatment (17% increase), but by 24 hr the difference between treatments was marginally non-significant (Fig 4-1A; Table 4-1). Additionally, we found positive correlations between JA concentration and Si accumulation 6 ($p = 0.042$, $R = 0.593$) and 24 ($p = 0.026$, $R = 0.647$) hr after MeJA treatment (Fig 4-1B).
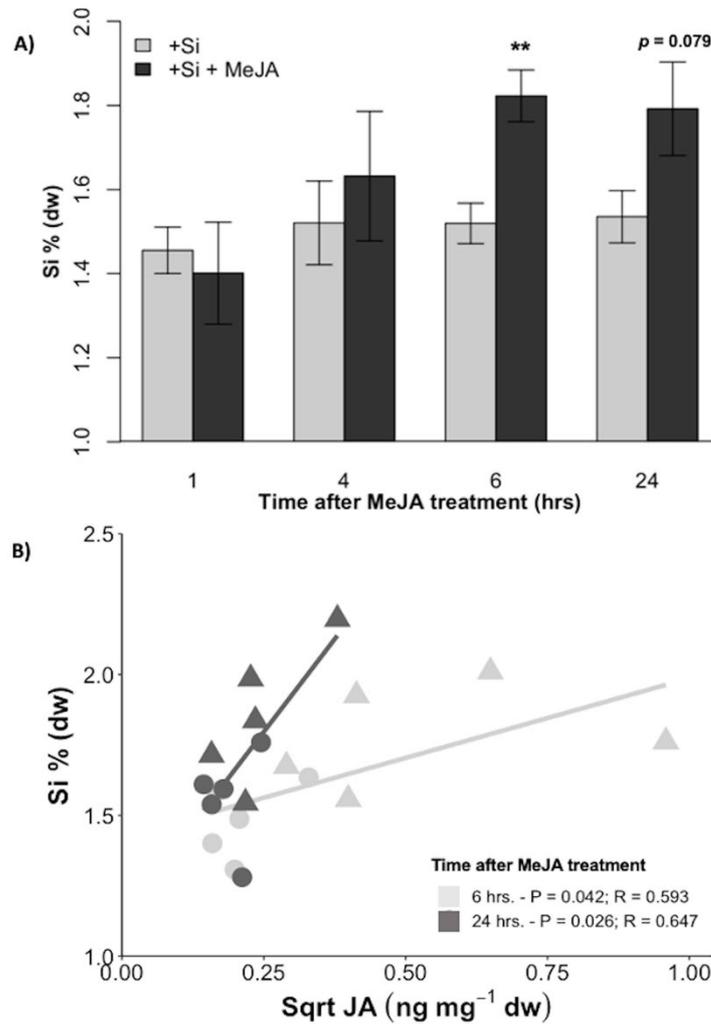
Figure 4-1. Silicon (Si) accumulation is induced by methyl jasmonate (MeJA) and jasmonic acid (JA). Differences in Si concentration between MeJA treated (+Si + MeJA) and control (+Si) plants 1, 4, 6 and 24 hr after MeJA treatment (A; n = 6-7). Differences between treatments were determined at each time point using Welch's two-sample tests (** =p < 0.01 at 95% confidence interval). Si concentration was regressed against square root (sqrt) JA concentrations 6 and 24 hr after MeJA treatment (B; n = 5). For (B) the solid lines represent linear regression through all data points of a given time point. Light grey points= 6 hr after MeJA treatment, and dark grey points = 24 hr after MeJA treatment. Circles are +Si plants and triangles are +Si + MeJA plants. All plants used in these analyses were supplemented with Si.

4.4.2 *Effects of MeJA and Si on phytohormone concentrations over time*

MeJA treatment significantly increased JA concentration 1, 6 and 12 hr after treatment (Fig 4-2A; Table 4-1), with a particularly large increase after 1 hr. This increase occurred irrespective of Si status after 1 and 6 hr (Fig 4-2A; Table 4-1), though after 12 hr, there was an interaction effect of MeJA and Si, whereby +Si and all MeJA-treated plants had higher JA concentrations than -Si plants (Fig 4-2A; Table 4-1). After 24 hr +Si plants had higher JA concentrations than –Si plants, regardless of MeJA treatment (Fig 4-2A; Table 4-1). Both Si

supplementation and MeJA treatment significantly decreased SA concentrations after 24 hr (Fig 4-2B; Table 4-1). Except at the 6-hr timepoint, +Si plants had significantly lower SA concentrations than -Si plants regardless of MeJA treatment (Fig 4-2B; Table 4-1). Across all time points, we found a significant negative relationship between Si and SA in +Si + MeJA plants ($p = 0.015$, $R = -0.575$; Fig 4-3B). This relationship, however, was not observed in +Si plants not treated with MeJA ($p = 0.842$, $R = -0.055$; Fig 4-3A). Additionally, there were no significant correlations between JA and SA concentration detected overall, across treatments or across time points (Table S4-1).
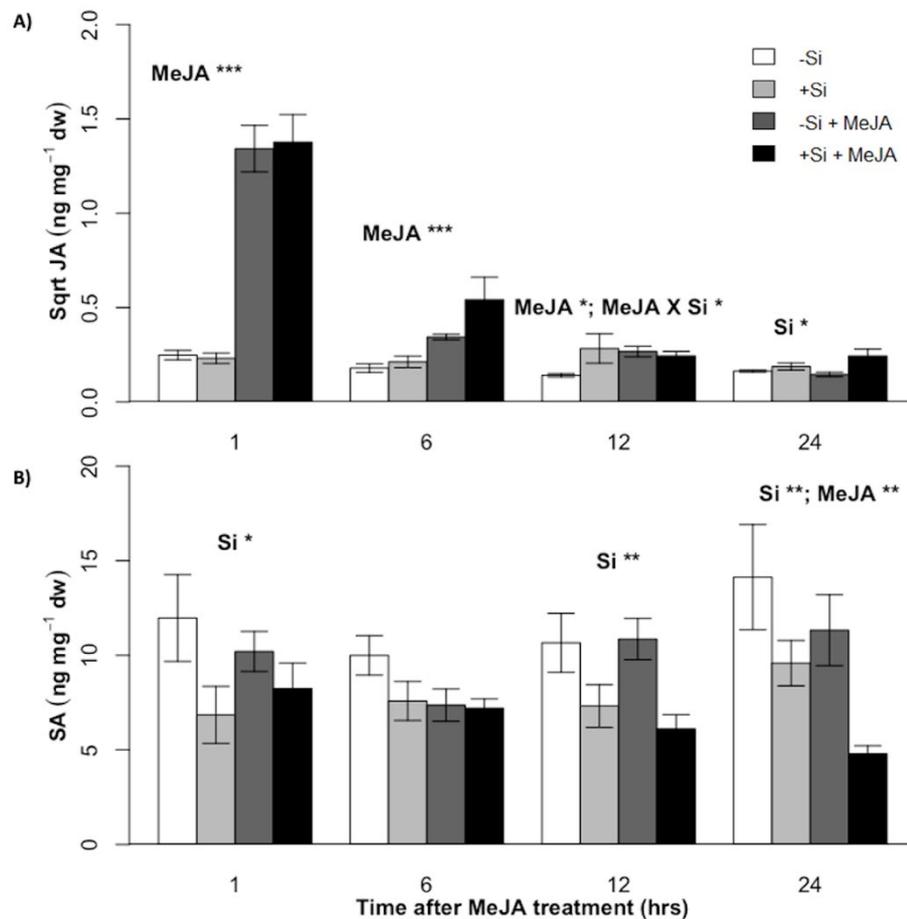
Figure 4-2. Silicon (Si) affects phytohormone concentrations over time. Concentrations of (A) square root (sqrt)-transformed jasmonic acid (JA) and (B) salicylic acid (SA) 1, 6, 12 and 24 hr after methyl jasmonate (MeJA) treatment ($n = 4$-5). White bars = control plants (-Si), light grey bars = Si-supplemented plants (+Si), dark grey bars = plants treated with MeJA (-Si + MeJA), and black bars = plants supplemented with Si and treated with MeJA (+Si + MeJA). Differences between treatments was determined within each time point for each phytohormone using two-way ANOVAs or Scheirer-Ray-Hare tests (Table 4-1; * =$p < 0.05$, ** =$p < 0.01$, *** =$p < 0.001$ at 95% confidence intervals).
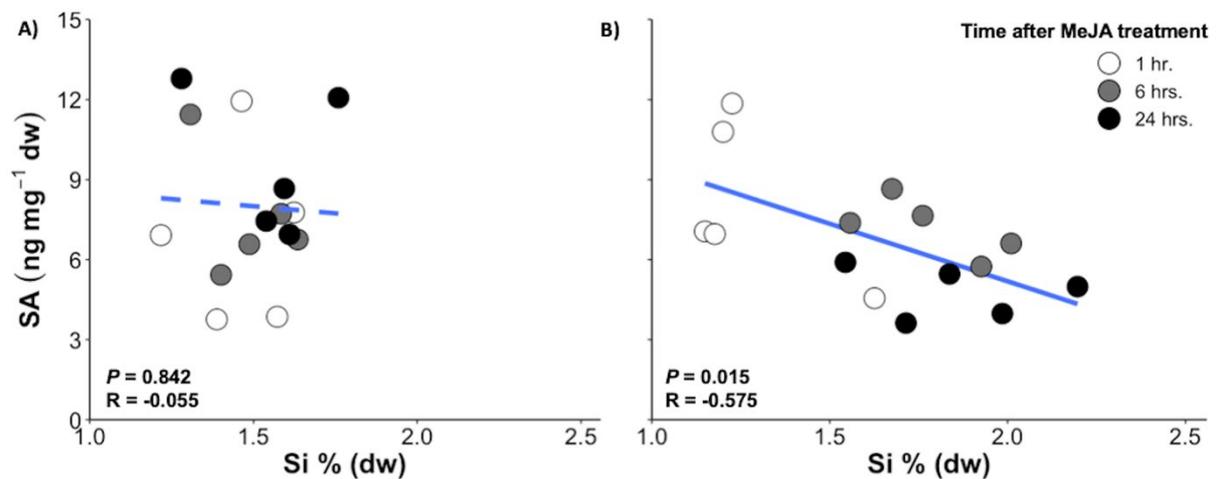
Figure 4-3. Salicylic acid (SA) regressed against silicon (Si) concentration. SA regressed against Si in Si-supplemented plants in (A) untreated plants (+Si) and (B) plants treated with methyl jasmonate (+Si + MeJA). The solid line in (B) represents linear regression through all data points (no significant linear relationship was observed in (A), as marked by a dashed line). White dots = 1 hr after MeJA treatment, grey dots = 6 hr after MeJA treatment, and black dots = 24 hr after MeJA treatment.

4.4.3 Effects of MeJA and Si on carbon-based defence responses

Overall, irrespective of time point, MeJA significantly increased total phenolics ($F_{1,42}$ = 9.196, p = 0.004) and Si significantly reduced total phenolics ($F_{1,42}$ = 7.631, p = 0.008). MeJA treatment, although marginally insignificant, increased total phenolics at 6 and 24 hr specifically (Fig 4-4A; Table 4-1). Si treatment significantly reduced total phenolics after 24 hr (Fig 4-4A; Table 4-1). MeJA increased phenylalanine concentration 1 and 6 hr after treatment, narrowly missing statistical significance at 24 hr (Fig 4-4B; Table 4-1). At 6 hr, +Si plants also had higher phenylalanine concentrations (Fig 4-4B; Table 4-1). In +Si plants without MeJA, there was a significant negative relationship between total phenolics and Si concentrations which was not observed in +Si + MeJA plants (p = 0.013 and 0.119 respectively; Fig 4-4C). Additionally, there was a positive correlation between phenylalanine and total phenolics concentration in all plants without Si (-Si and -Si + MeJA), whereas there was no such relationship in +Si and +Si + MeJA plants (p = 0.037 and 0.704, respectively; Fig 4-4D).

After 12 hr, there was a significant increase in polyphenol oxidase (PPO) activity in plants treated with MeJA, however this was largely driven by the increase in –Si plants (Fig S4-1; Table 4-1). After 24 hr, PPO activity was highest in both –Si and +Si plants treated with MeJA (Fig S4-1; Table 4-1). We did not observe effects of either Si supplementation or MeJA treatment on PPO activity at 1 and 6 hr.
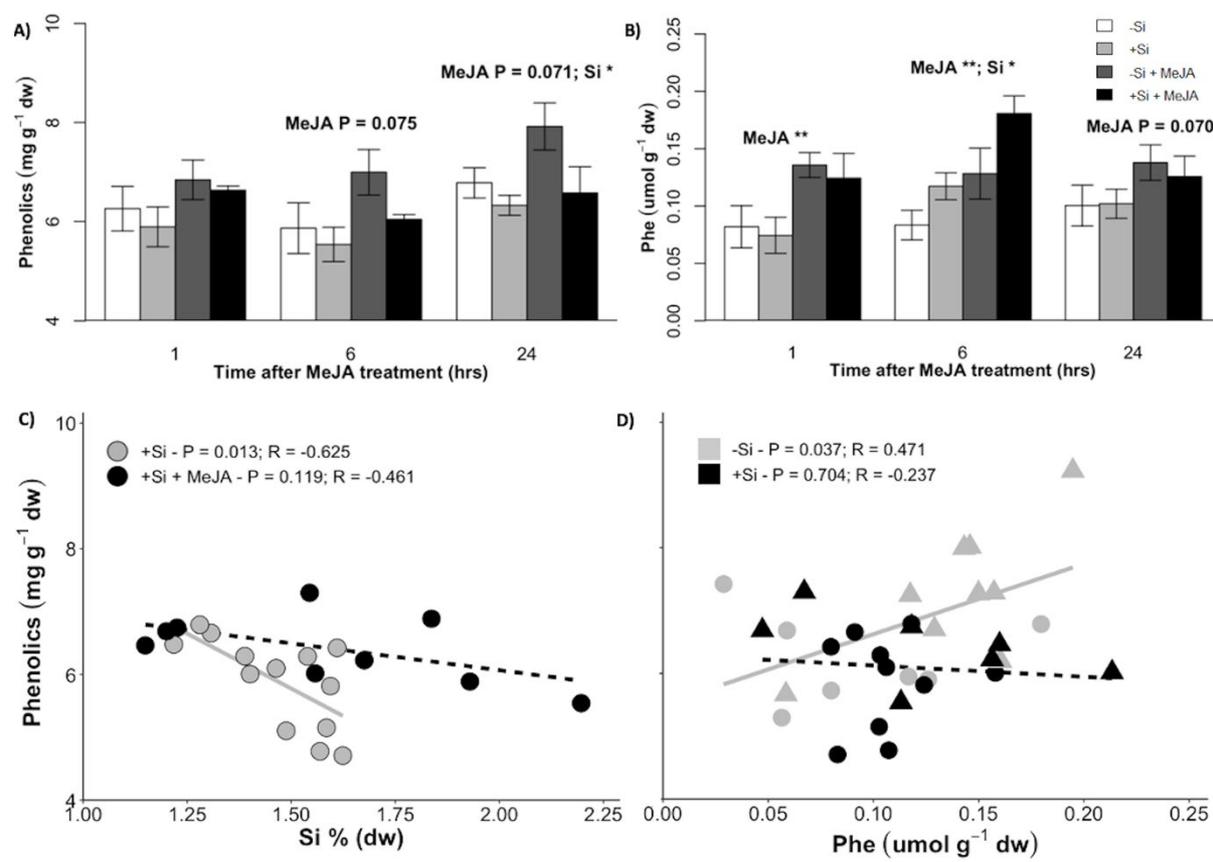
Figure 4-4. Effects of silicon (Si) and methyl jasmonate (MeJA) on total phenolics and phenylalanine over time. (A) concentrations of total phenolics 1, 6 and 24 hr after MeJA treatment ($n = 3\text{-}5$) and (B) concentrations of phenylalanine (Phe) 1, 6 and 24 hr after MeJA treatment ($n = 5\text{-}7$). White bars = control plants (-Si), light grey bars = Si-supplemented plants (+Si), dark grey bars = Plants treated with MeJA (-Si + MeJA), and black bars = plants supplemented with Si and treated with MeJA (+Si + MeJA). Differences in phenylalanine and total phenolics between treatments were determined within each time point using two-way ANOVAs (* $= p < 0.05$, ** $= p < 0.01$, *** $= p < 0.001$ at 95% confidence intervals). (C) Total phenolics concentration regressed against Si concentration, (D) phenylalanine concentration regressed against total phenolics concentration. For (C), only Si supplemented plants were used and light grey points = control plants (+Si) and black points = MeJA treated plants (+Si + MeJA). For D, light grey circles = -Si, black circles = +Si, light grey triangles = -Si + MeJA, and black triangles = +Si + MeJA. For both (C) and (D) solid trend lines indicate a significant relationship between x- and y-axis variables and dashed lines indicate that no significant relationship was observed.

4.5 Discussion:

We demonstrate that a grass species induces silicon (Si) accumulation following simulated herbivory in as little as 6 hr. Further, we found that Si accumulation plateaus between 6 and 24 hr after single-entry (non-continuous) simulated herbivory. The correlation between jasmonic acid (JA) concentration and Si accumulation lends further support to Si-based defences being regulated by the JA pathway (Hall et al. 2019). Moreover, our results indicate that Si suppresses salicylic acid (SA) under simulated chewing herbivory, which could have implications on further Si accumulation and downstream herbivore defences. Further, we did not see the effect of methyl jasmonate (MeJA) on SA concentration until 24 hr after treatment, suggesting that the effects of MeJA on SA suppression may be less rapid than the effects of MeJA on JA. We demonstrate that MeJA treatment might decouple the negative relationship between Si accumulation and total phenolic concentration, which highlights the potential utility of phenolics during herbivory, even in Si-treated plants. Finally, Si might reduce the conversion of phenylalanine to phenolic compounds, as Si increased soluble phenylalanine concentration but decreased total phenolics and disrupted the positive relationship between phenylalanine and phenolics observed in -Si plants (Fig 4-5).
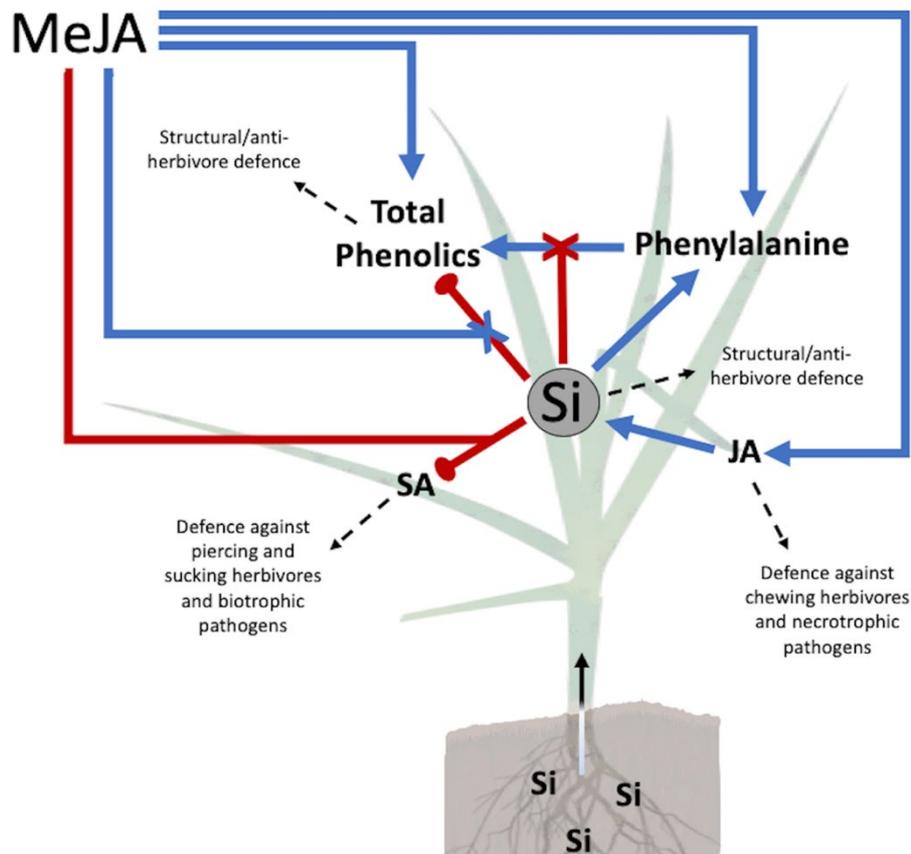
Figure 4-5. Summary of the effects of silicon (Si) and methyl jasmonate (MeJA) on the Si- and carbon-based defence machinery. Positive effects are denoted by blue pointed arrows and negative effects by red rounded arrows. Arrows with X's at the end indicate an obstruction of the relationship between two defences (blue = disruption of negative effect and red = disruption of a positive effect). MeJA increased jasmonic acid (JA), total phenolics and phenylalanine. MeJA induced Si accumulation via increased JA concentrations. Si increased phenylalanine concentration and decreased salicylic acid (SA) and phenolics. Si also disrupted the positive relationship between phenylalanine and total phenolics. MeJA disrupted the negative relationship between Si and phenolics.

4.5.1 Si accumulation is a rapidly induced defence

Other studies have also observed increased Si accumulation in response to stimulation of the JA pathway (Hall et al. 2019, Hall et al. 2020b). Additionally, Ye et al. (2013) report that three critical proteins for active uptake and transport of Si into and through tissues (Lsi1, Lsi2 and Lsi6) can be activated through stimulation of the JA pathway. By emphasising earlier timepoints for Si quantification, our results confirm the role of MeJA in inducing Si accumulation, but also indicate that significant amounts of Si (20% increase beyond unstressed levels) can be accumulated between 4 and 6 hr after MeJA treatment. This trend

persisted through to 24 hr, however at this time differences were marginally insignificant, although, these differences may have been significant if there was more replication, as the overall positive relationship between JA and Si persisted through to 24 hr. Nevertheless, the levels of replication used in the present study are well-aligned with other similar time series experiments (Peñuelas et al. 1996, Ye et al. 2013, Chen et al. 2019, Hall et al. 2020b).

A previous study showed that a *c.* 15% increase in Si concentration significantly reduced grasshopper herbivory in rescuegrass (*Bromus catharticus*), albeit this reduction in herbivory occurred even at Si concentrations substantially lower than the present study (Mir et al. 2019). Our findings suggest that Si might be a particularly effective defence as it can be deployed very rapidly, in a matter of hours, but once deposited as silica-rich structures such as phytoliths, it is also known to remain in tissues and serve as an effective herbivore defence for, at least, many months (Epstein 1994, Reynolds et al. 2012). In fact, Si accumulation can occur at an even faster rate than well described chemical defences. For example, serine protease inhibitors, commonly measured herbivore defences due to their anti-digestive characteristics, were only induced after 24 hr following MeJA treatment (Hartl et al. 2010). Further, we found polyphenol oxidase (PPO), likewise known to interfere with herbivore digestion (Felton et al. 1989), was not induced until 12 hr after MeJA treatment.

4.5.2   *Si in relation to plant defence machinery*

One hypothesis as to how Si supplementation affects JA concentrations is by maintaining higher levels of JA under ambient conditions, or after herbivory signals wear off (Kim et al. 2014, Jang et al. 2018, Hall et al. 2019). Our results support these findings as Si supplementation maintained higher JA concentrations after the effects of MeJA treatment subsided. After 1, 6 and 12 hr, we found a significant effect of MeJA on JA concentration; in

contrast to other studies, however (Ye et al. 2013, Kim et al. 2014, Hall et al. 2020b), we show that the effects of Si on JA concentration are minimal, and only present once the effects of MeJA subside (i.e. 24 hr after treatment). Although Si can change leaf surface morphology in *B. distachyon* (Hall et al. 2020a), these changes are fairly modest (e.g. larger, but less numerous prickle cells). Additionally, in *B. distachyon*, Si is primarily deposited in the leaf hairs and not the leaf cuticle, so we do not consider that Si impedes MeJA perception (Głazowska et al. 2018b). In support of this, both -Si + MeJA and +Si + MeJA plants had equally strong increases in JA when the effects of MeJA on JA were strongest (1 hr after treatment). Considering MeJA is known to directly stimulate JA biosynthesis we can conclude that differences in plant defences between -Si and + Si plants are not influenced by different levels of MeJA perception (Tamogami et al. 2008).

Unlike JA, evidence of the impact of Si on the levels of SA following herbivore signals is sparse in the literature. SA and JA are generally considered to induce different defence pathways, for example, JA has been shown primarily to activate defences most effective against chewing herbivores and necrotrophic pathogens, while SA is considered to activate many defences against piercing and sucking insects (e.g. aphids) and biotrophic pathogens (Reymond and Farmer 1998, Erb et al. 2012, Pieterse et al. 2012). Interestingly, in Si supplemented plants, only after MeJA treatment was there a significant negative relationship between Si and SA concentration, suggesting Si-mediated SA suppression might be heightened when a threat of chewing herbivory is perceived (Traw and Bergelson 2003). In general, though, we found that Si decreased SA concentration independent of MeJA treatment, suggesting Si may suppress SA regardless of whether or not a threat of herbivory is perceived. Further, after 24 hr SA was suppressed by MeJA suggesting that the induction of the JA pathway can reduce SA, perhaps through molecular crosstalk (Pieterse et al. 2009).

However, considering we did not observe any linear correlations between JA and SA (even at 24 hr), the negative relationship between Si and SA is likely direct and not one that only results from antagonism between SA and JA. Considering SA is itself a phenolic compound, it is possible that the negative relationship between Si and SA could be due to a reduction in SA precursors, perhaps underpinned by the negative relationship between Si and total phenolics, however further investigation into the relationship between Si and specific compounds in the SA biosynthesis pathway is necessary in order to make this determination (Chen et al. 2009).

Previous studies have shown negative relationships between phenolic compounds and Si accumulation, partially explained by differences in carbon assimilation and resource allocation (Cooke and Leishman 2012, Frew et al. 2016, Johnson and Hartley 2018). As in previous studies, we found Si supplementation decreased total phenolics (Cooke and Leishman 2012, Frew et al. 2016, Johnson and Hartley 2018). However, to our knowledge, this is the first study to show that the negative relationship between Si and phenolics is disrupted in response to activation of the JA pathway. Overall, we report that MeJA significantly increased total phenolics, and although marginally insignificant (perhaps due to relatively low levels of replication), MeJA increased phenolics after 6 and 24 hr specifically, primarily driven by -Si + MeJA plants. The decoupling of the negative relationship between Si and phenolics in Si-treated plants by MeJA suggests that, under stress, plants might utilise both Si and phenolic-based defences. Si is often considered an energetically cheap alternative to carbon-based defences (Cooke and Leishman 2011b), and it is therefore possible that plants supplemented with Si can employ Si-based structural defences and preferentially utilise less constitutive phenolics in the absence of herbivory.

Induction of phenylalanine synthesis has been observed in response to herbivory, as it is an essential precursor to defence compounds of the phenylpropanoid pathway (Bernards and Båstrup-Spohr 2008). Downstream compounds of this pathway including phenolics, flavonoids, phytoalexins, and stilbenes are all derived from phenylalanine and can play an important role in combatting herbivory (Deng and Lu 2017). We found that, in contrast to phenolics, Si supplementation can increase phenylalanine concentration. This combined with evidence of a positive relationship between phenylalanine and total phenolics in plants without Si (with no such correlation in Si-supplemented plants), suggests that despite the apparent trade-off between Si and phenolics, there is no trade-off between Si and phenolic precursors such as phenylalanine. This might be due to the fact that in Si-supplemented plants, less available phenylalanine is converted to phenolics, as demonstrated by the loss of a positive correlation between phenolics and phenylalanine in +Si plants. In light of this, Si supplementation might maintain higher levels of phenylalanine, which could be utilised for critical metabolic processes independent of combatting herbivory including protein synthesis (Deng and Lu 2017).

Although we show that MeJA can induce rapid Si accumulation, thus modifying plant defence responses, whether or not authentic herbivory can do this remains to be seen. Nevertheless, the MeJA treatment employed in the present study has been shown to yield similar results to authentic herbivory. For example, Ye et al. (2013) found no difference in JA concentration between MeJA and authentic caterpillar herbivory as soon as 3 hr after treatment. Johnson et al. (2021) also found no differences in Si accumulation between MeJA and caterpillar feeding using a very similar hydroponic system to the present study. Additionally, MeJA allows us to make determinations not afforded by true herbivory. Specifically, insects are known to modify their feeding behaviour based on the quality of leaf

tissue; considering Si is known to reduce the palatability of leaves to insects, it is likely that insects would not damage +Si plants to the same extent as -Si plants, which would make it impossible to discern if differences in defence responses were due to direct effects of Si, or simply due to inconsistencies in damage across Si treatments (Ryalls et al. 2017, Andama et al. 2020). Standardising the exact timing of herbivory is also critical for measuring short-term chemical defence signalling; the precise timing necessary for this experiment could not have been achieved with live insects, as individual insects would not have started to feed at exactly the same time or at the same rate (Waterman et al. 2019). With MeJA, we can not only determine exactly how much time has passed since the treatment was applied but also ensure that the extent of herbivore-signals is identical across all plants and treatments. Nevertheless, future work that investigates the temporal dynamics of Si-accumulation and its effects on various plant defences in natural ecosystems will prove instrumental for understanding the ecological role of Si accumulation as a response to insect herbivores.

4.6 Conclusions

Our study demonstrates the rapid induction of Si accumulation in response to MeJA treatment. We show that this induction occurs as early as 6 hr after exposure to MeJA via increased JA concentration. Additionally, we found that the relationship between Si and chemical defences changes depending on whether signals of herbivory are perceived. In addition to its well-documented role as a long-term defence against herbivores, we demonstrate here that, over a short-term temporal scale, Si accumulation responds to simulated herbivory and impacts on the defence machinery in plants.

Table 4-1. Statistical analyses on the effects of silicon supplementation (Si) and methyl jasmonate treatment (MeJA) on defence responses in *Brachypodium distachyon*.

Response Variable	MeJA $F^a/H^b/t^c$	MeJA *p*-value	MeJA d.f.	Si F^a/H^b	Si *p*-value	Si d.f.	MeJA X Si F^a/H^b	MeJA X Si *p*-value	MeJA X Si d.f
Si conc. (Fig 1A)									
1 hr. [c]	0.407	0.694	8	-	-	-	-	-	-
4 hrs. [c]	-0.607	0.557	9	-	-	-	-	-	-
6 hrs. [c]	-3.880	**0.002**	11	-	-	-	-	-	-
24 hrs. [c]	-2.016	<u>0.079</u>	7	-	-	-	-	-	-
[‡]*JA conc. (Fig 2A)*									
1 hr. [a]	107.915	**< 0.001**	1,15	0.136	0.718	1,15	0.056	0.817	1,15
6 hrs. [b]	11.760	**< 0.001**	1,15	0.910	0.340	1,15	0.037	0.847	1,15
12 hrs. [b]	4.167	**0.041**	1,15	1.428	0.232	1,15	4.018	**0.045**	1,15
24 hrs. [b]	0.107	0.744	1,15	4.795	**0.029**	1,15	1.795	0.180	1,15
SA conc. (Fig 2B)									
1 hr. [a]	0.004	0.953	1,15	5.037	**0.040**	1,15	1.056	0.320	1,15
6 hrs. [a]	2.587	0.129	1,15	2.285	0.151	1,15	1.581	0.228	1,15
12 hrs. [a]	0.156	0.698	1,15	11.911	**0.004**	1,15	0.354	0.561	1,15
24 hrs. [a]	11.605	**0.004**	1,15	10.256	**0.006**	1,15	0.243	0.629	1,15
Phenolics conc. (Fig 4A)									
1 hr. [a]	2.755	0.125	1,11	0.559	0.470	1,11	0.039	0.847	1,11
6 hrs. [a]	3.880	<u>0.075</u>	1,11	2.360	0.153	1,11	0.584	0.461	1,11
24 hrs. [a]	3.923	<u>0.071</u>	1,12	4.927	**0.046**	1,12	1.359	0.266	1,12
Phe conc. (Fig 4B)									
1 hr. [a]	8.333	**0.009**	1,19	0.297	0.592	1,19	0.011	0.916	1,19
6 hrs. [a]	10.096	**0.006**	1,17	6.637	**0.020**	1,17	0.299	0.592	1,17
24 hrs. [a]	3.657	<u>0.070</u>	1,21	0.120	0.733	1,21	0.178	0.677	1,21
[+]*PPO activity (Fig S1)*									
1 hr. [a]	0.915	0.348	1,25	1.174	0.289	1,25	0.533	0.472	1,25
6 hrs. [a]	3.318	<u>0.080</u>	1,27	0.411	0.527	1,27	2.281	0.143	1,27
12 hrs. [a]	13.758	**0.001**	1,24	0.923	0.346	1,24	5.967	**0.022**	1,24
24 hrs. [a]	22.733	**< 0.001**	1,25	0.000	1	1,25	0.080	0.780	1,25

a = Analysed using a two-way ANOVA; b = Analysed using a Scheirer-Ray-Hare test; c = Analysed using a Welch's two-sample t-test; ‡ = Analysed on square-root transformed data; + = Analysed on Log transformed data. Abbreviations: JA = jasmonic acid, SA = salicylic acid, phe = phenylalanine, PPO = polyphenol oxidase. Bold values = P < 0.05 and underlined values = P < 0.1 at a 95% confidence interval.

4.7 Acknowledgements

5 Chapter 5: Short-term exposure to silicon rapidly enhances plant resistance to herbivory

5.1 Abstract:

Silicon (Si) can adversely affect insect herbivores, particularly in plants that evolved the ability to accumulate large quantities of Si. Very rapid herbivore-induced accumulation of Si has recently been demonstrated, but the level of protection against herbivory this affords plants remains unknown. *Brachypodium distachyon*, a model Si hyperaccumulating grass, was exposed to the chewing herbivore, *Helicoverpa armigera,* and grown under three conditions: supplied Si over 34 days (+Si), not supplied Si (-Si), or supplied Si once herbivory began (-Si➔+Si). We evaluated the effectiveness of each Si treatment at reducing herbivore performance and measured Si-based defenses and phenolics (another form of defense often reduced by Si). Although Si concentrations remained lower, within 72 hr of exposure to Si, -Si➔+Si plants were as resistant to herbivory as +Si plants. Both +Si and -Si➔+Si treatments reduced herbivore damage and growth, and increased mandible wear compared to -Si. After 6 hr, herbivory increased filled Si cell density in -Si➔+Si plants, and within 24 hr, -Si➔+Si plants reached similar filled Si cell densities to +Si plants, although decreased phenolics only occurred in +Si plants. We demonstrate that plants with short-term Si exposure can rapidly accumulate Si-based anti-herbivore defenses as effectively as plants with long-term exposure.

Keywords: *Helicoverpa armigera*, herbivory, plant defense, silica cells, silicon

5.2 Introduction:

An understanding of the mechanisms through which plants defend themselves against herbivory is critical, as insect herbivores can have substantial impacts on ecosystem function and agricultural production (Bradshaw et al. 2016). The functional role of silicon (Si) uptake and deposition in plants, particularly the grasses, in ecological interactions between plants and their antagonists has been demonstrated at scales from individual plants to grassland ecosystems (Hartley and DeGabriel 2016). Si also alleviates many of the biotic and abiotic stressors affecting plants in natural communities, including pests, pathogens, nutrient deficiency or toxicity, and extreme climatic conditions (Cooke and Leishman 2016). The utility of Si as an anti-herbivore defense has been particularly well-studied (Reynolds et al. 2009, Leroy et al. 2019). Si accumulation and subsequent deposition has been shown to confer physical resistance to herbivory through the formation of discrete Si-based physical structures (e.g., phytoliths) on the leaf surface (Hartley et al. 2015). Some plants deposit Si in specialized epidermal Si cells which are actively filled with Si and can play a role in Si-based anti-herbivore defense (Kumar et al. 2017a, Hall et al. 2020a). These Si structures can interfere with herbivore feeding and reduce herbivore fitness through a range of mechanisms, including mandibular wear (Massey and Hartley 2009), decreased growth (Massey and Hartley 2009, Johnson et al. 2021), and interference with digestion (Hunt et al. 2008, Massey and Hartley 2009, Andama et al. 2020).

Recently, we reported that foliar Si accumulation could be induced in as little as 6 hr (Waterman et al. 2021b), but whether the amount of Si accumulated during such rapid induction is sufficient to modify plant–herbivore interactions remained unknown. Additionally, Si supplementation can lead to a trade-off between Si and carbon-based phenolic defenses (Cooke and Leishman 2012, Simpson et al. 2017, Waterman et al. 2021b).

Whether this relationship is maintained after short-term exposure to Si, or if it only occurs after long periods of exposure, remains unclear. In the present study, we exposed the chewing herbivore and global agricultural pest, *Helicoverpa armigera* (Lepidoptera: Noctuidae), to the model grass species and high Si accumulator, *Brachypodium distachyon* (L.) P. Beauv. Herbivore-treated plants were either supplied with Si throughout their growth period, not supplied any Si, or only supplied Si at the onset of herbivory. Plants and herbivores were harvested over three time points within a 72-hr period; we measured *H. armigera* feeding, growth and mandible wear, and compared plant responses including Si-based defenses and total phenolics.

5.3   Materials and methods:

5.3.1   *Plant treatments*

Nine-day-old *Brachypodium distachyon* plants germinated in perlite in a glasshouse (22/18° C day/night; Appendix 2: Section S1) were transferred to nested disposable cups containing 330 mL nutrient solution and held in place with a foam disk as per Hall et al. (2020b). Roughly one quarter of the plants (41) were supplemented with 2 mM potassium silicate (+Si). The remaining plants were not supplemented with Si (-Si), and instead potassium chloride was added to the solution to balance the potassium availability between treatments. HCl was added to each solution to bring the pH to 5.5. Fresh solution was given to each plant once per week. Nutrient solutions across treatments were identical to those used in Hall et al. (2020b). After 34 days, 66 -Si plants were switched to the +Si solution (-Si➜+Si); 41 plants remained in -Si solution. At the same time, individual 4th instar *H. armigera* larvae were placed on 33 plants within each Si treatment (99 herbivore-treated plants), leaving 33 -Si➜+Si plants, 8 +Si and 8 -Si plants herbivore free (148 total plants). *Helicoverpa armigera* movement was restricted using whole-plant cages, as per Johnson et al. (2021). Cages were

also used on herbivore-free plants to account for any effects of cages on plant responses. Plants were harvested 6, 24 and 72 hr after commencing the -Si➔+Si treatment and *H. armigera* herbivory, except for the 8 herbivore-free +Si and -Si plants, which were only harvested after 72 hr. Although 11 larvae per time point within each Si treatment (33 total for each treatment) were placed on plants, some larvae escaped their cages during the experiment and, along with their respective plants, were excluded from analysis; thus, the number of plants harvested per treatment per time point ranged from 8–11. None of the larvae used molted during the experiment (i.e., all larvae remained 4th instar at the time of harvest).

5.3.2   *Helicoverpa armigera performance*

Helicoverpa armigera larvae reared on artificial diet (CSIRO, Narrabri, Australia) at 22°C were starved for 24 hr prior to placement on plants. Relative growth rate (RGR) and relative consumption (RC) were measured for the eight *H. armigera* larvae that damaged the most foliar tissue within each treatment after 24 and 72 hr of exposure to plants. Larvae from the 6-hr time point were not included considering negligible tissue consumption occurred at 6 hr across all treatments. RGR was defined for each larva as ((final larval mass after exposure to plants - initial larval mass after starvation)/initial larval mass after starvation)/number of days of exposure to plants. RC was defined for each larva as area of foliar tissue consumed/mean larval weight throughout herbivory period. Larvae were stored at -80°C.

5.3.3   *Quantification of foliar damage and Si concentrations*

Herbivore-damaged leaves were placed on white paper inside a drawn 12 cm × 12 cm square and imaged from a fixed position directly above the leaves with a Nikon Coolpix P900 digital camera. Using the dimensions of the drawn square as a reference, total area of tissue removed from all damaged leaves on a given plant was measured using ImageJ (National Institutes of

Health, Bathesda MD, USA; Version 2.0.0). Damaged and undamaged leaves were stored at -80°C until being freeze-dried. Freeze-dried and ground undamaged leaves of herbivore-treated plants were used to quantify Si concentrations. Si was quantified using X-ray fluorescence spectrometry (Reidinger et al. 2012), applied as described in Waterman et al. (2021b).

5.3.4 Scanning electron microscopy (SEM)

To determine silicified (filled) leaf surface Si cell density, the abaxial side of three freeze-dried fully expanded undamaged leaves (technical replicates) of 3-4 plants per treatment and herbivory duration combination was imaged using a Phenom XL scanning electron microscope (SEM; Phenom XL G2 Desktop SEM, Thermo Fisher Scientific, Waltham MA, USA) with a backscattered electron detector (Appendix 2: Section S1). Images were used to calculate filled Si cell density using ImageJ (Appendix 2: Section S2: Fig S5-1A).

To ensure mandibles measured made substantial contact with leaf tissue (i.e., that active feeding occurred), mandibles from the four *H. armigera* larvae that consumed the most tissue within each Si treatment at 24 and 72 hr of herbivory were dissected from the heads of larvae. At 6 hr of herbivory, mandibles were dissected from four of the six larvae that consumed the most tissue within each Si treatment. The inner surface of each mandible was positioned to face upwards and was imaged using the same SEM methods described above. Previous mandible wear quantification methods (e.g., Massey and Hartley 2009) were not sufficiently sensitive for this insect–host plant combination over the time period of this study. We used a 4-point scale-based measurement approach, with 4 being the most severe damage and 0 being no damage, to score the severity of mandible wear (Appendix 2: Section S1 and Section S2: Fig S5-2).

5.3.5   Total phenolics quantification

Total phenolics were quantified in the damaged leaves of the six most damaged plants within each Si treatment at 24 and 72 hr after herbivory using a modified version of the Prussian blue assay described in Waterman et al. 2021b (Appendix 2: Section S1). Phenolics were not quantified in damaged leaves after 6 hr because negligible tissue had been damaged at this stage. Phenolics were also measured in leaves from six randomly selected herbivore-free plants within each Si treatment (harvested at 72 hr).

5.3.6   Statistical Analyses

All analyses were conducted on plants with herbivores unless otherwise specified. Data were analyzed using one- or two-way ANOVA, however differences in foliar damage at 6 hr were determined using a Kruskal-Wallis test and filled Si cell density between -Si➔+Si and +Si plants at 72 hr was analyzed using Welch's t-test. Linear or logarithmic regressions were used to determine the relationship between foliar damage and Si concentration. All statistical analyses were conducted in R version 4.0.2 (R Core Team 2020). ANOVAs were run using the R package 'car' (Fox and Weisberg 2019). Pairwise comparisons of Estimated Marginal Means between groups were performed using the 'emmeans' R package (Lenth 2020). See Appendix 2: Section S1 for additional details on statistical analyses.

5.4   Results:

5.4.1   Si concentration and herbivore performance

Plants only exposed to Si once *H. armigera* herbivory began (-Si➔+Si) showed an increase in foliar Si concentration compared to plants not supplied with Si (-Si) in as little as 6 hr, and nearly doubled in Si concentration by 72 hr (from 0.69% to 1.35% foliar Si; Fig 5-1A; Table 5-1). Although -Si➔+Si concentrations were lower than those in plants exposed to Si for

over 34 days (+Si), there was no difference in foliar damage between these two treatments (Fig 5-1B; Table 5-1). Foliar damage significantly increased between 24 and 72 hr of herbivory in -Si but not in -Si➔+Si or +Si plants (Fig 5-1B). There were no differences in the amount of foliar damage between any treatments after 6 hr of feeding ($\chi^2 = 0.048$, $p = 0.976$; Fig 5-1B inset). At both 24 and 72 hr there was a significant negative relationship between foliar damage and Si concentration (Fig 5-1C and D, respectively).
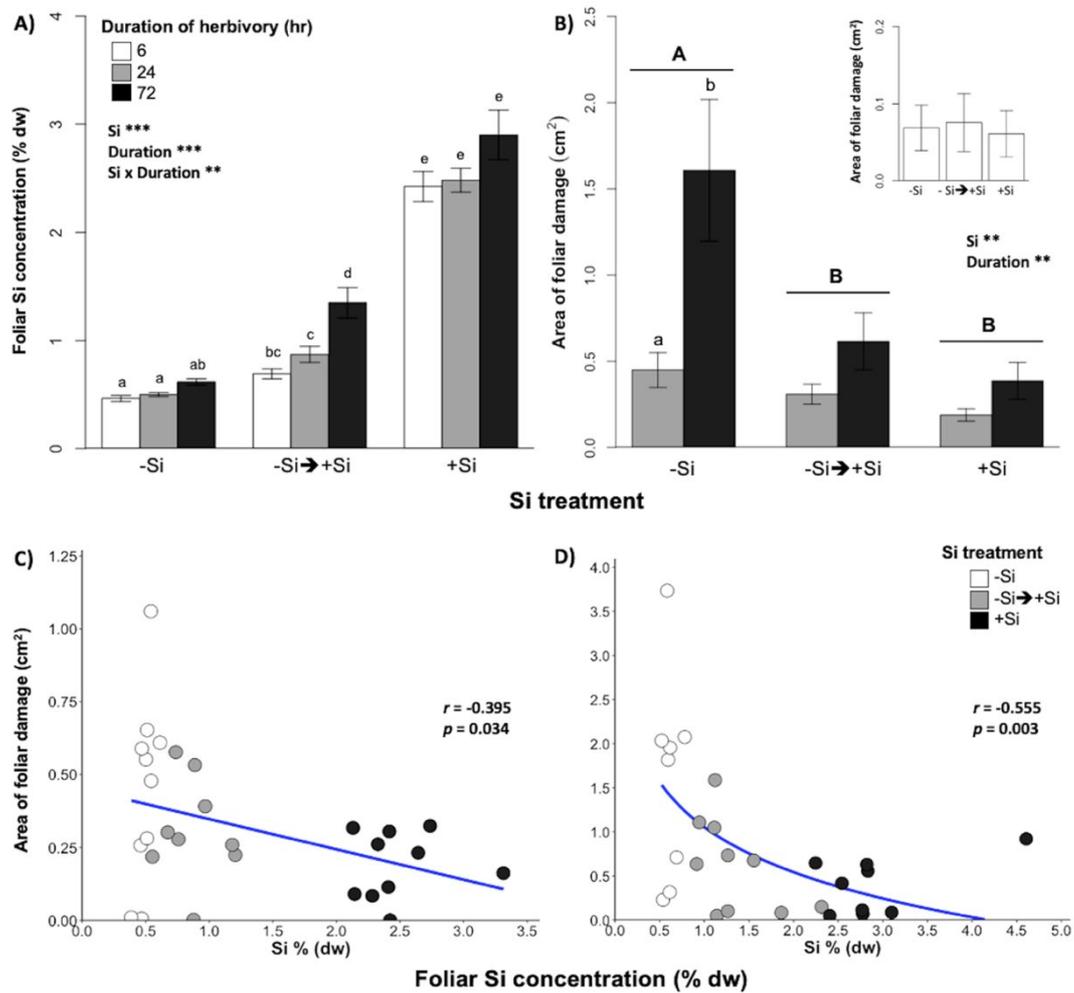
Figure 5-1. Effects of Si treatment on (A) *Brachypodium distachyon* foliar Si concentration and (B) foliar damage after 24 and 72 hr of *Helicoverpa armigera* herbivory. Inset in (B): amount of foliar damage after 6 hr of *H. armigera* herbivory. For (A), letters above bars indicate significant differences across all Si treatments and herbivory durations and for (B), uppercase and lowercase letters indicate significant differences between Si treatments or herbivory durations within a Si treatment, respectively. ** = $p < 0.01$, *** = $p < 0.001$, ANOVA. The linear (C) and logarithmic (D) relationship between foliar damage and Si concentrations (C) 24 and (D) 72 hr after herbivory began. -Si = plants not treated with Si, -Si➔+Si = plants only treated with Si once herbivory began and +Si = plants exposed to Si long term.

Helicoverpa armigera relative growth rate (RGR) was significantly higher when fed on -Si plants compared to both -Si➜+Si and +Si plants, whereas there were no differences between -Si➜+Si and +Si plants (Fig 5-2A; Table 5-1). *Helicoverpa armigera* fed on +Si plants had significantly lower relative consumption (RC) than -Si plants, whereas -Si➜+Si plants did not significantly differ from either -Si or +Si plants (Fig5- 2B; Table 5-1). There were no differences in initial mass of *H. armigera* larvae between treatments across all herbivory durations (Appendix 2: Section S5-2: Fig S5-3).

5.4.2 Helicoverpa armigera *mandible wear*

Overall, -Si➜+Si- and +Si-fed larvae had significantly increased mandible wear compared to -Si-fed larvae (Fig 5-2C and D; Table 5-1). Mandible wear significantly increased over time in -Si➜+Si and +Si but not in -Si plants (Fig 5-2C).

5.4.3 *Rapid silicification in -Si➜+Si plants*

In -Si➜+Si plants, there was a significant effect of Si exposure duration on filled Si cell density, whereby plants had higher filled Si cell density after 24 and 72 hr compared to 6 hr ($F_{2,17} = 22.492$, $p < 0.001$; Fig 5-3A and B). *Helicoverpa armigera* herbivory also significantly increased filled Si cell density compared to herbivore-free plants at 6 hr of herbivory, but not subsequently ($F_{1,17} = 6.412$, $p = 0.021$; Fig 5-3A and B). Additionally, when Si cell density was compared between damaged -Si➜+Si and +Si plants after 72 hr of herbivory, no significant differences were detected ($t_{4.364} = -0.116$, $p = 0.913$).

5.4.4 *Effect of short-term Si exposure on phenolics*

Damaged leaves of +Si plants had significantly lower total phenolic concentrations than those of -Si plants and -Si➜+Si phenolic concentrations were not significantly different from

either -Si or +Si plants (Table 5-1; Appendix 2: Section S2: Fig S5-4A). In herbivore-free plants, after 72 hr, +Si plants had significantly and marginally insignificantly lower phenolic concentrations than -Si➔+Si and -Si plants, respectively (Table 5-1; Appendix 2: Section S2: Fig S5-4B).
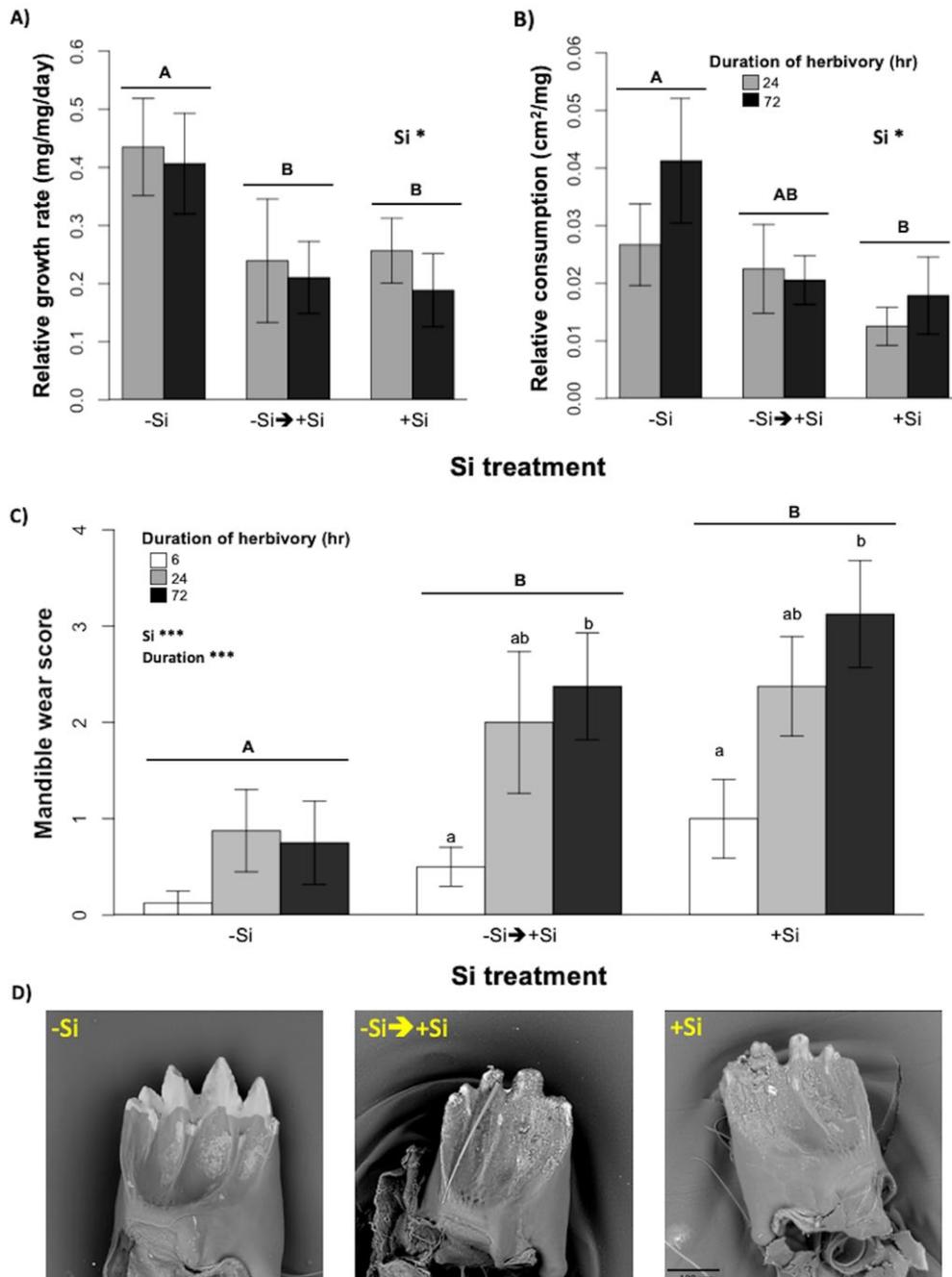
Figure 5-2. Impacts of Si treatment on *Helicoverpa armigera* (A) relative growth rate, (B) relative consumption and (C) mandible wear. Images in (D) are exemplar mandibles from each Si treatment. Uppercase and lowercase letters indicate significant differences between Si treatments or herbivory durations within a Si treatment, respectively. * = $p < 0.05$, *** = $p < 0.001$, ANOVA. -Si = plants not treated with Si, -Si➔+Si = plants only treated with Si once herbivory began and +Si = plants exposed to Si long term.
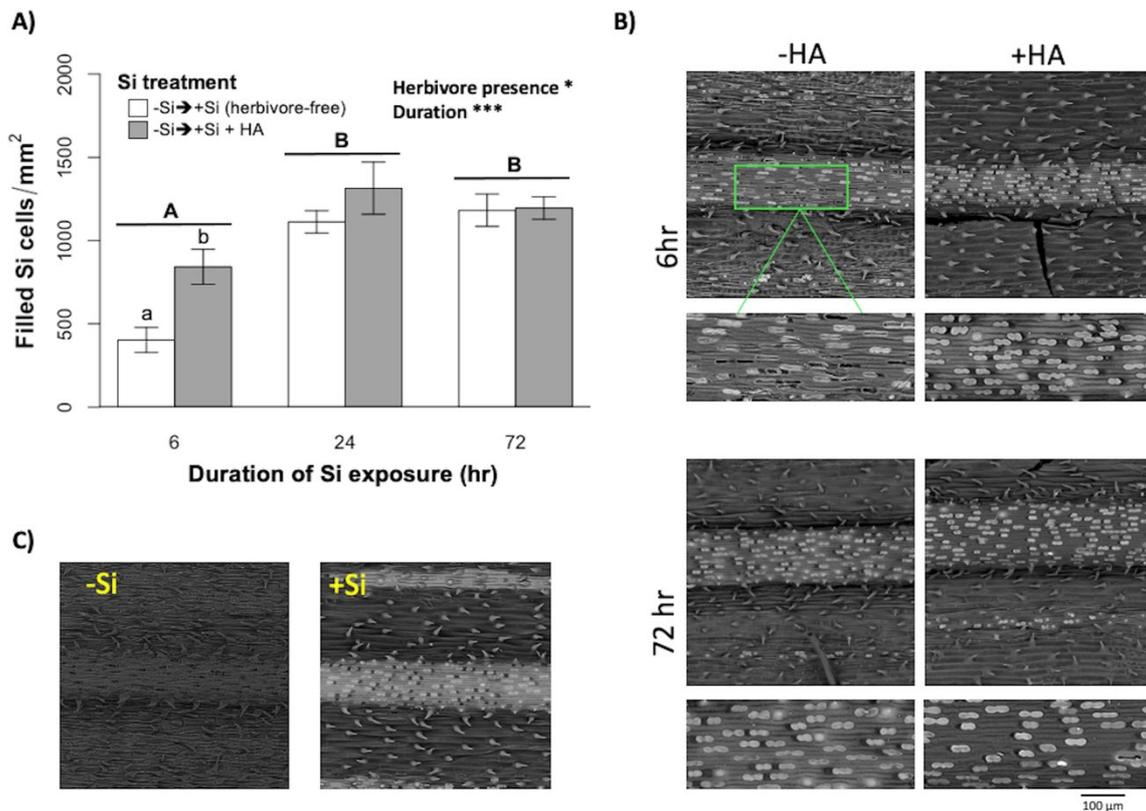
Figure 5-3. Filled Si cell density in (A) herbivore-free and *Helicoverpa armigera* (HA) treated *Brachypodium distachyon* plants only exposed to Si at the start of herbivory (-Si➔+Si and -Si➔+Si + HA, respectively). Scanning electron micrographs of *B. distachyon* abaxial surfaces highlighting (B) the differences in Si deposition across Si exposure periods and (C) herbivore-free plants not treated with Si (-Si) and exposed to Si long term (+Si). For (A), uppercase letters above bars indicate significant differences between Si exposure durations. Lowercase letters indicate significant differences between HA-treated and herbivore-free plants within a Si exposure duration. * = $p < 0.05$, *** = $p < 0.001$, ANOVA. The 100 μm bar beneath (B) is scaled to the lower magnification images (upper images at each Si exposure duration in (B) and both images in (C)).

5.5 Discussion:

5.5.1 Short-term exposure to Si reduces herbivore performance

Here we report that within 72 hr of exposure to Si, plants are equally well defended against herbivory compared to plants exposed to Si over much longer time periods. Despite having lower Si concentrations there were no differences in herbivore feeding, relative growth rate, relative consumption, or mandible wear between +Si and -Si➔+Si treatments. It has been previously reported that Si can reduce *Helicoverpa* spp. performance (Frew et al. 2019, Hall et al. 2020a, Hall et al. 2020b), and that increases in foliar Si accumulation in response to

herbivore signals occur rapidly (Waterman et al. 2021b), but this is the first demonstration of how quickly plants previously unexposed to Si incorporate it into their tissues as an effective anti-herbivore defense. A recent study by Wang et al. (2020) found that *Chilo suppressalis* (Lepidoptera) growth was equally suppressed when feeding on plants supplemented with either 0.5 mM or 2 mM calcium silicate solution compared to non-supplemented plants, despite the fact that plants grown in 2 mM solution had over 1.5 times higher stem Si concentrations. Plants might only require a certain amount of Si accumulation to reach the same level of resistance as would be achieved with much higher concentrations, and here we report that this threshold may be reached much faster than previously envisaged.

5.5.2   *Brief exposure to Si wears down mandibles*

Although the species and measurement techniques used in this study differ, our result that Si can wear down mandibles is consistent with other findings (Massey and Hartley 2009, Mir et al. 2019). Here we show that mandibular wear can occur within 72 hr of herbivory, a similar time frame to Massey & Hartley (2009), where mandible wear occurred within the duration of a single instar of *Spodoptera exempta*. Additionally, we provide new evidence that, within 72 hr of plants being exposed to Si, plant tissues can wear down herbivore mandibles to a similar extent as plants exposed to Si for over 34 days. We saw minor impacts on mandibles in herbivores feeding on -Si plants, not unexpected as feeding on low Si-accumulating plants can cause wear (Raupp 1985, Hodson et al. 2005), however wear was far more severe on mandibles of herbivores exposed to Si, even when fed on plants that only had brief Si exposure. This is despite the fact that herbivores in +Si and -Si➔+Si treatments consume considerably less tissue than those fed on -Si plants. Such rapid impacts of Si may have critical implications for herbivore fitness; as herbivores replace their mandibles after each

molt (Massey and Hartley 2009), wear occurring early in the instar might be particularly problematic for insect growth.

5.5.3 *Short-term Si exposure approaches maximum Si-deposition*

We found that after 6 hr of herbivory, -Si➔+Si plants had higher filled Si cell density than undamaged plants, even though very minimal tissue damage occurred at this stage, suggesting that subtle herbivore stimulation is enough to induce Si cell filling (phytolith formation). Beyond 6 hr, -Si➔+Si plants reached +Si levels of Si cell density independent of herbivory. Si cells are among the first sites for Si deposition (Kaufmian et al. 1969, Kumar et al. 2017a), and it is likely that, considering Si concentrations in -Si➔+Si plants remained lower than +Si plants, longer periods are required to reach maximum Si deposition throughout foliar tissue (Hartley et al. 2015). Interestingly, Si cell densities were nearly identical between +Si and -Si➔+Si plants, though total Si concentrations were markedly different, as found in sugarcane (de Tombeur et al. 2020), where plants grown in low-Si soils had similar Si cell densities compared to plants grown in high-Si soils, despite having much lower total Si concentrations. Because Si cells specifically have been shown to have minimal influence on leaf erectness, whereas total Si concentration has substantial influence (de Tombeur et al. 2021a), it is likely that although Si cells might not impact the plant's physical stability, they do play a critical role in Si-based defense against chewing herbivory.

Though the active filling of Si cells has been documented (Kumar et al. 2017a, Kumar et al. 2017b, Hall et al. 2020a), here we provide new evidence that Si cells become filled within hours of first exposure to Si, and that this process can be accelerated by and provide resistance against herbivory under short-term exposure to Si. Si deposition is also considered

to be irreversible; once Si is incorporated into tissues, it becomes immobilized, and therefore Si defenses can be both rapid and long-lasting (Epstein 1994).

5.5.4 *Short-term Si exposure does not alter carbon-based defenses*

The trade-off between Si and phenolics has been well documented (Cooke and Leishman 2012, Simpson et al. 2017), and here we provide further evidence for this trade-off; overall, in +Si plants, phenolic concentrations were reduced. Interestingly, although short-term exposure to Si has similar negative impacts on herbivores compared to long-term exposure, it does not reduce foliar phenolics. Therefore, unlike +Si plants, it is possible that reduced herbivore performance from -Si➜+Si plants might be enhanced by retention of phenolic defenses.

5.6 Conclusions:

Within 72 hr of exposure to Si, plants reduced feeding and growth, and increased mandibular wear in *Helicoverpa armigera* as effectively as plants with long-term Si exposure. Additionally, we demonstrate *in vivo* that herbivory can induce rapid Si deposition within 6 hr of exposure to Si, and that with only brief exposure to Si, plants have similar filled Si cell densities to plants exposed to Si for much longer periods, which likely plays a critical role in the associated increase in resistance to herbivory. Further, in contrast to long-term Si exposure, the increase in resistance to herbivory associated with short-term exposure does not come at a cost to phenolics. Our findings lend support to the ecological concept that some plants are adapted to use Si as a 'metabolically cheap solution' for defensive roles (Cooke and Leishman 2012), particularly under herbivore attack. The fact that plants can deploy Si defenses so rapidly points to both their effectiveness and the strong selective pressure from herbivory for grasses to utilize Si. Moreover, there is increasing interest in maximizing Si

uptake through breeding and Si fertilizer application to enhance yield, particularly in cereal crops such as maize, rice and wheat (Haynes 2017). However, the rapid accumulation and effectiveness of relatively low amounts of Si highlights its potential utility even when Si supply, and thus ability to reach maximum levels of Si uptake, are limited.

Table 5-1. Summary of ANOVA results

Type of ANOVA	Response Variable	Figure	Si treatment			Duration of herbivory			Si x Duration		
			F	p	df	F	p	df	F	p	df
Two-way	[†]Si %	1A	450.065	**< 0.001**	2,78	26.224	**< 0.001**	2,78	3.699	**0.008**	4,78
	[†]Foliar damage	1B	5.544	**0.007**	2,50	8.278	**0.006**	1,50	1.623	0.207	2,50
	RGR	2A	4.229	**0.021**	2,42	0.430	0.515	1,42	0.042	0.959	2,42
	[‡]RC	2B	4.211	**0.022**	2,42	1.029	0.316	1,42	0.446	0.643	2,42
	Mandible wear	2C	9.228	**< 0.001**	2,27	9.644	**< 0.001**	2,27	0.827	0.519	4,27
	Phenolics (damaged)	S4A	3.937	**0.030**	2,30	1.478	0.234	1,30	2.641	<u>0.088</u>	2,30
One-way	Phenolics (no herb, 72 hr)	S4B	4.353	**0.032**	2,15	-	-	-	-	-	-

Note: Bold values: $p < 0.05$; Underlined values: $p < 0.1$

Abbreviations: RGR, *Helicoverpa armigera* relative growth rate; RC, *H. armigera* relative consumption; no herb, leaves from herbivore-free plants

† = Analysed using log-transformed data

‡ = Analysed using square-root-transformed data

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5.7 Acknowledgements:

6 Chapter 6: Meta-analysis shows that simulated herbivory can imitate short-term plant defences induced by real herbivory

To be submitted to *Nature Plants*

6.1 Abstract

Plant defence responses to herbivory can be challenging to disentangle considering inherent unpredictability and biases introduced by individual herbivores. In addition to variation in behaviour and feeding patterns between different individuals, phenotypic responses are the product of disparate herbivore-associated signals perceived by the plant. Simulated herbivory allows researchers to standardise treatments and separate herbivore signals to determine which responses are associated with which signal(s). Nevertheless, the comparability of plant defence response induction and variability between simulated herbivory and true herbivory remains unquantified. We conducted multi-level meta-analysis of > 1,600 defence response measurements from 110 studies to address this. We found that simulated herbivory can accurately replicate true herbivory in the short-term (< 24 hours), but overall, it begins to underestimate (-34%) defensive responses beyond this. The most accurate forms of simulated herbivory considered multiple herbivore signals and, particularly at later timepoints, are contingent upon the specific type of defence or order of herbivore included.

6.2 Introduction

Plants and herbivores have been engaged in an evolutionary arms race for upwards of 300 million years (Hartley and Jones 1997), over which time plants have evolved a plethora of complex responses to defend themselves (Rosenthal and Berenbaum 1991, Stamp 2003). Considering the importance of plant–herbivore interactions in natural and managed ecosystems, developing an understanding of how plants are best able to defend themselves against herbivores is at the forefront of both ecological and agricultural research (Oerke 2005, Deutsch et al. 2018). Nevertheless, there are complexities associated with herbivory that make plant-herbivore interactions a difficult topic to study. Plants are exposed to many discrete signals during herbivory that can trigger defence responses, including mechanical stimulation, tissue damage, chemical elicitation, transmission of microbes and microenvironment alteration (Waterman et al. 2019). Understanding how these stimuli affect plant defences is confounded by the fact that studies with herbivores introduce uncontrolled bias. This can stem from variation in feeding patterns, intensity of damage, and the introduction of biotic and abiotic signals in a non-standardised way (Mithöfer et al. 2005, Robin et al. 2017, Waterman et al. 2021a). The use of stimulated herbivory, whereby researchers apply herbivore stimuli artificially, can reduce bias introduced by authentic herbivores and identify which specific responses are associated with each stimulus (Schmelz et al. 2006, Waterman et al. 2019, Steinbrenner et al. 2020, Waterman et al. 2021b). Simulated herbivory techniques range from simple mechanical damage (Heil et al. 2012, Dillon et al. 2020) to more sophisticated techniques such as coupling mechanical damage with herbivore saliva or oral secretions (OS), and thus chemical signals (Peiffer and Felton 2009, Machado et al. 2017, Sobhy et al. 2017, De Lange et al. 2020). In rare cases, highly refined methods that aim to replicate the temporal, spatial, and chemical patterns associated with herbivore feeding are used (Mithöfer et al. 2005, Bricchi et al. 2010, Li et al. 2019).

One of the major drawbacks of simulated herbivory is that plant defence responses are considered to be quite sensitive to timing, and their abundance can change rapidly over time (Waterman et al. 2019); certain defences might be induced within seconds or minutes of herbivory (Toyota et al. 2018, Steinbrenner et al. 2020) but others may take hours or even days to reach maximum levels of induction (Erb et al. 2015, Liu et al. 2021, Waterman et al. 2021b). In addition to the intensity of a given response, variation in plant defence responses can contribute to the effectiveness of plant resistance to herbivores (Pearse et al. 2018). Some studies use simulated herbivory as a tool to induce responses and then subsequently feed plant tissue to herbivores as a means of determining the effects of previous 'herbivory' on future herbivores (Xu et al. 2015, Weeraddana and Evenden 2019, Hall et al. 2020b). However, whether induced responses are comparable to live herbivores in terms of intensity and variability of responses, or are just useful for mechanistic decoupling, requires further, systematic, evaluation.

To understand how simulated herbivory-induced responses used compare to responses induced by true herbivory, we conducted multi-level meta-analysis with the objective of providing insights on how well simulated herbivory can replicate responses induced by true herbivory. Further, we investigated if the comparability of simulated and true herbivory is dependent on the timing of measurements, technique, and herbivore taxon used, as well as the type of defence response being measured.

6.3   Methods:

6.3.1   *Literature searching and screening*

We followed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) and PRISMA-EvoEvo reporting guidelines (Moher et al. 2009, O'Dea et al. 2021)

for reporting systematic study selection and data extraction (Extended data Figure 6-1). In brief, in April 2021 we conducted comprehensive searches in the Web of Science (WoS) and MEDLINE databases using the Boolean search string:

'((((artificial* OR mechanical*) AND (wound* OR damage*) AND "plant") AND (herbiv or* OR insect*)) OR ("simulat* herbivor*"))'

After removing duplicates, 3,831 studies were screened. Screening was conducted first on abstracts and then, where applicable, full texts, assuming they met the following pre-defined inclusion criteria: 1) studies measured the same defence response (e.g., volatile organic compound induction) between true herbivory and simulated herbivory, 2) timing of measurements was identical between both simulated and true herbivory, and 3) responses were related to biochemical plant defences (physiological responses such as growth, gas exchange, etc., were excluded). In total, 110 studies were selected for inclusion (Extended data Figure 6-1). When studies utilised mutants or transgenic plants, only wildtypes were included. Further, there were instances where multiple herbivore taxa or simulated herbivory techniques were used, or multiple defence responses were measured in a single study. In those instances, only the techniques/responses appropriate for the herbivore were included in this study. For example, responses to mechanical wounding and application of lepidopteran-specific elicitors would not be compared to live hemipteran herbivore-induced responses, even if both were measured in the same study.

6.3.2   *Data extraction and compilation*

From each study, we extracted the following data: 1) descriptive statistics of biochemical defence responses (mean, standard deviation, sample size), 2) time elapsed after treatments

before responses were measured, 3) technique used to simulate herbivory, 4) herbivore order being simulated, 5) the type of defence response measured and 6) plant species used in experiments. Additionally, to ensure standardisation across studies, when responses were measured across more than three time points, data were extracted from only three representative time points (one early, one middle, and one late). Data were extracted either manually from tables or from figures using the R package 'metaDigitise' (Pick et al. 2019). Data were only extracted and included when all descriptive statistics were extractible from the main text. For all meta-analysis and meta-regression, true herbivory was considered as the control group and simulated herbivory the treatment group unless otherwise stated. True control (i.e., untreated/undamaged plant) data were extracted when possible, however in some instances no true controls were present. For example, studies that utilised gene expression often reported induced expression levels relative to baseline (where baseline was considered to be 0).

In statistical modelling, either duration of treatments, technique used to simulate herbivory, herbivore order, or type of defence response was included as a predictor variable (i.e., moderator). Duration of treatments was delineated into two separate groups: < 24 hr or ≥ 24 hr, as this enabled us to divide the data set into two relatively even sample-sized groups and separate rapidly induced responses from delayed, extended responses. Simulated herbivory technique was divided into six categories based on the type of tool used to simulate herbivory: 1) salicylate application (induces anti-hemipteran responses), 2) mechanical wounding (whereby tissues were either separated from the remainder of the plant or substantially damaged), 3) needle punctures (typically to simulate fluid feeding herbivory, very localised damage), 4) elicitors + mechanical wounding (wounding in addition to chemical signals of herbivory), 5) MecWorm (mechanical robot that enables the temporal and

spatial dynamics of chewing herbivores to be mimicked in a standardised way, see Mithöfer et al. 2005), and 6) jasmonate application (induces a generic herbivore response). Studies included in this meta-analysis contained measurements of responses induced by five herbivore orders (Lepidoptera, Coleoptera, Hemiptera, Hymenoptera and Trombidiformes). Additionally, the type of response measured was broken into six commonly delineated types of defence (enzyme/protein, gene expression, specialised metabolite, phytohormone, early signalling molecule, and volatile organic compound).

6.3.3 Choosing and calculating effect sizes

Our data showed strong correlations between mean responses and standard deviations in plants treated with true herbivory and plants treated with simulated herbivory (Fig S6-1). We therefore used the natural logarithm of response ratio (lnRR) as opposed to the standardised mean difference, as the latter assumes homogeneity of variance (Nakagawa et al. 2015). Additionally, we calculated effect sizes for variation around the mean (natural logarithm of the ratio of the standard deviations; lnVR (Nakagawa et al. 2015)) to measure the variability between simulated and true herbivory, as increased response variability is considered to impact plant resistance to arthropod herbivory (Pearse et al. 2018). The first effect size, lnRR and its sampling variance are defined as follows:

$$lnRR = ln\left(\frac{\bar{x}_S}{\bar{x}_T}\right),$$ (eqn 1)

$$\text{var}(lnRR) = \frac{sd_T^2}{n_T \bar{x}_T^2} + \frac{sd_S^2}{n_S \bar{x}_S^2}.$$ (eqn 2)

where $\bar{x}_S$ and $\bar{x}_T$ are the means of simulated (S) and true herbivory (T), respectively. Further, sd_S and sd_T are the standard deviations from responses to simulated and true herbivory

and n_S and n_T are the sample sizes of simulated and true herbivory. The second effect size measurement, lnVR and its sampling variance are defined as:

$$lnVR = ln\left(\frac{sd_S}{sd_T}\right) + \frac{1}{2(n_S-1)} - \frac{1}{2(n_T-1)}, \qquad \text{(eqn 3)}$$

$$var(lnVR) = \frac{1}{2(n_T-1)} + \frac{1}{2(n_S-1)}. \qquad \text{(eqn 4)}$$

where symbols in terms on the righthand side of the equation are the same as those for the lnRR and var(lnRR) equations. Positive values for both lnRR and lnVR indicate that the response induced by simulated herbivory was greater than true herbivory and negative values indicate that the response induced by true herbivory was greater than simulated herbivory. All effect sizes and sampling variances were calculated as defined in Nakagawa et al. (2015).

Phylogenetic tree and correlation matrix

We created a phylogenetic tree for the plant species used in studies included in this meta-analysis (Fig S6-3) using the R package 'rotl' (Michonneau et al. 2016). We converted this tree to a correlation matrix assuming the Brownian motion mode of evolution using the R package 'ape' (Paradis and Schliep 2018).

6.3.4 Meta-analytic models

This meta-analysis included publications across 55 journals that utilised 56 different plant species. Additionally, some studies measured a multitude of defence responses, compared multiple simulated herbivory treatments, and/or used multiple herbivores. Inclusion of multiple effect sizes from identical or similar publications can invalidate model assumptions of independence (Noble et al. 2017, Bishop and Nakagawa 2021). The use of multi-level

meta-analytic models accounts for this dependence via random effects and sampling variance–covariance matrices using the same principles as linear mixed-effects models (Harrison et al. 2018). Meta-analysis and meta-regression were conducted using the 'rma.mv' function in the R package 'metafor' (Viechtbauer 2010). We fitted variance-covariance matrices that account for sampling variance as well as co-variance in sampling errors among effect sizes within a given publication (Noble et al. 2017). Test statistics and confidence intervals were computed using a t-distribution as it is more conservative than the commonly used z-distribution (IntHout et al. 2014).

To estimate the overall lnRR and lnVR of simulated herbivory, we used a multilevel meta-analytic model with random effects, determined as optimal by comparing the AIC of candidate models. Potential random effects included: 1) phylogenetic relationship between plants (estimated with the correlation matrix based on a phylogenetic tree), 2) plant species identity (non-phylogenetic effect), 3) publication from which each effect size was extracted, 4) individual effect size (unique for each row of data) and 5) the specific response measured within a study (termed 'item'); in other words, if a study used more than one type of true and/or simulated herbivory to measure the same response, each respective row of data would be assigned the same 'item' value. The optimal random-effects structure (based on AIC score) included plant species, publication, individual effect size and item (Table S6-1). Heterogeneity of the meta-analytic models was estimated using the multilevel version of I^2 (Higgins and Thompson 2002).

Additionally, we ran a univariate regression model for each of the following moderators: 1) timing of response measurements, 2) simulated herbivory technique used, 3) herbivore order, and 4) the type of defence response measured. Regression was also conducted on subsets of

data (e.g., only on data from a single level of a moderator). We added each predictor (moderator) as a fixed effect in models to assess how the various levels mediate effect sizes. In some instances, true controls (i.e., undamaged plants) used in publications were different for simulated and true herbivory treatments. Therefore, when possible (i.e., true control responses were reported), we applied overall comparison and meta-regression that separately compared true and simulated herbivory to their respective controls to further highlight trends shown between simulated and true herbivory.

6.3.5   *Removal and analysis of outliers*

We removed 118 observations (7%) from the main meta-analytic models for lnRR and 155 observations (9%) for lnVR due to the magnitude of effect size. Between 91-93% of data had effect sizes between -10 and 10, but the excluded effect sizes were either under -30 or over 30 (i.e., the ratio of 10 to the power of 13-fold increase). This occurred due to the response being highly induced by true herbivory and only marginally induced by simulated herbivory, or *vice versa*. The primary reason of exclusion was for visualization, and overall, the direction of the outliers matched that of the remaining data; meta-analytic models showed very similar trends regardless of whether outliers were excluded (Extended data Figure 6-9). For lnRR, three additional data points were removed due to extreme var(lnRR) values that prevented models from running appropriately. After removal of extreme values, 1672 observations for lnRR and 1638 for lnVR remained. Outliers were also analysed separately using models/model selection methods described above. Of the 118 observations excluded from the main models for lnRR, 116 of them were from measurements of volatile organic compound (VOC) emission.

6.3.6 Publication bias and sensitivity analysis

Publication bias can occur when small and non-significant effects are less likely to be published than larger and more significant effects (Rosenthal 1979). Publication bias can inflate overall meta-analytic estimates, so to control for potential publication bias we conducted three types of analyses. First we examined publication bias with contour-enhanced funnel plots (Peters et al. 2008) of residuals from a multivariate meta-regression model containing each of the three aforementioned moderators: simulated herbivory technique, herbivore taxa and type of defence response induced (Egger et al. 1997, Nakagawa and Santos 2012) (Fig S6-4). Second, we tested deviations caused by funnel asymmetry using a multilevel version of Egger regression tested on both univariate and multivariate meta-regression models (Egger et al. 1997, Moreno et al. 2009)(Fig S6-5). Finally, we conducted regression-based tests for time-lag bias to check whether effect size changed based on publication date using univariate and multivariate meta-regression models (Nakagawa and Santos 2012) (Fig S6-6).

6.3.7 Interpretation of results

We present model point estimates and their 95% confidence intervals (CIs) from all models used in the main text in Tables 6-1 and 6-2. Estimates were considered statistically significant if CIs did not span zero (i.e., $p < 0.05$) (Nakagawa and Cuthill 2007). Figures of model estimates, raw effect-size data, 95% CIs and prediction intervals (Figs 6-1:3 and Extended data Figures 6-2:9) were produced using the R package 'orchRd' (Nakagawa et al. 2021).

6.4 Results

6.4.1 Overall trends

Overall, considering all 1672 observations of lnRR, simulated herbivory induced defence responses to a significantly lesser extent than true herbivory (Fig 6-1a; lnRR: -0.2620, 95% CI: -0.4883 to -0.0356). Although not significant, including all 1638 observations of lnVR, the variation in responses was lower when using simulated herbivory (Fig 6-1b; lnVR: -0.1757, 95% CI: -0.3921 to 0.0406). Additionally, responses to both simulated and true herbivory were significantly higher than respective control (undamaged) plants in terms of both mean (Fig S6-2a; lnRR: 0.7372, 95% CI: 0.5872 to 0.8872 and 0.8860, 95% CI: 0.7117 to 1.0203, respectively) and variation (Fig S6-2b; lnVR: 0.6035, 95% CI: 0.4343 to 0.7727 and 0.5915, 95% CI: 0.4184 to 0.7647, respectively).

Figure 6-1. Overall effect size (a) natural logarithm of response ratio (lnRR) and (b) natural logarithm of the ratio of the standard deviations (lnVR) for simulated herbivory in comparison to true herbivory. Coloured points represent raw data (i.e., individual observations, k), black-outlined coloured circles represent model estimates, thick black lines represent 95% confidence intervals (CIs) and thin black lines represent prediction intervals. Asterisks depict significance (i.e., a 95% CI that does not overlap 0): ** = $p < 0.01$. Figures on the right-hand side depict the data presented in left-hand side figures with effect sizes between -1 and 1.

6.4.2   Temporal effects

Herbivory is continuous and is often challenging to temporally replicate using most simulated

herbivory techniques (Mithöfer et al. 2005), even if they are repeated multiple times over the

course of an experimental period. Additionally, many defence responses, particularly those

induced by chewing herbivores, spike within 24 hr of inflicting stress; however, if stress is

continuous, these responses may be sustained for longer periods (Erb et al. 2015, Xin et al.

2017, Waterman et al. 2021b). Because of this, we investigated how timing of measurement

after treatments impacted effect sizes. We divided data into responses that were induced the

same day plants were treated (within 24 hr) and those that were induced long-term ($\geq$ 24 hr

after treatment), which resulted in a near 50/50 split of the data. Prior to 24 hr, the intensity

(mean) of defence responses was virtually the same between simulated and true herbivory

(Fig 6-2a; lnRR: -0.0255, 95% CI: -0.2846 to 0.2337), however, after 24 hr the mean

response induced by simulated herbivory was significantly lower than true herbivory (Fig 6-

2a; lnRR: -0.4155, 95% CI: -0.6583 to -0.1727). Similarly, within 24 hr the variability of

responses in plants treated by simulated herbivory was comparable to those of plants exposed

to true herbivory (Fig 6-2b; lnVR: - 0.0408, 95% CI: -0.2951 to 0.2134), whereas variability

in responses was significantly lower in simulated herbivory treated plants measured $\geq$ 24 hr

after treatment (Fig 6-2b; lnVR: -0.2678, 95% CI: -0.5023 to -0.0333). In comparison to

undamaged control plants, true herbivory induced mean responses both within and after 24

hr, however the mean was higher after 24 hr (Extended data Figure 6-2a; lnRR: 0.7654, 95%

CI: 0.5180 to 1.0128 compared to lnRR: 1.0569, 95% CI: 0.8277 to 1.2861). In contrast,

although simulated herbivory also induced higher mean responses in comparison to controls,

responses were greater within 24 hr (Extended data Figure 6-2b; lnRR: 0.8003, 95% CI:

0.6185 to 0.9822) compared to after 24 hr (Extended data Figure 6-2b; lnRR: 0.6992, 95% CI: 0.5369 to 0.8615).
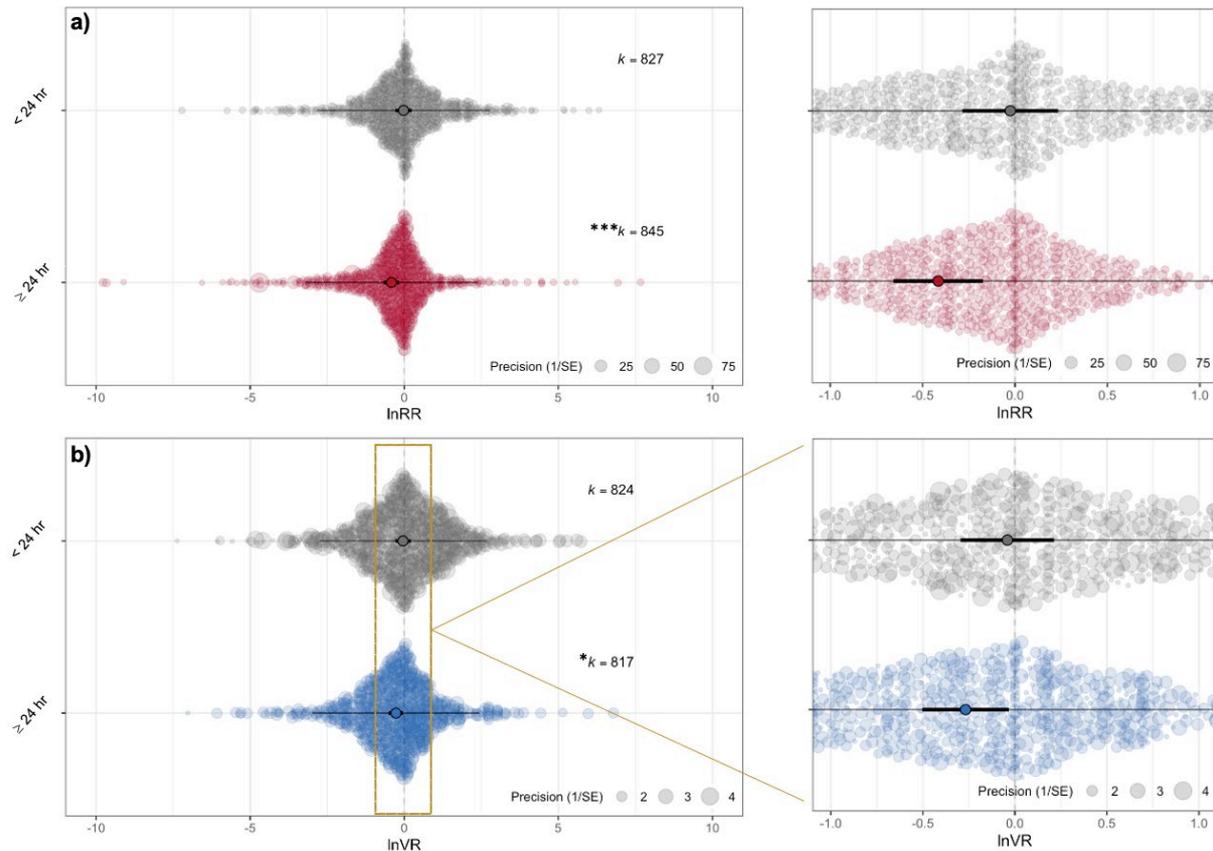
Figure 6-2. Effect size (a) natural logarithm of response ratio (lnRR) and (b) natural logarithm of the ratio of the standard deviations (lnVR) for simulated herbivory in comparison to true herbivory. Grey points represent raw effect-size data (i.e., individual observations, *k*) measured prior to 24 hr after treatments (both simulated and true herbivory) begin and coloured points represent raw data measured ≥ 24 hr after treatments. Black-outlined coloured circles represent model estimates, thick black lines represent 95% confidence intervals (CIs) and thin black lines represent prediction intervals. Asterisks depict significance (i.e., a 95% CI that does not overlap 0): * = $p < 0.05$, *** = $p < 0.001$. Figures on the right-hand side depict the data presented in left-hand side figures with effect sizes between -1 and 1.

6.4.3 Effects of modifiers

<u>Technique</u>

For all simulated herbivory techniques, means of defence responses (lnRR) were lower than true herbivory except for needle piercing (Table 6-1, Fig 6-3a). However, only simple mechanical wounding (tissue removal) was significant. Jasmonate application and mechanical wounding were the two most common forms of simulated herbivory (25% and

34% of all effect sizes, respectively), and interestingly, are among the least sophisticated based on what is known about the complexities associated with real herbivore feeding. For example, simple mechanical damage alone does not expose the plant to any of the numerous foreign chemical signals known to be introduced when herbivores are feeding (Peiffer and Felton 2009, Waterman et al. 2019). Interestingly, needle piercing induced defence responses to a greater extent than respective true herbivory treatments (Table 6-1; Fig 6-3a). Both the application of herbivore-derived elicitors + mechanical wounding as well as the use of MecWorm most accurately mimicked responses to true herbivory, though both treatments had effect sizes that trended towards negative (Table 6-1, Fig 6-3a).
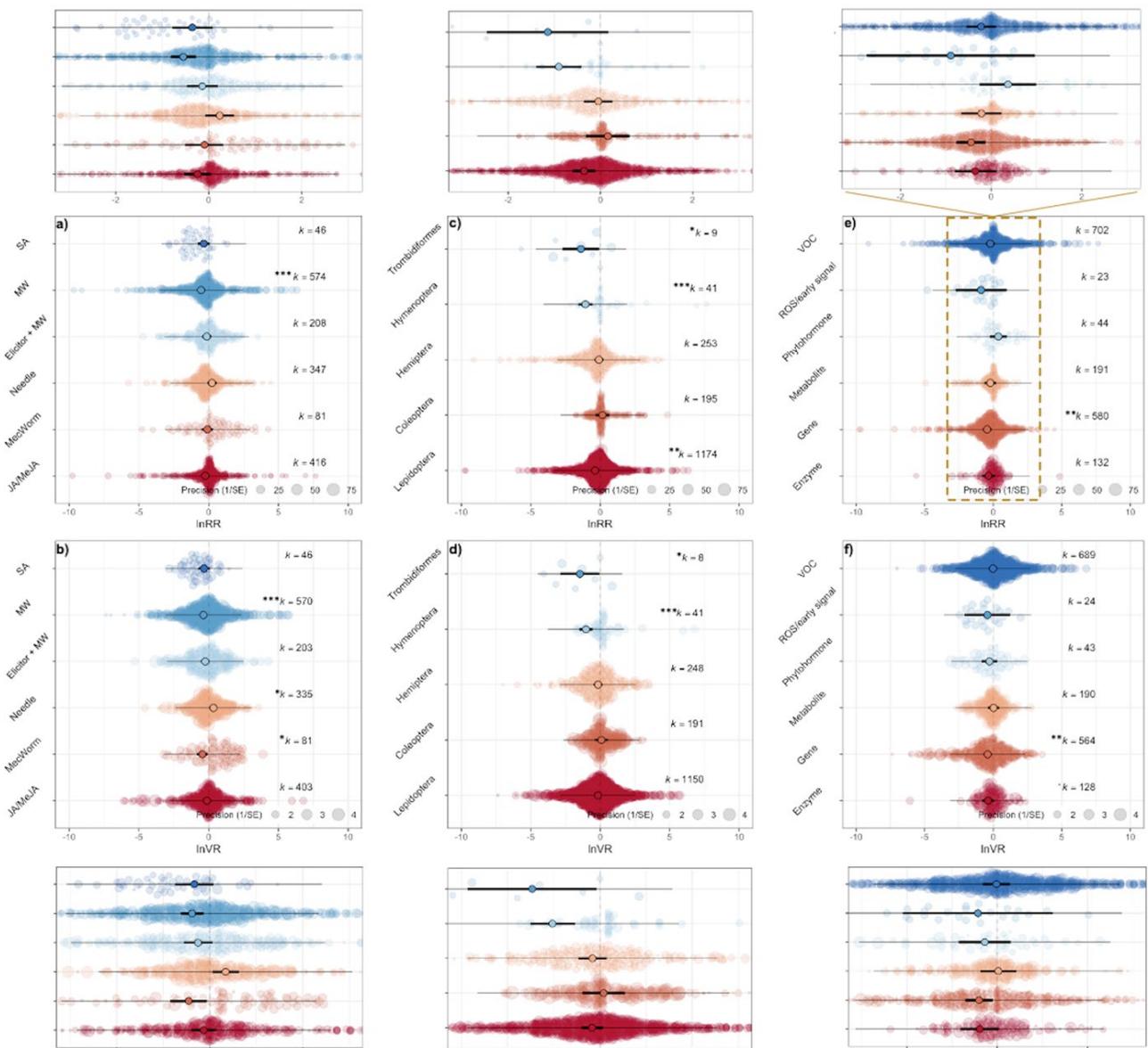
Figure 6-3. Effect sizes of raw data and model outputs from meta-regression with each moderator. Natural logarithm of response ratio (lnRR) and natural logarithm of the ratio of the standard deviations (lnVR) for simulated herbivory in comparison to true herbivory by simulated herbivory technique (a and b), herbivore taxa (c and d) and type of defence response (e and f). Coloured points represent raw effect size (i.e., individual observations, *k*), black-outlined coloured circles represent model estimates, thick black lines represent 95% confidence intervals (CIs) and thin black lines represent prediction intervals. Asterisks depict significance (i.e., a 95% CI that does not overlap 0): . = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Small figures above (lnRR) or below (lnVR) main figures depict the data presented in central figures with effect sizes between -3 and 3. JA/MeJA = jasmonate, MW = mechanical wounding, SA = salicylate, VOC = volatile organic compound.

In contrast, only jasmonate application, elicitors + mechanical wounding, and salicylates accurately replicated variation introduced by real herbivores. MecWorm and simple mechanical wounding had significantly lower variability in defence responses (lnVR) compared to true herbivory, whereas needles induced significantly higher variability (Table 6-1; Fig 6-3b). MecWorm is one of the more sophisticated simulated herbivory techniques as it can mimic the timing of real herbivory while applying identical amounts of damage over a specified amount of time across replicates (Mithöfer et al. 2005, Bricchi et al. 2010). Considering this, the associated reduction in variation is expected given its application for standardising the amount of damage between individual plants and treatments, even though MecWorm can accurately replicate the mean of responses induced by true herbivory.

When mechanical wounding was used as the simulated herbivory technique, studies that used either Lepidoptera or Hemiptera had lower defence responses induced by simulated herbivory than true herbivory (Table S6-2, Extended data Figure 6-3a). Additionally, only simulated hemipteran herbivory had significantly lower variation in comparison to true herbivory, although Lepidoptera, Hymenoptera and Trombidiformes had marginally insignificantly lower variation under simulated herbivory (Table S6-2, Extended data Figure 6-3b). Of all mechanically wounded observations, 77% were from simulated lepidopteran herbivory. Both gene expression and volatile organic compound (VOC) responses were not induced to the same extent by mechanical wounding as they were by true herbivory both in terms of mean

and variation, although this was only significant for mean and marginally insignificant for variation (Table S6-2, Extended data Figure 6-3c-d).

Within 24 hr, only jasmonates and mechanical wounding had lower mean responses (Table 6-2; Fig 6-4a). While tissue removal was also significant within 24 hr, the disparity with true herbivory increased after 24 hr (Table 6-2; Fig 4b). There were minimal differences in variation as well, with only jasmonate application having marginally insignificantly lower variation compared to true herbivory (Table 6-2; Fig 6-4a). After 24 hr, salicylate application, mechanical wounding, MecWorm and jasmonate application induced significantly lower responses compared to true herbivory (Table 6-2; Fig 6-4b). Mechanical wounding, MecWorm and salicylate application (marginally insignificant) also had lower variation.

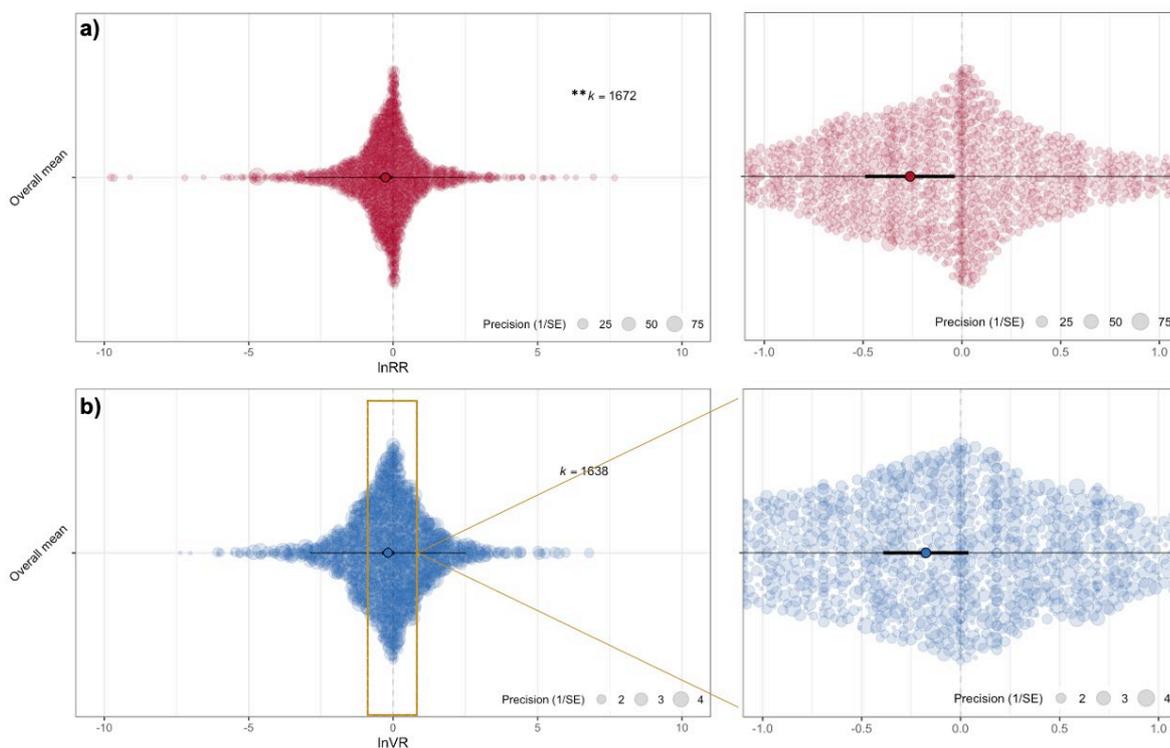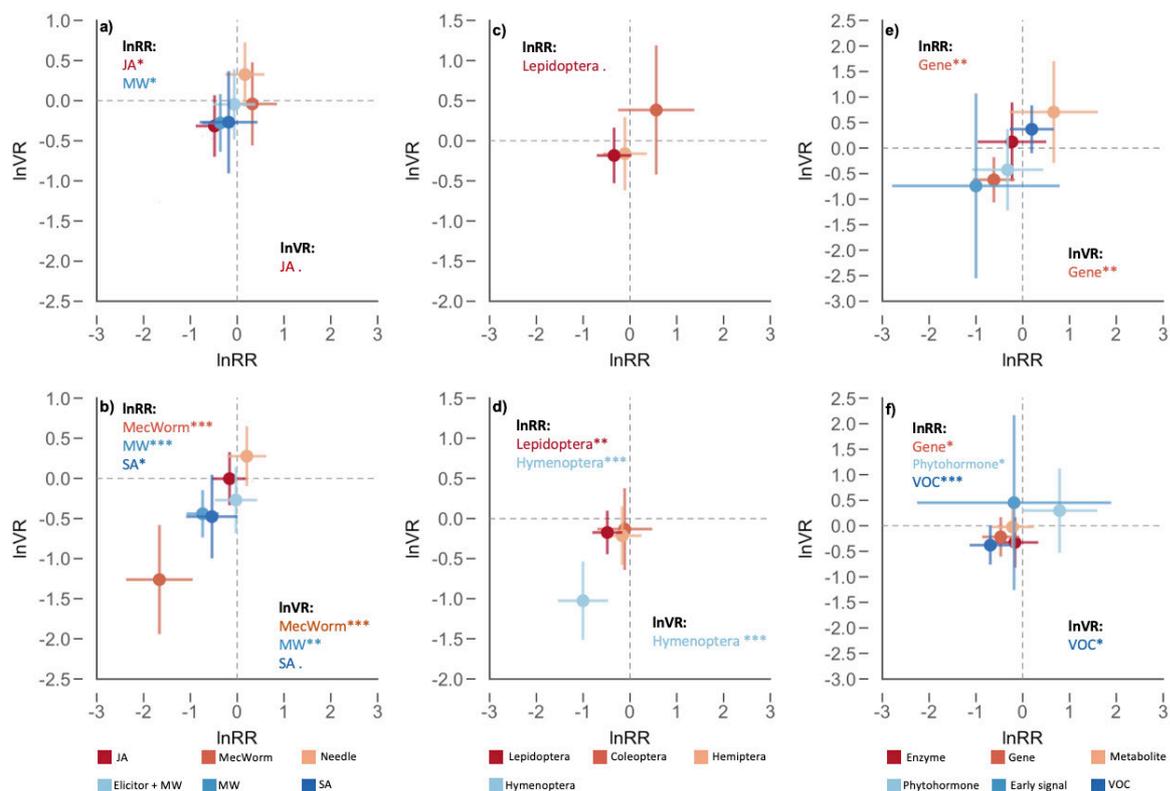**Figure 6-4**. Galaxy plots of model estimates and 95% confidence intervals (CIs) for both natural logarithm of response ratio (lnRR) and natural logarithm of the ratio of the standard deviations (lnVR) from meta-regression with each modifier from responses measured within 24 hr or ≥ 24: simulated herbivory technique (a and b), herbivore taxa (c and d) and type of defence response (e and f). Coloured circles represent and coloured lines represent 95% CIs for lnRR (horizontal) and lnVR (vertical). Asterisks depict significance (i.e., a 95% CI that does not overlap 0) for either lnRR or lnVR: . = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. JA/MeJA = jasmonate, MW = mechanical wounding, SA = salicylate, VOC = volatile organic compound.

Herbivore taxa

The accuracy of simulated herbivory at replicating true herbivory is also dependent on the type of herbivore being simulated, as many herbivore orders have vastly different feeding strategies and behaviours (Novotny et al. 2010). Studies that simulated lepidopteran and hymenopteran feeding induced significantly lower defence responses compared to authentic herbivory (Table 6-1; Fig 6-3c). Interestingly, over 70% of observations (1136 for lnRR and 1116 for lnVR) used in this meta-analysis were from studies that compared simulated and true lepidopteran herbivory. Although Hymenoptera and Trombidiformes were also significant, these orders only accounted for 2.5% and < 1% of total observations, respectively. Hymenoptera and Trombidiformes were the only taxa where simulated herbivory had significantly lower variability in defence responses overall (Table 6-1, Fig 6-3d).

Considering studies using Lepidoptera as the live herbivore order made up 70% of overall data, trends for Lepidoptera were similar to overall trends for both mean and variation (i.e., when all taxa were included; Fig 6-3c-d). Mechanical wounding underestimated true lepidopteran herbivory in terms of both mean response and variation. Further, needle wounding had significantly higher variation than true herbivory (Table S6-3, Extended data Figure 6-4a-b). Additionally, gene expression was significantly lower for simulated lepidopteran herbivory in terms of both mean response and variation (Table S6-3, Extended data Figure 6-4c-d). Overall, true lepidopteran herbivory induced responses to a significantly higher degree than undamaged control plants (Extended data Figure 6-5a).

Jasmonate and salicylate application underestimated responses induced by hemipteran herbivory but were only significant in terms of variation (Table S6-4; Extended data Figure

6-6a-b). Only enzymatic responses were significantly lower for simulated herbivory than true herbivory, but only in terms of mean (Table S6-4; Extended data Figure 6-6c-d). Interestingly, overall, true hemipteran herbivory did not induce responses to a significantly higher degree than controls (Extended data Figure 6-5b).

Within 24 hr, only simulated lepidopteran herbivory had significantly lower mean compared to true herbivory and there were no differences between simulated and true herbivory for any arthropod order in terms of variation (lnVR) (Table 6-2; Fig 6-4c). After 24 hr, both simulated hymenopteran and lepidopteran herbivory had significantly lower mean response and variation compared to true herbivory (Table 6-2; Fig 6-4d). There were no instances of hymenopteran simulated herbivory within 24 hr of treatment. Simulated Trombidiformes herbivory also did not induce defences as much as true herbivory, however results should be interpreted with caution as there were very few observations for this taxon (Table 6-2; k < 10).

Type of defence

Overall, gene expression was the only defence response category significantly underestimated by simulated herbivory, although all other categories except for the phytohormones had negative effect sizes (Table 6-1, Fig 6-3e). Similarly, gene expression was the only response with significantly lower variation in response to simulated herbivory, however enzyme activity induced by simulated herbivory was only marginally insignificantly lower (Table 6-1, Fig 6-3f). This negative response appeared to be primarily driven by mechanical wounding and jasmonate application, both of which underrepresented true herbivory overall (Table S6-5; Fig 6-3a-b; Extended data Figure 6-7a-b). Further, over 60% of gene expression measurements were from observations where either mechanical wounding

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or jasmonate application was used as the simulated herbivory technique. Additionally, 67% of gene expression measurements were from observations using lepidopteran herbivory, and only studies from Lepidoptera resulted in significant differences in mean or variability of responses between simulated and true herbivory (Table S6-5; Extended data Figure 6-7c-d).

Although 42% of observations were from measurements of VOCs, there were some differences in comparison to overall trends. Specifically, for VOC measurements, jasmonate application induced significantly higher responses than true herbivory both in terms of mean response and variation (Table S6-6; Extended data Figure 6-8a-b). Like overall trends, mechanical wounding induced a lesser VOC response than true herbivory in terms of mean response, however needle, elicitors + mechanical wounding, and mechanical wounding also induced responses to a lesser degree than true herbivory, albiet these were marginally insignificant (Table S6-6; Extended data Figure 6-8a-b). Additionally, simulated hemipteran herbivory induced VOC responses to a lesser extent than true herbivory (significant for lnVR marginally insignificant for lnRR) (Table S6-6; Extended data Figure 8c-d).

Within 24 hr, only gene expression had significantly lower means in simulated herbivory compared to true herbivory (Table 6-2; Fig 6-4e). Simulated herbivory also had lower variation in gene expression responses at this time frame (Table 6-2; Fig 4e). After 24 hr, both gene expression and VOCs had lower mean response and variation compared to true herbivory (Table 6-2; Fig 6-4f). After 24 hr enzyme activity had marginally insignificantly lower variability compared to true herbivory (Table 6-2; Fig 6-4f).

6.4.4 Outliers

Without removal of outliers the trends in the data were nearly identical, albeit absolute values were different (Extended data Figure 6-9a; lnRR: -1.2528, 95% CI: -2.1642 to -0.3414). Outliers were removed primarily for visualization and the direction of the outliers themselves also matched the remaining data (Extended data Figure 6-9b; lnRR: -17.3754, 95% CI: -33.1425 to -1.6083).

6.4.5 Publication bias

Although funnel plot asymmetry was not very apparent visually (Fig S6-4), estimates for both univariate and multivariate Egger regression were significant, suggesting that there is in fact significant asymmetry in the funnel plot, indicating the possibility of publication bias (Fig S6-5a-b), however there are many other factors that could also result in asymmetry (Egger et al. 1997). Asymmetry can also be due to true heterogeneity in data as opposed to publication bias (Sterne et al. 2011). Stanley and Doucouliagos (2012) have suggested that when an Egger regression test finds significant funnel asymmetry, one can fit sampling variance (rather than sampling standard error, as is done in Egger regression). In this model, the intercept (i.e., when sampling variance = 0) is an unbiased estimate of meta-analytic mean. This analysis suggests that, even if asymmetry is due to publication bias, main model estimates are virtually unaffected because the intercept value of this model (lnRR = -0.28) is almost identical to the original meta-analytic model (lnRR = -0.26). Additionally, there was no effect of year of publication on effect sizes in either univariate or multivariate models (Fig S6-6).

6.5 Discussion

6.5.1 General trends in simulated herbivory

Overall, simulated herbivory induced lower responses compared to herbivores, likely because simulated herbivory often does not contain all stimuli that would be present under a real herbivory scenario (Waterman et al. 2019). Nevertheless, the range in effect sizes were quite large (-46.6207 to 47.5183, prior to removal of extreme outliers), highlighting that there are inconsistencies across the literature, perhaps in how treatments are implemented. For example, when plants are treated with needle pricks, the diameter of needles and the number of needle pricks can vary substantially, from a few to multiple hundred, which also coincides with variation in the number of live herbivores plants are exposed to (Mozoruk et al. 2006, Zhou et al. 2014). In other words, there are no standard practices for the precise amount of damage being done and number of herbivores used, which generates a lack of comparability between studies; although these decisions are often study-specific based on estimations of the extent of damage herbivores are predicted to do (Chung et al. 2013). Additionally, reduced variation is often a primary rationale for use of simulated herbivory when the objective of the experimental procedure is to elucidate the specific mechanism of a defence response (Tiffin and Inouye 2000, Reese et al. 2016). However, as evidenced by Pearse et al. (2018), variation in defence responses can have substantial impacts on herbivore performance. Therefore, the likelihood of reduced variation in responses to simulated herbivory should be considered when designing ecological studies in which plants previously damaged by simulated herbivory are later exposed to herbivores.

It is likely that simulated herbivory is more accurate at earlier timepoints because it is often implemented in systematic, non-continuous intervals (e.g., at the start, middle or end of experimental period), whereas insects typically feed continuously and have sporadic feeding

patterns (Mithöfer et al. 2005, Waterman et al. 2019). Even when simulated herbivory treatments are replicated multiple times throughout experiments, the responses induced do not always reflect those induced by true herbivory, perhaps due to the inability to replicate temporal patterns of herbivore feeding (Singh et al. 2008, Rodriguez-Saona et al. 2013). In longer-term experiments (i.e., ≥ 24 hr) herbivore feeding patterns are likely to be more complex (i.e., have more potential for feeding variation) and are therefore more difficult to emulate with most simulated herbivory techniques. Regarding variation (lnVR), when responses to simulated herbivory are measured ≥ 24 hr after damage, responses are less intense (i.e., may only be marginally induced relative to control plants), therefore the lower variation may be a consequence of absolute responses being lower, especially considering we observed a strong correlation between mean and standard deviation. Although temporal considerations are critical, they are not the only contributing factor to differences between simulated and true herbivory; when using MecWorm, a robot designed to accurately replicate the spatial and temporal patterns of herbivory, responses are not always induced to the same degree as herbivores, likely due to the lack of additional patterns involved in herbivory such as foreign chemical signals (Mithöfer et al. 2005, Bricchi et al. 2010, Li et al. 2019).

6.5.2   Technique, herbivore taxa and type of defence matter in the context of time
Often, multiple herbivore-associated signals are required to induce effective plant defence responses (Erb and Reymond 2019, Waterman et al. 2019), and therefore simulated herbivory is most accurate when more than one signal is considered. For example, simple mechanical wounding only introduces plants to wound signals, which are not exclusively associated with herbivory (Gardiner et al. 2016). Further supporting this is the fact that the simulated herbivory treatments that included complex stimuli patterns (MecWorm and elicitor + mechanical wounding) most accurately represented the mean responses induced by true

herbivory (Fig 6-5). However, MecWorm, while accurately representing mean responses, induced lower variation in responses relative to true herbivory, presumably because it allows for identical timing and spatial patterns in damage across individual replicates and treatments, which is something not afforded by live herbivores (Mithöfer et al. 2005).

Considering the reduction in accuracy in terms of mean and variation from techniques such as mechanical wounding and MecWorm (wound signals only) after 24 hr, the importance of herbivore-specific chemical signals may become increasingly critical over time. Plants have a limited number of resources at their disposal and often have to 'choose' between diverting said resources to either defence compounds or growth and reproduction (Züst and Agrawal 2017). It is possible that plants deploy generic wound responses to plant-derived signals in the short term, regardless of herbivore-specific signals (Heil et al. 2012). However, once they determine they are not under herbivore attack due to lack of herbivore-specific signals (as is the case with mechanical wounding and MecWorm), they might reduce defences to conserve resources. Further support for this hypothesis is provided by the fact that when chemical signals are present (i.e., elicitors + mechanical wounding), responses are very similar to those induced by true herbivory, regardless of timing. Additionally, SpitWorm, a version of MecWorm that allows for the introduction of herbivore oral secretions, induced VOC emission and JA responsive gene expression more similarly to lepidopteran herbivory than MecWorm, albeit treatments lasted less than 24 hr (Li et al. 2019).
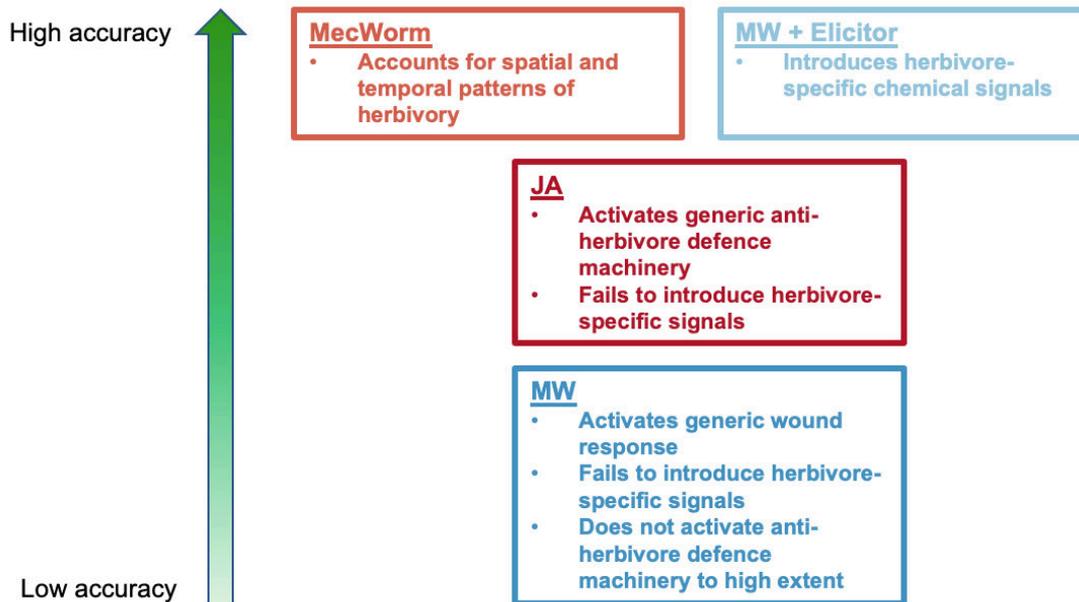
Figure 6-5. The accuracy of commonly used simulated herbivory techniques at replicating responses induced by herbivores. Higher accuracy techniques are those that incorporate herbivore specific signals whether spatial, temporal, or chemical. Lower accuracy techniques fail to relay herbivore-specific signals to plants. JA = jasmonate, MW = mechanical wounding.

Interestingly, needle damage was the only technique to induce defence responses to a greater degree than true herbivory. We hypothesized that this might be because needle damage was a common way of replicating hemipteran herbivory, as needles are often substantially larger (i.e., wider) than hemipteran mouth parts and are likely to induce more physical damage to tissues than typical of live herbivores (Mozoruk et al. 2006, Guerrieri and Digilio 2008). However, needle damage tended to overestimate lepidopteran herbivory more so than hemipteran herbivory, particularly in terms of response variation. The fact that simulated herbivory is (generally) accurately replicating hemipteran herbivory is perplexing given the clear dissimilarities between damaged tissue techniques and hemipteran feeding. The likely explanation for this is that, overall, responses induced by true hemipteran herbivory did not significantly differ from those in undamaged controls. Therefore, it is possible that the specific responses measured in studies simulating hemipteran herbivory were less sensitive to treatments (both true and simulated). This is clearly not the case for taxa such as Lepidoptera,

which could explain why the differences between simulated and true lepidopteran herbivory are, generally, more apparent.

Unlike Hemiptera, that insert very narrow mouthparts (Mozoruk et al. 2006, Brożek et al. 2015) in between cells to reach the phloem and/or xylem and cause minimal cell damage (Züst and Agrawal 2016), Lepidoptera consume large areas of tissue by removing it from the rest of the plant, making it easy to quantify the extent of tissue damage (Getman-Pickering et al. 2020). Nevertheless, many attributes of lepidopteran herbivory known to impact plant defences are often ignored. For example, insect movement around tissues is known to induce a response as are the sounds associated with lepidopteran chewing (Appel and Cocroft 2014, Cazzonelli et al. 2014, Kollasch et al. 2020). Additionally, it has been shown that within 10 min of feeding, caterpillars can regurgitate several nanolitres of OS (Peiffer and Felton 2009). When herbivores feed for longer periods, in addition to the amount of damage increasing over time, the absolute amount of chemical signals (OS) the plant is exposed to also increases; as time goes on it becomes harder to quantify the amount of OS deposited onto plant tissues as herbivores often 'eat their evidence' by re-consuming regurgitated material (Peiffer and Felton 2005, 2009); despite tissues being consumed, plants might still mount a response to more than just the OS present on wounds at the end of an herbivore feeding bout (Schittko et al. 2000). For this reason, herbivory may become more distinguishable from simulated herbivory as time goes on.

Gene expression was the only type of defence response to be, overall (independent of timing), induced to a lesser extent by simulated than true herbivory in terms of both mean and variability. As with overall trends, this was driven primarily by mechanical wounding, but also seemed to be driven by jasmonate application. Gene expression, in contrast to overall

trends, was more accurately induced by simulated herbivory ≥ 24 hr after damage. It is known that there are often substantial temporal differences between gene expression and subsequent production of metabolites (Gulati et al. 2013), so it is possible that, in contrast to downstream defences, gene expression is induced shortly after treatments begin but diminishes with longer periods of herbivore signals. VOCs were induced to a much lesser extent by mechanical wounding compared to true herbivory, however in contrast to gene expression, jasmonate application induced mean and variability of VOCs to a greater extent than true herbivory. Depending on the response measured, jasmonate concentration may have a substantial impact on the degree to which responses are induced. Nevertheless, there are instances where this isn't the case; for example, Kautz et al. (2014) showed that 0.001 and 1 mM jasmonate application induce β-glucosidase activity (enzymatic) and biosynthesis of cyanogenic compounds (metabolic) to the same degree, and both induce these defences to a greater degree than observed in untreated plants.

Together, gene expression and VOC responses made up 78% of total observations. Interestingly, the overall similarity in VOC responses induced by simulated and true herbivory is a result of the fact that jasmonate application induced VOCs to a greater degree than true herbivory, considering all other simulated herbivory techniques had negative effect sizes. Further, VOCs showed strong contrast between early and late timepoints, where lnRR and lnVR both trended towards positive values within 24 hr and were both significantly negative after 24 hr. This makes sense as the timing of VOC production is known to be sensitive, and the specific blend and composition can change on minute timescales (Erb et al. 2015). The continuous nature of herbivory makes it a challenge to spatially and temporally mimic VOC emission over longer timescales, perhaps explaining why MecWorm (very

temporally controlled) was the most accurate at inducing VOC responses in comparison to true herbivory (Mithöfer et al. 2005).

6.5.3   Biases, caveats, and conclusions

Relatively few observations of simulated herbivory were found for Hymenoptera, Trombidiformes and Coleoptera; therefore, measurements from these taxa should be considered with caution as their use associated with simulated herbivory is much less prevalent in the literature. For example, all observations for both Hymenoptera and Trombidiformes each came from only three studies each, and Coleoptera came from 10 studies, whereas 83 studies used Lepidoptera and 26 used Hemiptera. Most of the observations of unsophisticated techniques such as mechanical wounding were from simulated lepidopteran herbivory, which might explain why Lepidoptera appeared to be harder to replicate than other insect orders. The discrete objectives of the use of simulated herbivory are also critical to consider. For example, although mechanical wounding clearly tends to underestimate responses induced true herbivory, many studies implement mechanical wounding to highlight the importance of considering factors such as chemical signals or the spatial and temporal aspects of herbivory, as opposed to replicate the responses induced by authentic herbivory (Turlings et al. 1993, Engelberth et al. 2012, Sobhy et al. 2017, Waterman et al. 2020). Additionally, we found extensive variation in individual effect sizes across the literature. Although overall trends suggest simulated herbivory underestimates true herbivory, there are still many individual instances in which simulated herbivory induced a higher response than true herbivory; for lnRR, 736 (out of 1793) observations were positive (i.e., responses were more greatly induced by simulated herbivory). However, overall, we highlight that simulated herbivory techniques can be utilised to replicate defence responses induced by true herbivory and minimise variability between treatments. Generally, simulated

herbivory techniques that account for multiple herbivore-associated stimuli are most accurate, and the lack of comparability between simulated and true herbivory is largely driven by unsophisticated techniques such as mechanical wounding. Finally, we show that careful consideration of the sensitivity and temporal trends of defence responses is essential to ensure the effective implementation of simulated herbivory.

Table 6-1. Meta-regression model outputs for all effect-sizes. Lower 95% confidence interval = L CI, Upper 95% confidence interval = U CI

		ln RR				*ln VR*			
Modifier	*Level*	*Estimate*	*p-value*	*L CI*	*U CI*	*Estimate*	*p-value*	*L CI*	*U CI*
Technique	JA	-0.2414	0.1042	-0.5326	0.0498	-0.1293	0.3406	-0.3954	0.1367
	MecWorm	-0.0944	0.6505	-0.5031	0.3143	**-0.4549**	**0.0225**	**-0.8455**	**-0.0642**
	Needle	0.2353	0.1395	-0.0769	0.5476	**0.3401**	**0.0204**	**0.0526**	**0.6277**
	Elicitor + MW	-0.1401	0.4110	-0.4741	0.1940	-0.2511	0.1083	-0.5576	0.0554
	MW	**-0.5480**	**< 0.0001**	**-0.8192**	**-0.2767**	**-0.3792**	**0.0023**	**-0.6230**	**-0.1353**
	SA	-0.3538	0.1135	-0.7921	0.0845	-0.3314	0.1177	-0.7466	0.0839
Taxa	Lepidoptera	**-0.3742**	**0.0054**	**-0.6375**	**-0.1110**	-0.1708	0.1561	-0.4069	0.0653
	Coleoptera	0.1634	0.5174	-0.3314	0.6581	0.0802	0.7331	-0.3808	0.5411
	Hemiptera	-0.0949	0.5679	-0.4206	0.2308	-0.1624	0.2930	-0.4652	0.1404
	Hymenoptera	**-1.0690**	**< 0.0001**	**-1.5787**	**-0.5593**	**-1.0312**	**< 0.0001**	**-1.5132**	**-0.5491**
	Trombidiformes	**-1.3933**	**0.0390**	**-2.7165**	**-0.0701**	**-1.4690**	**0.0400**	**-2.8707**	**-0.0673**
Defence	Enzyme	-0.3517	0.1283	-0.8050	0.1017	<u>-0.3849</u>	<u>0.0752</u>	<u>-0.8090</u>	<u>0.0392</u>
	Gene	**-0.4448**	**0.0066**	**-0.7654**	**-0.1243**	**-0.4033**	**0.0092**	**-0.7066**	**-0.1000**
	Metabolite	-0.2142	0.3350	-0.6498	0.2214	0.0249	0.9028	-0.3747	0.4244
	Phytohormone	0.3715	0.2443	-0.2541	0.9971	-0.2761	0.3482	-0.8530	0.3009
	Early signal	-0.8891	0.3486	-2.7490	0.9708	-0.4261	0.6137	-2.0816	1.2293
	VOC	-0.2206	0.1773	-0.5411	0.1000	-0.0172	0.9098	-0.3152	0.2808

Table 6-2. Meta-regression model outputs for effect-sizes for each time point delineation. Lower 95% confidence interval = L CI, Upper 95% confidence interval = U CI, Nd = no data

Time	Modifier	Level	ln RR				ln VR			
			Estimate	p-value	L CI	U CI	Estimate	p-value	L CI	U CI
		JA	**-0.4850**	**0.0107**	**-0.8570**	**-0.1131**	<u>-0.3158</u>	<u>0.0906</u>	<u>-0.6817</u>	<u>0.0501</u>
		MecWorm	0.3233	0.2025	-0.1742	0.8207	-0.0408	0.8732	-0.5425	0.4609
	Technique	Needle	0.1657	0.4001	-0.2206	0.5520	<u>0.3264</u>	<u>0.0948</u>	<u>-0.0567</u>	<u>0.7094</u>
		Elicitor + MW	-0.0634	0.7685	-0.4861	0.3593	-0.0460	0.8302	-0.4668	0.3748
		MW	**-0.3593**	**0.0458**	**-0.7119**	**-0.0067**	-0.2776	0.1114	-0.6195	0.0643
		SA	-0.1829	0.5414	-0.7704	0.4046	-0.2685	0.3953	-0.8884	0.3513
		Lepidoptera	<u>-0.3398</u>	<u>0.0506</u>	<u>-0.6805</u>	<u>0.0008</u>	-0.1829	0.2763	-0.5124	0.1467
		Coleoptera	0.5609	0.1610	-0.2238	1.3456	0.3836	0.3395	-0.4042	1.1713
< 24 hr	Taxa	Hemiptera	-0.1081	0.6273	-0.5450	0.3288	-0.1607	0.4739	-0.6007	0.2794
		Hymenoptera	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
		Trombidiformes	-0.0626	0.9608	-2.5637	2.4384	-0.2324	0.8494	-2.6349	2.1700
		Enzyme	-0.2303	0.5204	-0.9333	0.4727	0.1226	0.7464	-0.6213	0.8665
		Gene	**-0.6179**	**0.0037**	**-1.0344**	**-0.2013**	**-0.6208**	**0.0036**	**-1.0381**	**-0.2035**
	Defence	Metabolite	-0.6615	0.1562	-0.2534	1.5763	0.7055	0.1534	-0.2635	1.6745
		Phytohormone	-3239	0.3838	-1.0535	0.4057	-0.4246	0.2795	-1.1948	0.3455
		Early signal	-0.9999	0.2654	-2.7606	0.7609	-0.7411	0.4148	-2.5241	1.0420
		VOC	0.1921	0.3922	-0.2483	0.6325	0.3697	0.1014	-0.0728	0.8122
		JA	-0.1634	0.3570	-0.5114	0.1846	-0.0041	0.9796	-0.3186	0.3104
		MecWorm	**-1.6630**	**< 0.0001**	**-2.3461**	**-0.9799**	**-1.2619**	**0.0002**	**-1.9249**	**-0.5989**
	Technique	Needle	0.2042	0.2995	-0.1818	0.5902	0.2766	0.1262	-0.0781	0.6313
		Elicitor + MW	-0.0274	0.8988	-0.4498	0.3950	-0.2699	0.1792	-0.6640	0.1242
		MW	**-0.7381**	**< 0.0001**	**-1.0542**	**-0.4220**	**-0.4415**	**0.0018**	**-0.7182**	**-0.1648**
		SA	**-0.5398**	**0.0412**	**-1.0580**	**-0.0215**	<u>-0.4765</u>	<u>0.0637</u>	<u>-0.9802</u>	<u>-0.0272</u>
		Lepidoptera	**-0.4842**	**0.0011**	**-0.7746**	**-0.1937**	-0.1744	0.1780	-0.4284	0.0796
		Coleoptera	-0.1121	0.6929	-0.6693	0.4450	-0.1321	0.5985	-0.6242	0.3601

≥ 24 hr	Taxa	Hemiptera	-0.1653	0.3939	-0.5456	0.2150	-0.2144	0.2262	-0.5620	0.1331
		Hymenoptera	**-1.0037**	**0.0001**	**1.5120**	**-0.4954**	**-1.0251**	**< 0.0001**	**-1.4966**	**-0.5535**
		Trombidiformes	**-1.6519**	**0.0277**	**-3.1216**	**-0.1821**	**-2.0234**	**0.0057**	**-3.4573**	**-0.5895**
		Enzyme	-0.1667	0.4841	-0.6342	0.3007	-0.3264	0.1699	-0.7927	0.1400
		Gene	**-0.4708**	**0.0121**	**-0.8382**	**-0.1034**	-0.2174	0.2369	-0.5778	0.1431
	Defence	Metabolite	-0.2180	0.3109	-0.6400	0.2040	-0.0232	0.9073	-0.4138	0.3674
		Phytohormone	**0.7868**	**0.0473**	**0.0095**	**1.5640**	0.2971	0.4648	-0.5004	1.0946
		Early signal	-0.1861	0.8581	-2.2295	1.8572	0.4527	0.5980	-1.2320	2.1375
		VOC	**-0.6934**	**0.0010**	**-1.1039**	**-0.2830**	**-0.3793**	**0.0351**	**-0.7321**	**-0.0265**

6.6 Extended data Figures
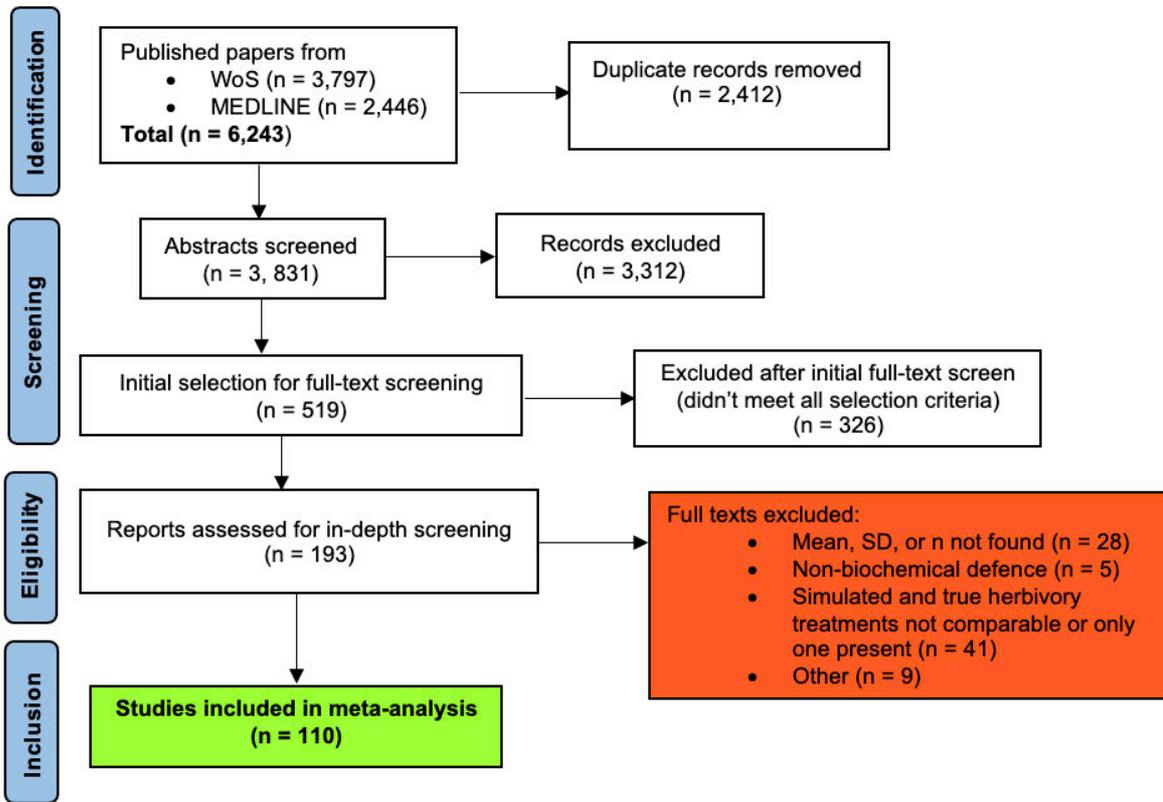
Extended data Figure 6-1. PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses) flowchart detailing the data-collection process. *n* = the number of studies remaining after each stage.
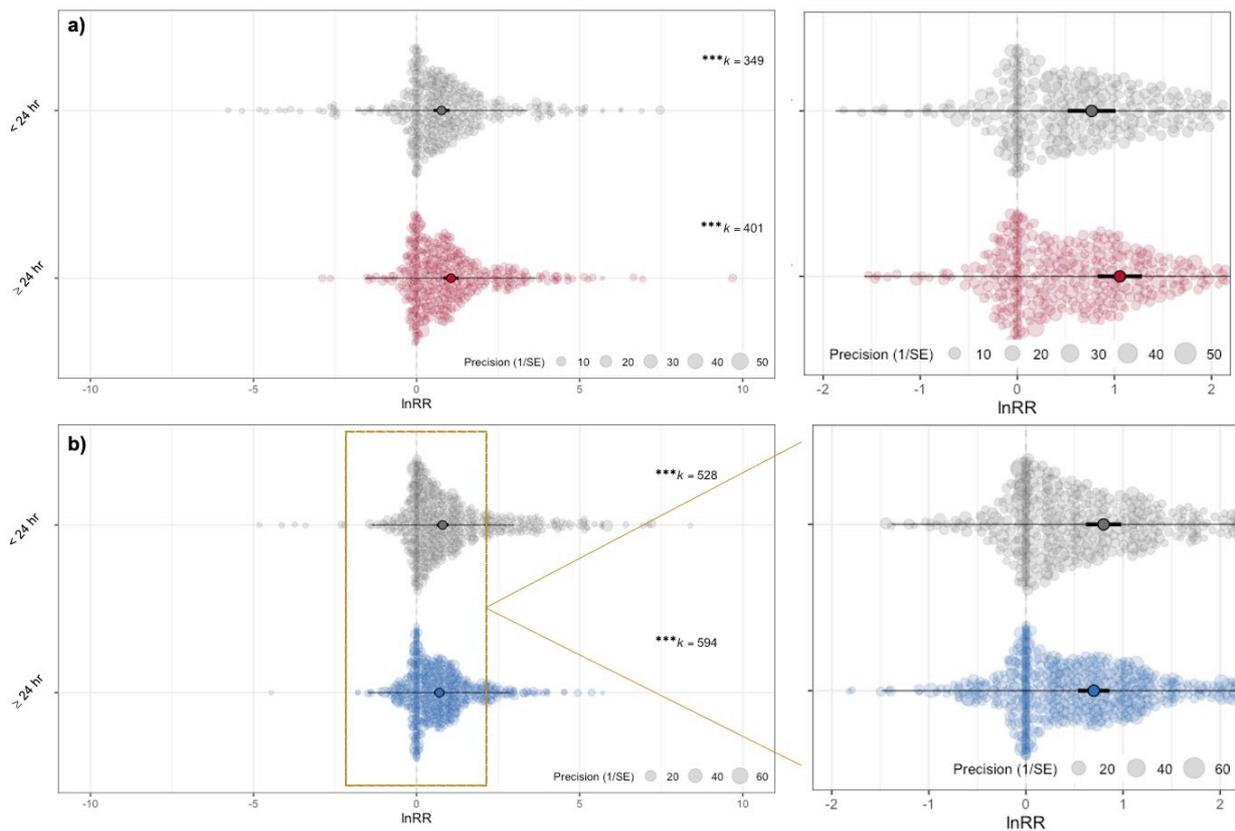
Extended data Figure 6-2. Natural logarithm of response ratio (lnRR) (a) for true herbivory and (b) simulated herbivory in comparison to control (untreated) plants. Grey points represent raw effect data (i.e., individual observations, *k*) measured prior to 24 hr after treatments (both simulated and true herbivory) begin and coloured points represent raw effect-size data measured ≥ 24 hr after treatments. Black-outlined coloured circles represent model estimates, thick black lines represent 95% confidence intervals (CIs) and thin black lines represent prediction intervals. Asterisks depict significance (i.e., a 95% CI that does not overlap 0): *** = *p* < 0.001. Figures on the right-hand side depict the data presented in left-hand side images with effect sizes between -2 and 2.
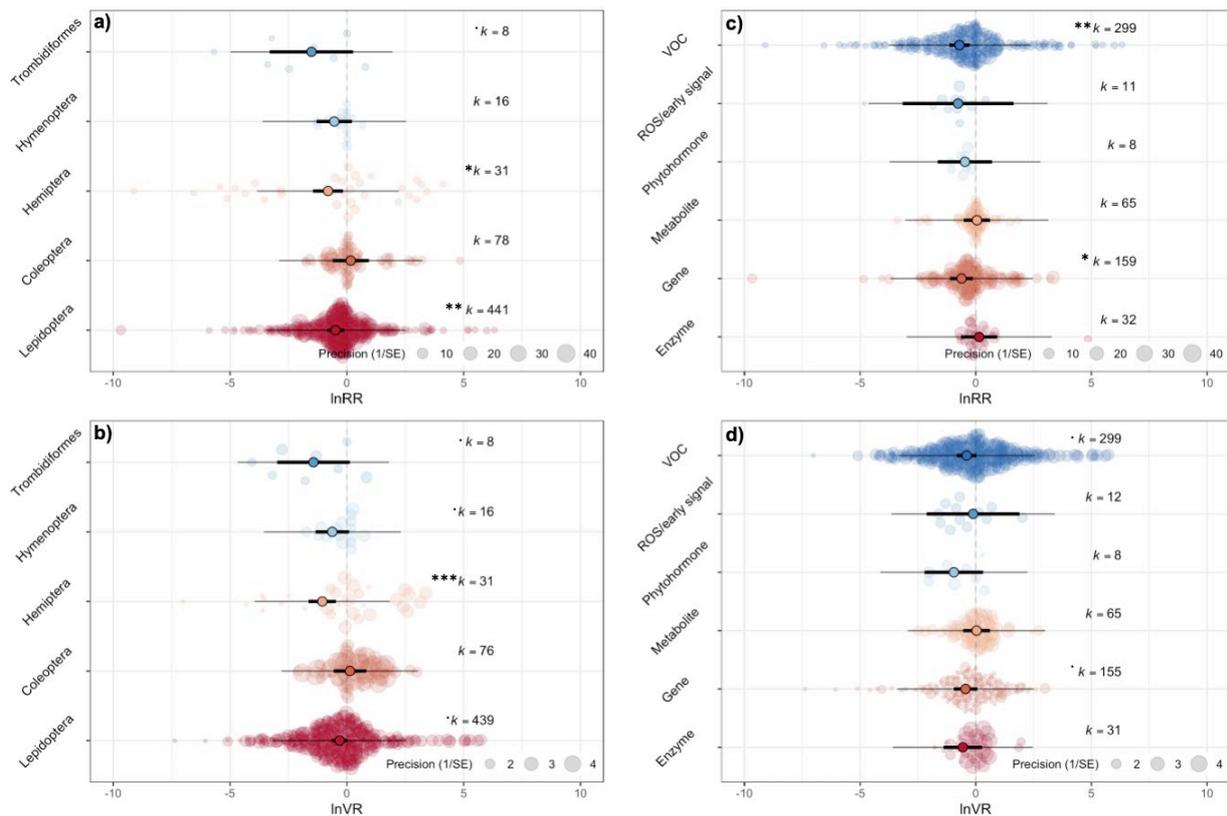
Extended data Figure 6-3. Effect sizes of responses induced by mechanical wounding and model outputs from meta-regression. Natural logarithm of response ratio (lnRR) and natural logarithm of the ratio of the standard deviations (lnVR) for simulated herbivory in comparison to true herbivory by herbivore taxa (a and b) and type of defence response (c and d). Coloured points represent raw data (i.e., individual observations, k), black-outlined coloured circles represent model estimates, thick black lines represent 95% confidence intervals (CIs) and thin black lines represent prediction intervals. Asterisks depict significance (i.e., a 95% CI that does not overlap 0): . = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. VOC = volatile organic compound.

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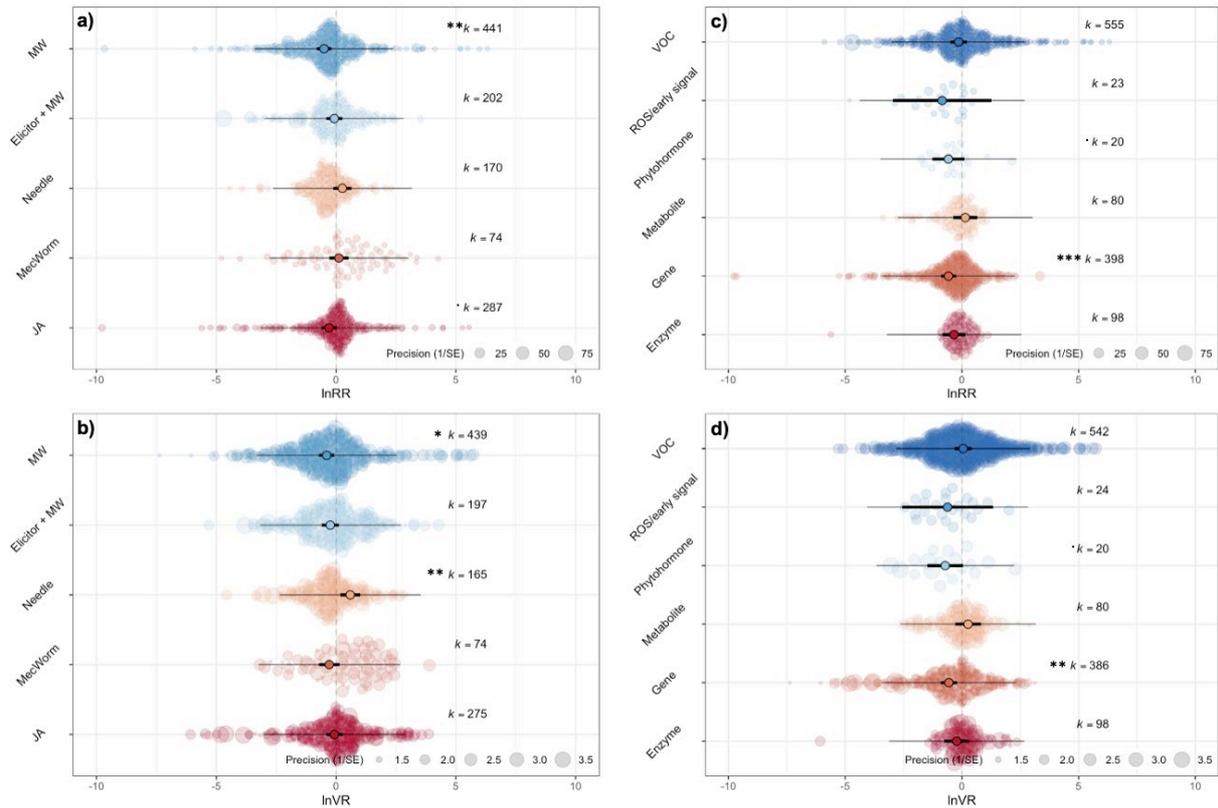
Extended data Figure 6-4. Effect sizes of responses when lepidopteran herbivory was simulated and model outputs from meta-regression. Natural logarithm of response ratio (lnRR) and natural logarithm of the ratio of the standard deviations (lnVR) for simulated herbivory in comparison to true herbivory by simulated herbivory technique (a and b) and type of defence response (c and d). Coloured points represent raw data (i.e., individual observations, k), black-outlined coloured circles represent model estimates, thick black lines represent 95% confidence intervals (CIs) and thin black lines represent prediction intervals. Asterisks depict significance (i.e., a 95% CI that does not overlap 0): . = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. JA = jasmonate, MW = mechanical wounding, VOC = volatile organic compound.

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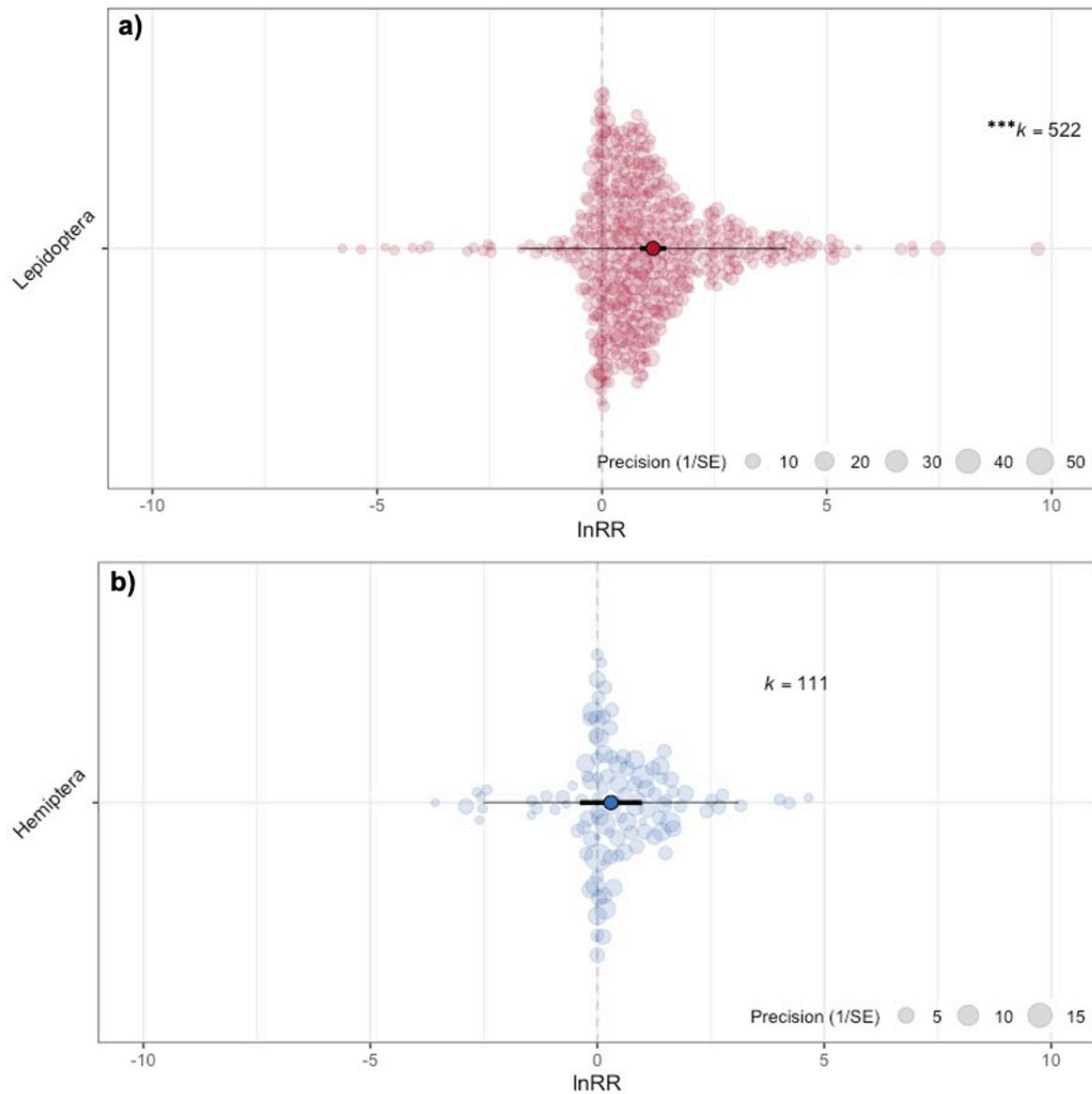
Extended data Figure 6-5. Natural logarithm of response ratio (lnRR) for (a) lepidopteran and (b) Hemipteran herbivory compared to undamaged controls. Coloured points represent raw effect-size data (i.e., individual observations, k), black-outlined coloured circles represent model estimates, thick black lines represent 95% confidence intervals (CIs) and thin black lines represent prediction intervals. Asterisks depict significance (i.e., a 95% CI that does not overlap 0): *** = $p < 0.001$.
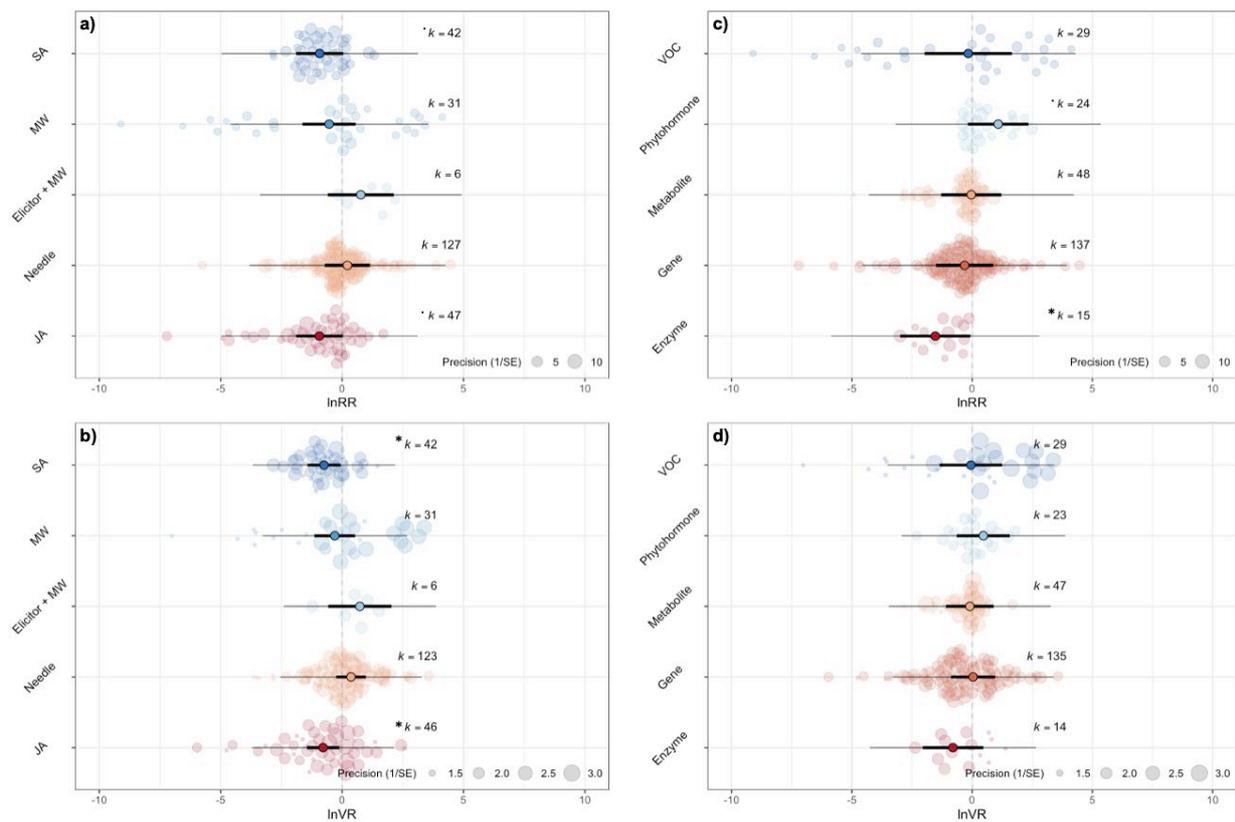
Extended data Figure 6-6. Effect sizes of responses when hemipteran herbivory was simulated and model outputs from meta-regression. Natural logarithm of response ratio (lnRR) and natural logarithm of the ratio of the standard deviations (lnVR) for simulated herbivory in comparison to true herbivory by simulated herbivory technique (a and b) and type of defence response (c and d). Coloured points represent raw effect-size data (i.e., individual observations, k), black-outlined coloured circles represent model estimates, thick black lines represent 95% confidence intervals (CIs) and thin black lined represent prediction intervals. Asterisks depict significance (i.e., a 95% CI that does not overlap 0): . = $p < 0.1$, * = $p < 0.05$. JA = jasmonate, MW = mechanical wounding, SA = salicylate, VOC = volatile organic compound.
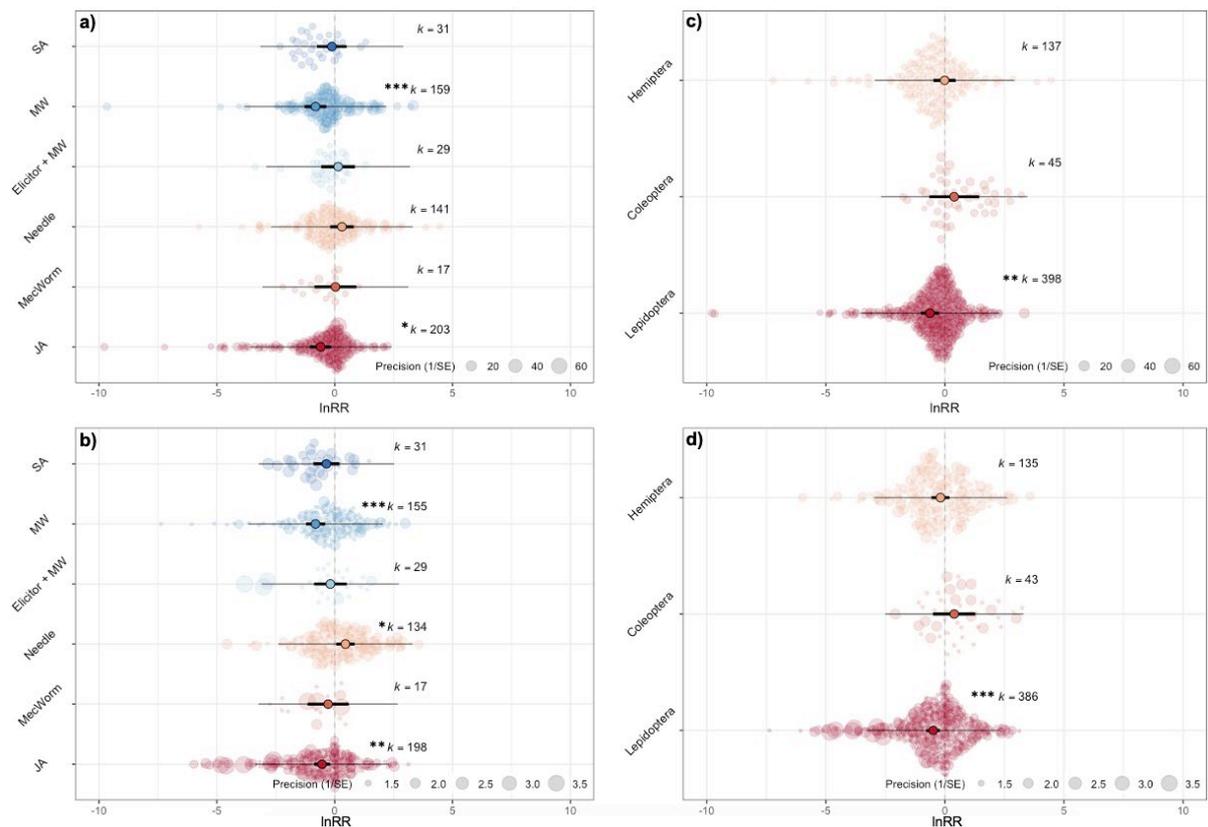
Extended data Figure 6-7. Effect sizes of gene expression responses for simulated herbivory in comparison to true herbivory and model outputs from meta-regression. Natural logarithm of response ratio (lnRR) and natural logarithm of the ratio of the standard deviations (lnVR) for simulated herbivory in comparison to true herbivory by simulated herbivory technique (a and b) and herbivore taxa (c and d). Coloured points represent raw effect-size data (i.e., individual observations, k), black-outlined coloured circles represent model estimates, thick black lines represent 95% confidence intervals (CIs) and thin black lines represent prediction intervals. Asterisks depict significance (i.e., a 95% CI that does not overlap 0): * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. JA = jasmonate, MW = mechanical wounding, SA = salicylate.

Extended data Figure 6-8. Effect sizes of volatile organic responses (VOC) responses for simulated herbivory in comparison to true herbivory and model outputs from meta-regression. Natural logarithm of response ratio (lnRR) and natural logarithm of the ratio of the standard deviations (lnVR) for simulated herbivory in comparison to true herbivory by simulated herbivory technique (a and b) and herbivore taxa (c and d). Coloured points represent raw effect-size data (i.e., individual observations, k), black-outlined coloured circles represent model estimates, thick black lines represent 95% confidence intervals (CIs) and thin black lines represent prediction intervals. Asterisks depict significance (i.e., a 95% CI that does not overlap 0): * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. JA = jasmonate, MW = mechanical wounding, SA = salicylate.

Extended data Figure 6-9. Natural logarithm of response ratio (lnRR) for simulated herbivory in comparison to true herbivory with (a) all data extracted from studies (i.e., including outliers) and (b) only outliers from dataset. Coloured points represent raw effect-size data (i.e., individual observations, *k*), black-outlined coloured circles represent model estimates, thick black lines represent 95% confidence intervals (CIs) and thin black lined represent prediction intervals. Asterisks depict significance (i.e., a 95% CI that does not overlap 0): * = $p < 0.05$, ** = $p < 0.01$. Inset in (a) depicts the data presented with effect sizes between -5 and 5.

7 Chapter 7: General Discussion

In this thesis I investigated the impacts of simulated and true herbivory on plant responses, including senescence, specialised metabolites, and Si-based defences. This was initially examined by assessing the role of *Helicoverpa armigera* oral secretions (OS), and microbes contained therein, in inducing senescence around plant wounds and wound closure (chapter 3). Further investigations focused on how methyl jasmonate application (simulated herbivory) impacts Si accumulation over short-term temporal scales, and how, over similar amounts of time, Si and simulated herbivory integrate to modify anti-herbivore defence machinery (chapter 4). Subsequently, based on evidence of rapidly induced Si-accumulation in chapter 4, the impacts of brief Si exposure on Si accumulation and deposition patterns were investigated in *Brachypodium distachyon* (chapter 5). Additionally, as part of chapter 5 fitness and performance metrics of herbivores (*H. armigera*) fed on plants with brief exposure to Si were measured. Finally, responses induced by simulated herbivory from across the literature were compared using meta-analysis to highlight how simulated herbivory compares to true herbivory in terms of intensity and variation in responses and inform future studies that aim to incorporate simulated herbivory into experiments (chapter 6). The key findings from each chapter are summarised in Fig 7-1:

Key Findings

Chapter 2
- Simulated herbivory is a critical tool for understanding the mechanistic basis of biochemical defence responses in plants

Chapter 3
- Lepidopteran oral secretions (OS) induce senescence around plant wounds and wound closure
- Microbes within OS play a secondary role in induction of senescence

Chapter 4
- Silicon (Si) accumulation is induced by herbivore signals in as little as 6 hr
- Si accumulation is integrated with jasmonic acid signaling
- Si suppresses the salicylic acid signaling pathway
- Although there is a tradeoff between Si and phenolic defences in the absence of stress, simulated herbivory counteracts this relationship by inducing phenolics

Chapter 5
- Only brief exposure to Si is needed for effective Si-based defences against chewing herbivores
- Plants with < 72 hr of exposure to Si reduce herbivore performance (feeding, growth) and increase mandible wear to the same degree as plants with long-term exposure to Si
- Si cells are fully filled within 24 hr of exposure to Si and this is accelerated by herbivory

Chapter 6
- Simulated herbivory is, overall, a conservative estimate of true herbivory
- Simulated herbivory can induce responses similarly to true herbivory, however timing, technique, herbivore taxa and type of response measured all influence comparability between the two

Figure 7-1. Key findings of thesis chapters 2–6.

7.1 Plant defences are signal specific

Plants are exposed to many signals during an herbivory event making it a challenge to discern which specific herbivore stimulus (or stimuli) plants are responding to (chapter 2). Cell senescence is a stress response most often considered as an anti-microbial response (Guo and Gan 2012, Häffner et al. 2015), however I found this response to be induced primarily by herbivore-specific (i.e., not derived from microbes) signals, although microbial signals played a secondary role (chapter 3). Further, any induction of senescence in the absence of

herbivore- or microbe-specific signals (i.e., simple wounding) was very minor, suggesting that, in *Brachypodium distachyon*, senescence may be an evolved response to herbivores specifically, as opposed to generic wounds. Although research into senescence as a plant defence response to biotic stress is most often in the context of pathogen prevention, it might also affect herbivore feeding behaviour and performance. It has been shown that during senescence, nutritional quality of plant tissues may be increased, which can have beneficial effects on feeding insects (Steinbauer et al. 2014). Considering this, it could be advantageous for insects to associate with microbes that induce senescence in plants to enhance nutritional quality of plant tissues. During the wound response, senescence is also associated with increases in anti-herbivore compounds such as lignin (Cui et al. 2013), which could, alternatively, increase plant resistance to chewing herbivores like *H. armigera* through feeding deterrence resulting from increases in toughness and reduction in nutritional quality of tissues (War et al. 2012). Cell senescence occurs as a response to an oxidative burst from the production of hydrogen peroxide, which is a critical signalling molecule for many defence responses and well documented to be induced by herbivory (Bi and Felton 1995, Gough and Cotter 2011). Although oxidative bursts are induced by simple mechanical wounding (Prasad et al. 2020), they can be enhanced further by contents (e.g., oxidative enzymes and insect-derived elicitors) found within herbivore OS (Bricchi et al. 2010, Block et al. 2018). Chapter 3 highlights that this oxidative burst associated with OS substantially induces cell senescence in addition to wound closure (production of lignin, callose, etc.). However, simple wounding alone is not enough to trigger the response and, therefore, in the context of herbivory it is likely that plants rely on herbivore-specific signals to initiate responses such as cell senescence and wound closure, which can have significant impacts on both insects and introduced microbes.

7.2 A deeper understanding of how Si integrates with C-based defences

The majority of terrestrial plants are anchored in place and therefore are incapable of evading predators (Chai et al. 2005). As such, plants must either resist (reduce or prevent) or tolerate (negate the impacts of) herbivore feeding (Núñez-Farfán et al. 2007). In order to resist herbivores, plants often rely on a complex network of chemical defences to combat herbivory (Agrawal and Fishbein 2006). However in plants with limited capacity to produce metabolites (Defossez et al. 2021), alternative strategies must be employed, such as Si accumulation and deposition (Moore and Johnson 2017). Considering Si may have benefits to plants even in the absence of stress (Korndörfer and Lepsch 2001, Frew et al. 2018), the degree to which Si accumulation is under the control of plant defence machinery is an outstanding question in the field. The results from chapter 4 of this thesis not only provide strong support for the inducibility of Si accumulation and its integration with the JA pathway (increases in foliar Si concentration were tightly correlated with higher foliar JA levels) but also context to the temporal scale at which these inductions occur. The work done as part of this thesis also reveals that, even in the absence of stress, Si uptake and ecologically relevant levels of Si deposition occur rapidly, albeit those levels are reached faster in the presence of herbivore stress (chapter 5).

To ensure the appropriate defence responses are mounted in response to a given stressor, multiple phytohormones work in tandem and can heighten or suppress one another (Altmann et al. 2020). Several studies have focused on the interactions between Si and JA, however few other phytohormones have been examined in this context. Specifically, salicylic acid (SA) may be particularly relevant due to its known antagonism with JA (Thaler et al. 2012, Phuong et al. 2020). Although this antagonism is taxa-specific (Thaler et al. 2012, Fabisch et al. 2019), in *Brachypodium distachyon* (plant species used throughout this thesis), Johnson et

al. (2020a) found that phloem-feeding herbivore-induced increases in SA corresponded with subsequent decreases in JA. Furthermore, results from this thesis suggest that Si accumulation in response to chewing herbivore signals is associated with concurrent increases in JA. Results also suggest that Si directly suppresses SA, potentially heightening JA-dependent responses, resulting in a positive feedback loop between Si and JA (chapter 4).

Although the negative relationship between Si and C-based defences such as phenolics has been extensively demonstrated, whether this relationship is maintained under herbivory stress remained unclear. In chapter 4 of this thesis, I found that, upon simulated herbivory, the negative relationship between phenolics and Si is nullified due to inductions in phenolics by simulated herbivory; even in the presence of Si, phenolic defences were inducible. This suggests that plants might be able to preferentially utilise constitutive Si-based defences in place of C-based defences, however upon perception of relevant stress signals plants might be able to utilise induced C- and Si-based defences for a more robust and effective response. Although Si is metabolically more efficient (i.e., metabolically cheaper per unit) than C, there may still be potential costs to Si accumulation that might impact plant fitness, particularly in the absence of stress, for reasons such as increased tissue density (requiring additional resource investment in strength and anchorage), lack of ability to cross-link with C-based cell wall constituents, and lack of water repellence (Raven 1983, Cooke and Leishman 2011b, a, Kumar et al. 2017b). Nevertheless, despite potential trade-offs associated with Si, the overall effect of Si on plants, especially in terms of stress prevention, has been beneficial, as grasses are considered to be the most evolutionarily successful plant family in terms of global occurrence and dominance, in large part due to their ability to persist in ecosystems under challenging conditions (Linder et al. 2018).

7.3 Rethinking the temporal scale of Si defences

The timing at which induced defences are deployed can have substantial impacts on herbivore resistance, both against the present attacking herbivore and future herbivores (Karban and Myers 1989). Many defence responses occur rapidly (Block et al. 2018, Toyota et al. 2018), whereas others build up over time (Reynolds et al. 2012, Wang et al. 2014). To date, to the best of my knowledge, all studies (other than chapter 5 of this thesis) have determined the impacts of Si on herbivores in plants that were exposed to Si for a substantial period (at least several weeks) prior to herbivore treatments (Hall et al. 2020b, Johnson et al. 2021). Therefore, upon exposure to herbivores plants have already built-up substantial quantities of Si. Even in high Si-accumulating species (e.g*., B. distachyon*), plants can reach a maximum level of deposition in certain tissues, even at relatively low absolute foliar Si concentrations (chapter 5). In natural and agricultural systems, the biological availability of Si ranges from 0.01 mM to 2 mM (Karathanasis 2002, Haynes 2014). Therefore, the findings of chapters 4 and 5 highlight the importance of Si over short periods of stress and show that only brief exposure to Si can confer the same level of resistance to herbivory as with over a month of exposure. Recently, Si-based fertilisers have gained traction as a means to promote increases in crop yield and resistance to biotic and abiotic stressors (Haynes 2017), and if plants are able to rapidly accumulate recently supplied Si (e.g., in 6 hr, chapter 5) when previous levels of soil Si are negligible, that could have substantial implications on effective pest management, even in crops that are under ongoing herbivore attack (i.e., herbivores are already feeding on foliar tissues). This may be particularly important for mitigating the detrimental effects of agricultural pests such as *Helicoverpa armigera*, that are particularly difficult to manage using conventional methods such as chemical pesticides (Jones et al. 2019), but quite negatively impacted by Si deposition (Kvedaras et al. 2010, Hall et al. 2020a, Johnson et al. 2020b, Johnson et al. 2021, Vandegeer et al. 2021).

7.4   Important considerations to improve simulated herbivory

There are many modes of chemical plant defences, ranging from anti-herbivore enzymes to toxic chemicals and indirect volatile organic compounds that attract natural enemies (Steppuhn et al. 2004, Zong and Wang 2007, Clavijo McCormick et al. 2012); simulated herbivory may not consistently impact each the same way. In this thesis I demonstrated that the effectiveness of simulated herbivory at inducing comparable responses to those induced by herbivores can depend greatly on the simulated herbivory technique used and specific type of herbivore being simulated (chapter 6). Through millions of years of evolution plants have evolved a highly acute ability to detect herbivores and distinguish them from one another and from other forms of stress (Steinbrenner et al. 2020). Therefore, close attention to the temporal and chemical patterns of defence responses should be considered when developing simulated herbivory techniques for both comparative and pragmatic reasons.

Simulated herbivory is widely used as a technique to reduce biases associated with herbivores and to elucidate the mechanisms of defence responses. Specifically, simulated herbivory can decouple the mechanisms of responses to herbivory and is a standardised alternative to herbivores in experimental settings, as the extent of damage is more consistent between individuals and treatments (Tian et al. 2012, Li et al. 2019). Nevertheless, meta-analysis in this thesis revealed that simulated herbivory does not necessarily reduce variation in defence responses (e.g., the differences between replicates do not differ between simulated and true herbivory treatments), particularly when simulated herbivory that incorporates multiple herbivore signals is used (chapter 6). Even though the amount of damage might be inconsistent when using herbivores (Ryalls et al. 2017), responses may not necessarily be sensitive to such differences when comprehensive herbivore signals (wounding and chemical

signals) are present (Kautz et al. 2014). Nevertheless, in the absence of chemical signals (elicitors) variation tends to be reduced and it is therefore possible that in the absence of herbivore-specific signals plant defence capacity is reduced considerably (see also chapter 3 of this thesis). This alone is likely to reduce variation considering the strong positive relationship between intensity (mean) of response and variation in response highlighted in chapter 6. It has recently been demonstrated that variation in nutritional quality and defensive chemistry of plant tissues can have substantial impacts on herbivore performance (Wetzel et al. 2016, Pearse et al. 2018). Therefore, particularly in ecological studies, it is important to consider whether reduction in variation is a desired outcome when selecting simulated herbivory techniques.

7.5 Limitations and future work

Many organisms, including most insect herbivores, possess a resident microbiome, whereby certain microbial taxa are incorporated into the gut microbiome independent of environment and diet (Engel and Moran 2013). Interestingly, lepidopteran herbivores such as *Helicoverpa armigera* do not have resident microbiomes, and instead their gut microbial makeup is determined primarily by diet and environment (Hammer et al. 2017). Additionally, the overall abundance of microbial organisms in lepidopteran guts is often considerably lower than other insects (Hammer et al. 2017). Therefore, in Lepidoptera specifically, the implications for how gut-associated microbes modify plant defences during herbivory would be expected to change under differing environmental and dietary regimes. Nevertheless, despite certain signals being taxa-specific (Chung et al. 2013, Wong et al. 2020), plants are known to respond to generic microbial signals as a preventative measure against possible pathogen infection (Chisholm et al. 2006). Additionally, defence responses can be induced in plants from self-recognition of metabolites released from their cells during tissue damage

(Heil et al. 2012). Therefore, some wound responses induced by oral secretions (OS) might also be, in part, due to self-recognition patterns associated with plant-derived compounds found within OS in addition to insect- and microbe-derived signals (Wang et al. 2017, Block et al. 2018). Future endeavours that aim to elucidate and identify specific elicitors and OS microbial isolates from lepidopteran larvae collected from various locations that have fed on a diversity of diets and to directly apply them to plant wounds will delve deeper into the mechanisms of the wound responses identified in chapter 3.

Simulated herbivory can be a useful tool for uncoupling mechanisms of plant–herbivore interactions, however, to date the variety of herbivore taxa that have been simulated is sparse. Most studies simulate lepidopteran herbivory; further work is required to develop a comprehensive understanding of the effectiveness of simulated herbivory across taxa. For example, beyond invertebrates, through a systematic literature search (chapter 6), no instances of comparison between biochemical responses induced by simulated and true mammalian herbivory were found, despite the importance of mammalian herbivores in agricultural and natural ecosystems (Bayani et al. 2016, Linder et al. 2018, Tuomi et al. 2019). Another challenge is that true herbivory is very difficult to replicate temporally using artificial techniques, therefore increased accessibility of tools such as MecWorm and SpitWorm (Mithöfer et al. 2005, Li et al. 2019), that allow for accurate temporal and spatial replication of herbivory (and in the case of SpitWorm, introduction of chemical signals), can help elucidate unanswered questions pertinent to the biochemistry of plant–herbivore interactions.

The results from experimental chapters within this thesis (3–5) were all conducted in hydroponic systems to control Si supply. Although hydroponic-based studies provide an

invaluable tool for elucidating mechanisms of Si-based defence in a controlled setting (i.e., controlling Si inputs and nutrient regime), it will be critical to also test the hypotheses from this thesis in more natural, soil-based systems as well as in systems that supply soil- or hydroponic solution-Si at multiple concentrations in order to determine if belowground substrate and supplied Si concentration impacts the rate of Si accumulation and deposition. In soil-based systems there are multiple ways in which Si can be deployed in agricultural settings, that take advantage of an array of carrier elements (K, Na, Ca, etc.) and depending on which is used, plant resistance to herbivory may vary. Specifically, when calcium carbonate ($CaCO_3$) was used, although absolute Si concentrations were similar, herbivore performance on these plants was much higher than when sodium chloride (NaCl) and potassium chloride (KCl) were used, perhaps underpinned not by Si effects but by changes in nutritional quality of plant tissues (Cohen 2004, Johnson et al. 2020b). Additionally, studies within this thesis were conducted in a single model grass (*Brachypodium distachyon*), and mechanisms of Si-based defences have been shown to vary considerably between species and even varieties within the same species (Hartley et al. 2015, McLarnon et al. 2017), particularly those with starkly differing Si-accumulation strategies such as high and low capacity to uptake Si (Frew et al. 2019, Putra et al. 2020, Acevedo et al. 2021). Expanding upon this work by testing these hypotheses on a diversity of plant and herbivore taxa combinations will also be useful to identify the nature of these mechanisms in a broader context.

This thesis provides a mechanistic understanding of the ways Si is rapidly incorporated into plant tissue, modifies alternative defence machinery and impacts chewing herbivores at rates much faster than previously envisaged. However, a deeper, molecular understanding of these processes may be of critical importance for understanding the evolutionary rationale behind

these processes and possibly for the development of technologies to exploit these traits in agriculture. For example, determining how expression of Si transport genes (Lsi1, Lsi2, Lsi6) change over short periods of time or in response to novel exposure to Si could support the evidence provided by this thesis. Additional experiments are currently underway to further test the mechanisms of Si-based defences in response to simulated and authentic herbivory and show that the impacts of Si on said responses change depending on whether the plant is stressed (as highlighted in chapter 4). Specifically, using true herbivory, in support of findings in chapter 4 of this thesis, the negative trade-off between phenolics is observed only in the absence of herbivore stress in an additional grass species, *Festuca arundinaceae* (Fig 7-2), suggesting that this is not a phenomenon specific to *B. distachyon*. Considering many phenolic compounds may be constitutive, and the majority of work relating Si to phenolics has focused on general or total phenolics, identification of specific inducible phenolic compounds and their relationship with Si will inform with greater detail how Si integrates with C-based defences both under and in the absence of stress (Rehman et al. 2012). Additionally, Si deposition patterns in *Festuca arundinaceae*, for example, are markedly different from those in *B. distachyon*, both in terms of total concentration and Si-based structures (Fig 7-3). The mechanisms of short-term Si accumulation in plants like *F. arundinaceae* remain unstudied, despite Si-structures being identified in multiple *Festuca* species and being linked to reductions in herbivore performance (Hartley et al. 2015, McLarnon et al. 2017). Additional experiments are also underway that investigate Si deposition at specific sites around wounded tissue to identify whether Si is locally accumulated at higher concentrations in immediately damaged tissues (Fig 7-4).
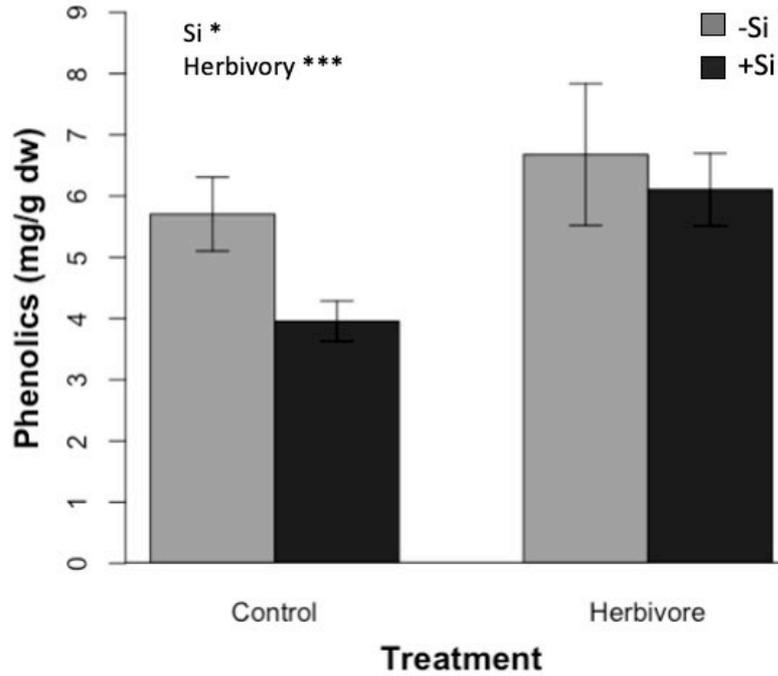
Figure 7-2. Total phenolics concentration in *Festuca arundinaceae* plants supplemented with silicon (+Si) or grown in the absence of Si (-Si) that were ether left undamaged (control) or were damaged by the chewing herbivore, *Helicoverpa armigera* (herbivore).
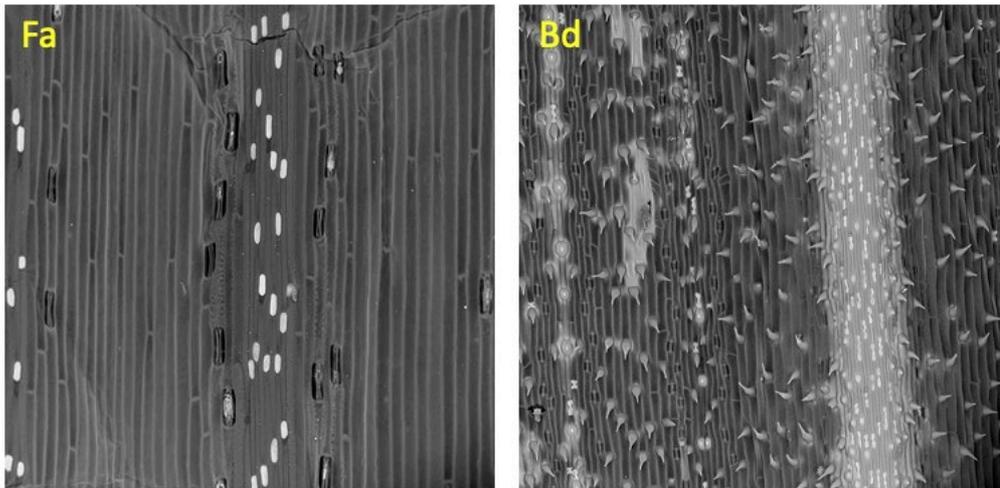
Figure 7-3. Backscatter electron (BSE) Scanning electron microscope (SEM) images of abaxial midribs of *Festuca arundinaceae* (Fa) and *Brachypodium distachyon* (Bd). Light grey/white parts of image are silicon-rich structures. Images from Waterman, Cibils-Stewart…Johnson, In prep.
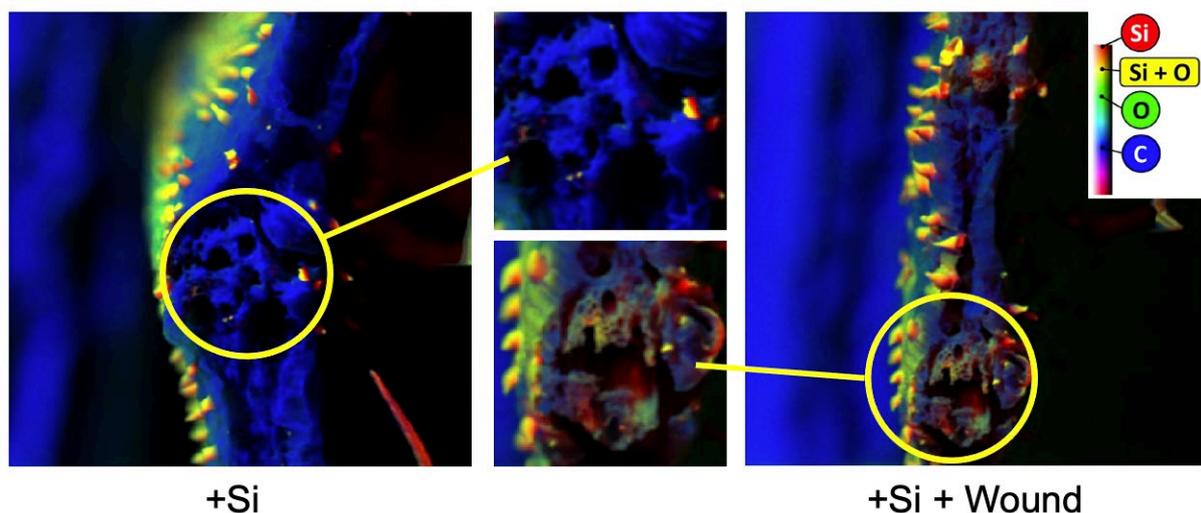
Figure 7-4. Pseudo-coloured elemental X-ray maps of cross sections of *Brachypodium distachyon* plants grown in silicon and either left undamaged (+Si) or wounded with scissors and allowed 24 hr to heal (+Si + Wound). Zoomed-in areas highlight solid silicon dioxide deposition in vascular tissue. Images from Waterman, Cibils-Stewart…Johnson, In prep.

7.6 Conclusions

The work presented in this thesis identified the key factors responsible for differences in responses induced between simulated and true herbivory as well as how each can be integrated to understand the mechanisms of anti-herbivore defence, particularly in the context of inducible Si-based defences. This work found that plant responses to wounding are greatly enhanced in the presence of herbivore signals (as opposed to generic wounding) and are even further enhanced when microbial signals are present, which could have substantial implications on the defence networks regulating the dynamics of plant–insect–microbe interactions. Also demonstrated in this thesis is that Si-accumulation is integrated with the jasmonic acid (JA) pathway and responds to herbivore signals within 6 hr of perception, with wider implications on C-based defences and alternative, JA-antagonistic, phytohormonal pathways. This work highlights that Si accumulation and deposition in plants previously unexposed to Si occurs within hours of exposure to Si, and within < 72 hr these plants are as resistant to chewing herbivory as plants exposed to Si for much longer periods. These findings are of considerable relevance to agriculture and point to the application of Si in

agricultural systems as a more effective alternative to conventional pest management strategies such as pesticide application, particularly for species such as *Helicoverpa armigera* that threaten global agricultural sustainability due to their prevalence and high pesticide resistance. Broadly, the findings of this work not only shine light on the ecological rationale of Si-based defences in the context of herbivory, but also inform possible avenues for facilitating increased plant resistance to insect herbivores.

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Appendix 1 - Chapter 3 supplementary information

Recipes

Antibiotic cocktail

The antibiotic cocktail contained three antibacterial (0.3 mg/mL neomycin sulfate, 1.5 mg/mL aureomycin, 0.12 mg/mL streptomycin sulfate) and two antifungal (0.8 mg/mL methyl paraben, 0.6 mg/mL sorbic acid) agents. All antibiotics were purchased from Sigma Aldrich, MO USA.

Nutrient solution

The nutrient solution was comprised of 2 mM KNO_3, 2 mM $Ca(NO_3)_2.4H_2O$, 2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 200 μM NaCl, 30 μM H_3BO_3, 1 μM $MnCl_2.4H_2O$, 1.4 μM $ZnSO_4.7H_2O$, 1.6 μM $Na_2MoO_4.2H_2O$, 1.6 μM $CuSO_4.5H_2O$, 100 μM NaFe(III) EDTA. All chemicals were purchased from Sigma Aldrich, MO USA.

Artificial Helicoverpa armigera *diet*

Artificial diet was made to 800 mL containing 65 g Soya flour, 30 g wheat germ, 26.5 g brewer's yeast, 1.65 g ascorbic acid, 0.85 g sorbic acid, 1.65 g methyl paraben and 10 g agar. The mixture was made up to 800 mL with water.

Materials and Methods

Figure S3-2.

Triticum aestivum var. Coolah (wheat) seeds were obtained from Australian Grain Technologies (NSW, AU) and were grown in Osmocote professional seed raising and cutting mix (Scotts; NSW, AU) potting mix. Plants were grown for 63 days in a naturally lit glasshouse under identical conditions to those described in the main text. *Helicoverpa*

armigera eggs from the Commonwealth Scientific and Industrial Research Organisation (CSIRO, Narrabri, Australia) were hatched and fed on *T. aestivum* for the duration of their development. Larvae were reared in 30 mL plastic containers containing 1% agar to maintain leaf moisture and incubated at 25°C. No larvae were fed antibiotics in this experiment. Leaves were damaged and measured in an identical fashion to those described in the main manuscript, however no antibiotics were used in this experiment. Senescence measurement and quantification were also done identically to techniques described in the main manuscript. Additionally, feeding duration and quantity was identical to methods described in the main text. Oral secretions (OS) were collected in an identical fashion to methods described in the main text, however for the OS (filtered) treatment, OS was passed through a 0.22 μm PES membrane (Merck Millipore, IRL) to sterilise OS by removing all particles larger than 0.22 μm, including microbial cells.

Figure S3-3.

In order to determine the effectiveness of the sterile filtration, 1/100 dilutions of OS and OS (filtered) were plated onto 1x LB media and incubated at 28°C for 36 hours. Microbial colony forming units were counted to determine the abundance of microbes within the OS. Water was also plated as a control to ensure that there was no contamination of materials from non-OS microbes.

Figure S3-4

Relative growth rate was calculated by subtracting the weight of larvae before feeding treatment from the weight post-treatment and dividing the resulting value by the pre-treatment weight. This value was then divided by the duration of the feeding trial (4 days).

Figure S3-5

Instead of water we used phosphate buffered saline (PBS) as a control.

Figures

Figure S3-1. The number of colony forming units (CFUs)/mL in *Helicoverpa armigera* oral secretions (OS) fed on *Brachypodium distachyon*. Letters above each bar indicate significant differences between treatments (Welch two sample t-test; $t_{(2)}$ = -39.55, $P < 0.001$). Abbreviations: OS+M = ½ dilution OS with normal levels of microbial abundance, OS-M = ½ dilution OS with significantly reduced microbial abundance from antibiotic-fed larvae (*n* caterpillars = 7, *n* plates = 3).

Figure S3-2. The effects of sterile *Helicoverpa armigera* oral secretions (OS) on the area of senescence around *Triticum aestivum* wounds. Abbreviations: OS+M = ½ dilution OS with normal levels of microbial abundance and OS (filtered) = ½ dilution sterile OS filtrate. Values are mean ± SE ($n = 12$). Letters above each bar indicate significant differences between treatments (ANOVA, P < 0.05 followed by an HSD test. One-way ANOVA showed that the effect of treatment on area of senescence was significant ($F_{(2, 32)} = 120.21$, $P < 0.0001$). No antibiotics were used in this experiment.
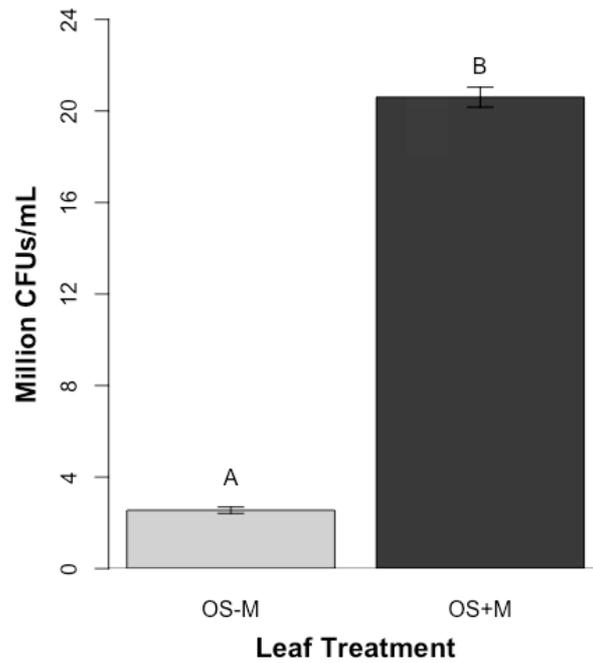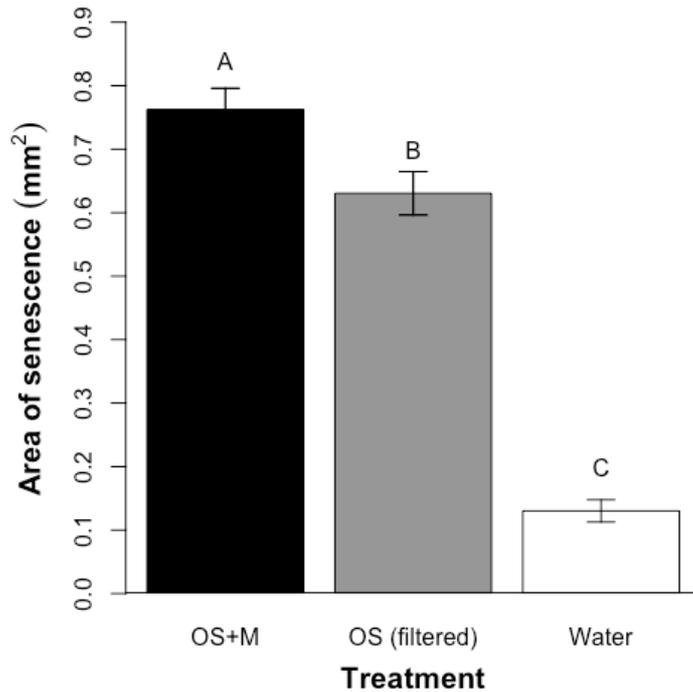

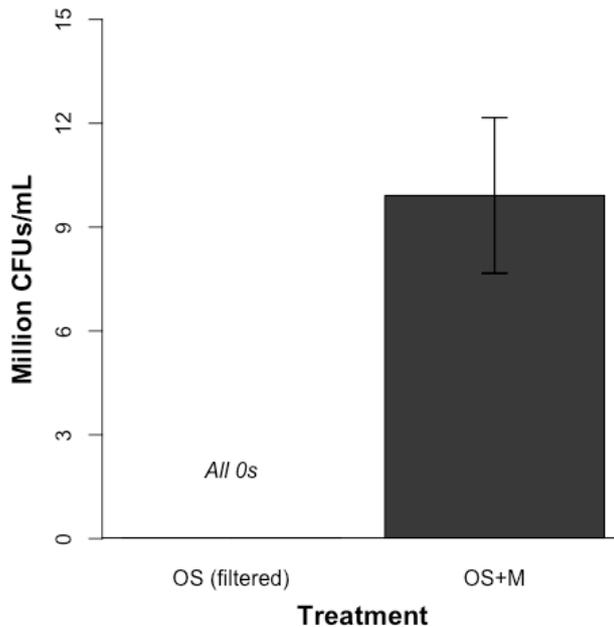**Figure S3-3.** The number of colony forming units (CFUs)/mL in *Helicoverpa armigera* oral secretions (OS) fed on *Triticum aestivum*. Letters above each bar indicate significant differences between treatments. Abbreviations: OS+M = ½ dilution OS with normal levels of microbial abundance, OS (filtered) = ½ dilution sterile OS filtrate (n caterpillars = 19, n plates = 3). No antibiotics were used in this experiment.
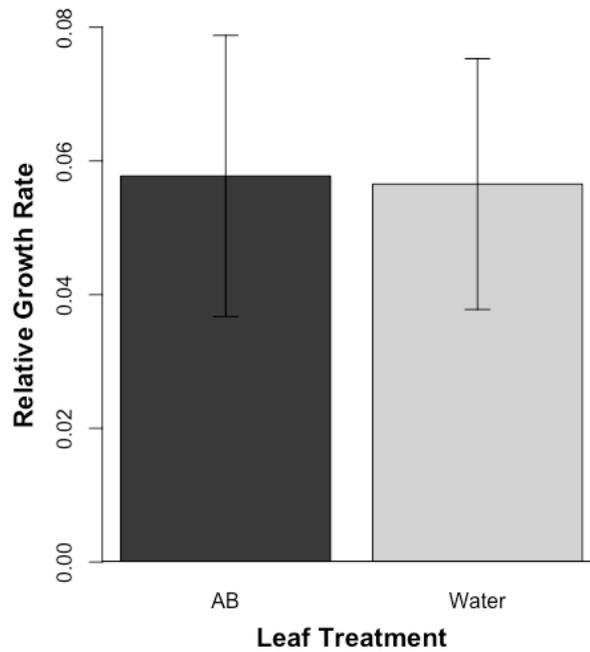
Figure S3-4. The effects of antibiotic treatment on the relative growth rate of *Helicoverpa armigera*. Values are mean ± SE (*n* = 7). Welch's two sample t-test showed no significant difference between 2 and 14 days ($t_{(11)}$ = 0.07, *P* = 0.94). AB = antibiotic-fed.

Figure S3-5. The effects of Phosphate buffered saline (PBS) on opening size over 14 days. Values are mean ± SE (*n* = 12). Welch's two sample t-test showed no significant difference between 2 and 14 days ($t_{(17)}$ = 1.65, *P* = 0.12).

Appendix 2 – Chapter 4 Supplementary information

Table S4-1. Relationship between endogenous JA and SA

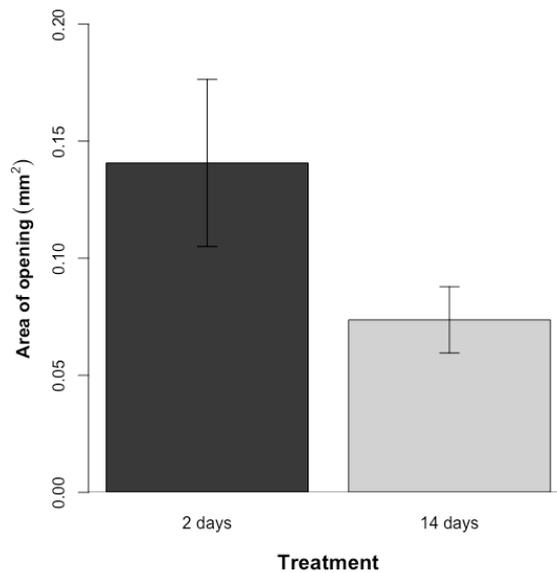|	R^2	P-Value
Overall		
	-0.012	0.761
All Si supplemented plants		
	0.009	0.255
All Si devoid plants		
	-0.009	0.421
All MeJA treated plants		
	-0.003	0.351
All untreated plants (no MeJA)		
	-0.004	0.365
By time after MeJA treatment		
1 hr		
	-0.058	0.887
6 hr		
	-0.055	0.171
12 hr		
	-0.017	0.416
24 hr		
	-0.045	0.192
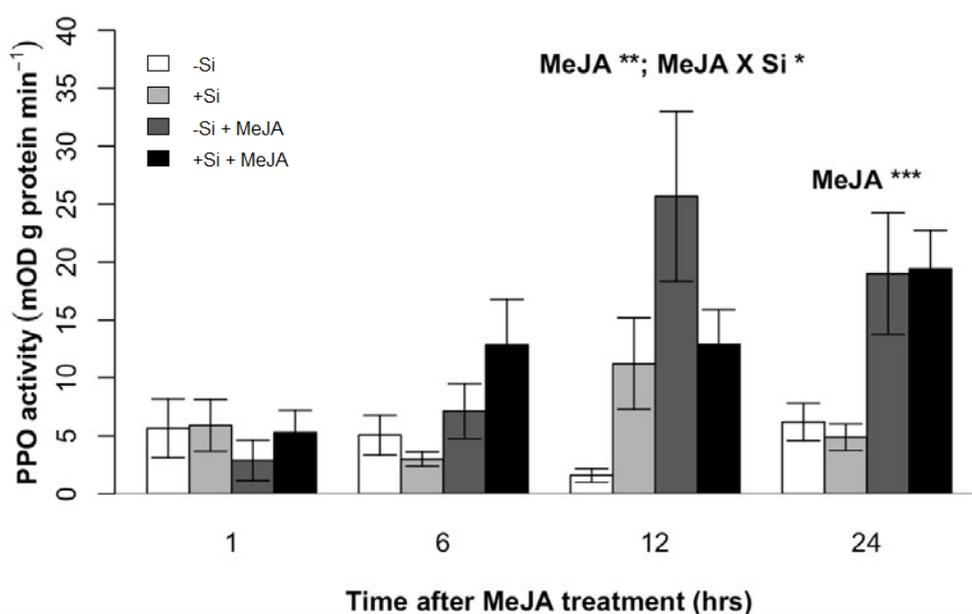
Figure S4-1. Polyphenol oxidase (PPO) activity 1, 6, 12 and 24 hr after methyl jasmonate (MeJA) treatment. White bars = control plants (-Si), light grey bars = Si-supplemented plants (+Si), dark grey bars = plants treated with MeJA (-Si + MeJA), and black bars = plants supplemented with Si and treated with MeJA (+Si + MeJA). Differences in PPO activity between treatments were determined within each time point using two-way ANOVAs (* = P < 0.05, ** = P < 0.01, *** = P < 0.001 at 95% confidence intervals).

Appendix 3 – Chapter 5 Supplementary information

Section S1: Materials and Methods

Brachypodium distachyon germination

Brachypodium distachyon seeds obtained from the French National Institute for Agricultural Research (INRA, Versailles, FR) were sterilized in 1% bleach (NaOCl) and stratified in wet perlite at 4° C for 7 days. After stratification, seeds were transferred to a glasshouse (22/18° C day/night) for germination.

Scanning electron microscopy (SEM)

The abaxial side of the freeze-dried leaf samples were mounted face up on aluminium scanning electron microscope (SEM) stubs with conductive adhesive carbon tape. Imaging was carried out in variable pressure mode at a chamber pressure of 10 to 60 Pa and an accelerating voltage of 15 kV. The elemental composition of Si cells was confirmed using energy-dispersive X-ray spectroscopy (EDS) (Section S2: Fig S1B-E) (Mason and Wuhrer 2017).

Mandible wear

Methods such as those developed by Kvedras et al. (2007) and Massey and Hartley (2009) were not sensitive enough to show differences in mandible wear in this study, despite being obviously detectable when looking at images of mandibles. Therefore, we developed a scale-based method that accommodated the nature of the mandible wear observed in this study. The scale accounted for two separate categories (2 points total each). The first category was for visible incisor surface wear and the second was for the pointedness of the incisors. The details of which are illustrated below:

Visible surface wear:

0 – No visible surface wear

0.5 – Minor abrasions on incisor surface

1 – Abrasions slightly more apparent and found in multiple locations on incisors

1.5 – Majority of incisors with apparent surface wear

2 – All incisors with very visible surface abrasions

Pointedness:

0 – No visible loss of pointedness to incisors

0.5 – Minor loss of pointedness to incisor (either at tip or edges)

1 – Edges or tip of incisor rounded, although shape remains the same

1.5 – Loss of pointedness to incisor and deformation of shape

2 – Completely flat top to incisor and original shape completely deformed

Total phenolics quantification

Using identical methods to Waterman et al. (2021), total phenolics were quantified in the damaged leaves of the six most damaged plants within each Si treatment at 24 and 72 hr after herbivory. Phenolics were not quantified in damaged leaves after 6 hr because negligible tissue had been damaged at this stage. Phenolics were also measured in leaves from six randomly selected herbivore-free plants within each Si treatment (harvested at 72 hr). In brief, 10 mg of tissue was extracted twice in 70% acetone and measured using a version of the Prussian blue assay (Graham 1992) modified for a 96-well microplate.

Statistical analyses

Differences in Si concentrations, foliar damage, herbivore relative growth rate and relative consumption, mandible wear, filled Si cell density in -Si➔+Si plants, phenolic concentrations in damaged leaves and initial larval mass were determined using two-way ANOVAs (Type = II). Data were log-transformed when they did not meet the assumptions of normality. Differences in phenolics in herbivore-free plants were analysed using a one-way ANOVA. ANOVAs were run using the 'Anova' function in the R package 'car' (Fox and Weisberg 2019). Foliar damage was analysed using heteroscedasticity-consistent standard errors, obtained using a White-adjusted ANOVA (White 1980), by adding the 'white.adjust' argument within the 'Anova' function. A linear regression was used for the relationship between foliar damage and Si concentration 24 hr after treatments began. For the relationship between foliar damage and Si concentration 72 hr after treatments began a logarithmic regression was used, as decreases in foliar damage occur rapidly along the x-axis at lower Si concentrations but slow as Si increases. Model selection was determined by comparing significance level and r^2.

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Graham, H. D. (1992). Stabilization of the Prussian blue color in the determination of polyphenols. Journal of Agricultural and Food Chemistry **40**: 801–805.

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Massey, F. P., & Hartley, S. E. (2009). Physical defences wear you down: progressive and

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Test for Heteroskedasticity. Econometrica **48**:817-838.

Section S2: Figures
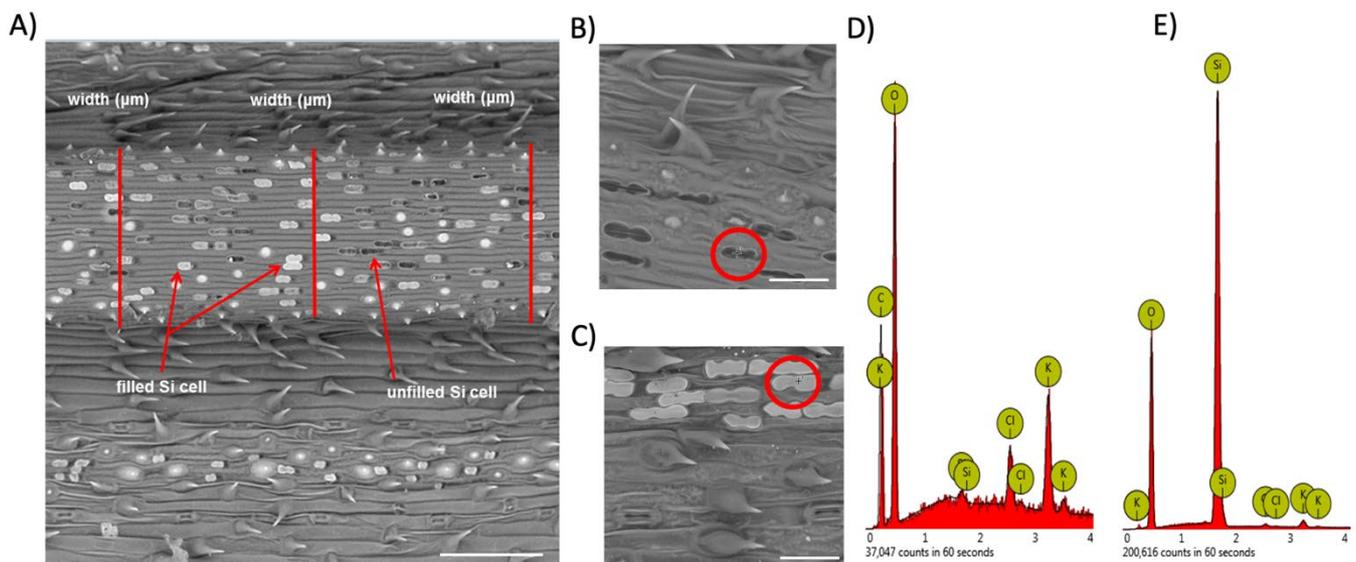

Figure S5-1. SEM micrographs of *Brachypodium distachyon* abaxial leaf surface A) indicating silicified (filled) and empty (unfilled) Si cells (500x; Scale bar = 100 μm), and close-ups (1000x; Scale bar = 30 μm) of B) an unfilled cell and C) a filled cell, along with the EDS analysis of the Si cell selected in the red circle confirming the absence (B, D) and presence (C, E) of Si.
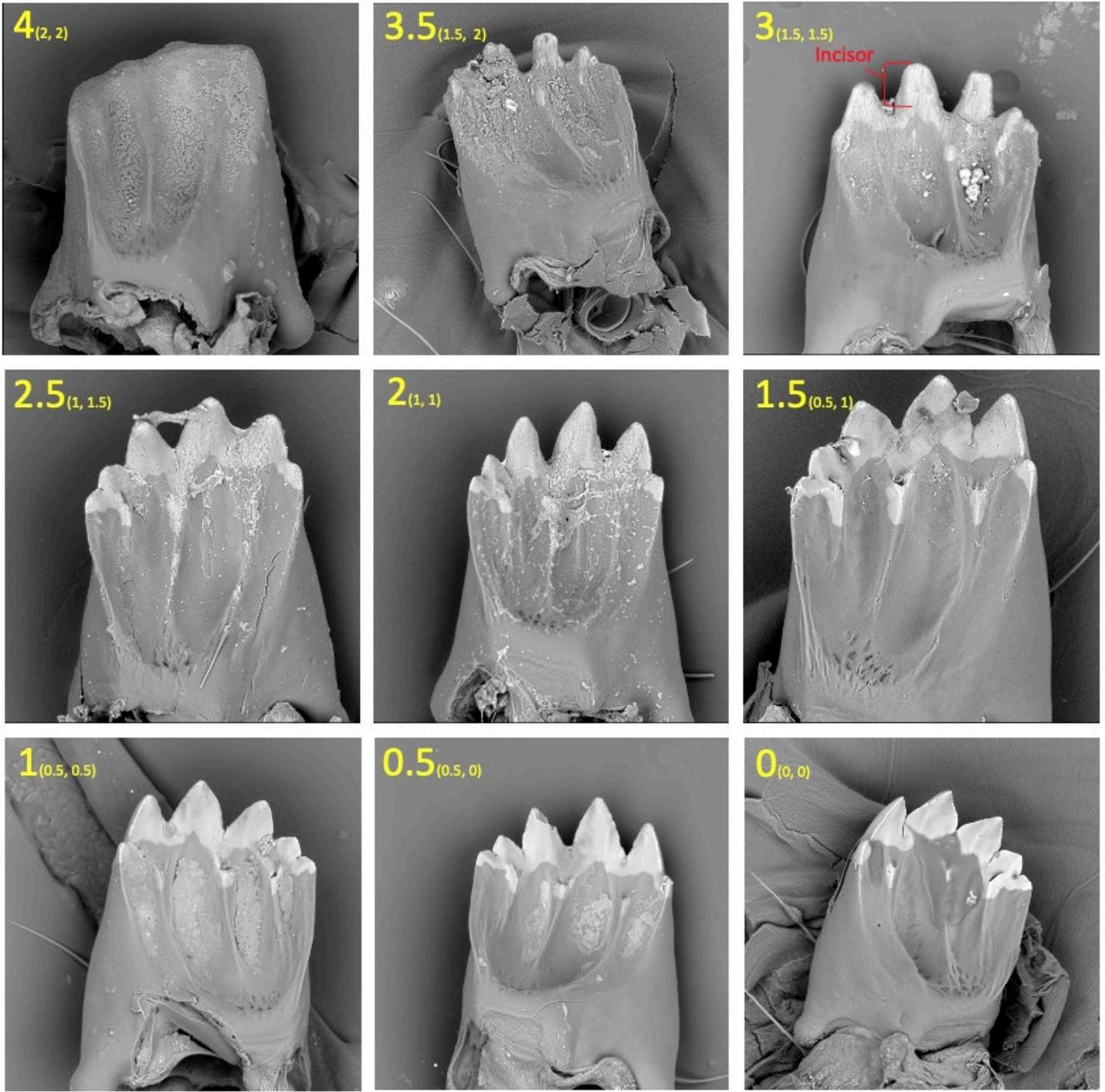
Figure S5-2. Example images of *Helicoverpa armigera* mandibles receiving each of the possible total scores for both incisor surface wear and pointedness. Large yellow numbers in the left-hand corner of each image indicate the overall score given to the mandible, and small yellow numbers in brackets indicate the score for each category (surface wear, pointedness).
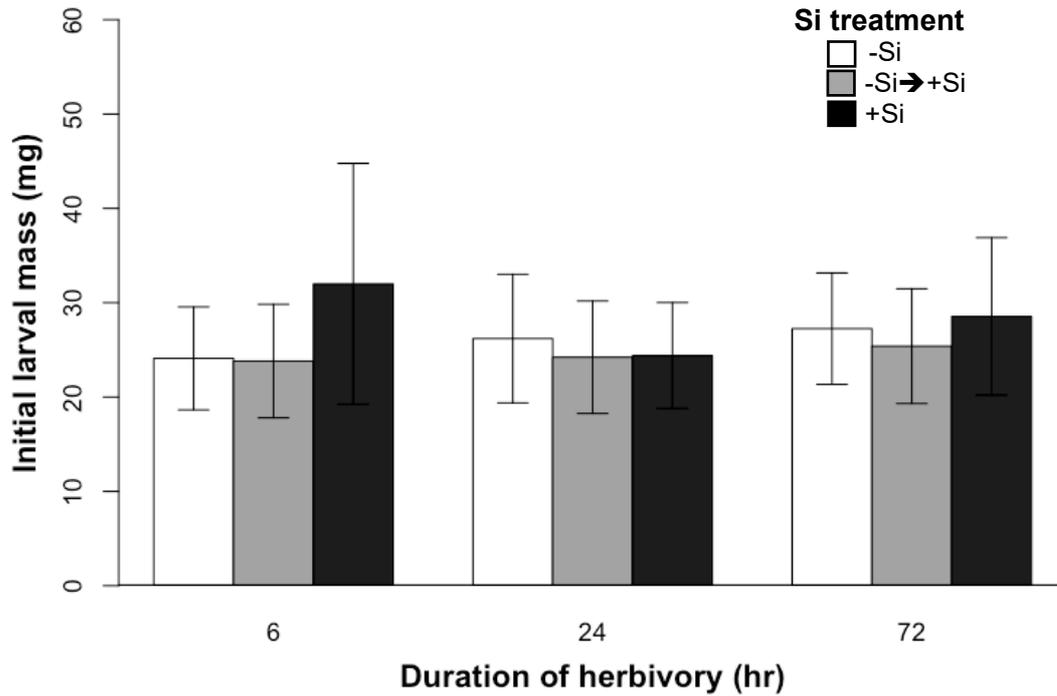
Figure S5-3. Initial larval masses (after starvation) of *Helicoverpa armigera* (HA) larvae used for each herbivory duration and Si treatment combination. Initial mass values were log-transformed to meet the assumptions of normality. No differences were determined between Si treatments and HA feeding durations ($F_{2,78} = 0.132$, $p = 0.877$ and $F_{2,78} = 0.129$, $p = 0.879$, respectively). There was also no interactive effect ($F_{4,78} = 0.050$, $p = 0.995$). -Si = plants not treated with Si, -Si➔+Si = plants only treated with Si once herbivory began and +Si = plants exposed to Si long term.

Figure S5-4. Total phenolics concentration A) 24 and 72 hr after *Helicoverpa armigera* herbivory began in herbivore-damaged *Brachypodium distachyon* leaves and B) in leaves of herbivore-free plants at 72 hr. Different letters above bars indicate significant differences between silicon (Si) treatments ($p < 0.05$). Asterisks indicate significant ANOVA results (. = $p < 0.1$, * = $p < 0.05$). -Si = plants not treated with Si, -Si➔+Si = plants only treated with Si once herbivory began and +Si = plants exposed to Si long term.
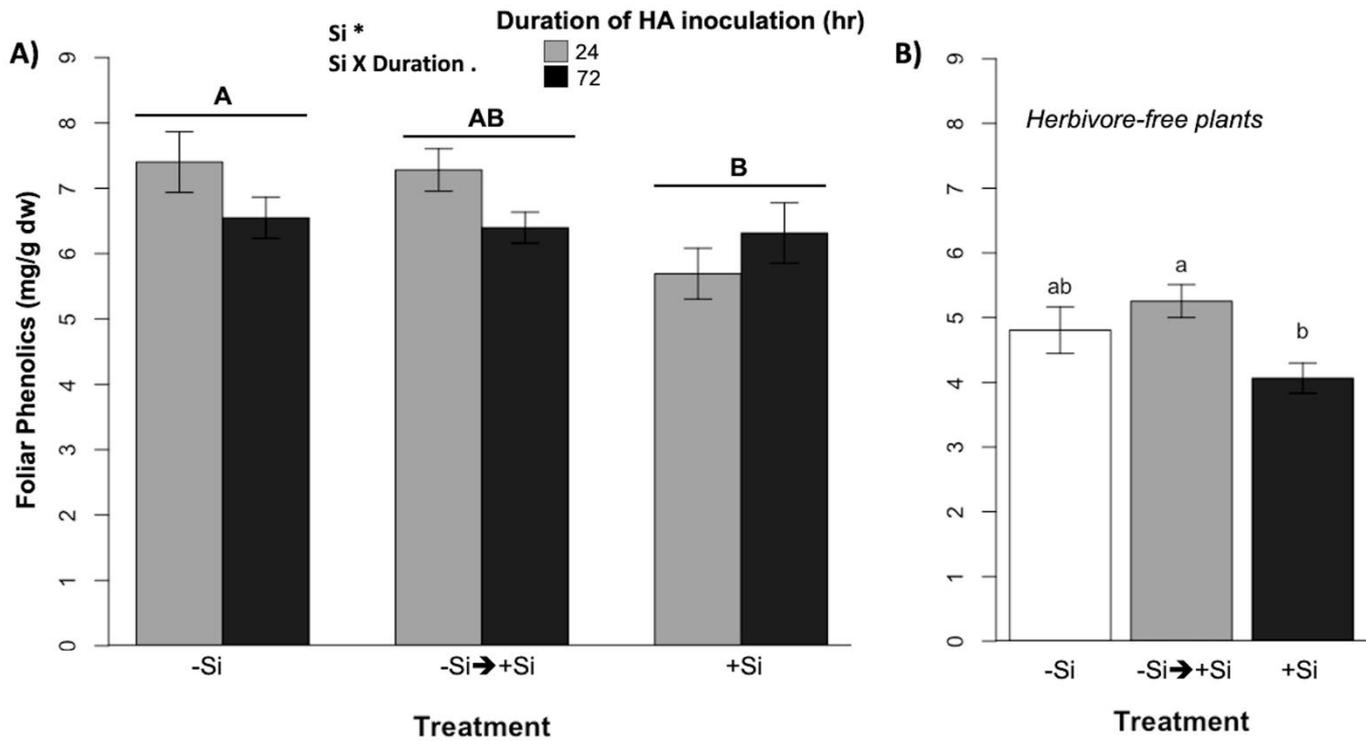
Appendix 4 – Chapter 6 Supplementary Information
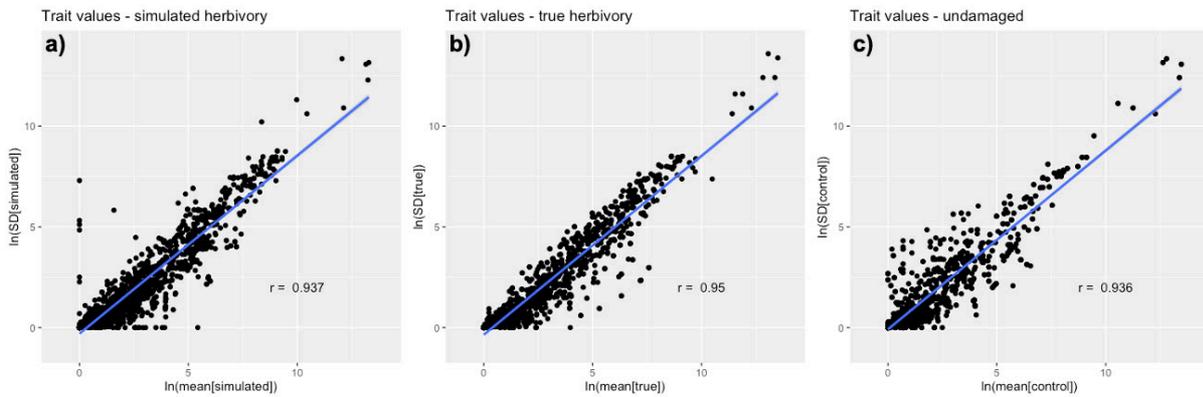
Figure S6-1. Relationship between the natural logarithm of standard deviation (SD) and mean for (a) simulated herbivory, (b) true herbivory and (c) undamaged controls.
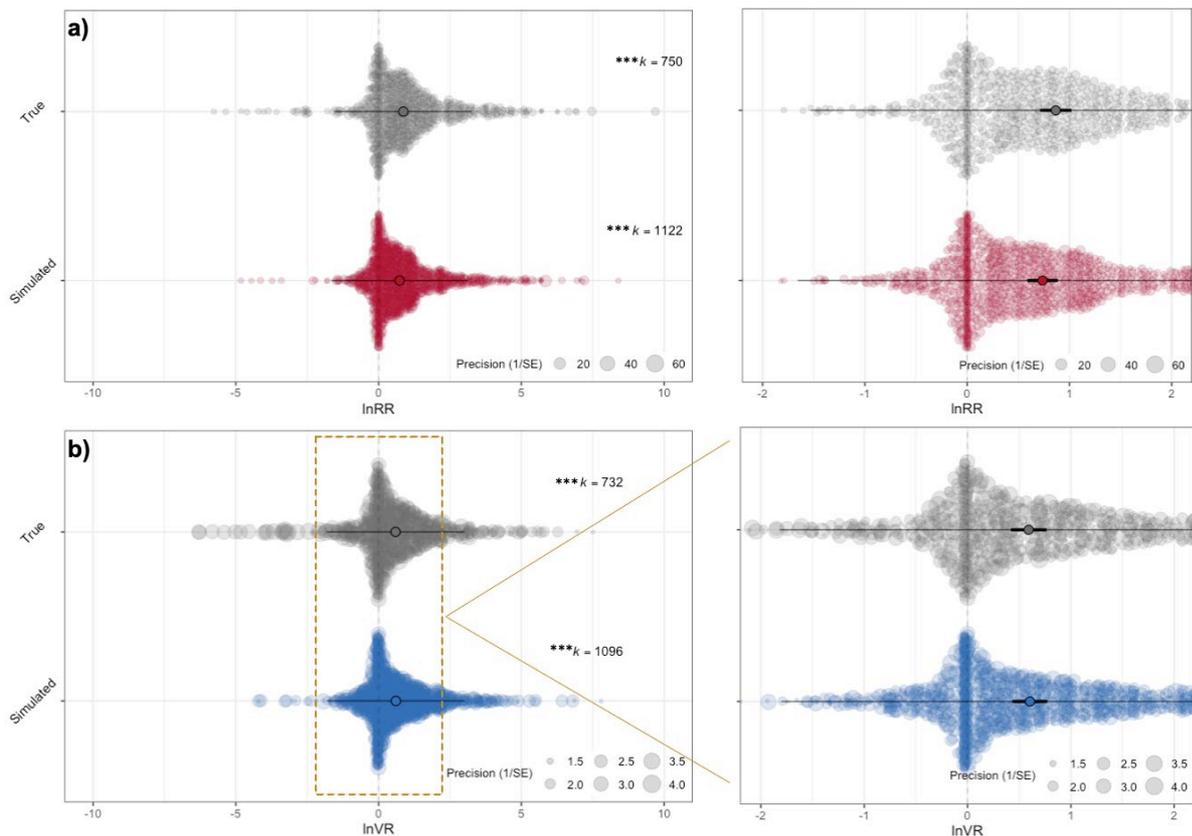
Figure S6-2. Natural logarithm of response ratio (lnRR; a) and natural logarithm of the ratio of the standard deviations (lnVR; b) for simulated herbivory or true herbivory in comparison to control (untreated) plants. Grey points represent raw data (i.e., individual observations, k) measured prior to 24 hr after treatments (both simulated and true herbivory) began and coloured points represent raw data measured ≥ 24 hr after treatments. Black-outlined coloured circles represent model estimates, thick black lines represent 95% confidence intervals (CIs) and thin black lined represent prediction intervals. Asterisks depict significance (i.e., a 95% CI that does not overlap 0): * = p < 0.05, *** = p < 0.001. Figures on the right-hand side depict the data presented in left-hand side images with effect sizes between -2 and 2.
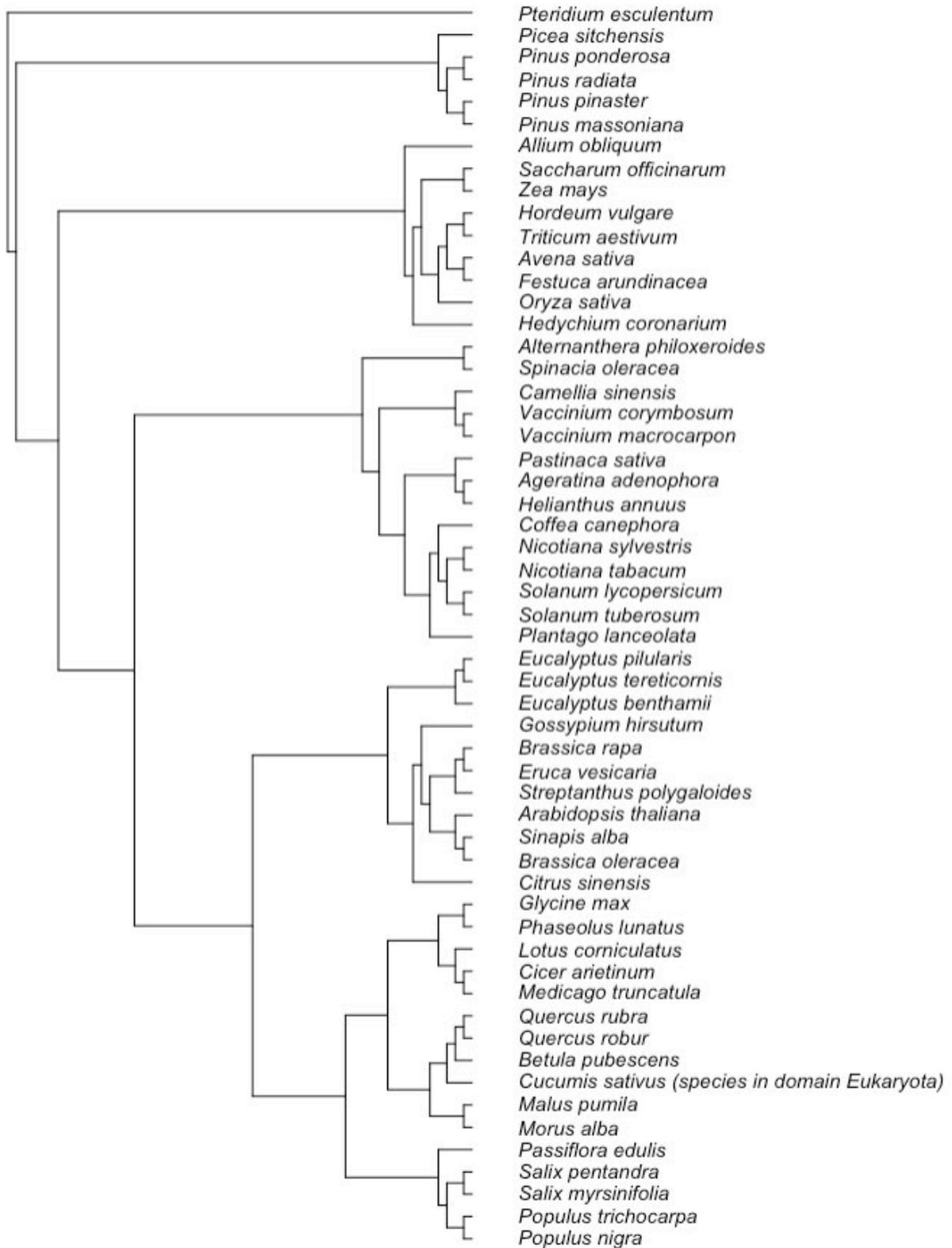
Figure S6-3. Phylogenetic tree of plant species from studies used for meta-analysis.
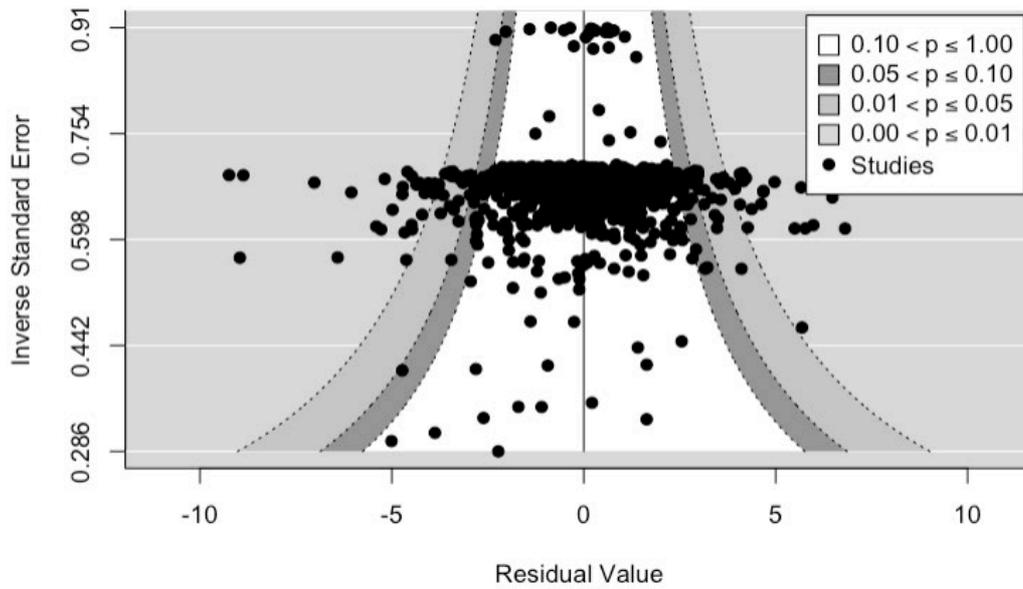
Figure S6-4. Funnel plot showing inverse SE as a function of Effect size (lnRR). Raw effect sizes are plotted against their precision (inverse of the square root of standard error). Funnel plots are useful to detect the presence of small-study effects, whereby studies with smaller sample sizes have larger effect sizes (Nakagawa et al. 2021). This can be indicative of publication bias; however, asymmetry can also result several other factors, such as heterogeneity. However, the funnel plot depicted is not clearly asymmetrical suggesting that both publication bias and heterogeneity are either minimal or not present at all.
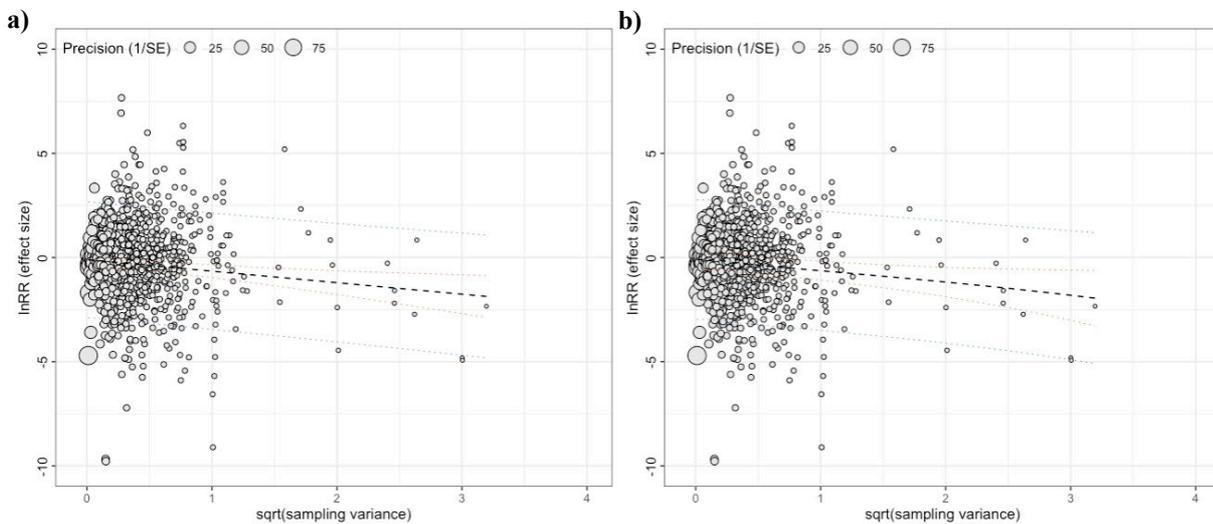
Figure S6-5. Egger regression plotting lnRR as a function of the sqrt of sampling variance for (a) univariate and (b) multivariate models. Estimates for both univariate and multivariate Egger regression were significant ($p = 0.001$ and 0.002, respectively), suggesting that there is in fact significant asymmetry in the funnel plot, perhaps due to publication bias.
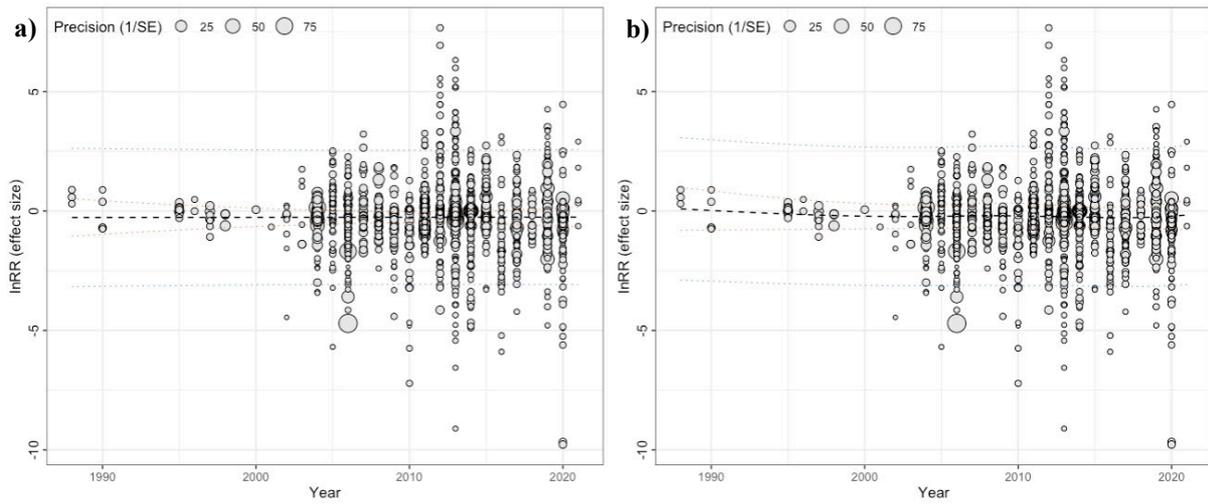
Figure S6-6. Time-lag bias showing lnRR as a function of year of publication for (a) univariate and (b) multivariate models. There was no effect of year of publication on effect sizes in either univariate or multivariate models ($p = 0.975$ and 0.675, respectively).

Table S6-1. Model outputs used to determine the optimal random effects structure for models based on Akaike's information criterion (AIC) scores

Random effects structure	I^2 (total)	AIC score
Phylogeny, Plant, Study, Item, Observation	0.9945	5432.47
Phylogeny, Study, Item, Observation	0.9944	5433.64
Plant, Study, Item, Observation	0.9945	5430.47
Study, Item, Observation	0.9944	5431.54

Table S6-2. Model outputs from mechanical wounding data only. Lower 95% confidence interval = L CI, Upper 95% confidence interval = U CI

Modifier	Level	LnRR Estimate	p-value	L CI	U CI	LnVR Estimate	p-value	L CI	U CI
	Lepidoptera	**-0.4814**	**0.0090**	**-0.8420**	**-0.1209**	-0.3111	0.0780	-0.6571	0.0350
	Coleoptera	0.1646	0.6769	-0.6108	0.9400	0.1314	0.7140	-0.5726	0.8353
Taxa	Hemiptera	**-0.8045**	**0.0144**	**-1.4481**	**-0.1609**	**-1.0526**	**0.0005**	**-1.6400**	**-0.4652**
	Hymenoptera	-0.5385	0.1630	-1.2957	0.2187	-0.6293	0.0850	-1.3456	0.0870
	Trombidiformes	-1.5165	0.0960	-3.3027	0.2698	-1.4300	0.0712	-2.9842	0.1241
	Enzyme	0.1465	0.7141	-0.6383	0.9312	-0.5586	0.1885	-1.3920	0.2748
	Gene	**-0.6150**	**0.0149**	**-1.1097**	**-0.1203**	-0.4444	0.0903	-0.9589	0.0700
Defence	Metabolite	0.0512	0.8614	-0.5241	0.6264	0.0362	0.9030	-0.5466	0.6189
	Phytohormone	-0.4653	0.4375	-1.6413	0.7108	-0.9469	0.1428	-2.2143	0.3205
	Early signal	-0.7648	0.5311	-3.1617	1.6322	-0.1140	0.9112	-2.1218	1.8938
	VOC	**-0.7041**	**0.0019**	**-1.1470**	**-0.2612**	-0.3903	0.0740	-0.8187	0.0380

Table S6-3. Model outputs from Lepidoptera data only. Lower 95% confidence interval = L CI, Upper 95% confidence interval = U CI

Modifier	Level	LnRR				LnVR			
		Estimate	*p-value*	*L CI*	*U CI*	*Estimate*	*p-value*	*L CI*	*U CI*
Tool	JA	<u>-0.2941</u>	<u>0.0702</u>	<u>-0.6125</u>	<u>0.0242</u>	-0.0699	0.6878	-0.4112	0.2714
	MecWorm	0.1180	0.5648	-0.2841	0.5201	-0.2845	0.1994	-0.7192	0.1502
	Needle	0.2625	0.1728	-0.1150	0.6400	**0.5915**	**0.0055**	**0.1744**	**1.0086**
	Elicitor + MW	-0.0752	0.6610	-0.4114	0.2611	-0.2423	0.1875	-0.6027	0.1181
	MW	**0.5017**	**0.0012**	**-0.8054**	**-0.1981**	**-0.3961**	**0.0153**	**-0.7160**	**-0.0761**
Defence	Enzyme	-0.3381	0.1774	-0.8298	0.1535	-0.2215	0.4117	-0.7507	0.3077
	Gene	**-0.5749**	**0.0006**	**-0.9014**	**-0.2485**	**-0.5582**	**0.0018**	**-0.9081**	**-0.2082**
	Metabolite	0.1447	0.5828	-0.3719	0.6613	0.2621	0.3516	-0.2897	0.8139
	Phytohormone	<u>-0.5796</u>	<u>0.0998</u>	<u>-1.2700</u>	<u>0.1107</u>	<u>-0.7161</u>	<u>0.0665</u>	<u>-1.4812</u>	<u>0.0489</u>
	Early signal	-0.8464	0.4310	-2.9542	1.2615	-0.6158	0.5353	-2.5638	1.3323
	VOC	-0.1456	0.4209	-0.5004	0.2092	0.0540	0.7689	-0.3063	0.4142

Table S6-4. Model outputs from Hemiptera data only. Lower 95% confidence interval = L CI, Upper 95% confidence interval = U CI

Modifier	Level	LnRR Estimate	p-value	L CI	U CI	LnVR Estimate	p-value	L CI	U CI
	JA	<u>-0.9370</u>	<u>0.0565</u>	<u>-1.8999</u>	<u>0.0260</u>	**-0.7868**	**0.0214**	**-1.4562**	**-0.1174**
	Needle	0.2191	0.6448	-0.7157	1.1539	0.3639	0.2418	-0.2470	0.9748
Tool	Elicitor + MW	0.7689	0.2661	-0.5897	2.1274	0.7298	0.2716	-0.5747	2.0343
	MW	-0.5354	0.3387	-1.6355	0.5647	-0.3030	0.4789	-1.1448	0.5387
	SA	<u>-0.9241</u>	<u>0.0624</u>	<u>-1.8965</u>	<u>0.0483</u>	**-0.7461**	**0.0332**	**-1.4323**	**-0.0599**
	Enzyme	**-1.5312**	**0.0404**	**-2.9949**	**-0.0675**	-0.7975	0.2149	-2.0608	0.4586
	Gene	-0.3124	0.6053	-1.5013	0.8766	0.0396	0.9325	-0.8811	0.9604
Defence	Metabolite	-0.0358	0.9552	-1.2879	1.2163	-0.0982	0.8459	-1.0922	0.8959
	Phytohormone	<u>1.0843</u>	<u>0.0909</u>	<u>-0.1740</u>	<u>2.3426</u>	0.4646	0.4084	-0.6404	1.5696
	VOC	-0.1633	0.8594	-1.9761	1.6496	-0.0509	0.9390	-1.3575	1.2558

Table S6-5. Model outputs from gene expression data only. Lower 95% confidence interval = L CI, Upper 95% confidence interval = U CI

Modifier	Level	LnRR				LnVR			
		Estimate	*p-value*	*L CI*	*U CI*	*Estimate*	*p-value*	*L CI*	*U CI*
	JA	**-0.5973**	**0.0150**	**-1.0544**	**-0.1402**	**-0.5359**	**0.0023**	**-0.8793**	**-0.1925**
	MecWorm	0.0290	0.9490	-0.8604	0.9184	-0.2804	0.5299	-1.1568	0.5959
Tool	Needle	0.3070	0.2298	-0.1945	0.8085	**0.4548**	**0.0235**	**0.0615**	**0.8481**
	Elicitor + MW	0.1471	0.6870	-0.5696	0.8638	-0.1834	0.6040	-0.8775	0.5107
	MW	**-0.8149**	**0.0006**	**-1.2803**	**-0.3494**	**-0.8125**	**< 0.0001**	**-1.2140**	**-0.4111**
	SA	-0.1224	0.7014	-0.7493	0.5044	-0.3526	0.2144	-0.9062	0.2010
	Lepidoptera	**-0.6302**	**0.0013**	**-1.0136**	**-0.2468**	**-0.4948**	**0.0006**	**-0.7781**	**-0.2115**
Taxa	Coleoptera	0.3871	0.4681	-0.6600	1.4342	0.3890	0.3891	-0.4976	1.2757
	Hemiptera	-0.0136	0.9546	-0.4820	0.4548	-0.1849	0.3395	-0.5648	0.1950

Table S6-6. Model outputs for volatile organic compound (VOC) data only. Lower 95% confidence interval = L CI, Upper 95% confidence interval = U CI

Modifier	Level	LnRR Estimate	p-value	L CI	U CI	LnVR Estimate	p-value	L CI	U CI
	JA	**1.0884**	**0.0001**	**0.5367**	**1.5402**	**1.1243**	**< 0.0001**	**0.6029**	**1.6457**
	MecWorm	-0.0959	0.7433	-0.6709	0.4790	-0.3918	0.1560	-0.9334	0.1498
Tool	Needle	-0.3802	0.2104	-0.9755	0.2152	_-0.4724_	_0.0971_	_-1.0306_	_0.0859_
	Elicitor + MW	_-0.4081_	_0.0922_	_-0.8833_	_0.0671_	_-0.3935_	_0.0834_	_-0.8392_	_0.0522_
	MW	**-0.6885**	**0.0018**	**-1.1204**	**-0.2566**	_-0.3915_	_0.0558_	_-0.7927_	_0.0098_
	Lepidoptera	-0.2483	0.3284	-0.7467	0.2501	-0.0191	0.9336	-0.4683	0.4301
	Coleoptera	0.1420	0.8255	-1.1217	1.4056	0.2303	0.6867	-0.8902	1.3507
Taxa	Hemiptera	_-0.7882_	_0.0684_	_-1.6359_	_0.0596_	**-1.0165**	**0.0080**	**-1.7668**	**-0.2662**
	Hymenoptera	**-1.2642**	**0.0012**	**-2.0252**	**-0.5032**	**-1.4762**	**< 0.0001**	**-2.1733**	**-0.7790**
	Trombidiformes	_-1.6413_	_0.1000_	_-3.5977_	_0.3151_	_-1.5059_	_0.0897_	_-3.2457_	_0.2338_

List of Publications

Waterman, J.M., Cibils-Stewart, X., Cazzonelli, C.I., Hartley, S.E., Johnson, S.N. (2021). Short-term exposure to silicon rapidly enhances plant resistance to herbivory. *Ecology*. e03438. LINK

Johnson, S.N., **Waterman, J.M.**, Wuher. R., Rowe, R.C., Hall, C.R., Cibils-Stewart, X. (2021). Siliceous but not nutritious: nitrogen limitation increases anti-herbivore silicon defences in a model grass. *Journal of Ecology*. DOI: 10.1111/1365-2745.13755. LINK

Waterman, J.M., Hall, C.R., Mikhael, M., Cazzonelli, C.I., Hartley, S.E., Johnson, S.N. (2021). Short-term resistance that persists: Silicon anti-herbivore defence is rapidly induced and affects carbon-based defence responses. *Functional Ecology*. **35**: 82–92. LINK

Waterman, J.M., D'Amato, A.W., Foster, D.R., Orwig, D.A., Pederson, N. (2020). Historic forest composition and structure across an old-growth landscape in New Hampshire, USA. *The Journal of the Torrey Botanical Society*. **147**: 291-303 LINK

Waterman, J.M., Mann, T.J., Cazzonelli. C.I., Hartley, S.E., Johnson, S.N. (2020). Microbes in *Helicoverpa armigera* oral secretions contribute to increased senescence around plant wounds. *Ecological Entomology*. **45**: 1224-1229. LINK

Johnson, S.N., **Waterman, J.M.,** Hall, C.R. (2020). Increased insect herbivore development under elevated CO_2 is associated with lower plant defence signaling and minimal declines in nutritional quality. *Scientific Reports*. **10**: 14553, doi:10.1038/s41598-020-70823-3. LINK

Hall, C.R., Dagg, V., **Waterman, J.M.**, Johnson, S.N. (2020). Silicon alters leaf morphology and suppresses insect herbivory in a model grass species. *Plants.* **9**: 643. LINK

Waterman, J.M., Cazzonelli, C.I., Hartley, S.E., Johnson, S.N. (2019). Simulated herbivory: the key to disentangling plant defence mechanisms. *Trends in Ecology & Evolution.* **34**: 447-458. LINK

Hall, C.R., **Waterman, J.M.**, Vandegeer, R.K., Hartley, S.E., Johnson, S.N. (2019). The role of silicon in anti-herbivore phytohormonal signaling. *Frontiers in Plant Science*. **10**:1131. LINK