

Try it with fire and lime: phytochemical responses to prescribed fire, soil amendments, and simulated herbivory

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Abstract

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Phylogenetic defensive compounds mediate important multitrophic interactions in terrestrial ecosystems, yet we have limited understanding of how the methods used to restore and maintain degraded ecosystems influence subsequent ecosystem chemical ecology. To elucidate the chemical ecology of applied ecological restoration management techniques and phytochemical mechanisms of biotic interactions, I carried out an observational and an experimental study in a restored grassland. In my observational study, I investigated the effects of prescribed fire regime (quantity of historical burns and time since burn) on plant defensive chemistry. In my experimental study, I tested the effects of simulated herbivory, prescribed burning, and fast-acting soil lime on plant defensive chemistry at three phenological time steps. I tested foliar tissues across two growing seasons in two perennial forbs: (1) *Castilleja levisecta* (observational and experimental study), a hemiparasite Pacific Northwest native that produces the defensive iridoid glycosides aucubin, catalpol, macafadienoside, and, putatively, methyl shanzhiside; and (2) *Plantago lanceolata* (experimental study only), a European exotic that produces the defensive iridoid glycosides aucubin and catalpol. In my observational study, quantity of historical burns was a significant factor for *C. levisecta* iridoid glycoside concentrations. Total

iridoid glycoside concentrations were negatively related to quantity of historical burns. Time since most recent burn was not a significant factor for total iridoid glycosides. In my experimental study, unique patterns emerged in response to simulated herbivory for one constituent iridoid glycoside in each of my two plant species. A prescribed autumn burn had no effect on *C. levisecta* iridoid glycoside concentrations, but had a lessening effect on foliar iridoid glycoside concentrations in *P. lanceolata*. Phenology significantly modified some of these patterns. Fast-acting soil lime had no significant short-term effect on iridoid glycoside concentrations in either species. Understanding how these landscape scale land management techniques interact with phenology and biotic factors to affect plant defensive chemistry is crucial to developing restoration and management plans rooted in sound chemical ecology theory. My research confirms that environmental factors, phenology, and land management techniques have the potential to create important chemical legacies that should be planned and monitored alongside other response variables in long-term terrestrial restoration projects.

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“Tell me of what plant-birthday a man takes notice, and I shall tell you a good deal about his vocation, his hobbies, his hay fever, and the general level of his ecological education.”

-Aldo Leopold

Chapter One: Background and overview

This thesis is about applications of chemical ecology to ecological restoration. Two main chapters cover an observational and an experimental study investigating how restored grassland landscape scale management techniques and simulated herbivory affect defensive chemistry in plant species central to species recovery efforts. Both chapters aim to bridge gaps between chemical ecology research and applied ecological restoration, and to advance chemical ecology theory while also answering practical questions related to ecological restoration and land management. I use as my study system two perennial angiosperms central to species recovery efforts in this system: the threatened hemiparasitic *Castilleja levisecta*, and the European exotic *Plantago lanceolata*. Both species produce iridoid glycosides, defensive compounds that are sequestered and utilized by larvae of the endangered Taylor's checkerspot butterfly (Haan et al., 2017). In this thesis I assess the foliar concentrations of constituent iridoid glycosides (aucubin, catalpol, macfadienoside, and methyl shanzhiside¹) across two growing seasons in response to prescribed burning, soil lime amendments, and simulated herbivory. My results provide novel insight into the phytochemical ramifications of grassland restoration and management techniques.

¹ The identity of this iridoid glycoside has not been confirmed. Throughout this chapter, I use the term methyl shanzhiside to refer to putative methyl shanzhiside. See full explanation in the "Sample preparation and analysis" sections of Chapters Two and Three.

Background

Ecological restoration is defined by the Society for Ecological Restoration as “an intentional activity that initiates or accelerates the recovery of an ecosystem with respect to its health, integrity, and sustainability.” Ecosystems requiring restoration are those that have been degraded, transformed, or even destroyed by anthropogenic factors (SER, 2002). If our reason for restoring a given ecosystem is to provide the ecosystem infrastructure needed to aid the recovery of threatened and endangered species, we must have effective and responsive protocols to achieve complex restoration goals and benchmarks.

In designing management and restoration plans, restoration practitioners often strategize to reintroduce ecosystem functional and structural attributes (SER, 2002). Collecting data to monitor progress toward these goals is important, and may focus on the collection of tangible data. Biodiversity and ecosystem structure are, after all, concrete variables we can measure with relative ease. Trees, grasses, mammals, and insects can be seen, identified, tallied, and assessed with the human eye and hand, and are therefore easily written into restoration design and monitoring plans, as well as into subsequent management protocols. It is the more elusive ecosystem components that tend to be glossed over—the crustose lichens too cryptic to identify with certainty, the hidden endophytes that confer traits once ascribed to phylogenetic origins (Ludwig-Müller, 2015), or the complex biochemical pathways mediating interactions between organisms of disparate phylogenetic kingdoms (Haan et al., 2017).

Chemical ecology, as applied to ecological restoration, is a more neglected area of research, in part because it is both challenging and expensive to assess the abundances and long-term patterns

of ecosystem phytochemicals (Hunter, 2016). However, overcoming these barriers to deepening our understanding is important, as phytochemicals play crucial roles in driving ecological feedback loops and evolutionary processes through their effects on trophic interactions, nutrient cycling, seed germination, and other ecological dynamics (Covelo and Gallardo, 2004; Dietz, 2013; Hunter, 2016). Ecological restoration practices that are informed by sound chemical ecology principles will help researchers and land managers make informed predictions about future ecological functions mediated by phytochemistry (Hunter, 2016).

Just as 19th century botanist Asa Gray urged scientists of his day in a Letter to the Editor of the Torrey Botanical Club Bulletin to “make haste more slowly” in their conclusions about genera and species identity (Harris and Ladd, 2008), my research urges us to take a step back and reconsider what we hope to achieve when we design, implement, and monitor ecological restoration projects. To date, restoration and management protocols typically do not include clear goals and benchmarks for chemical ecological function. However, the need to do so is clear when endangered species depend upon essential phytochemical processes (Haan et al., 2017). Techniques used in management of restored ecosystems that alter soil nutrients available to plants or soil pH, such as prescribed burning or applications of lime, may have lasting phytochemical legacies that land managers will want to keep in mind (Gibson, 2009; Matyssek, 2012).

The objective in my research was to investigate some of the abiotic and biotic mechanisms driving phytochemical dynamics. I focused on the iridoid glycoside defensive compound class.

Iridoid glycosides

Iridoid glycosides are cyclopentanoid monoterpene-derived plant defensive compounds (Bowers, 1991). Globally, there are over 600 different iridoid glycosides from 57 different plant families, primarily within the order Lamiales (Bowers, 1991; Yamane et al., 2010). In plants, iridoid glycosides are synthesized via the mevalonic acid pathway, where they are derived from 10-hydroxygeraniol, via epi-iridodial and epi-deoxy-loganic acid (Inouye and Uesato, 1986; Jensen, 1992). Although some specialist insects have evolved to tolerate and even rely on iridoid glycosides, these compounds are toxic to most generalist insects, and have a denaturing effect on nucleic acids, amino acids, and proteins (Baden, 2016; Bowers, 1991).

Understanding the impact of landscape scale management techniques on iridoid glycosides is important to restoration and species recovery efforts. For example, these phytochemicals are relied upon by the larvae of Taylor's checkerspot (*Euphydryas editha taylori*) butterflies, which sequester these compounds from plant leaf tissues (Haan et al., 2017). Land managers need to be aware of the potential consequences of their management actions on iridoid, since adult female butterflies who use iridoid glycosides as a cue for oviposition tend to select oviposition plants that are higher in iridoid glycoside content (Prudic et al., 2005). Moreover, variation in iridoid glycoside concentrations may have fitness consequences for specialist checkerspot larvae that sequester iridoid glycosides for their own defense (Haan et al., 2017; Prudic et al., 2005).

The mechanisms by which ecosystem management tools might affect iridoid glycoside concentrations depend on which environmental factors management tools affect. Prescribed burning regimes, for example, have a profound impact on the nutrient dynamics that influence phytochemistry. Following a prescribed fire, terrestrial NH_4^+ pools can nearly double, creating a

“nutrient pulse”, after which they gradually decline to pre-fire levels over the course of a year (Gibson, 2009; Wan et al., 2001). NO_3^- levels also increase after a fire, but on somewhat delayed schedule compared to NH_4^+ , peaking within six months to a year after a prescribed burn before declining (Wan et al., 2001). Frequent burning depletes ecosystem nitrogen pools, as nitrogen is depleted more rapidly by fire-induced volatilization than it can be replaced (Gibson, 2009; Ojima et al. 1990; Wan et al., 2001). Although nitrogen is not a direct component of iridoid glycoside molecular structure, it may be indirectly important to resource allocation dynamics in general defensive compound metabolism (Matyssek, 2012)

I wanted to learn whether prescribed burning and lime amendments—two landscape scale treatments land managers might apply to achieve various management goals—might also influence iridoid glycoside concentrations. I addressed the following research questions in my observational and experimental studies: (1) Does time since most recent burn have an effect on foliar iridoid glycoside concentrations? (2) Does quantity of historical burns have an effect on foliar iridoid glycoside concentrations? (3) Do soil lime amendments affect foliar iridoid glycoside concentrations in *Castilleja levisecta* and *Plantago lanceolata*? To better understand temporal dynamics and interactions between herbivores and iridoid glycoside producing plants, I also addressed the following research questions: (4) Does simulated herbivory have a direct effect on foliar iridoid glycoside concentrations in *Castilleja levisecta* and *Plantago lanceolata*? (5) Do iridoid glycoside responses to simulated herbivory differ across phenology?

In Chapter Two I present an observational study that assesses the direct effects of prescribed fire regime on foliar iridoid glycoside concentrations in a restored grassland. I show that prescribed fire regime has an especially noteworthy influence on concentrations of foliar iridoid glycosides.

Chapter Three covers an experimental study in which I elucidate the effects of simulated herbivory, autumn prescribed fire, and soil lime amendment across three phenological time steps. This study reveals how complex environmental and land management factors interact with phenology to produce variation in foliar iridoid glycoside patterns.

The findings of these two studies are synthesized as a launching point for future research in Chapter Four, where I propose future topics of research and recommendations for land managers who wish to consider phytochemical effects of landscape scale management techniques.

Note to readers

Chapters Two and Three are presented in manuscript form for the sake of proper formatting for submission to peer-reviewed scientific journals. Thus, there is redundancy between the two chapters, as they are meant for eventual publication as standalone articles.

Works Cited

- Baden, C.U. (2016). *Phylogeny and sequestration of iridoid glycosides in selected genera of the Mecininae (Coleoptera, Curculionidae) with particular focus on their host plant relationship* (Doctoral Dissertation).
- Baer, S. G., Blair, J. M., Collins, S. L., & Knapp, A. K. (2003). Soil resources regulate productivity and diversity in newly established tallgrass prairie. *Ecology*, 84(3), 724-735.
- Bowers, M.D. (1991). Iridoid glycosides. In: Rosenthal, G.A., & Berenbaum, M.R. [eds.] *Herbivores: their interactions with secondary plant metabolites*. Second Edition, Vol. 1: the chemical participants. Academic Press, Sandiego, CA.
- Covelo, F., & Gallardo, A. (2004). Green and senescent leaf phenolics showed spatial autocorrelation in a *Quercus robur* population in northwestern Spain. *Plant and Soil*, 259(1-2), 267-276.
- Dietz, M., Machill, S., Hoffmann, H. C., & Schmidtke, K. (2013). Inhibitory effects of *Plantago lanceolata* L. on soil N mineralization. *Plant and Soil*, 368(1-2), 445-458.
- Fajer, E. D., Bowers, M. D., & Bazzaz, F. A. (1992). The effect of nutrients and enriched CO₂ environments on production of carbon-based allelochemicals in *Plantago*: a test of the carbon/nutrient balance hypothesis. *The American Naturalist*, 140(4), 707-723.
- Gibson, D. J. (2009). *Grasses and grassland ecology*. Oxford University Press.
- Haan, N. L., Bakker, J. D., & Bowers, M. D. (2017). Hemiparasites can transmit indirect effects from their host plants to herbivores. *Ecology*.
- Harris, R. C., & Ladd, D. (2008). The lichen genus *Chrysothrix* in the Ozark ecoregion, including a preliminary treatment for eastern and central North America. *Opuscula Philolichenum*, 5, 29-42.
- Hunter, M. D. (2016). *The Phytochemical Landscape: Linking Trophic Interactions and Nutrient Dynamics*. Princeton University Press.
- Inouye, H., & Uesato, S. (1986). Biosynthesis of iridoids and secoiridoids. In *Fortschritte der Chemie Organischer Naturstoffe/Progress in the Chemistry of Organic Natural Products* (pp. 169-236). Springer Vienna.
- Jamieson, M. A., Quintero, C., & Blumenthal, D. M. (2013). Interactive effects of simulated nitrogen deposition and altered precipitation patterns on plant allelochemical concentrations. *Journal of Chemical Ecology*, 39(9), 1204-1208.

Jensen, S. R. (1992). Systematic implications of the distribution of iridoids and other chemical compounds in the Loganiaceae and other families of the Asteridae. *Annals of the Missouri Botanical Garden*, 284-302.

Ludwig-Müller, J. (2015). Plants and endophytes: equal partners in secondary metabolite production?. *Biotechnology Letters*, 37(7), 1325-1334.

Miller, J. O. (2016). *Soil PH and Nutrient Availability*. UME. FS-1054.

Mohan, J. E., Ziska, L. H., Schlesinger, W. H., Thomas, R. B., Sicher, R. C., George, K., & Clark, J. S. (2006). Biomass and toxicity responses of poison ivy (*Toxicodendron radicans*) to elevated atmospheric CO₂. *Proceedings of the National Academy of Sciences*, 103(24), 9086-9089.

Prudic, K. L., Oliver, J. C., & Bowers, M. D. (2005). Soil nutrient effects on oviposition preference, larval performance, and chemical defense of a specialist insect herbivore. *Oecologia*, 143(4), 578-587.

Society for Ecological Restoration Science & Policy Working Group. (2002). *The SER Primer on Ecological Restoration*. www.ser.org/. Accessed Dec. 10, 2017.

Wan, S., Hui, D., & Luo, Y. (2001). Fire effects on nitrogen pools and dynamics in terrestrial ecosystems: A meta-analysis. *Ecological Applications*, 11(5), 1349-1365.

Chapter Two: Sources of variation in *Castilleja levisecta* foliar iridoid glycoside concentrations: an observational study of restored grassland management techniques

Abstract

Defensive compounds derived from plants mediate important multitrophic interactions in terrestrial ecosystems, yet we have limited understanding of how the methods used to maintain restored ecosystems influence subsequent autotroph chemical ecology. To build upon growing interest in phytochemistry as an important consideration for applied ecological restoration, I investigated the effects of quantity of historical burns and time since burn on plant defensive chemistry in a grassland restoration experiment. I measured the concentration of foliar iridoid glycosides (aucubin, catalpol, macfadienoside, and, putatively, methyl shanzhiside) across two growing seasons in the perennial hemiparasite *Castilleja levisecta*. Quantity of historical burns was negatively related to total iridoid glycoside concentration. Time since most recent burn was not a significant factor for total iridoid glycoside concentrations. Foliar concentrations of constituent iridoid glycosides responded differently in magnitude to quantity of historical burns and time since most recent burn. Aucubin, macfadienoside, and methyl shanzhiside concentrations were negatively related to quantity of historical burns, with stronger effects seen in the 2016 growing season. Time since most recent burn was a marginally significant factor for methyl shanzhiside concentrations, which showed a slight increase in the year following a prescribed burn. These results confirm that methods used to maintain restored ecosystems have the potential to create important chemical legacies that should be monitored long-term alongside other measures of restoration success.

Key Words

Grassland restoration, iridoid glycosides, fire ecology, chemical ecology, ecological legacies

Introduction

Restoration of ecosystems degraded by anthropogenic activity has become an increasingly important priority of applied ecology (Benayas et al., 2009; SER, 2002). Restored habitat is the crucial infrastructure that reinstates ecosystem services and makes success in species recovery efforts possible (Benayas et al., 2009). In terrestrial restoration projects, researchers and land managers use iterative design approaches to fine tune protocols for meeting restoration benchmarks (Nassauer and Opdam, 2008). Effective monitoring of terrestrial restoration projects is necessary to assess progress toward goals of ecological function and to shape adaptive management strategies to support those goals (Benayas et al., 2009; SER, 2002). Assessing the complex ecological legacies of commonly used restoration and management techniques by applying theories from many ecological disciplines can aid the iterative design process, enabling land managers to achieve long-term goals of species recovery, ecosystem resilience, and ecological function (Benayas et al., 2009; Nassauer and Opdam, 2008; SER, 2002).

Assessing chemical ecological ramifications of restoration and management practices is not a typical component of monitoring and adaptive management strategies. Because chemical ecology aims to elucidate the biochemical mechanisms underlying multitrophic interactions (Hunter, 2016), it has great potential as a powerful tool in applied ecological restoration.

Chemical ecology as applied to ecological restoration is a neglected area of research, in part because it is both challenging and expensive to assess the abundances and long-term patterns of ecosystem phytochemicals (Hunter, 2016). However, overcoming these barriers to deepening

our understanding is important, as phytochemicals play crucial roles in driving ecological feedback loops and evolutionary processes through their effects on trophic interactions, nutrient cycling, seed germination, and other ecological dynamics (Covelo and Gallardo, 2004; Dietz, 2013; Hunter, 2016). Ecological restoration practices that are informed by sound chemical ecology principles will help researchers and land managers make informed predictions about future ecological functions mediated by phytochemistry (Hunter, 2016).

Prescribed burning is one example of an applied ecological restoration tool that has profound impacts on chemical ecology through its impacts on environmental factors such as nutrient cycling (Gibson, 2009; Wan et al., 2001). Following a prescribed fire, terrestrial NH_4^+ pools can nearly double, creating a “nutrient pulse”, after which they gradually decline to pre-fire levels over the course of a year (Gibson, 2009; Wan et al., 2001). NO_3^- levels also increase after a fire, but on somewhat delayed schedule compared to NH_4^+ , peaking within six months to a year after a prescribed burn before declining (Wan et al., 2001). Frequent burning depletes ecosystem nitrogen pools, as nitrogen is depleted more rapidly by fire-induced volatilization than it can be replaced, and this has important consequences phytochemistry (Gibson, 2009; Ojima et al. 1990; Wan et al., 2001). However, most monitoring of prescribed burn effects on grassland restoration experiments entails collecting data on soil qualities, species diversity, and presence of invasive species. There is no standard set of protocols for assessing the effects of prescribed burns on chemical ecological function, despite its importance to the multitrophic interactions of threatened and endangered species.

An example of an ecologically important phytochemical that is found in grasslands where prescribed burns are an ecological restoration tool are iridoid glycosides. Iridoid glycosides are cyclopentanoid monoterpene-derived plant defensive compounds (Bowers, 1991). Globally,

there are over 600 different iridoid glycosides from 57 different plant families, primarily within the order Lamiales (Bowers, 1991; Yamane et al., 2010). In plants, iridoid glycosides are synthesized via the mevalonic acid pathway, where they are derived from 10-hydroxygeraniol, via epi-iridodial and epi-deoxy-loganic acid (Inouye and Uesato, 1986; Jensen, 1992). Although some specialist insects have evolved to tolerate and even rely on iridoid glycosides, these compounds are toxic to most generalist insects, and have a denaturing effect on nucleic acids, amino acids, and proteins (Baden, 2016; Bowers, 1991). Iridoid glycosides play multiple important roles in terrestrial ecological communities, from conferring defensive compounds to endangered insects (Bowers, 1991) to influencing soil nutrient cycling and seed germination (Adam et al., 1979; Dietz et al., 2013). Developing a set of protocols for monitoring iridoid glycoside ecology in terrestrial restoration projects would provide helpful insight for adaptive restoration design and the use of management techniques such as prescribed fire.

Prescribed fire has the potential to alter the environmental factors that influence plant iridoid glycoside metabolism (Gibson, 2009; Matyssek, 2012; Wan, 2001). Following a prescribed fire, terrestrial NH_4^+ pools can nearly double, creating a “nutrient pulse”, after which they gradually decline to pre-fire levels over the course of a year (Gibson, 2009; Wan et al., 2001). NO_3^- levels also increase after a fire, but on somewhat delayed schedule compared to NH_4^+ , peaking within six months to a year after a prescribed burn before declining (Wan et al., 2001). This might lead us to expect a reduction in iridoid glycoside concentrations in the months after a prescribed burn, since increased soil nitrogen has been found to lower foliar iridoid glycoside concentrations (Jamieson, 2013; Prudic et al., 2005). In the long run, frequent burning depletes ecosystem nitrogen pools, as nitrogen is depleted more rapidly by fire-induced volatilization than it can be replaced (Gibson, 2009; Ojima et al. 1990; Wan et al., 2001). We might then expect higher

iridoid glycoside concentrations in plants growing in frequently burned environments, since plants growing in low nutrient conditions have been found to have higher concentrations of carbon-based allelochemicals, such as iridoid glycosides (Fajer et al., 1992).

Research objectives

My objective in this study was to investigate the effects of prescribed burning on plant defensive chemistry, focusing specifically on foliar iridoid glycoside concentrations in *Castilleja levisecta*. Special attention was given to macfadienoside, since it is the most dominant iridoid glycoside in this species at this site. I addressed two research questions: (1) Does time since most recent burn have an effect on foliar iridoid glycoside concentrations? (2) Does quantity of historical burns have an effect on foliar iridoid glycoside concentrations?

I hypothesized that prescribed burning would decrease iridoid glycoside concentrations in the first year after a fire. I predicted that plants growing in plots with a high quantity of historical prescribed burns would have lower foliar iridoid glycoside concentrations than plants growing in plots with a low quantity of historical prescribed burns.

Methods

Study sites and system

This research occurred at the Black River-Mima Prairie-Glacial Heritage Preserve in glacial outwash lowlands near the Puget Sound. My experiment was set up on the western edge of the 459-ha preserve (46.8712° Latitude, -123.0529° Longitude), on a section of abandoned

agricultural land with Nisqually loamy fine sand soil type, which has been restored over the past decade to native grassland (Bakker et al., 2013).

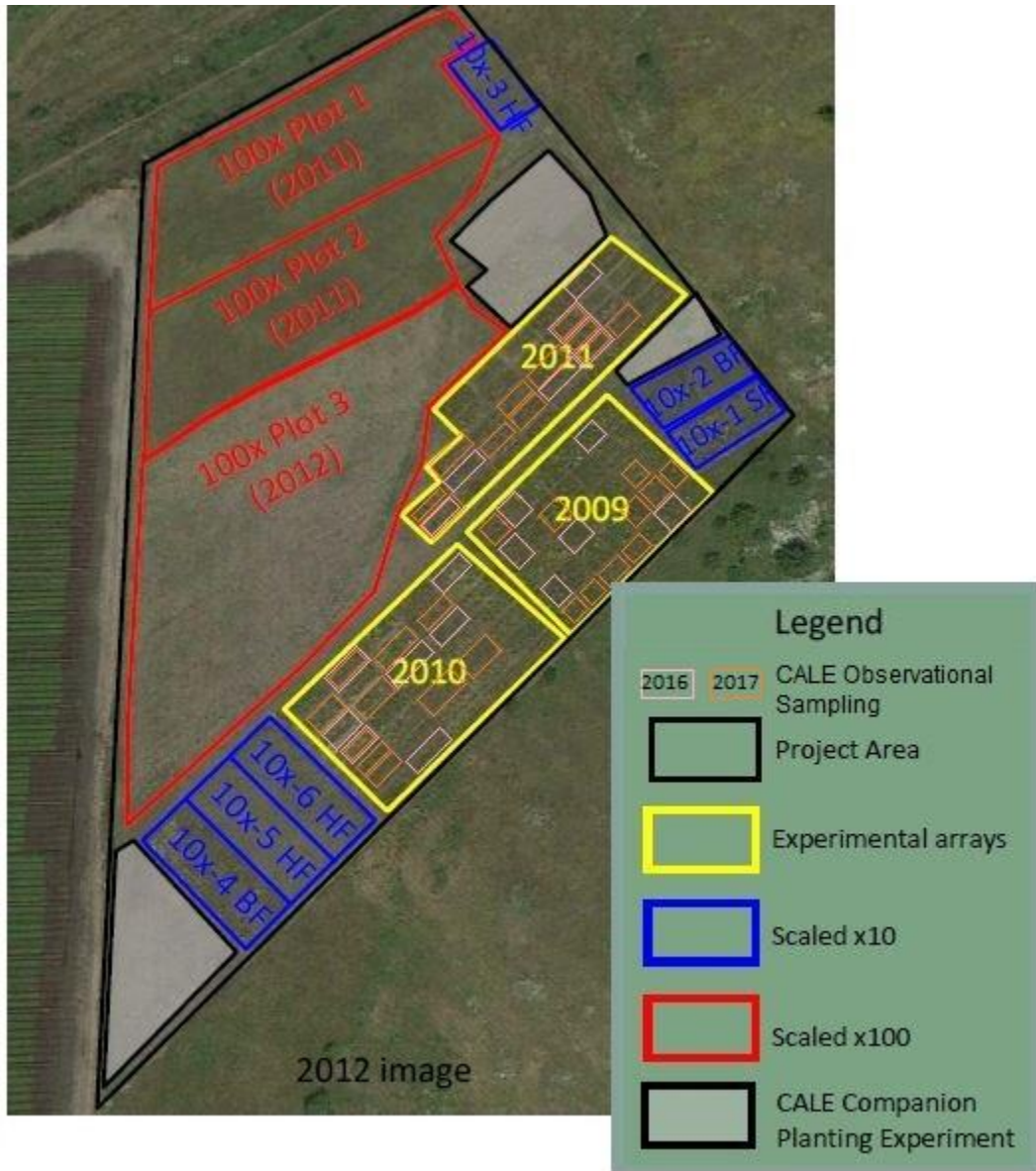


Figure 2.1: *C. levisecta* observational plots at Glacial Heritage Preserve. Original image from Bakker et al. 2013.

I used a subset of existing experimental grassland plots from a previous study for my experiment, as shown in Figure 2.1. This site is one of several Pacific Northwest lowland prairie restoration projects in which former agricultural fields have been restored using remnant native prairies of

the region as seed sources and reference sites, in an effort to expand and reconnect a greatly diminished and fragmented regional ecosystem (Bakker et al., 2013; Krueger et al., 2014).

My research species at this site was *Castilleja levisecta* (Orobanchaceae), a hemiparasitic angiosperm native to British Columbia, Washington, and Oregon. This species was chosen not only for its trait of iridoid glycoside production, but also for its central role in restoration and recovery efforts in this study system (Haan et al., 2017). *C. levisecta* has been listed as threatened since 1997 at the federal level (United States Dept. of Fish & Wildlife Service), and is one of a few known host species for larvae of the endangered Taylor's checkerspot (*Euphydryas editha* ssp. *taylori*) butterfly (Haan et al., 2017). The iridoid glycoside secondary metabolites produced by *C. levisecta* are sequestered and utilized by Taylor's checkerspot larvae in their own chemical defense (Haan et al., 2017). My study builds on recent multitrophic biochemical research on *C. levisecta* (Haan et al., 2017), and aims to help land managers monitor effectiveness of land management techniques for the creation of suitable biochemical species recovery habitat.

Experimental design

To assess foliar iridoid glycoside composition and concentration in response to prescribed burn regime, I collected five to six mature leaves from 30 *Castilleja levisecta* plants in each of three experimental arrays (established in 2009, 2010, and 2011), for a total of 90 samples in each growing season. Because there is a history of unintended hybridization between *Castilleja levisecta* and *C. hispida* at this site (Fisher et al., 2015; Kaye and Blakeley-Smith 2008), I was careful to select plants that exhibited standard *C. levisecta* phenotype, although this method

cannot guarantee that genetic hybrids are completely avoided. I consistently sampled mature leaves from the mid-stem level, since iridoid glycoside concentrations vary across different types and ages of tissues within an individual plant (Mead and Stermitz, 1993). I chose five to six leaves from the mid-stem area, taking as few leaves from any one stem as possible to avoid creating an herbivory-like event through defoliation. *C. levisecta* leaves separate easily from stems, and so were pinched off by hand. I collected as few leaves from any one stem as possible to avoid any possible risk of inducing widespread defensive chemical responses through herbivory-like defoliation (Fuchs and Bowers, 2004).

Each experimental array contained plots with several different burn histories ranging from one to five historical burns in the past six years (see Figure 2.2, Table 2.1). I did not sample burn regimes evenly. Within each array, plants were selected systematically along west to east transect lines bisecting at least six different plots representing different burn regimes. I sampled anywhere between one and five plants per plot, depending on how many *C. levisecta* plants could be found. I duplicated this method in two consecutive growing seasons, 2016 and 2017, for a total of 180 samples.

In 2016, I sampled leaves late in the growing season, when nearly all *C. levisecta* plants were nearing senescence and had been heavily browsed by deer. I sampled only plants that had signs of moderate deer herbivory, to improve consistency in circumstances where almost no unbrowsed plants could be found. In 2017, *C. levisecta* samples were collected in the early growing season, before any plants had evidence of deer herbivory. Thus, I was able to also collect data on plant height, anthocyanin pigmentation (Haan et al., 2017), and quantity of flowering stems for this second growing season.

Table 2.4: Prescribed fire schedule for experimental arrays at Glacial Heritage Preserve. Numbers in each cell represent burn quantity for each treatment area within each array. Sampling in both years occurred prior to burns scheduled for those years. From Bakker and Dunwiddie, 2017, unpublished fire frequency study.

Array	Treatment	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020		
GH 2009	Annual Early Burn			1 (Aug)			2	3	4	5	6	7	8		
	Annual Late Burn								3	4	5	6	7	8	
	Triannual Early Burn										3				4
	Triannual Late Burn										3				4
	Mowed Annually														
GH 2010	Annual Early Burn			1 (Sum)			2	3	4	5	6	7			
	Annual Late Burn								3	4	5	6	7		
	Triannual Early Burn											3			
	Triannual Late Burn											3			
	Mowed Annually														
GH 2011	Annual Early Burn					1 (Sum)		2 (Sept)		3	4	5	6		
	Annual Late Burn										3	4	5	6	
	Triannual Early Burn												3		
	Triannual Late Burn												3		
	Mowed Annually														
Total # of Plots	Burned	0	0	35	35	35	35	49	63	56	56	56	56		
	Mowed	0	0	0	0	0	0	0	14	21	21	21	21		
	Untreated (not burned or mowed)	105	105	70	70	70	70	56	28	28	28	28	28		

Notes: 2015: no mowing, and burns all occurred on the same day (no early vs. late distinction).

2016: early burn on June 30, 2016; mowing on August 18, 2016; late burn on September 16, 2016. All burns other than early burns were sprayed afterwards.

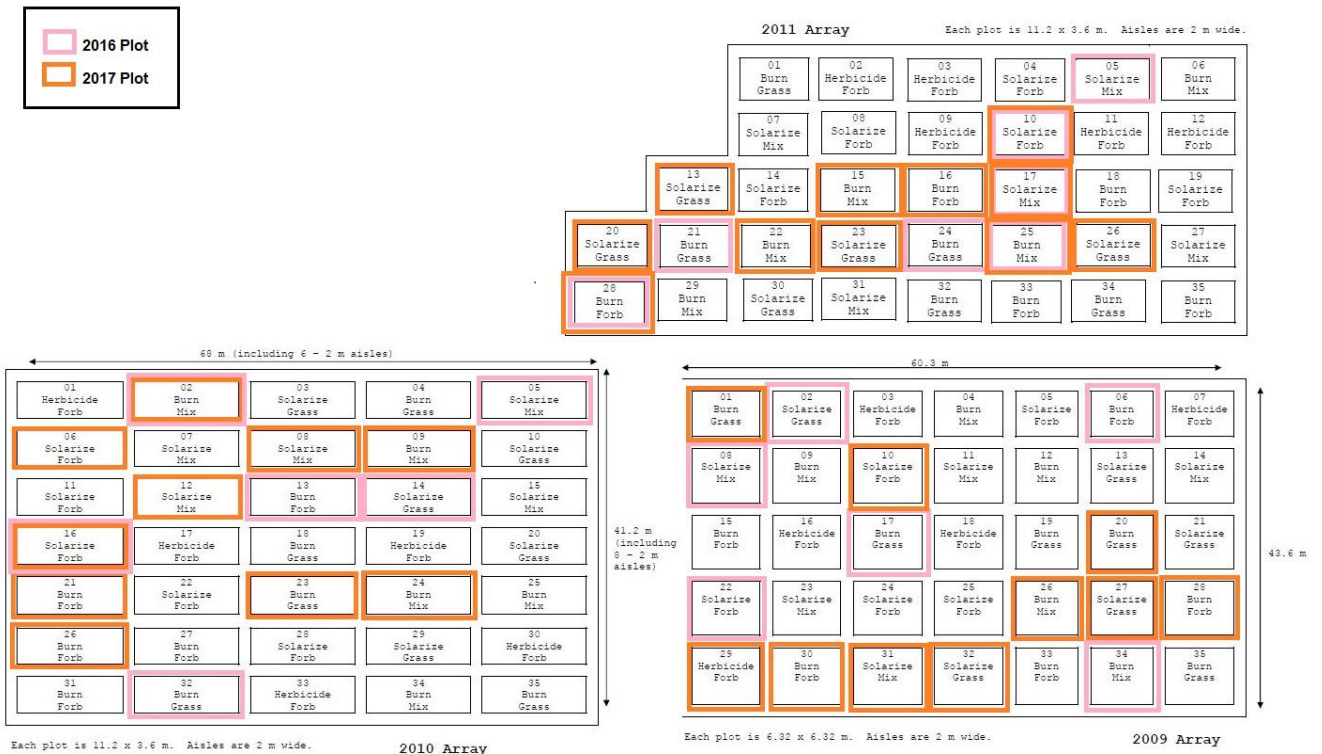


Figure 2.2: Map of plots sampled in the 2009, 2010, and 2011 arrays at Glacial Heritage Preserve. Original images from Bakker et al. 2013.

Sample preparation and analyses

Foliar concentrations (% dry weight) of the iridoid glycosides aucubin, catalpol, macfadienoside, and methyl shanzhiside² (Haan et al., 2017) were assessed by the University of Colorado Boulder Bowers Laboratory using gas chromatography. Iridoid glycoside analysis methods followed Bowers and Stamp (1997) and Bowers (2003). Within twelve hours of collection, leaf tissues were oven dried at 50°C for 48 hours. Dried tissues were ground using mortar and pestle and then frozen to await shipment. To process thawed samples, a 25 mg aliquot was extracted in 95% methanol for 24 hours. Plant tissue solids were then filtered out, and the methanol was evaporated completely. 1.0 mL of the internal standard phenyl-β-D-glycopyranoside (PBG, 0.500 mg/mL; Sigma Aldrich, St. Louis, Missouri, USA) was added, and each sample was partitioned with diethyl ether to remove hydrophobic compounds. The ether layer was removed and the iridoid glycoside infused water layer evaporated. The remaining residue was suspended in 1.0 mL methanol, and a 100 μL aliquot was taken for analysis. The methanol was evaporated and the residue was derivatized using Tri-Syl-Z (Thermo-Fisher Chemical Company) in pyridine before injection into an Agilent 7890A gas chromatograph equipped with a DB-1 column (30 m, 0.320 mm, 0.25 μm particle size) and using flame-ionization detection.

Amounts of aucubin, catalpol, and macfadienoside were quantified using ChemStation B-03-01 software. A fourth iridoid glycoside, putatively methyl shanzhiside (Haan et al., 2017), was present, but no standard was available to identify it clearly. Methyl shanzhiside concentrations were therefore estimated with a conversion factor based on the internal standard, PBG, since the

² The identity of this iridoid glycoside has not been confirmed. Throughout this chapter, I use the term methyl shanzhiside to refer to putative methyl shanzhiside. See full explanation in the “Sample preparation and analysis” section.

original amount of this compound in each sample was known. Therefore, my reported values for concentrations of this fourth compound may not be exact, and are based on the assumption that the unknown compound behaves identically to PBG.

Statistical analyses

I used R 3.4.2 for all analyses (R Core Development Team 2017), using total and constituent iridoid glycoside concentrations as response variables. (See Appendices 2 and 3 for R code and raw data.) I first analyzed a combined dataset from both sample years to test sample year, fire variables, and spatial unit (plot). I used a stepwise Akaike Information Criterion (AIC) function to test multiple variables and identify best-fit models among several potential models (Burnham and Anderson, 2004). The simplest model tested included just an intercept, and the most complex model included interaction effects between sample year and fire effects, with spatial unit included as a main effect. I then tested the significance ($\alpha < 0.05$) of best-fit models using ANOVA (Anderson, 2001). Because ANOVA is robust in the absence of normality (Läärä, 2009), data were not transformed.

Second, I tested additional models to assess factors that relate to individual-plant iridoid glycoside production. Plant-level data were only available for 2017, so I restricted these analyses to data from that year. I began with the best-fit models identified previously (but excluding the sample year factor), and added to these models various individual plant metrics (plant height, pigmentation, and quantity of flowering stems). I used ANOVA to compare models with and without these individual plant metrics to determine whether their inclusion improved model fit.

Results

Overview

Four iridoid glycosides were detected in my *Castilleja levisecta* samples: aucubin, catalpol, macfadienoside, and methyl shanzhiside³. However, catalpol was only detected in 2017.

Macfadienoside was the most abundant constituent iridoid glycoside in both years (Figure 2.3).

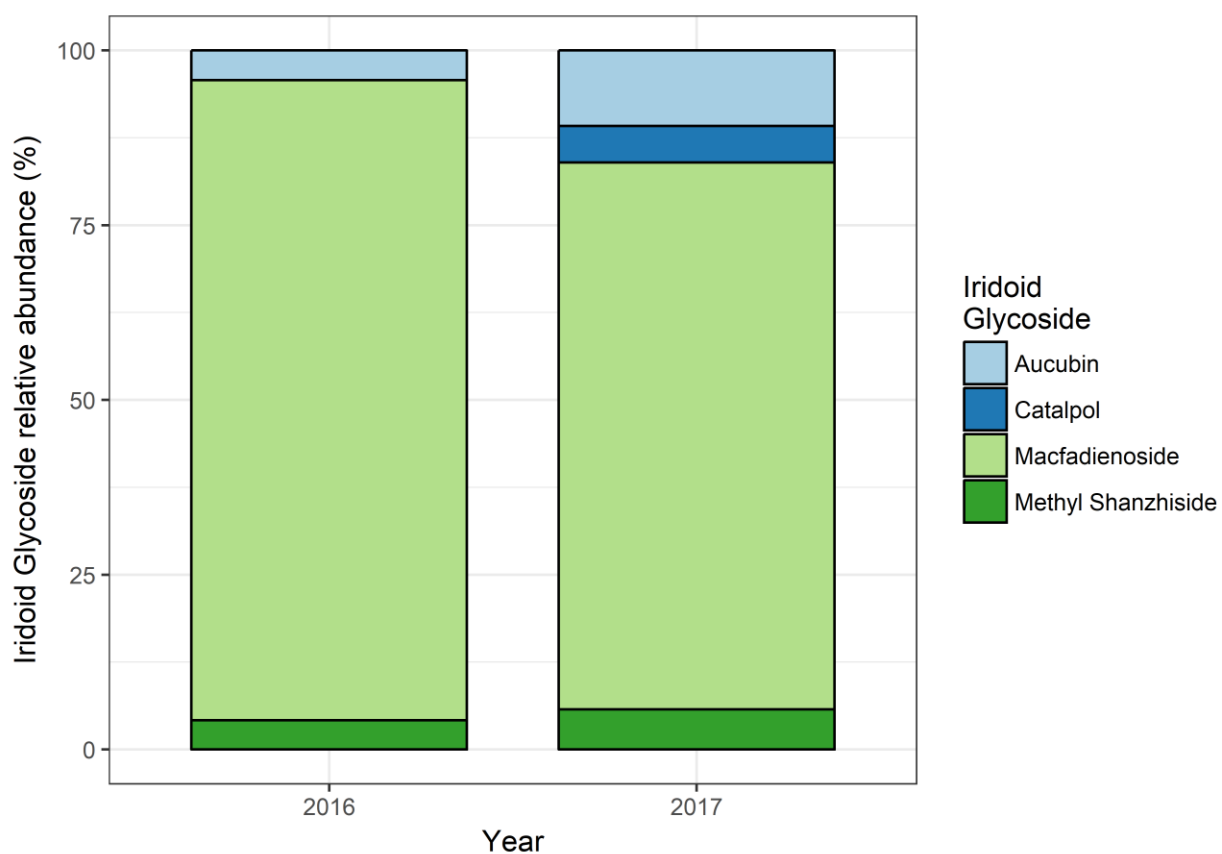


Figure 2.3: Composition of iridoid glycosides in *Castilleja levisecta* at Glacial Heritage Preserve.

³ Throughout this chapter, methyl shanzhiside refers to putative methyl shanzhiside. See “Sample preparation and analysis” for full explanation.

Mean iridoid glycoside concentrations were 3.78% dry weight (SE ± 0.21) in 2016 (late growing season) and 0.81% dry weight (SE ± 0.03) in 2017 (early growing season). Abundances of each constituent iridoid glycoside are shown in Figure 2.4.

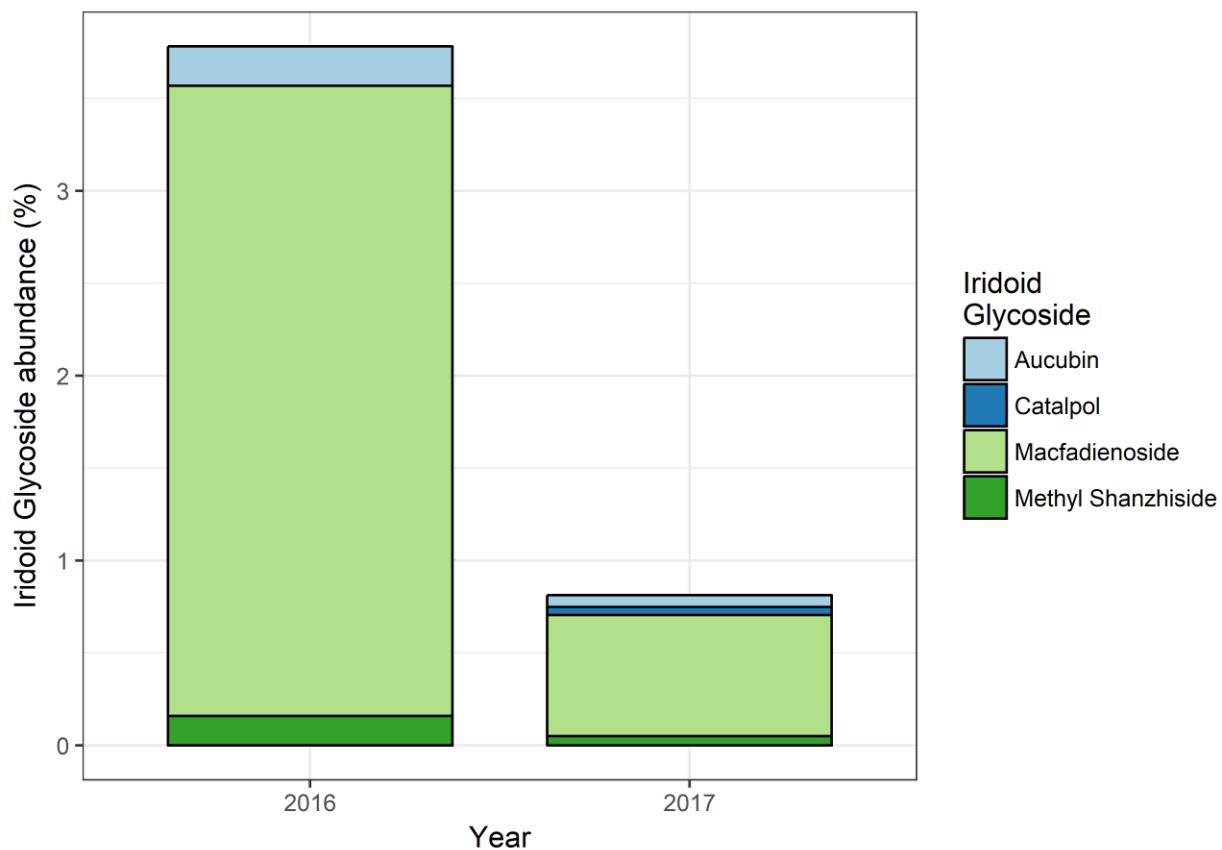


Figure 2.4: Total *C. levisecta* iridoid glycoside abundance by year.

Total and constituent iridoid glycoside concentrations responded differently in magnitude to time since prescribed fire, quantity of historical burns, and sample year, as will be discussed in this section. Best fit models are presented in Table 2.2, and ANOVA results for factors from these models are summarized in Table 2.3.

Table 5.2: Best fit models for *Castilleja levisecta* iridoid glycoside responses in 2016 and 2017. All models included plot as a random effect.

Response	Subset	Adjusted R ²	Model
<i>Total IGs</i>	All	0.529	~ SampleYear + QtyBurns + SampleYear: QtyBurns
	2017	0.024	~ QtyBurns
<i>Aucubin</i>	All	0.009	~ SampleYear
	2017	0.053	~ QtyBurns + Height
<i>Catalpol</i>	All	-	N/A (No catalpol detected in 2016)
	2017	-	~ (Intercept)
<i>Macfadienoside</i>	All	0.569	~ SampleYear + QtyBurns + SampleYear:Burns
	2017	-	None
<i>Methyl Shanzhiside</i>	All	0.091	~ SampleYear + QtyBurns
	2017	0.115	~ QtyBurns + TimeSinceBurn
<i>Relative abundance of Macfadienoside</i>	All	0.161	~ SampleYear + TimeSinceBurn + SampleYear:TimeSinceBurn
	2017	0.059	~ TimeSinceBurn + Height

Table 2.6: ANOVA table describing effects of factors on the concentrations of iridoid glycosides in *C. levisecta*. Significant terms ($P \leq 0.05$) are shown in bold font, and marginally significant terms ($P \leq 0.10$) are shown in italics.

Response	Subset	Factor	SS	df	F	p
<i>Total IGs</i> (% dry weight)	All	Sample Year	382.860	1, 170	189.449	<0.001
		Quantity of Burns	8.660	1, 170	4.287	0.040
		Sample Year × Burns	7.160	1, 170	3.543	<i>0.062</i>
	2017	Burns	0.227	1, 82	3.007	<i>0.087</i>
<i>Aucubin</i> (% dry weight)	All	Sample Year	0.975	1, 170	2.575	0.110
	2017	Quantity of Burns	0.004	1, 170	2.916	<i>0.092</i>
		Height	0.005	1, 170	3.806	<i>0.055</i>
<i>Catalpol</i> (% dry weight)	2017	Intercept	0.564	83	-	-
<i>Macfadienoside</i> (% dry weight)	All	Sample Year	329.430	1, 170	225.173	<0.001
		Quantity of Burns	4.590	1, 170	3.139	<i>0.078</i>
		Sample Year × Burns	4.290	1, 170	2.932	<i>0.089</i>
<i>Methyl Shanzhiside</i> (% dry weight)	All	Sample Year	0.519	1, 171	16.358	<0.001
		Quantity of Burns	0.095	1, 171	2.996	<i>0.085</i>
	2017	Quantity of Burns	0.017	1, 81	9.509	0.003
		Time Since Burn	0.006	1, 81	3.282	<i>0.074</i>
<i>Relative abundance of Macfadienoside</i>	All	Sample Year	0.770	1, 170	30.851	<0.001
		Time Since Burn	0.055	1, 170	2.202	0.140
		Sample Year × Time Since Burn	0.077	1, 170	3.094	<i>0.080</i>
	2017	Time Since Burn	0.130	1, 81	3.628	<i>0.060</i>
		Height	0.130	1, 81	3.607	<i>0.061</i>

Total Iridoid Glycosides

Both quantity of historical burns and sample year were significant factors ($p < 0.05$). Quantity of historical burns was negatively related to total iridoid glycoside concentration (Figure 2.5). The interaction between quantity of burns and sample year was also marginally significant ($p < 0.10$) because this decrease was more marked in 2016.

In 2017, quantity of burns was a marginally significant factor. Total iridoid glycoside concentrations were negatively related to quantity of historical burns.

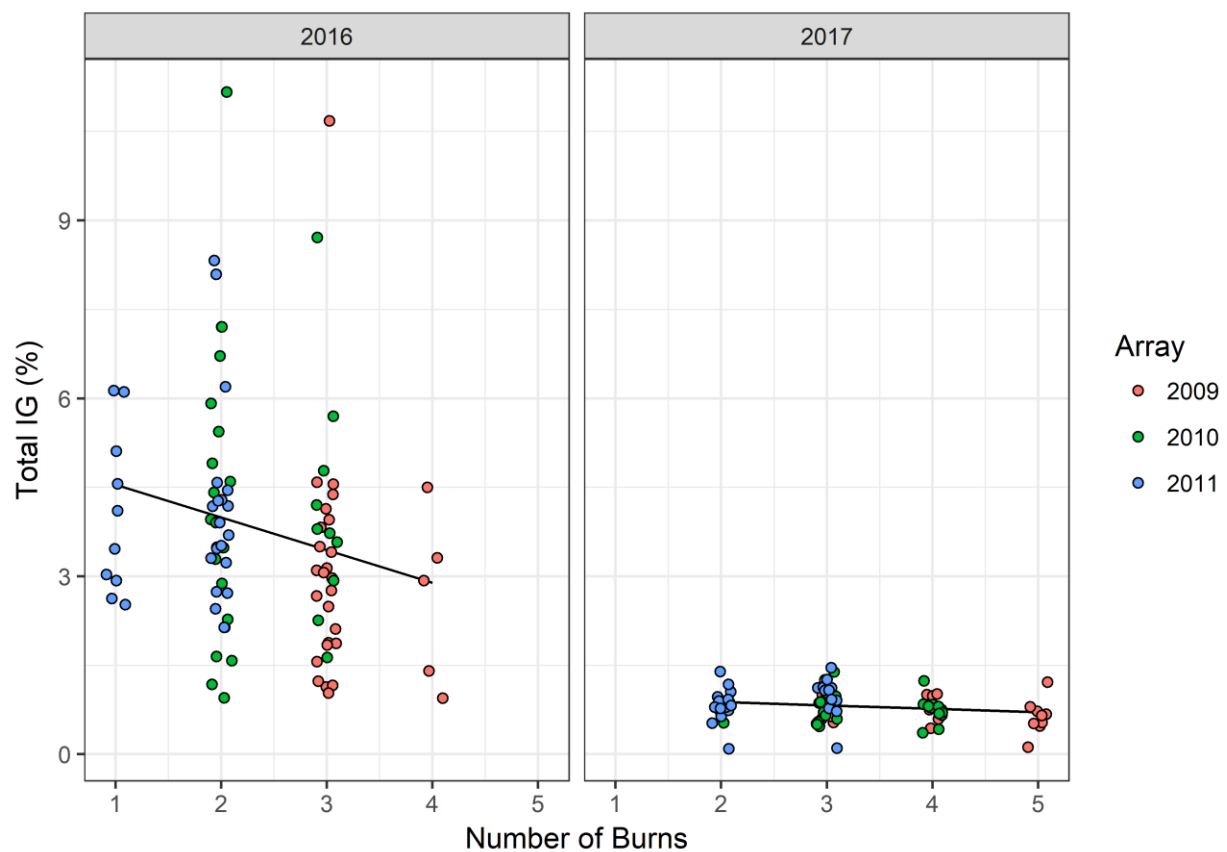


Figure 2.5: Total *C. levisecta* iridoid glycoside concentrations in response to quantity of historical prescribed burns.

Aucubin

Aucubin levels were not affected by sample year or fire variables when the entire data set was considered. Quantity of burns had a marginally significant effect for aucubin in models for 2017, with quantity of burns negatively related to aucubin concentrations. Height was a marginally significant factor for this subset as well, with aucubin concentrations negatively related to height.

Catalpol

No catalpol was detected in my 2016 samples. Catalpol was detected in 2017, but no recorded variables had a significant effect on catalpol concentrations.

Macfadienoside

Quantity of historical burns had a marginally significant effect on macfadienoside concentrations (Figure 2.6). Quantity of historical burns was negatively related to macfadienoside concentration abundance. Sample year was a significant factor, and there was also a marginally significant interaction effect between quantity of historical burns and sample year.

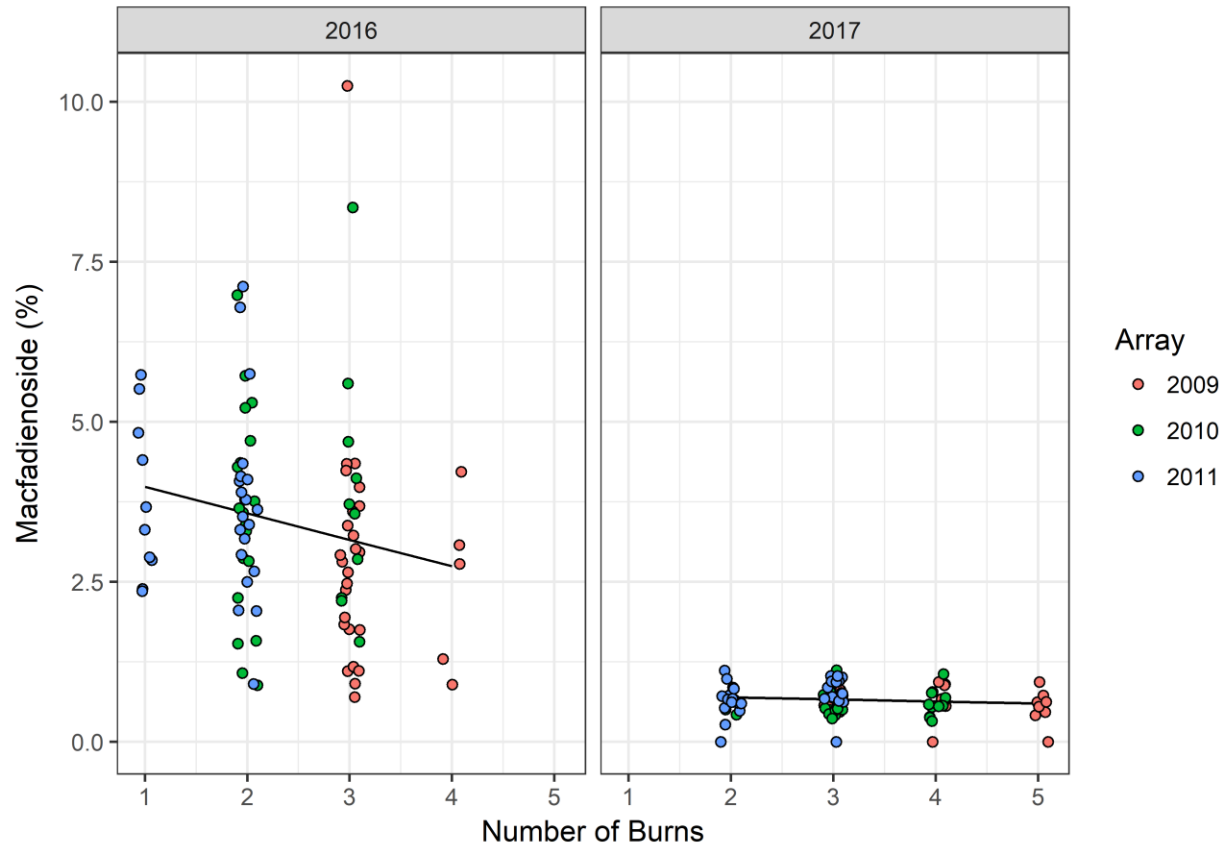


Figure 2.6: *C. levisecta* macfadienoside concentrations in response to quantity of historical prescribed burns.

Macfadienoside abundance

Time since most recent fire was a marginally significant factor for relative abundance of macfadienoside, especially in the 2017 growing season (Figure 2.7). Mean macfadienoside abundance was highest in plants three years after a prescribed burn, and lowest one year after a burn. Height was a significant factor for relative abundance of macfadienoside, with macfadienoside abundance negatively related to height (Figure 2.8).

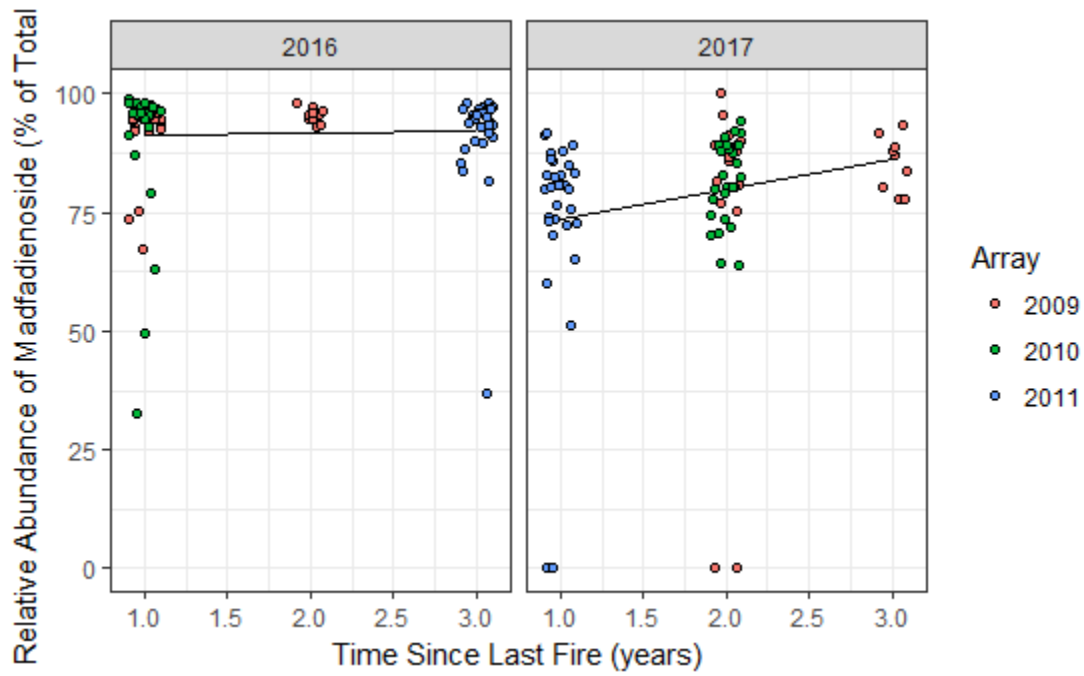


Figure 2.7: *C. levisecta* macfadienoside abundance in response to time since most recent burn.

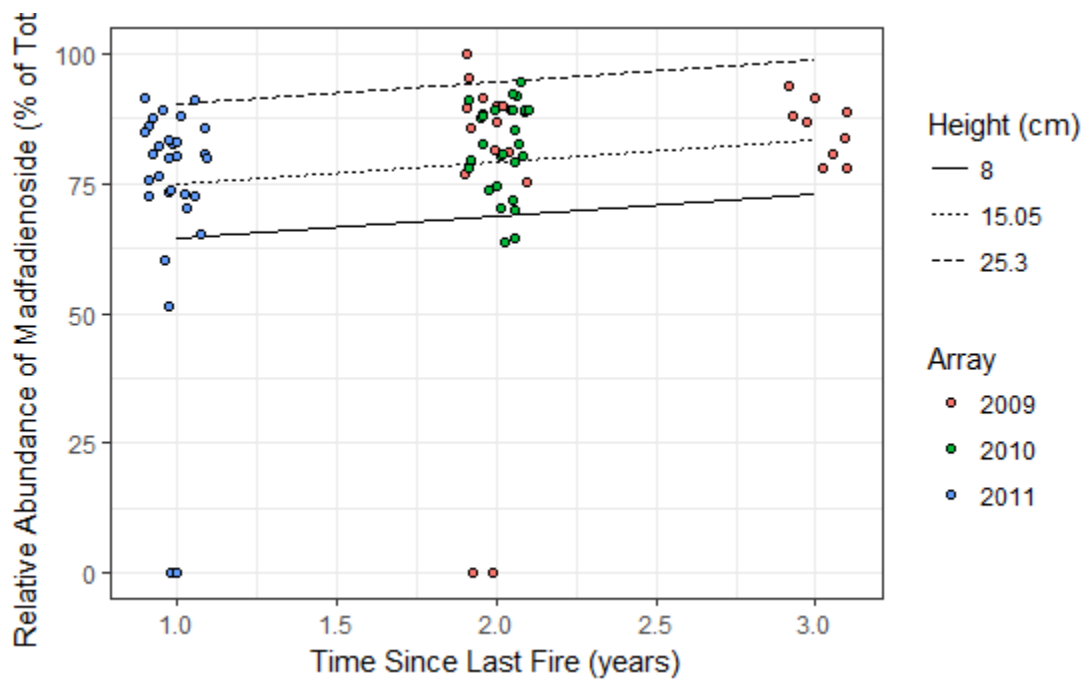


Figure 2.8: *C. levisecta* macfadienoside abundance by height in 2017 in response to time since most recent burn.

Methyl shanzhiside

Sample year was a significant factor for methyl shanzhiside concentrations. Quantity of historical burns and time since most recent burn were both marginally significant ($p < 0.1$) factors for methyl shanzhiside concentrations. Concentrations were negatively related to quantity of burns. Mean methyl shanzhiside concentrations were slightly higher in plants one year after a prescribed burn, and lower in plants two and three years after a burn.

When variables of plant height, pigmentation, and quantity of flowering stems were included in models for 2017, quantity of historical burns was a significant factor for methyl shanzhiside concentrations, resulting in lowered concentrations with increased quantity of burns. Response of methyl shanzhiside to quantity of burns is represented in Figure 2.9.

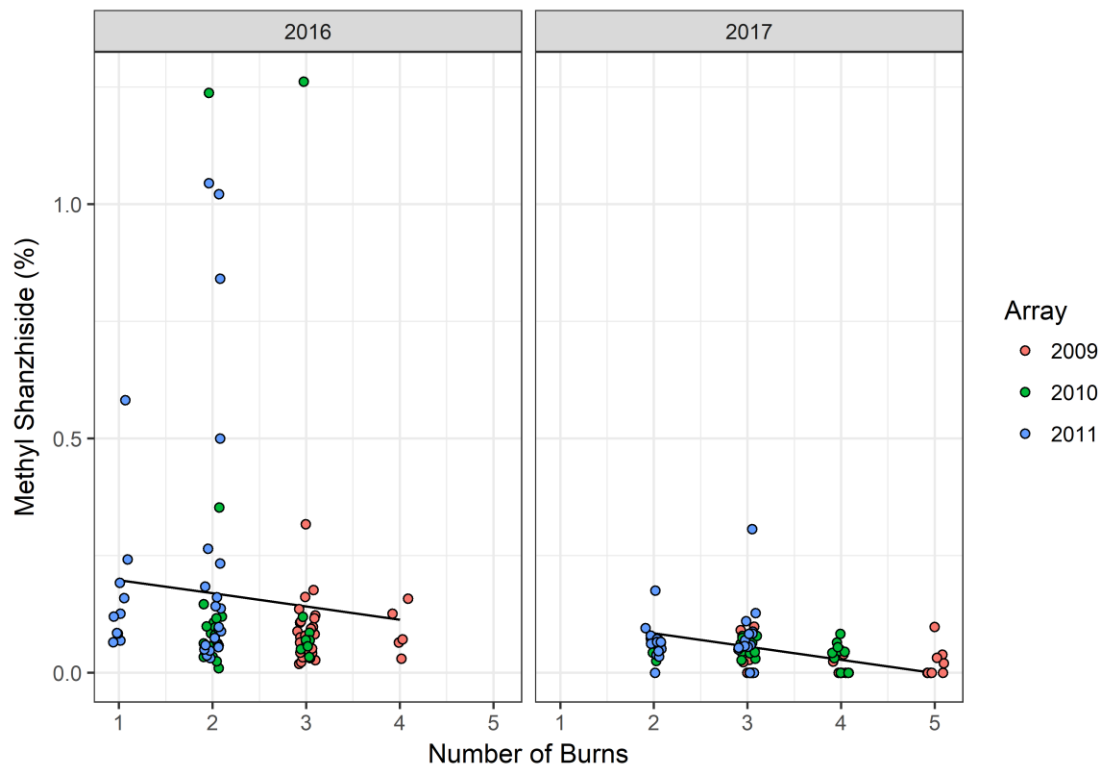


Figure 2.9: *C. levisecta* methyl shanzhiside concentrations in response to quantity of historical prescribed burns.

Discussion

My results reveal large variations in *Castilleja levisecta* iridoid glycoside concentrations among years. There are multiple possible explanations for this. The differences could be due to weather patterns: the early 2016 growing season was unusually warm and dry, which resulted in atypically rapid phenological progression, while the 2017 growing season followed more typical seasonal temperature and moisture patterns. Sampling also occurred at different phenological stages during the two years, with 2016 samples collected nearer senescence. Research from other plant families also documents peaks in some iridoid glycoside concentrations in mid- to late growing season (Bowers, 1991; Bowers et al., 1992).

Prescribed burning

The results in this study are in opposition to my initial hypotheses about iridoid glycoside responses to prescribed fire regime. I predicted that *Castilleja levisecta* iridoid glycoside concentrations would decrease in the months following a prescribed burn, but my results show almost no iridoid glycoside responses to time since most recent burn. The one constituent iridoid glycoside, methyl shanzhiside, that did show a marginally significant response to time since most recent burn responded in the opposite direction of what I predicted. I also predicted that iridoid glycoside concentrations would increase with increased quantity of burns, but the opposite turned out to be true for aucubin, macfadienoside, and putative methyl zhanzhiside concentrations. Although we might expect to see an impact on *C. levisecta* iridoid glycosides after an autumn burn, the effect of fire on plant secondary metabolism is more complex than the mere addition of a short lived nutrient pulse. Fire also alters soil pH, moisture, and temperature, as well as competition and light acquisition dynamics in the plant community (Gibson, 2009).

Some of these effects, such as altered soil moisture level, can complicate iridoid glycoside responses to increased soil nitrogen (Jamieson, 2013; Prudic et al., 2005).

Quantity of historical prescribed fires proved to be an important factor in this study, with iridoid glycoside concentrations negatively related to quantity of burns. Increased fire frequency is known to decrease the inorganic soil nitrogen and cumulative net nitrogen mineralization crucial to plant nutrient acquisition and metabolism, which could be a mechanism influencing secondary chemistry in *C. levisecta* (Blair, 1997). Frequent fires could influence iridoid glycosides directly through long-term ecological legacies in soil nutrient dynamics, or more indirectly by increasing plant community diversity (Gibson, 2009; Mraja et al., 2011). Aucubin levels were not affected by sample year or fire variables when the entire data set was considered, but quantity of burns had a marginally significant effect for aucubin in models for 2017, with quantity of burns negatively related to aucubin concentrations.

It is also worth noting that in my statistical analyses, experimental array was a spatial factor that could not be clearly distinguished from burn history. Because initial prescribed burn management regimes were applied on a whole-array basis, there would have been little ability to detect fire-related effects had I explicitly identified array as variable of interest.

It is unclear what caused quantity of historical burns to have a stronger effects in *C. levisecta* iridoid glycosides in 2016 than in 2017. This is perhaps due to sampling at different phenological stages in one growing season as compared to the other. Responses to time since most recent burn may have been modulated or obscured by phenological fluctuations in iridoid glycoside concentrations. Iridoid glycoside responses to environmental factors are more

pronounced at different phenological stages in species such as *Plantago lanceolata* (Darrow and Bowers, 1997), and this may be true for *C. levisecta* as well.

Macfadienoside abundance

Macfadienoside was the dominant constituent iridoid glycoside in my *C. levisecta* samples. It is not clear why macfadienoside is so much more abundant in *C. levisecta* than any other iridoid glycoside, and whether that is the case under all environmental conditions. Further research is needed to elucidate what macfadienoside abundance might mean for Taylor's checkerspot larvae feeding on this plant. Existing research shows that Taylor's checkerspot larvae do not require this particular iridoid glycoside for survival (Haan et al., 2017), but how macfadienoside abundance in host plant tissues might affect larval survival into adulthood is as of yet unknown. This topic warrants additional research, as conservation efforts for Taylor's checkerspot seek to identify ideal host plants for larvae (Haan et al., 2017; Severns, 2008).

Implications for land management

Understanding the impact of management decisions on iridoid glycoside concentrations is important. For example, female butterflies who use iridoid glycosides as a cue for oviposition tend to select oviposition plants that are higher in iridoid glycoside content (Prudic et al., 2005). Moreover, variation in iridoid glycoside concentrations may have fitness consequences for specialist larvae that sequester iridoid glycosides for their own defense (Haan et al., 2017; Prudic et al., 2005). Based on my findings, land managers, conservation biologists, and ethnobotanists should collaborate to create prescribed burning regimes that not only take into account historical precedent (Storm and Shebitz, 2006) and direct survival of Taylor's checkerspot larvae (Hill et

al., 2017), but also aim to achieve ideal phytochemical concentrations for maximizing recovery success of both threatened plants (*C. levisecta*), and endangered insects (*Euphydryas editha taylori*).

Because prescribed fire is an important management tool with strong potential for long-term ecological impact, additional research on the mechanisms underlying prescribed fire effects on iridoid glycoside concentrations is warranted. Carrying out prescribed fire experiments in a common garden setting could provide more control over potentially confounding plant genotype and age factors, which could not be controlled for in this study. Plant age and genotype influence iridoid glycoside patterns in other plant families (Bowers, 1992; Fuchs and Bowers, 2004) and may in Orobanchaceae as well. Future observational studies could also cover broader geographical ranges to ascertain how my findings compare to large scale phytochemical landscapes, and to add phytochemical information to existing biological maps (Hunter, 2016).

Conclusion

My results show that prescribed burn regimes can alter patterns of important plant defensive compounds that mediate multitrophic interactions with larvae of the endangered Taylor's checkerspot butterfly and other herbivores. While time since most recent burn was only a significant factor for one of four *C. levisecta* iridoid glycosides, quantity of historical burns was a significant factor for several iridoid glycosides, with total iridoid glycoside concentrations negatively related to quantity of historical burns. My results reveal that methods used in the maintenance of restored terrestrial ecosystems have the potential to create important chemical legacies that ought to be considered alongside other important response variables in long-term restoration projects. This knowledge will help provide chemical ecology context for land

managers, conservation biologists, and ethnobotanists to collaborate on developing prescribed burning regimes that achieve ideal phytochemical habitat goals to support species recovery efforts.

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Works Cited

- Adam, G., Khoi, N. H., Bergner, C., & Lien, N. T. (1979). Plant growth inhibiting properties of plumeride from *Plumeria obtusifolia*. *Phytochemistry*, 18(8), 1399-1400.
- Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral ecology*, 26(1), 32-46.
- Baden, C.U. (2016). *Phylogeny and sequestration of iridoid glycosides in selected genera of the Mecininae (Coleoptera, Curculionidae) with particular focus on their host plant relationship* (Doctoral Dissertation).
- Baer, S. G., Blair, J. M., Collins, S. L., & Knapp, A. K. (2003). Soil resources regulate productivity and diversity in newly established tallgrass prairie. *Ecology*, 84(3), 724-735
- Bakker, J. D., E. Delvin, and P. W. Dunwiddie. (2013). Prairie habitat restoration for endangered species: final report. Prepared for the U.S. Fish and Wildlife Service.
- Barton, K. E. (2007). Early ontogenetic patterns in chemical defense in *Plantago* (Plantaginaceae): genetic variation and trade-offs. *American Journal of Botany*, 94(1), 56-66.
- Benayas, J. M. R., Newton, A. C., Diaz, A., & Bullock, J. M. (2009). Enhancement of biodiversity and ecosystem services by ecological restoration: a meta-analysis. *science*, 325(5944), 1121-1124.
- Blair, J. M. (1997). Fire, N availability, and plant response in grasslands: a test of the transient maxima hypothesis. *Ecology*, 78(8), 2359-2368.
- Bowers, M.D. (1991). Iridoid glycosides. In: Rosenthal, G.A., & Berenbaum, M.R. [eds.] *Herbivores: their interactions with secondary plant metabolites*. Second Edition, Vol. 1: the chemical participants. Academic Press, Sandiego, CA.
- Bowers, M. D., Collinge, S. K., Gamble, S. E., & Schmitt, J. (1992). Effects of genotype, habitat, and seasonal variation on iridoid glycoside content of *Plantago lanceolata* (Plantaginaceae) and the implications for insect herbivores. *Oecologia*, 91(2), 201-207.
- Burnham, K. P., & Anderson, D. R. (2004). Multimodel inference: understanding AIC and BIC in model selection. *Sociological methods & research*, 33(2), 261-304.
- Covelo, F., & Gallardo, A. (2004). Green and senescent leaf phenolics showed spatial autocorrelation in a *Quercus robur* population in northwestern Spain. *Plant and Soil*, 259(1-2), 267-276.
- Craine, J. M. (2009). *Resource strategies of wild plants*. Princeton University Press.

- Darrow, K., & Bowers, M. D. (1997). Phenological and population variation in iridoid glycosides of *Plantago lanceolata* (Plantaginaceae). *Biochemical Systematics and Ecology*, 25(1), 1-11.
- Dietz, M., Machill, S., Hoffmann, H. C., & Schmidtke, K. (2013). Inhibitory effects of *Plantago lanceolata* L. on soil N mineralization. *Plant and Soil*, 368(1-2), 445-458.
- Fajer, E. D., Bowers, M. D., & Bazzaz, F. A. (1992). The effect of nutrients and enriched CO₂ environments on production of carbon-based allelochemicals in *Plantago*: a test of the carbon/nutrient balance hypothesis. *The American Naturalist*, 140(4), 707-723.
- Fisher, L., Bakker, J. D., & Dunwiddie, P. W. (2015). An Assessment of Seed Production and Viability of Putative *Castilleja levisecta* × *C. hispida* Hybrids. Report for the Center for Natural Lands Management.
- Fuchs, A., & Bowers, M. D. (2004). Patterns of iridoid glycoside production and induction in *Plantago lanceolata* and the importance of plant age. *Journal of chemical ecology*, 30(9), 1723-1741.
- Gibson, D. J. (2009). *Grasses and grassland ecology*. Oxford University Press.
- Haan, N. L., Bakker, J. D., & Bowers, M. D. (2017). Hemiparasites can transmit indirect effects from their host plants to herbivores. *Ecology*.
- Hill, K. C., Bakker, J. D., & Dunwiddie, P. W. (2017). Prescribed fire in grassland butterfly habitat: targeting weather and fuel conditions to reduce soil temperatures and burn severity. *Fire Ecology*, 13(3), 24-41.
- Hunter, M. D. (2016). *The Phytochemical Landscape: Linking Trophic Interactions and Nutrient Dynamics*. Princeton University Press.
- Inouye, H., & Uesato, S. (1986). Biosynthesis of iridoids and secoiridoids. In *Fortschritte der Chemie Organischer Naturstoffe/Progress in the Chemistry of Organic Natural Products* (pp. 169-236). Springer Vienna.
- Jamieson, M. A., Quintero, C., & Blumenthal, D. M. (2013). Interactive effects of simulated nitrogen deposition and altered precipitation patterns on plant allelochemical concentrations. *Journal of Chemical Ecology*, 39(9), 1204-1208.
- Jarzomski, C. M., Stamp, N. E., & Bowers, M. D. (2000). Effects of plant phenology, nutrients and herbivory on growth and defensive chemistry of plantain, *Plantago lanceolata*. *Oikos*, 88(2), 371-379.
- Jensen, S. R. (1992). Systematic implications of the distribution of iridoids and other chemical compounds in the Loganiaceae and other families of the Asteridae. *Annals of the Missouri Botanical Garden*, 284-302.

- Kaye, T. N., & Blakeley-Smith, M. (2008). An Evaluation of the Potential for Hybridization Between *Castilleja levisecta* and *C. hispida*. Unpublished report. Institute for Applied Ecology, Corvallis, OR.
- Läärä, E. (2009). Statistics: reasoning on uncertainty, and the insignificance of testing null. In *Annales Zoologici Fennici* (Vol. 46, No. 2, pp. 138-157).
- MacLean, D. A., Woodley, S. J., Weber, M. G., & Wein, R. W. (1983). Fire and nutrient cycling.
- Matyssek, R. (2012). Conclusions and perspectives. In *Growth and Defence in Plants* (pp. 453-457). Springer Berlin Heidelberg.
- Miller, J. O. (2016). *Soil PH and Nutrient Availability*. UME. FS-1054.
- Mraja, A., Unsicker, S. B., Reichelt, M., Gershenson, J., & Roscher, C. (2011). Plant community diversity influences allocation to direct chemical defence in *Plantago lanceolata*. *PLoS One*, 6(12), e28055.
- Nassauer, J. I., & Opdam, P. (2008). Design in science: extending the landscape ecology paradigm. *Landscape ecology*, 23(6), 633-644.
- Prudic, K. L., Oliver, J. C., & Bowers, M. D. (2005). Soil nutrient effects on oviposition preference, larval performance, and chemical defense of a specialist insect herbivore. *Oecologia*, 143(4), 578-587.
- Ringold, P. L., Alegria, J., Czaplewski, R. L., Mulder, B. S., Tolle, T., & Burnett, K. (1996). Adaptive monitoring design for ecosystem management. *Ecological applications*, 6(3), 745-747.
- Severns, P. M., & Warren, A. D. (2008). Selectively eliminating and conserving exotic plants to save an endangered butterfly from local extinction. *Animal Conservation*, 11(6), 476-483.
- Stamp, N. E., & Bowers, M. D. (1994). Effects of cages, plant age and mechanical clipping on plantain chemistry. *Oecologia*, 99(1-2), 66-71.
- Storm, L., & Shebitz, D. (2006). Evaluating the purpose, extent, and ecological restoration applications of indigenous burning practices in southwestern Washington. *Ecological Restoration*, 24(4), 256-268.
- United States. Dept. of Fish and Wildlife Service. Golden paintbrush (*Castilleja levisecta*) listing status. Environmental Conservation Online System. Web. 4 December 2017.
- Wan, S., Hui, D., & Luo, Y. (2001). Fire effects on nitrogen pools and dynamics in terrestrial ecosystems: A meta-analysis. *Ecological Applications*, 11(5), 1349-1365.

Chapter Three: Experimental sources of variation in *Castilleja levisecta* and *Plantago lanceolata* foliar iridoid glycoside concentrations: lime amendments, prescribed fire, and herbivory simulation across phenology

Abstract

Plant defensive compounds mediate multitrophic interactions between plants and insects in many ecosystems. Because phytochemistry is influenced by both biotic and abiotic factors, both herbivores and landscape scale land management techniques have the potential to alter plant defensive compounds in ecosystems. To investigate the effects of herbivory and land management techniques on ecosystem chemical ecology, I tested the effects of simulated herbivory, prescribed burning, and fast-acting soil lime on defensive foliar iridoid glycoside concentrations in perennial forbs *Castilleja levisecta* and *Plantago lanceolata*. I tested these effects across three phenological time steps in two consecutive growing seasons. Four iridoid glycosides—aucubin, catalpol, macfadienoside, and, putatively, methyl shanzhiside—were detected in *C. levisecta*, while only aucubin and catalpol were detected in *P. lanceolata*. Each of these constituent iridoid glycosides exhibited different phenological patterns and responded differently in direction and magnitude to the experimental treatments I applied. With the exception of methyl shanzhiside, herbivory simulation was not a significant factor for *C. levisecta* iridoid glycosides. Herbivory simulation treatment affected methyl shanzhiside concentrations differently in magnitude and direction in each growing season. In *P. lanceolata*, herbivory simulation was not a significant factor for aucubin concentrations, and was only significant for catalpol concentrations in interaction with other factors. A prescribed burn in autumn 2016 had no significant effect on 2017 *C. levisecta* iridoid glycosides, but was a significant factor for *P. lanceolata* iridoid glycoside concentrations, with lower iridoid glycoside

concentrations in plants from burned plots in 2017. Fast-acting soil lime had no significant effect on any iridoid glycoside concentration or abundance in either species. My research suggests that phenology, herbivory, and environmental factors are important considerations when designing land management protocols informed by sound chemical ecology goals.

Key Words

Grassland management, iridoid glycosides, phenology, fire effects, soil liming

Introduction

Native grasslands are among Earth's most imperiled ecosystems (White et al., 2000). Conversion to land for human use has degraded grasslands through domestic grazing, agriculture, and urbanization (Gibson, 2009). Lowland glacial outwash prairies of the Puget Sound region are no exception, and have been severely compromised, with losses of greater than 90% of historical prairie land (Dunwiddie and Bakker, 2011; Fitzpatrick, 2004). The ecological function of remnant prairie habitat in the Puget Sound has been disrupted by loss of habitat connectivity and invasive species, as well as by 20th century disruption of historical burning regimes and concomitant woody species encroachment (Fitzpatrick, 2004; Hamman et al., 2011).

Researchers and land managers work together to develop landscape scale management techniques, such as reintroduction of historical prescribed burning regimes, that can effectively achieve goals of restoring grassland ecological function (Bakker et al., 2013; Pyke et al. 2010; Storm and Shebitz, 2006). Additional management techniques, such altering pH to enhance competitiveness of native plants adapted to nitrogen-poor soils are of interest to some land

managers (Marty Chaney, USDA Washington Natural Resources Conservation Service, personal communication, March 20, 2017), and could affect plant chemical ecology by influencing plant nutrient acquisition (Matyssek et al., 2012; McCauley et al., 2009; Miller, 2016).

On Puget Sound prairies, the needs of sensitive species—such as Taylor’s checkerspot butterfly (*Euphydryas editha* ssp. *taylori*), golden paintbrush (*Castilleja levisecta*), Mazama pocket gopher (*Thomomys mazama*), streaked horned lark (*Eremophila alpestris* spp. *strigata*), and rare soil-dwelling prairie lichens—must all be considered and balanced when determining appropriate prescribed fire and other landscape scale management regimes (Calabria et al., 2016; Hill et al., 2017; Stinson, 2005). Land managers are tasked with implementing protocols that promote resilient ecological function and that avoid inadvertently harming one sensitive species in an attempt to aid the recovery of another (Dunwiddie and Rogers, 2016; Hill et al., 2017; Hobbs, 2007; Stanley, 2010). Understanding the complex ecological legacies of commonly used landscape scale management techniques can help land managers customize protocols to achieve goals of species recovery, ecosystem resilience, and ecological function.

Both fire and lime amendments have the potential to alter ecosystem chemical ecology through their effects on plant nutrient acquisition and competition (Gibson, 2009; Miller, 2016). Both cause a rise in pH, which enhances nitrate uptake (Gibson, 2009; Miller, 2016). The sudden nutrient pulse that occurs immediately after a fire also has a strong enhancing effect on plant metabolism (Baer et al., 2003; Gibson, 2009; Maclean, 1983). This has implications for plant defensive compounds. Increased soil nitrogen has been found to lower foliar concentrations of carbon-based defensive chemicals (Jamieson, 2013; Prudic et al., 2005). By contrast, plants

growing in low nutrient conditions have been found to have higher concentrations of carbon-based defensive chemicals (Fajer et al., 1992).

Incorporating theories of chemical ecology into assessments of landscape level management tools is a novel approach to this complex work. It is typically unheard of for restoration design and management plans to include benchmarks for chemical-ecological function. My research aims to begin to synthesize these disparate fields by investigating the outcomes of landscape scale management techniques on the defensive chemistry (specifically, iridoid glycosides) of plant species used in species recovery efforts. This holistic approach will supplement what is known about the ecological impacts of management techniques on sensitive species by illuminating subtle biochemical ramifications of those techniques.

In this study, I investigate how land management techniques influence the iridoid glycoside class of phytochemicals. Iridoid glycosides are cyclopentanoid monoterpene-derived plant defensive compounds (Bowers, 1991). Globally, there are over 600 different iridoid glycosides from 57 different plant families, primarily within the order Lamiales (Bowers, 1991; Yamane et al., 2010). In plants, iridoid glycosides are synthesized via the mevalonic acid pathway, where they are derived from 10-hydroxygeraniol, via epi-iridodial and epi-deoxy-loganic acid (Inouye and Uesato, 1986; Jensen, 1992). Although some specialist insects have evolved to tolerate and even rely on iridoid glycosides, these compounds are toxic to most generalist insects, and have a denaturing effect on nucleic acids, amino acids, and proteins (Baden, 2016; Bowers, 1991).

To fully understand how abiotic land management techniques impact iridoid glycoside metabolism, we must also understand how biotic factors, such as herbivory, may also influence these feedback processes. Herbivory induces defensive chemical pathways in plants that have

the ability to influence ecosystem processes like nutrient cycling as their phytochemicals make their way into soil through leaf litter (Bardgett et al., 1998). However, herbivory induced phytochemical responses may be short-lived, and their broader impacts may also depend on the phenological stage in which they occur (Bowers, 1991).

This study focuses on individual plant iridoid glycoside variation in two genera (*Castilleja* and *Plantago*) using an unusually large sample size. It is unique in characterizing parallel iridoid glycoside patterns in response to phenological, abiotic, and biotic factors in plants from two different families (Orobanchaceae and Plantaginaceae, respectively) growing under similar conditions at a single site. Patterns in response to phenology and environmental factors are increasingly well-represented in the scientific literature on *Plantago*, but few equivalent studies exist for *Castilleja*. Existing research on *Plantago* reveals complex patterns of phenological fluctuation and induction in response to abiotic and biotic factors in iridoid glycosides (Bowers, 1991; Bowers et al., 1992).

Research objectives

My objectives in this study were to investigate and characterize the effects of simulated herbivory, prescribed burning, and soil lime amendments on plant defensive chemistry in two different plant families, focusing specifically on foliar iridoid glycoside concentrations in *Castilleja levisecta* and *Plantago lanceolata*. Special attention was paid to macfadienoside and aucubin, since they are, respectively, the most dominant iridoid glycosides in *C. levisecta* and *P. lanceolata* at this site.

I addressed the following research questions: (1) Does simulated herbivory have a direct effect on foliar iridoid glycoside concentrations in *Castilleja levisecta* and *Plantago lanceolata*, and do effects differ across phenology? (2) Do prescribed fire and soil lime amendments affect foliar iridoid glycoside concentrations in *Castilleja levisecta* and *Plantago lanceolata*?

My hypotheses were as follows: (1) I hypothesized that herbivory simulation would induce a short-term rise in iridoid glycoside concentrations, with no detectable long-run differences. (2) I predicted that plants growing in plots that were burned in the previous autumn would have lower foliar iridoid glycoside concentrations than plants growing in unburned plots, and that fast-acting lime would also result in decreased iridoid glycoside concentrations in plants growing in experimental quadrats.

Methods

Study sites and system

This research occurred at the Black River-Mima Prairie-Glacial Heritage Preserve in glacial outwash lowlands near the Puget Sound. My experiment was set up on the western edge of the 459-ha preserve (46.8712° Latitude, -123.0529° Longitude), on a section of abandoned agricultural land with acidic Nisqually loamy fine sand soil type, which has been restored over the past decade to native grassland (Bakker et al., 2013). I used a subset of existing experimental grassland plots from a previous study for my experiment (Bakker et al., 2013), as shown in Figure 2.1. This site is one of several Pacific Northwest lowland prairie restoration projects in which former agricultural fields have been restored using remnant native prairies of the region as

seed sources and reference sites, in an effort to expand and reconnect a greatly diminished and fragmented regional ecosystem (Bakker et al., 2013; Krueger et al., 2014).

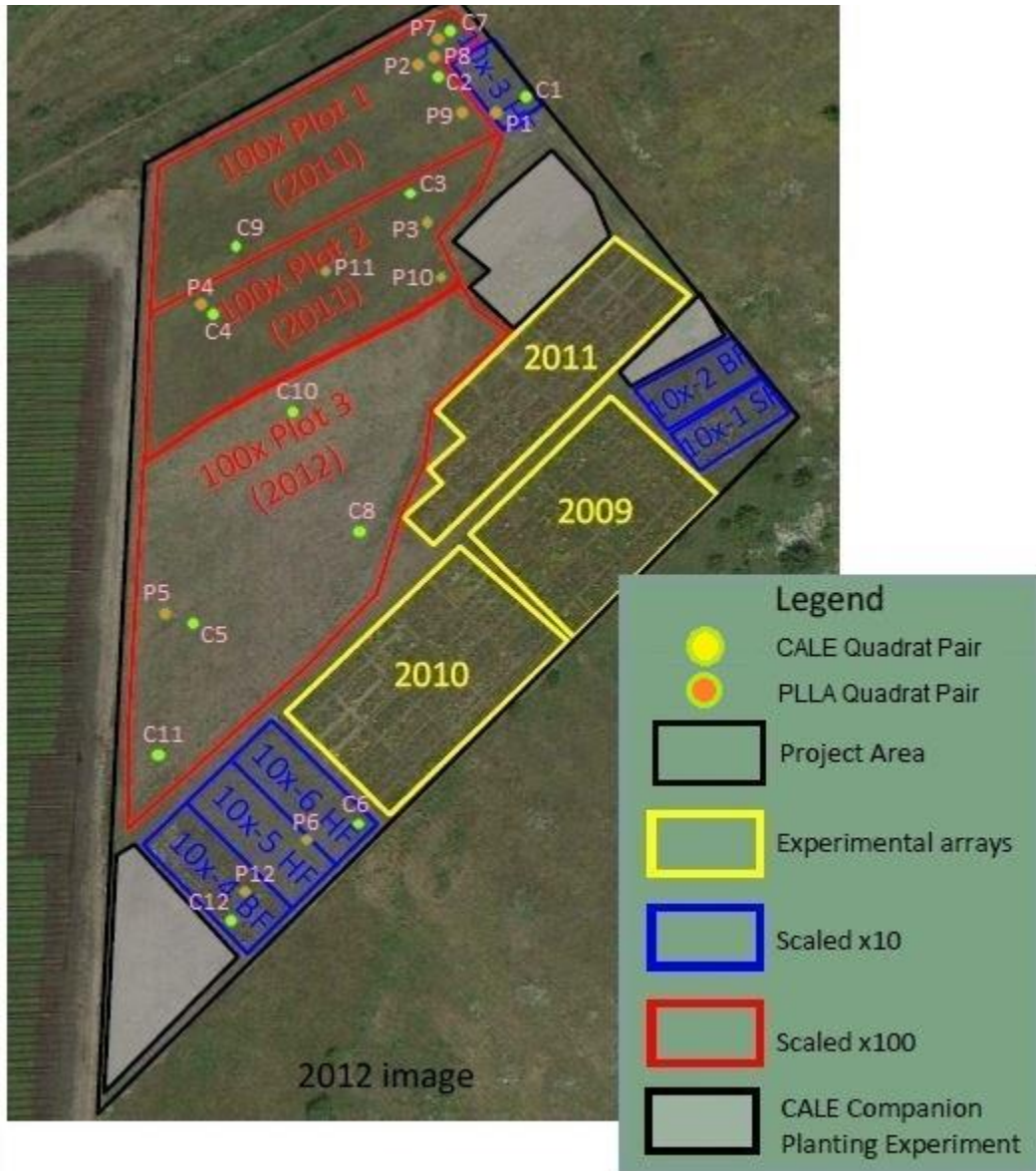


Figure 3.1: *C. levisecta* and *P. lanceolata* experimental plots at Glacial Heritage Preserve. Original image from Bakker et al. 2013.

My research focuses on two plant species found at Glacial Heritage Preserve: (1) *Castilleja levisecta* (Orobanchaceae), a hemiparasitic angiosperm native to British Columbia, Washington, and Oregon, and (2) *Plantago lanceolata* (Plantaginaceae), an exotic angiosperm native to

Europe (Cavers, 1980). These perennial species were chosen not only for their shared trait of iridoid glycoside production, but also for their central role in restoration and recovery efforts in this study system. *C. levisecta* itself has been listed as threatened since 1997 at the federal level (United States Dept. of Fish & Wildlife Service) and is one of three known suitable host species for larvae of the endangered Taylor's checkerspot (*Euphydryas editha* ssp. *taylori*) butterfly (Haan et al., 2017). Although considered weedy, *P. lanceolata* is the primary forage and oviposition species for Taylor's checkerspot larvae, and has become more widely used in recovery efforts (Severns, 2008). The iridoid glycoside secondary metabolites produced by *C. levisecta* and *P. lanceolata* are sequestered and utilized by Taylor's checkerspot larvae in their own chemical defense (Haan et al., 2017). My study builds on recent multitrophic biochemical research on *C. levisecta* and *P. lanceolata* (Haan et al., 2017), and aims to help land managers customize restoration and management protocols for the creation of suitable biochemical habitat for species recovery.

Experimental design

I created a factorial experimental design to investigate the effects of simulated herbivory and fast-acting lime on patterns of foliar iridoid glycoside concentration (% dry weight) across phenological stages (treatment dates). I established 24 plots consisting of pairs of 1m² quadrats and collected leaf samples during the 2016 and 2017 growing seasons. Twelve plots contained quadrats centered around at least four *Castilleja levisecta* plants, and 12 plots contained quadrats centered around at least four *Plantago lanceolata* plants. I established quadrats for both species in pairs to allow for one control and one experimental quadrat in each plot. (See Appendix 1 for photos.) Quadrats were separated by 1 to 1.5 m, in order to ensure similar environmental

conditions without undue influence from experimental treatments applied to a neighboring quadrat (Figure 2). Simple t-post and wire deer exclosures were erected around the *C. levisecta* plots because deer typically browse this species throughout the growing season.

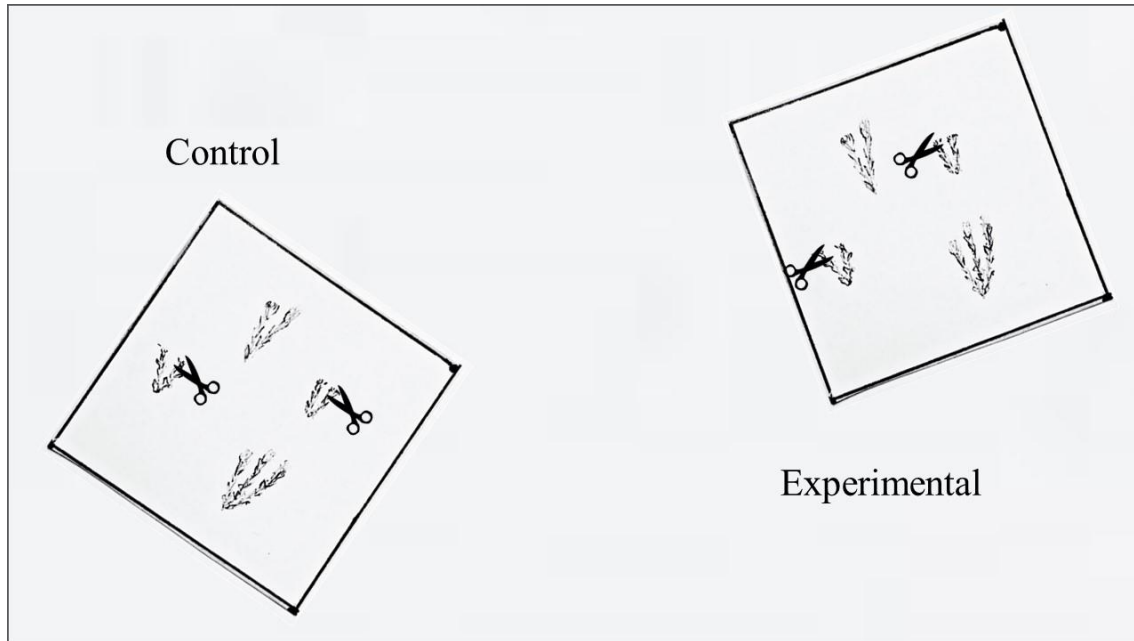


Figure 3.2: Diagram of 2016 *C. levisecta* plot design, with two 1m² quadrats per plot. Quadrats within a plot were separated by 1 to 1.5 m. Within quadrats, plants were selected systematically for herbivory simulation treatments in a clockwise, alternating manner. Note that in 2016, no experimental treatment was applied to quadrats designated “experimental”. In 2017, only one control plant and one experimental plant were sampled in each quadrat, and an experimental treatment (fast-acting lime) was applied.

Herbivory simulation

An herbivory simulation treatment was applied to all plots in both years using a factorial design, so that both control and experimental quadrats included control plants and plants receiving an herbivory simulation treatment (Figure 2). Plants were selected and designated experimental or control in a clockwise, alternating manner within each quadrat, avoiding plants growing at the quadrat edge.

Herbivory simulation was applied to experimental plants immediately after initial leaf sampling (pre-treatment), and was administered by clipping off the top ~30% of aboveground plant tissue, including flowers, with shears. Leaves were collected from the same individual plants prior to applying herbivory simulation treatments, and again at two additional post-simulation treatment time steps. This design was based on phenological interactions observed by Darrow and Bowers (1997) in the weeks following herbivory of *P. lanceolata*. Because iridoid glycoside concentrations can vary across different types and ages of tissues within an individual plant (Bowers, 1992; Mead and Stermitz, 1993), I sampled only mature leaves from a consistent position. For *C. levisecta* individuals, I chose five to six leaves from the mid-stem area, taking as few leaves from any one stem as possible to avoid creating an herbivory-like event through defoliation. *C. levisecta* leaves separate easily from stems, and so were pinched off by hand. In *P. lanceolata* individuals, I radially selected two to three mature leaves in an evenly spaced clockwise manner, clipping leaves at the base with shears. I recorded any signs of herbivore or pathogen foliar damage as potential explanatory variables.

In each *C. levisecta* quadrat, I marked four plants with paper clips, taking care to damage no leaves or stems, for a total of 192 plants. Because there is a history of unintended hybridization between *Castilleja levisecta* and *C. hispida* at this site (Fisher et al., 2015; Kaye and Blakeley-Smith 2008), I was careful to select plants that exhibited standard *C. levisecta* phenotype, although this method cannot guarantee that genetic hybrids are completely avoided. I sampled leaves from both control and experimental plants on May 25, 2016 (pre-treatment) and then immediately carried out an herbivory simulation treatment on experimental plants. I sampled leaves from these same plants again on June 1, 2016 (early) and June 14, 2016 (late). Most plants sampled on June 14 were near senescence.

To ensure that plants did not senesce before sampling could be completed, a reduced sample size for *P. lanceolata* was appropriate. In each *P. lanceolata* quadrat, I marked two plants with paper clips, taking care to damage no leaves or stems, for a total of 48 plants. I sampled leaves from both control and experimental plants on June 22, 2016 (pre-treatment), and then immediately carried out an herbivory simulation treatment on experimental plants. I sampled two to three leaves from these same plants again on June 29, 2016 (early). I also sampled a third plant from each plot on that same day to assess whether my sampling method on original control plants might be acting as an unintended minor herbivory simulation. No third round of sampling was feasible due to rapid desiccation of plant leaf tissues at the onset of late growing season drought.

In 2017, I used the same plots and quadrats used in 2016. I carried out initial leaf sampling, immediately followed by herbivory simulation treatment, for all *C. levisecta* plots on April 26, 2017 (pre-treatment), and for all *P. lanceolata* plots on May 2, 2017 (pre-treatment). Additional leaf samples were collected from *C. levisecta* plots on May 2 (early) and May 24, 2017 (late). Additional leaf samples were collected from *P. lanceolata* plots on May 9 (early) and May 30, 2017 (late). To ensure that plants did not senesce before all sampling rounds could be completed, only one control plant and one experimental plant were chosen in each quadrat for each species during this second year. Because winter weathering dislocated most of my 2016 paperclip markers, it was impossible in most cases to tell whether the plants I sampled in 2017 were the same plants I had sampled in 2016.

Lime amendment

I applied a soil liming treatment (Pennington Fast-Acting Lime, 480 g per 1m²) on March 30, 2017 to the experimental quadrat in each plot, while control quadrats were left unlimed. Lime

granules were distributed evenly over experimental quadrats by hand, and were not raked in to avoid disturbing existing plants.

Autumn prescribed burn

A prescribed fire to reduce the abundance of invasive *Hypochaeris radicata* occurred on some parts of my research site in September 2016. These burns affected nine of my *C. levisecta* plots and nine of my *P. lanceolata* plots, giving me three unburned control plots for each species to compare with the burned plots in 2017.

Sample preparation and analyses

Foliar concentrations of the iridoid glycosides aucubin (*P. lanceolata* and *C. levisecta*), catalpol, macfadienoside, and methyl shanzhiside (Haan et al., 2017) were assessed by the University of Colorado Boulder Bowers Laboratory using gas chromatography. Forty *C. levisecta* samples (2 from 2016, 38 from 2017) and two *P. lanceolata* samples (from 2017) were not analyzed due to missing sample, insufficient sample tissue, or because they were damaged in transit. Iridoid glycoside analysis methods followed Bowers and Stamp (1997) and Bowers (2003). Within twelve hours of collection, leaf tissues were oven dried at 50°C for 48 hours. Dried tissues were ground using mortar and pestle and then frozen to await shipment. To process thawed samples, a 25 mg aliquot was extracted in 95% methanol for 24 hours. Plant tissue solids were then filtered out, and the methanol was evaporated completely. 1.0 mL of the internal standard phenyl-β-D-glycopyranoside (PBG, 0.500 mg/mL; Sigma Aldrich, St. Louis, Missouri, USA) was added, and each sample was partitioned with diethyl ether to remove hydrophobic compounds. The ether layer was removed and the iridoid glycoside infused water layer evaporated. The remaining

residue was suspended in 1.0 mL methanol, and a 100 μ L aliquot was taken for analysis. The methanol was evaporated and the residue was derivatized using Tri-Syl-Z (Thermo-Fisher Chemical Company) in pyridine before injection into an Agilent 7890A gas chromatograph equipped with a DB-1 column (30 m, 0.320 mm, 0.25 μ m particle size) and using flame-ionization detection.

Amounts of aucubin, catalpol, and macfadienoside were quantified using ChemStation B-03-01 software. A fourth iridoid glycoside, putatively methyl shanzhiside (Haan et al., 2017), was present, but no standard was available to identify it clearly. Methyl shanzhiside concentrations were therefore estimated with a conversion factor based on the internal standard, PBG, since the original amount of this compound in each sample was known. Therefore, my reported values for concentrations of this fourth compound may not be exact, and are based on the assumption that the unknown compound behaves identically to PBG.

Statistical analyses

I used R 3.4.2 for all analyses (R Core Development Team 2017), using *Castilleja levisecta* and *Plantago lanceolata* iridoid glycoside concentrations as response variables. (See Appendices 2 and 3 for R code and raw data.) Given the large chemical differences among the species, they were analyzed separately. For each species, I first analyzed a combined dataset from both sample years to test sample year, phenology (factor, three levels), and herbivory simulation treatment as potential explanatory variables. I used linear mixed models (Lindstrom, 1988) with R package *lme4* (Bates et al. 2015), and compared Akaike information criterion (AIC) scores to select a best-fit model (Burnham and Anderson, 2004). Plot, quadrat, and plant were specified as

random effects in all models. In each case I compared a model containing sample year, treatment date, and herbivory simulation treatment to models that included each fixed effect. I used R package *r2glmm* to generate R^2 values for models (Jaeger, 2017). The significance of terms within the best-fit models was assessed using $\alpha = 0.05$, though p-values up to 0.10 were considered to be marginally significant. Because ANOVA is robust in the absence of normality (Läärä, 2009), data were not transformed. Significant results for factors or interactions with more than two levels were followed with pairwise contrasts using package *lmerTest* (Kuznetsova et al. 2017).

Second, I tested additional models by adding factors from my 2017 dataset (lime treatment and autumn 2016 prescribed burn) to significant combined dataset models in order to address variables that were only recorded in that year.

Results

Overview - Castilleja levisecta

Four iridoid glycosides were detected in my *Castilleja levisecta* leaf samples: aucubin, catalpol, macfadienoside, and methyl shanzhiside⁴. Total mean iridoid glycoside concentrations were 2.16% dry weight (SE ± 0.053) in 2016 (mid- to late growing season) and 1.00% dry weight (SE ± 0.04) in 2017 (early to mid-growing season). Macfadienoside was the most abundant constituent iridoid glycoside in both years. Abundances of each constituent iridoid glycoside are shown in Figure 3.3. Total and constituent iridoid glycoside concentrations responded differently in magnitude to sample year, phenology (treatment date), and herbivory simulation

⁴ Putative methyl shanzhiside. See full explanation in “Sample preparation and analysis” section.

treatment, as will be discussed in this section. Lime treatment and prescribed autumn burn were not significant factors for any *C. levisecta* iridoid glycoside category and thus are not discussed in the analyses below. Best fit models are presented in Table 3.1, and ANOVA results for factors from these models are summarized in Table 3.2.

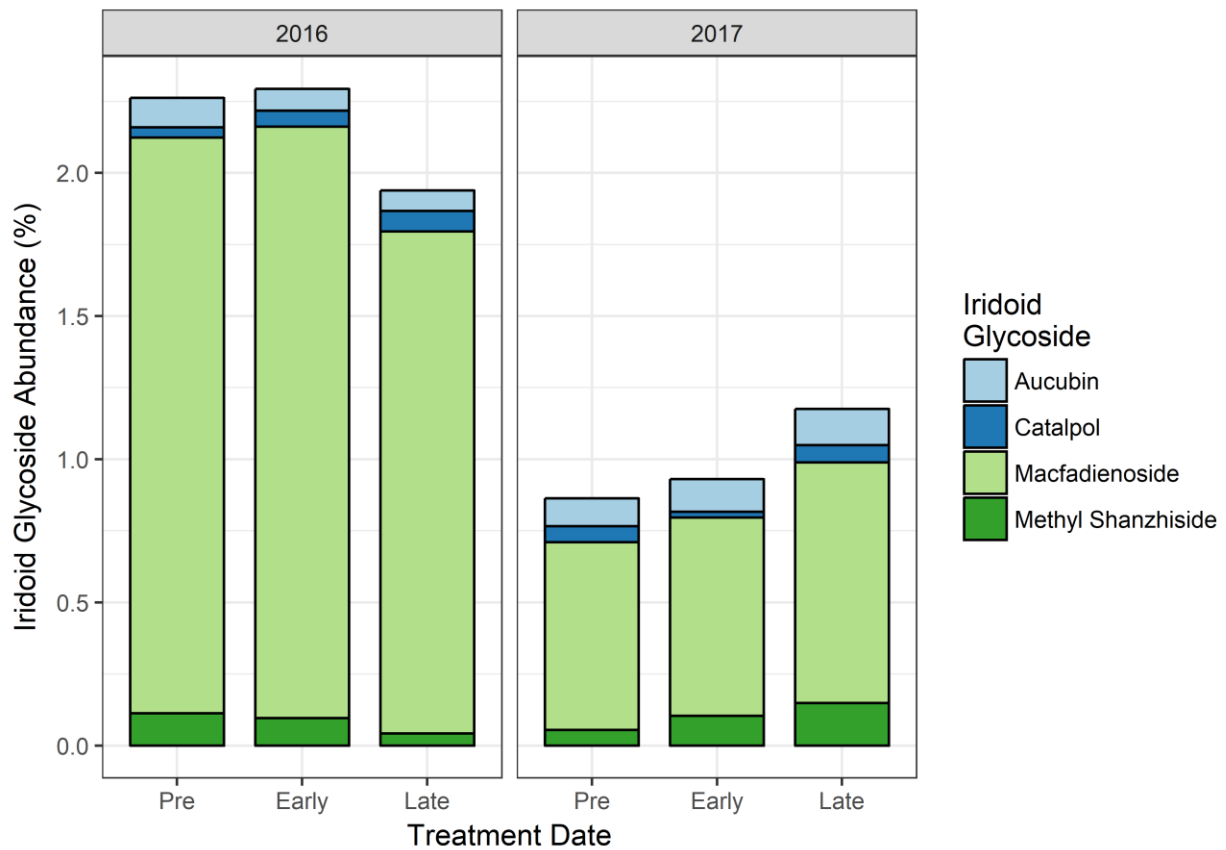


Figure 3.3: *C. levisecta* iridoid glycoside abundances in two growing seasons at three different time steps pre- and post-herbivory simulation treatment.

Table 3.1: Best fit models for *Castilleja levisecta* iridoid glycoside responses in 2016 and 2017.

Response	Adjusted R ²	Best Fit Models
<i>Total IGs</i> (% dry weight)	0.345	~ Year + Treatment Date + Year:Treatment Date
<i>Aucubin</i> (% dry weight)	0.038	~ Year + Treatment Date + Year:Treatment Date
<i>Catalpol</i> (% dry weight)	0.037	~ Year + Treatment Date + Year:Treatment Date
<i>Macfadienoside</i> (% dry weight)	0.375	~ Year + Treatment Date + Year:Treatment Date
<i>Methyl shanzhiside</i> (% dry weight)	0.212	~ Year + Treatment Date + Herbivory Simulation + Year:Treatment Date + Year:Herbivory + Year:Treatment Date:Herbivory
<i>Macfadienoside Abundance</i>	0.150	~ Year + Treatment Date

Table 3.2: ANOVA table describing effects of factors on the concentrations of iridoid glycosides in *C. levisecta* in 2016 and 2017. Alpha = 0.05. Reported P-values are lower P-values.

Response	Factor	SS	MS	df	F	p
<i>Total IGs</i> (% dry weight)	Year	42.502	42.502	1, 204	90.720	<0.001
	Treatment Date	2.454	1.227	2, 204	2.619	0.075
	Year × Treatment Date	6.946	3.473	2, 204	7.413	0.001
<i>Aucubin</i> (% dry weight)	Year	0.059	0.059	1, 204	6.417	0.012
	Treatment Date	0.019	0.009	2, 204	1.027	0.360
	Year × Treatment Date	0.056	0.028	2, 204	3.056	0.049
<i>Catalpol</i> (% dry weight)	Year	0.005	0.005	1, 204	0.694	0.406
	Treatment Date	0.057	0.029	2, 204	4.223	0.016
	Year × Treatment Date	0.042	0.021	2, 204	3.093	0.048
<i>Macfadienoside</i> (% dry weight)	Year	52.198	52.198	1, 204	118.730	<0.001
	Treatment Date	2.312	1.156	2, 204	2.629	0.075
	Year × Treatment Date	3.718	1.859	2, 204	4.228	0.016
<i>Methyl Shanzhiside</i> (% dry weight)	Year	0.008	0.008	1, 198	2.283	0.132
	Treatment Date	0.055	0.028	2, 198	7.501	0.001
	Herbivory Simulation	0.006	0.006	1, 198	1.513	0.220
	Year × Treatment Date	0.364	0.182	2, 198	49.666	<0.001
	Year × Herbivory Simulation	0.038	0.038	1, 198	10.318	0.002
	Year × Treatment Date × Herbivory Simulation	0.068	0.034	2, 198	9.267	<0.001
<i>Relative abundance of Macfadienoside</i>	Year	1.644	1.644	1, 206	46.299	<0.001
	Treatment Date	0.316	0.158	2, 206	4.446	0.013

Total iridoid glycosides - C. levisecta

Sample year was a significant factor ($p < 0.05$) for *C. levisecta* total iridoid glycoside concentrations, while phenology (treatment date) was a marginally significant ($p < 0.10$) factor. There was also a significant interaction effect between these two factors. Mean total iridoid glycoside concentrations were higher in 2016 (sampled mid- to late growing season) than in 2017 (sampled early to mid-growing season). In 2016, pre-treatment and early samples had approximately equal mean concentrations, while concentrations were lower in late samples. In 2017, mean concentrations were lowest in pre-treatment samples, intermediate in early samples, and highest in late samples (Figure 3.4).

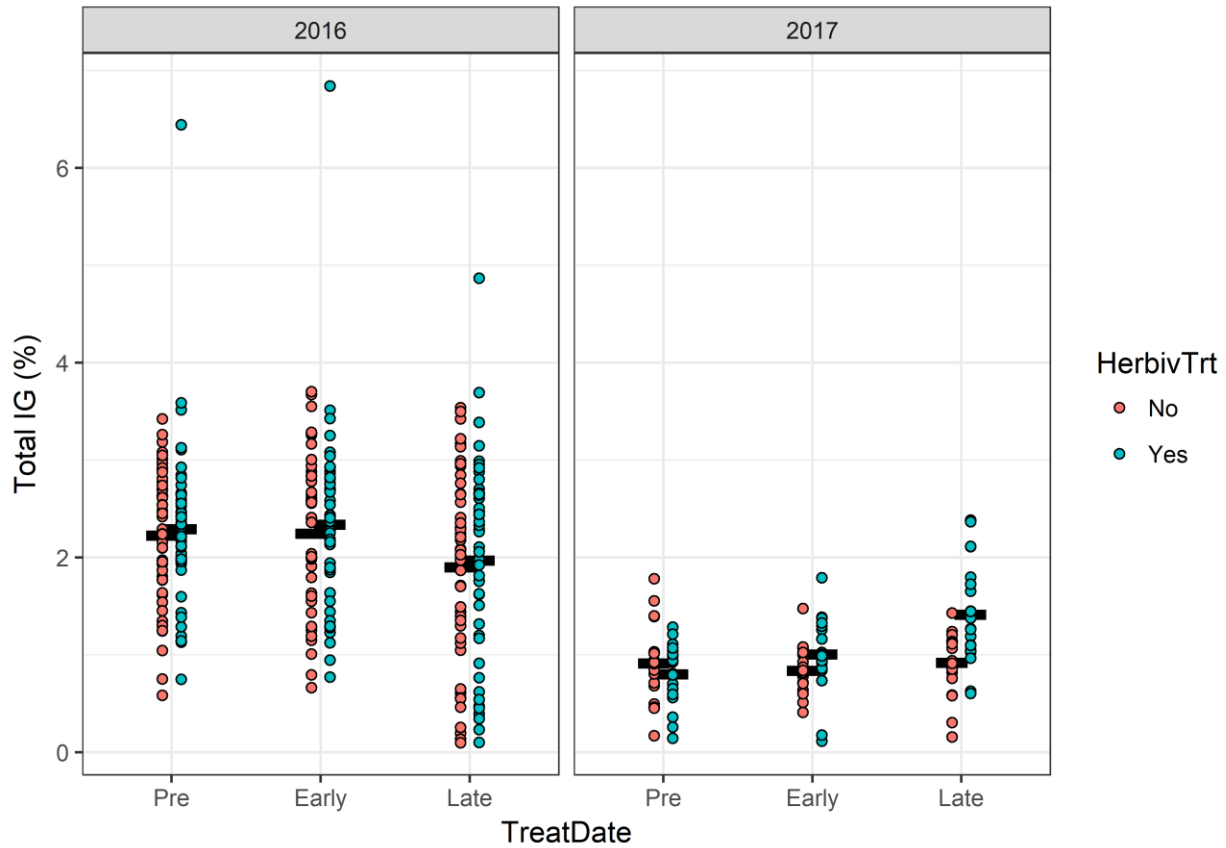


Figure 3.4: *C. levisecta* total iridoid glycosides pre- and post-herbivory simulation treatment.

Aucubin - C. levisecta

Sample year was a significant factor for *C. levisecta* aucubin concentrations, while phenology (treatment date) as a main effect was not. However, there was a significant interaction effect between sample year and phenology. Herbivory simulation treatment was not a significant factor for aucubin concentrations.

Mean aucubin concentrations were lower in 2016 (sampled mid- to late growing season) than in 2017 (sampled early to mid-growing season). In 2016, pre-treatment samples had the lowest mean aucubin concentrations, while early and late samples had approximately equal mean concentrations. In 2017, concentrations were lowest in pre-treatment samples, intermediate in early samples, and highest in late samples (Figure 3.5).

Catalpol - C. levisecta

Phenology (treatment date) was a significant factor for *C. levisecta* catalpol concentrations. There was also a significant interaction effect between sample year and phenology. Herbivory simulation treatment was not a significant factor for catalpol concentrations.

In 2016, catalpol concentrations were lowest in pre-treatment samples, intermediate in early samples, and highest in late samples. In 2017, concentrations were intermediate in pre-treatment samples, lowest in early samples, and highest in late samples (Figure 3.5).

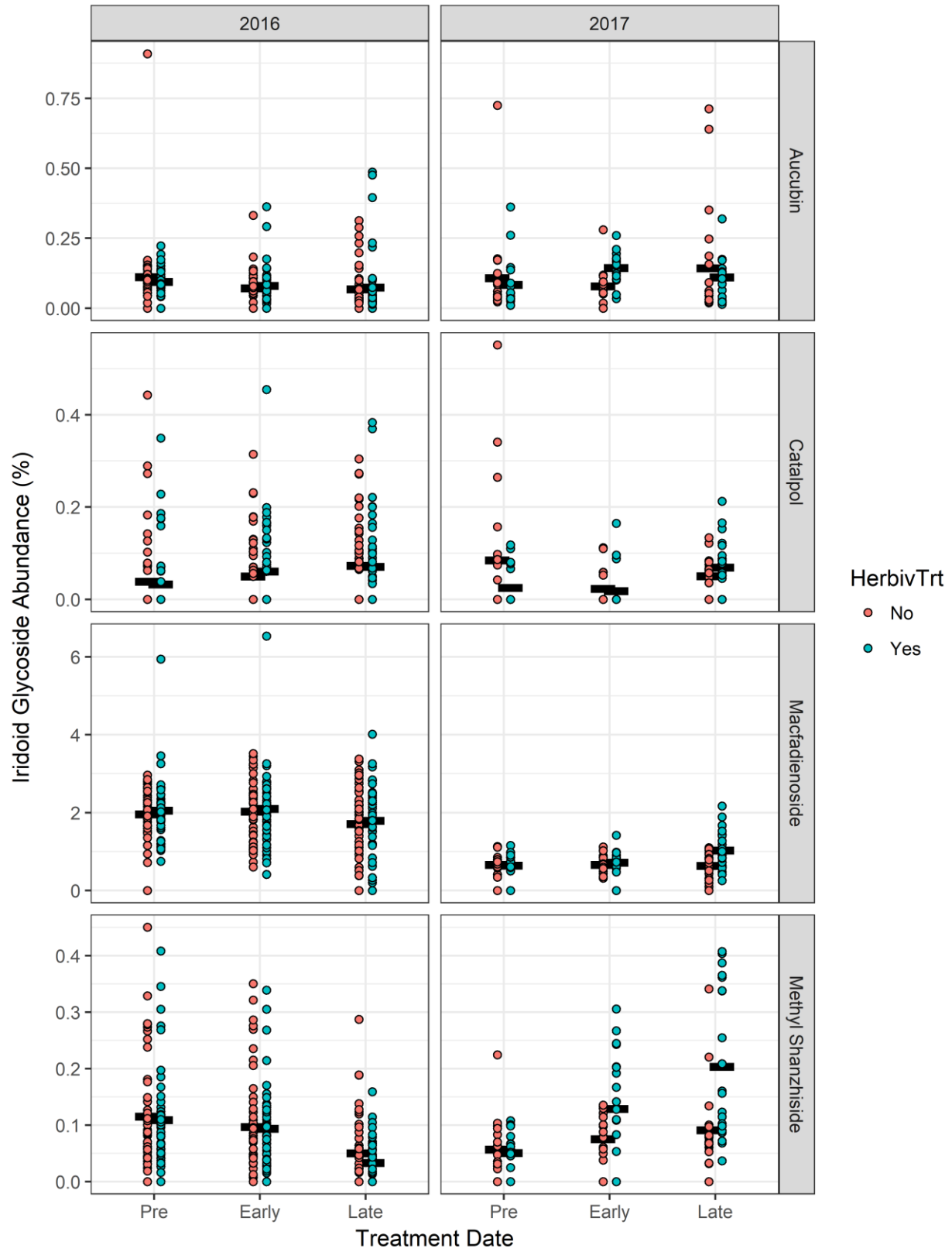


Figure 3.5: *C. levisecta* constituent iridoid glycosides pre- and post-herbivory simulation treatment.

Macfadienoside - C. levisecta

Sample year was a significant factor for *C. levisecta* macfadienoside concentrations, while phenology (treatment date) was a marginally significant factor. There was also a significant interaction effect between these two factors. Mean macfadienoside concentrations were higher in 2016 (sampled mid- to late growing season) than in 2017 (sampled early to mid-growing season). In both years, pre-treatment and early samples had approximately equal mean macfadienoside concentrations, while concentrations were lower in late samples (Figure 3.5). Herbivory simulation treatment was not a significant factor for macfadienoside concentrations.

Macfadienoside abundance

Sample year and phenology were both significant factors for macfadienoside abundance. Macfadienoside abundance was higher in 2016 than in 2017. In both years, pre-treatment and early samples had approximately equal macfadienoside abundance, while abundance was lower in late samples (Figure 3.6). Herbivory simulation treatment was not a significant factor for macfadienoside concentrations.

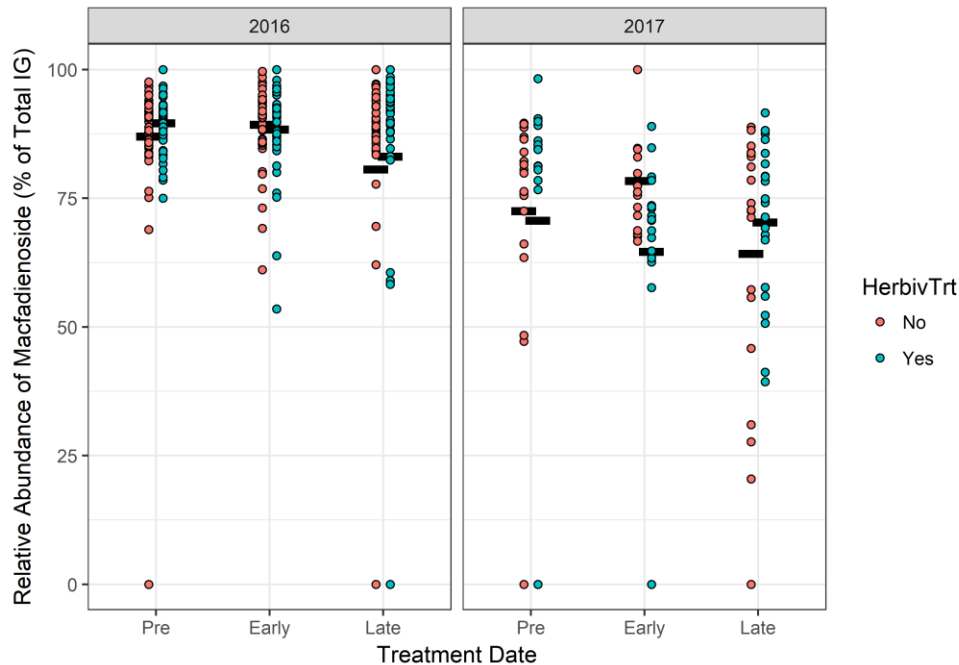


Figure 3.6: *C. levisecta* macfadienoside abundance pre- and post-herbivory simulation treatment.

Methyl shanzhiside - C. levisecta

Sample year, phenology (treatment date), and herbivory simulation treatment were significant factors for *C. levisecta* methyl shanzhiside concentrations. There was also a significant three-way interaction effect among these factors. Mean methyl shanzhiside concentrations were higher in 2016 (sampled mid- to late growing season) than in 2017 (sampled early to mid-growing season). In 2016, mean methyl shanzhiside concentrations were highest in pre-treatment samples, intermediate in early samples, and lowest in late samples.

In 2017, concentrations were lowest in pre-treatment samples, intermediate in early samples, and highest in late samples. Responses to herbivory simulation were stronger in 2017, with elevated methyl shanzhiside concentrations at the early and late samplings in plants that received herbivory simulation (Figure 3.5).

Overview - *Plantago lanceolata*

Two iridoid glycosides were detected in my *Plantago lanceolata* leaf samples: aucubin and catalpol. Total mean iridoid glycoside concentrations were 9.86% dry weight (SE \pm 0.36) in 2016 (mid- to late growing season) and 11.59% dry weight (SE \pm 0.37) in 2017 (early to mid-growing season). Aucubin was the most abundant constituent iridoid glycoside in both years. Abundances of aucubin and catalpol are shown in Figure 3.7. Total and constituent iridoid glycoside concentrations responded differently in magnitude to sample year, phenology (treatment date), herbivory simulation treatment, and recent prescribed burn, as will be discussed in this section. Lime treatment was not a significant factor for any iridoid glycoside category and thus is not discussed in the analyses below. Best fit models are presented in Table 3.3, and ANOVA results for factors from these models are summarized in Table 3.4.

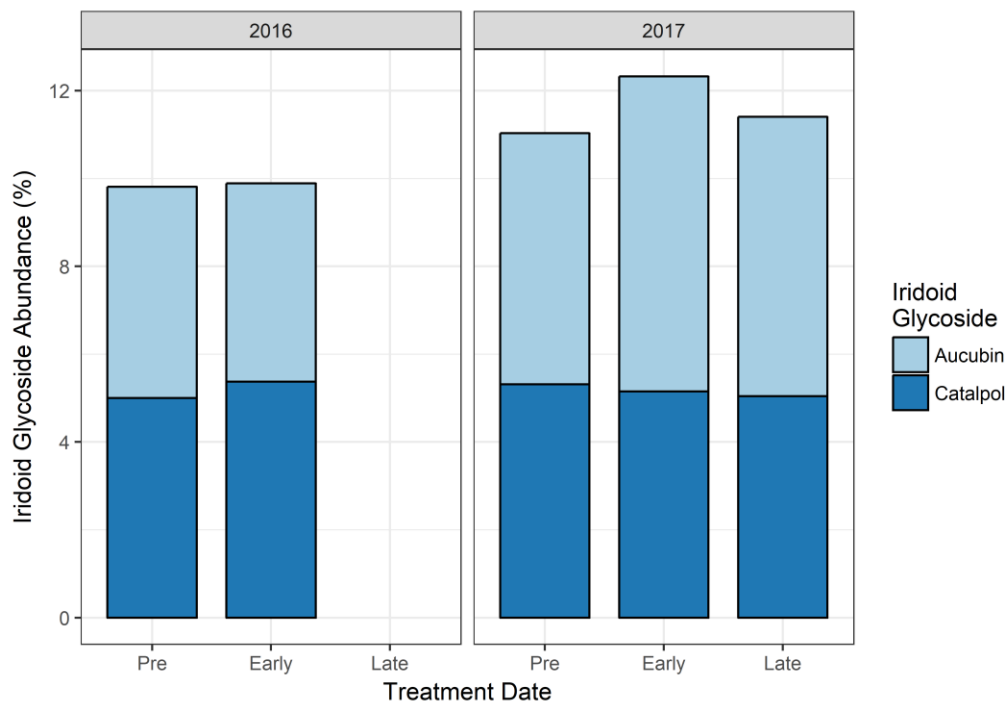


Figure 3.7: *P. lanceolata* total iridoid glycosides pre- and post-herbivory simulation treatment.

Table 3.3: Best fit models for *Plantago lanceolata* iridoid glycoside responses in 2016 and 2017.

Response	Subset	Adjusted R ²	Best Fit Models
<i>Total IGs</i> (% dry weight)	All	0.070	~ Year + Year:Treatment Date + Year: Herbivory + Treatment Date:Herbivory + Year:Treatment Date:Herbivory
	2017	0.192	~ Treatment Date + Herbivory + Treatment Date:Herbivory + Burned 2016
<i>Aucubin</i> (% dry weight)	All	0.140	~ Year + Treatment Date + Year:Treatment Date
	2017	0.117	~ Treatment Date + Burned 2016
<i>Catalpol</i> (% dry weight)	All	0.022	~ Year + Year:Treatment Date + Year: Herbivory + Treatment Date:Herbivory + Year:Treatment Date:Herbivory
	2017	0.128	~ Treatment Date:Herbivory + Burned 2016
<i>Aucubin Abundance</i>	All	0.099	~ Year + Herbivory + Treatment Date + Year:Treatment Date + Treatment Date:Herbivory

Table 3.4: ANOVA table describing effects of factors on the concentrations of iridoid glycosides in *P. lanceolata*. Alpha = 0.05. Reported P-values are lower P-values.

Response	Subset	Factor	SS	MS	df	F	p
<i>Total IGs</i> (% dry weight)	All	Year	125.815	125.815	1, 83	10.225	0.002
		Treatment Date	24.904	12.452	2, 83	1.012	0.368
		Herbivory Simulation	12.820	12.820	1, 83	1.042	0.310
		Year × Treatment Date × Herbivory Simulation	57.010	57.010	1, 83	4.633	0.034
	2017	Treatment Date	47.467	23.733	2, 83	1.690	0.190
		Herbivory Simulation	14.516	14.516	1, 83	1.034	0.317
		Treatment Date × Herbivory Simulation	37.338	18.669	2, 83	1.330	0.270
		Burned 2016	230.227	230.227	1, 83	16.396	0.002
<i>Aucubin</i> (% dry weight)	All	Year	91.759	91.759	1, 88	24.904	<0.001
		Year × Treatment Date	16.326	8.163	2, 88	2.216	0.115
	2017	Treatment Date	51.117	25.558	2, 88	5.624	0.005
		Burned 2016	28.993	28.993	1, 88	6.380	0.030
<i>Catalpol</i> (% dry weight)	All	Year	0.124	0.124	1, 83	0.031	0.861
		Treatment Date	1.946	0.973	2, 83	0.242	0.786
		Herbivory Simulation	0.335	0.335	1, 83	0.083	0.774
		Year × Treatment Date × Herbivory Simulation	18.179	18.179	1, 83	4.512	0.037
	2017	Treatment Date	1.304	0.652	2, 83	0.142	0.868
		Herbivory Simulation	1.205	1.205	1, 83	0.262	0.611
		Burned 2016	45.070	45.070	1, 83	9.808	0.003
<i>Relative abundance of Aucubin</i>	All	Year	0.081	0.081	1, 85	12.913	0.001
		Year:Treatment Date	0.075	0.075	1, 85	11.944	0.001
		Treatment Date × Herbivory Simulation	0.070	0.035	2, 85	5.578	0.005

Total iridoid glycosides - P. lanceolata

Sample year was a significant factor for *P. lanceolata* total iridoid glycoside concentrations, as was the three-way interaction among sample year (Figure 3.8), phenology (treatment date), and herbivory simulation. Prescribed burning in autumn 2016 was a significant factor for 2017 samples, with lower total iridoid glycoside concentrations in plants growing in plots that burned compared to those growing in plots that did not burn (Figure 3.9).

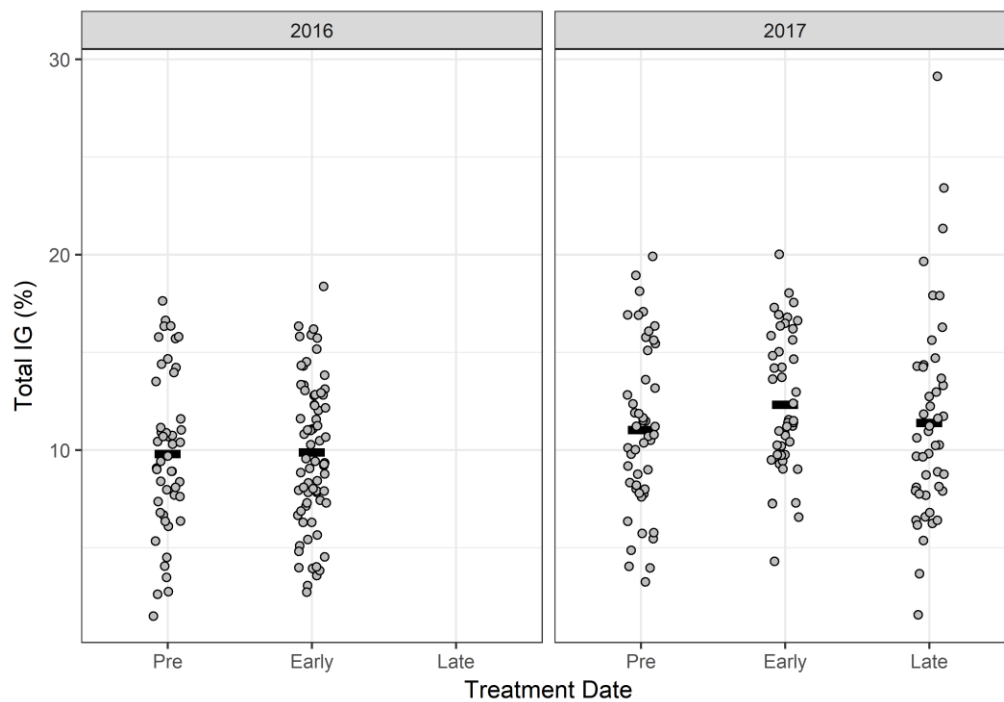


Figure 3.8: *P. lanceolata* total iridoid glycoside concentrations

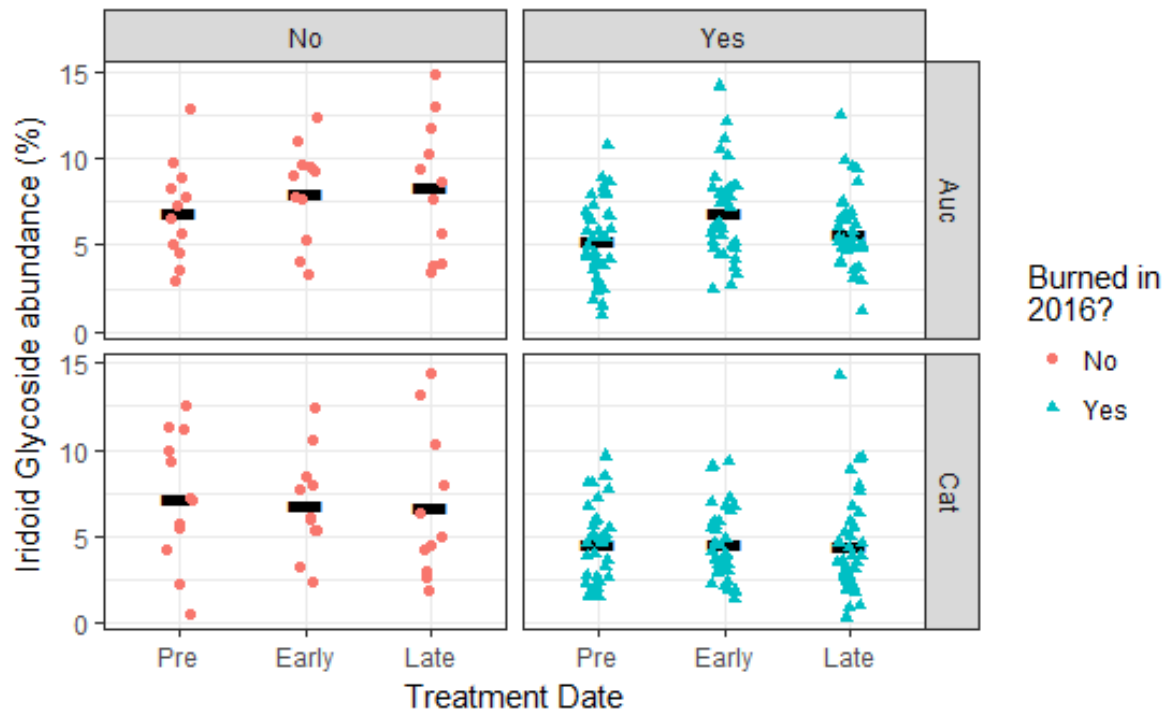


Figure 3.9: *P. lanceolata* total iridoid glycosides in burned and unburned plots, 2017.

Aucubin - P. lanceolata

Sample year was a significant factor for *P. lanceolata* aucubin concentrations, with higher concentrations in 2017 than in 2016, while phenology and herbivory simulation were not significant factors (Figure 3.10). Prescribed burning in autumn 2016 was a significant factor for 2017 samples, with lower aucubin concentrations in plants growing in plots that burned compared to those growing in plots that did not burn.

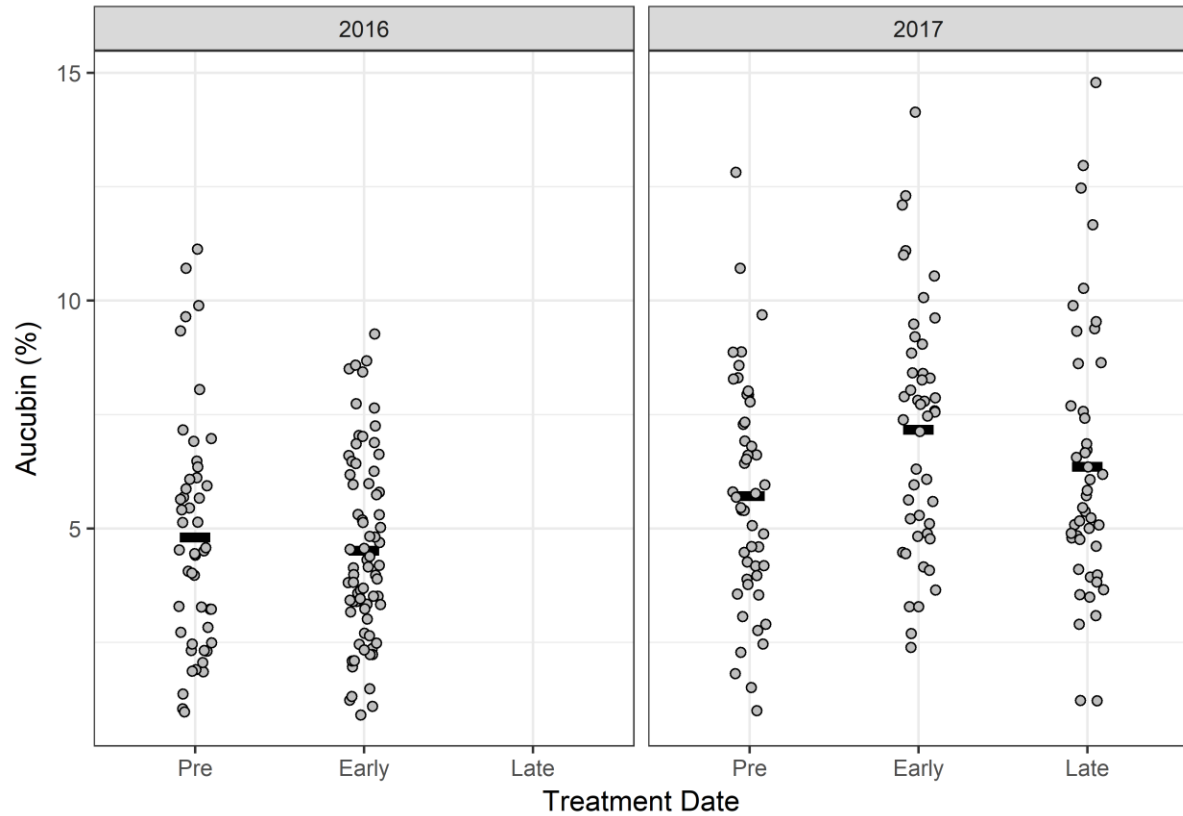


Figure 3.10: *P. lanceolata* aucubin concentrations pre- and post-herbivory simulation treatment.

Aucubin abundance

Sample year was a significant factor for *P. lanceolata* aucubin abundance, which was slightly higher in 2017 than in 2016 (Figure 3.11). There was also a significant interaction effect for aucubin abundance between sample year and phenology (treatment date), as well as between phenology and herbivory simulation. Abundance was slightly higher in plants receiving herbivory simulation treatments than in control plants. In 2016, aucubin abundance was higher at pre-treatment sampling than at early sampling. No late samples were collected in 2016. In 2017, aucubin abundance was lowest at pre-treatment sampling, highest at early sampling, and intermediate at late sampling.

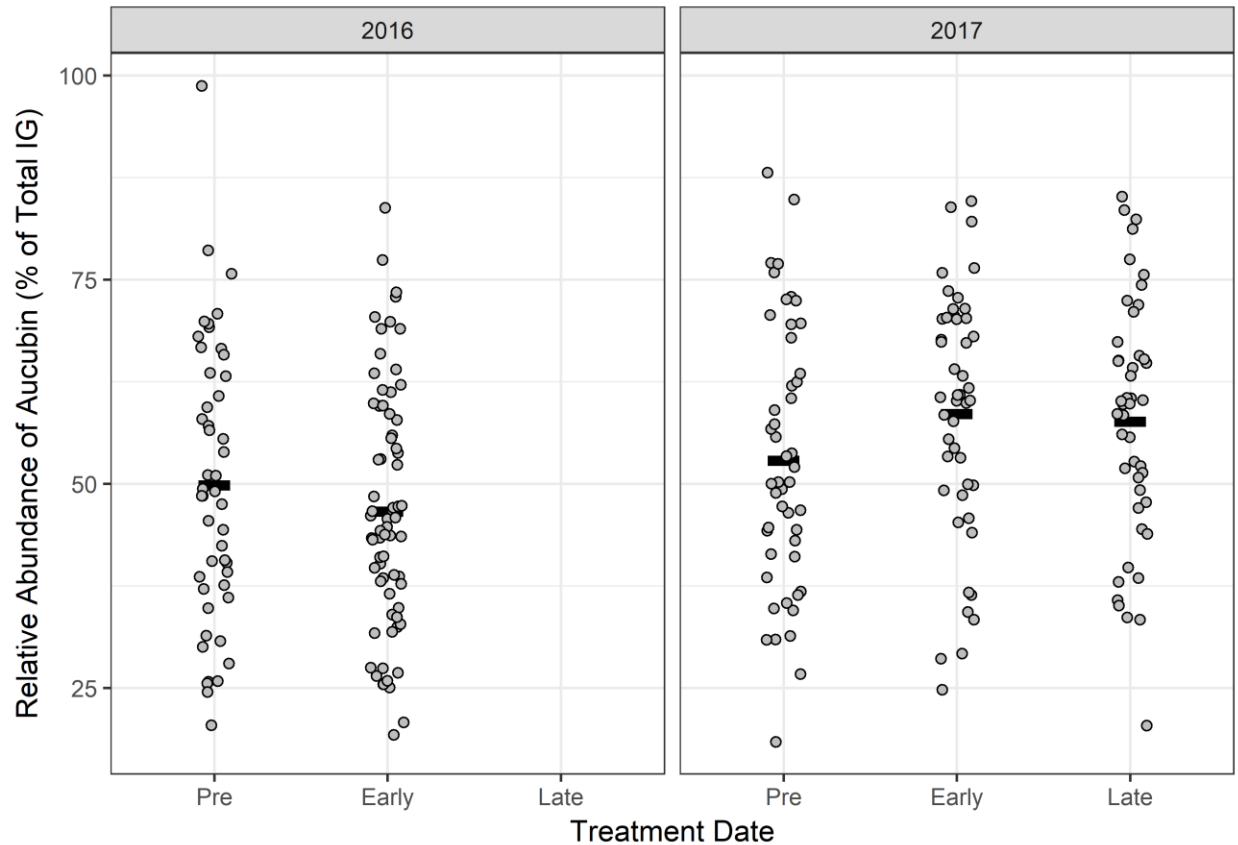


Figure 3.11: *P. lanceolata* aucubin abundance pre- and post-herbivory simulation treatment.

Catalpol - P. lanceolata

There was a significant three-way interaction effect among sample year, phenology (treatment date), and herbivory simulation for *P. lanceolata* catalpol concentrations. Mean catalpol concentrations were slightly higher in 2016 (sampled mid- to late growing season) than in 2017 (sampled early to mid-growing season). In 2016, catalpol concentrations were lowest in pre-treatment samples and highest in early samples. No late samples were collected in 2016.

In 2017, concentrations were highest in pre-treatment samples, intermediate in early samples, and lowest in late samples. Prescribed burning in autumn 2016 was a significant factor for 2017

P. lanceolata samples, with lower catalpol concentrations in plants growing in plots that burned compared to those growing in plots that did not burn (Figure 3.8).

Discussion

My results not only reveal large differences between *Castilleja levisecta* and *Plantago lanceolata* iridoid glycoside concentrations, but also reveal large variations in iridoid glycoside concentrations of both species among years. There are multiple possible explanations for this temporal variation. The differences could be due to weather patterns: the early 2016 growing season was unusually warm and dry, which resulted in atypically rapid phenological progression for both species, especially *C. levisecta*. By contrast, the 2017 growing season followed more typical seasonal temperature and moisture patterns, and phenological progression in both species was consistent with that of a typical growing season. Sampling also occurred at different phenological stages during the two years, with 2016 samples collected nearer senescence. Research from other plant families also documents peaks in some iridoid glycoside concentrations in mid- to late growing season (Bowers, 1991; Bowers et al., 1992).

Because my 2016 samples were collected in the mid- to late growing season, and my 2017 samples were collected in the early to mid-growing season, we might overlap them to propose a speculative complete growing season pattern for both species, in which total iridoid glycoside concentrations start out lower in the early growing season, gradually rise by mid-growing season, and then decline near senescence.

The insight my research provides into these phenological patterns may provide helpful insight for Taylor's checkerspot butterfly recovery efforts, to ensure appropriate timing for release of

larvae such that their chemical ecology needs are sufficiently supported by plant phenology (Haan,2017; Severns, 2008).

Herbivory simulation treatment

In *C. levisecta*, herbivory simulation only had a significant effect on methyl shanzhiside concentrations, with decreased concentrations in the late 2016 growing season, and a gradual rise over four weeks in the early to mid-growing season in 2017. In *P. lanceolata*, herbivory simulation treatment had a significant effect on total iridoid glycoside concentrations, aucubin abundance, and catalpol concentrations. More research is needed to understand what mechanisms cause constituent iridoid glycosides in different plant families to respond differently to herbivory simulation, as well as to actual herbivory. I distinguish throughout this chapter between simulated herbivory and actual herbivory, since simulated mechanical damage effects do not necessarily mimic the patterns of those induced by herbivores, and may be the reason not all constituent iridoid glycosides were affected. One reason for this may be that animal saliva proteins are known to interact with plant defensive chemicals in a variety of ways, though the ramifications of herbivore biomolecules for plants responding to herbivory are still unclear (Austin, 1989; Keefover-Ring, 2015). My results reveal some significant responses for simulated herbivory, and warrant future research on differences in these effects across plant families and constituent iridoid glycoside types. Insight into how precursor molecules are allocated between iridoid glycoside pathways in response to herbivore induction of defensive mechanisms would provide valuable insight for Taylor's checkerspot recovery efforts.

Prescribed burning

Autumn prescribed burning had no effect on *C. levisecta* total or constituent iridoid glycosides, but had a significant lowering effect on *P. lanceolata* total and constituent iridoid glycoside concentrations. Only the *P. lanceolata* results are consistent with my hypothesis, which was based on the findings of previous studies which found that nitrogen enrichment in soil, such as temporarily occurs following a prescribed burn, causes reductions in carbon-based defensive compounds (Fajer et al., 1992; Wan et al., 2001).

Although we might also expect to see an impact on *C. levisecta* iridoid glycosides after an autumn burn, the effect of fire on plant secondary metabolism is more complex than the mere addition of a short lived nutrient pulse. Fire also alters soil pH, moisture, and temperature, as well as competition and light acquisition dynamics in the plant community (Gibson, 2009). Some of these effects, such as altered soil moisture level, can complicate iridoid glycoside responses to increased soil nitrogen (Jamieson, 2013; Prudic et al., 2005).

If land managers use *P. lanceolata* as a forage species for Taylor's checkerspot butterfly recovery, it is important for them to know that prescribed fire may cause significant reductions in the iridoid glycosides that influence checkerspot butterfly oviposition preferences and larval metabolism (Bowers, 1991; Prudic, 2005). Future research on the effect of iridoid glycosides in these species should be carried out at multiple prairie sites that have experienced different burning regimes within each site to develop management standards for conservation goals across larger geographical regions.

Soil lime amendments

In this study, lime application had no significant effect on any iridoid glycoside concentrations in *C. levisecta* or *P. lanceolata*, but this should not rule it out for further research, as lime is an important aid to plant nutrient acquisition in acidic soils (McCauley et al., 2009), and may have significant indirect effects on plant secondary chemistry (Matyssek et al., 2012).

Moreover, because lime increases pH in the same way that fire does, it may give us a way to explore some of the mechanisms of fire underlying fluctuations in iridoid glycoside concentrations in a more controlled experimental setting. Multiple years of lime applications may produce more marked results, and may help simulate the soil pH effects of multiple years of burning.

Recommendations for future research

The fact that constituent iridoid glycosides of *Castilleja levisecta* (Orobanchaceae) and *Plantago lanceolata* (Plantaginaceae) respond differently to mechanical damage and experimental treatment factors across phenology creates a need for more insight into the more subtle mechanisms underlying their chemical ecology. Phenological considerations, and their implication for species recovery, will prove especially important with climate change (Cleland, 2007). To better characterize iridoid glycoside patterns across plant families and phenology, future research could replicate my methods in a common garden experiment, with additional protocols surrounding genotype and plant age. These two factors were unknowns in my research and no doubt created some confounding effects. Additional research could also elucidate the

mechanisms underlying resource allocation between different constituent iridoid glycosides during biosynthesis.

Macfadienoside and aucubin abundance

Macfadienoside was the dominant constituent iridoid glycoside in my *C. levisecta* samples, while aucubin was dominant in my *P. lanceolata* samples. Further research is needed to elucidate what this might mean for Taylor's checkerspot larvae feeding on these plant. Existing research shows that Taylor's checkerspot larvae do not require macfadienoside for survival (Haan et al., 2017), but how differences in macfadienoside and aucubin abundance in host plant tissues might affect larval survival into adulthood is as of yet unknown. This topic warrants additional research, as conservation efforts for Taylor's checkerspot seek to identify ideal host plants for larvae (Haan et al., 2017; Severns, 2008).

Conclusion

I tested the effects of simulated herbivory, prescribed fire, and fast-acting soil lime on foliar iridoid glycoside concentrations in *Castilleja levisecta* and *Plantago lanceolata* across two growing seasons, providing important insight into the mechanisms by which iridoid glycosides mediate crucial multitrophic interactions in imperiled grassland ecosystems. My results show that plant families can differ greatly in iridoid glycoside levels and constituent iridoid glycoside responses to phenology, simulated herbivory, and prescribed burning, and that landscape scale management techniques may be one method of achieving chemical ecology goals in the context of ecological restoration. Herbivory simulation only affected one constituent iridoid glycoside in each species. *Castilleja levisecta* iridoid glycoside concentrations were not affected by a recent

burn, while *Plantago lanceolata* iridoid glycoside concentrations were significantly affected. Fast-acting lime had no effect on iridoid glycosides in either species. This research confirms that environmental factors, phenology, and multitrophic interactions are all important considerations that can inform sound land management protocols.

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Works Cited

- Anderson, M. J. (2001). A new method for non-parametric multivariate analysis of variance. *Austral ecology*, 26(1), 32-46.
- Austin, P. J., Suchar, L. A., Robbins, C. T., & Hagerman, A. E. (1989). Tannin-binding proteins in saliva of deer and their absence in saliva of sheep and cattle. *Journal of Chemical Ecology*, 15(4), 1335-1347.
- Baden, C.U. (2016). *Phylogeny and sequestration of iridoid glycosides in selected genera of the Mecininae (Coleoptera, Curculionidae) with particular focus on their host plant relationship* (Doctoral Dissertation).
- Baer, S. G., Blair, J. M., Collins, S. L., & Knapp, A. K. (2003). Soil resources regulate productivity and diversity in newly established tallgrass prairie. *Ecology*, 84(3), 724-735
- Bakker, J. D., E. Delvin, and P. W. Dunwiddie. (2013). Prairie habitat restoration for endangered species: final report. Prepared for the U.S. Fish and Wildlife Service.
- Bardgett, R. D., Wardle, D. A., & Yeates, G. W. (1998). Linking above-ground and below-ground interactions: how plant responses to foliar herbivory influence soil organisms. *Soil Biology and Biochemistry*, 30(14), 1867-1878.
- Bates, D., Mächler, M., Bolker, B., & Walker, S. (2014). Fitting linear mixed-effects models using lme4. *arXiv preprint arXiv:1406.5823*.
- Bowers, M.D. (1991). Iridoid glycosides. In: Rosenthal, G.A., & Berenbaum, M.R. [eds.] *Herbivores: their interactions with secondary plant metabolites*. Second Edition, Vol. 1: the chemical participants. Academic Press, Sandiego, CA.
- Bowers, M. D., Collinge, S. K., Gamble, S. E., & Schmitt, J. (1992). Effects of genotype, habitat, and seasonal variation on iridoid glycoside content of *Plantago lanceolata* (Plantaginaceae) and the implications for insect herbivores. *Oecologia*, 91(2), 201-207.
- Burnham, K. P., & Anderson, D. R. (2004). Multimodel inference: understanding AIC and BIC in model selection. *Sociological methods & research*, 33(2), 261-304.
- Calabria, L. M., Petersen, K., Hamman, S. T., & Smith, R. J. (2016). Prescribed Fire Decreases Lichen and Bryophyte Biomass and Alters Functional Group Composition in Pacific Northwest Prairies. *Northwest Science*, 90(4), 470-483.
- Cavers, P. B., Bassett, I. J., & Crompton, C. W. (1980). The Biology of Canadian Weeds: *Plantago lanceolata* L. *Canadian Journal of Plant Science*, 60(4), 1269-1282.
- Cleland, E. E., Chuine, I., Menzel, A., Mooney, H. A., & Schwartz, M. D. (2007). Shifting plant phenology in response to global change. *Trends in ecology & evolution*, 22(7), 357-365.

- Covelo, F., & Gallardo, A. (2004). Green and senescent leaf phenolics showed spatial autocorrelation in a *Quercus robur* population in northwestern Spain. *Plant and Soil*, 259(1-2), 267-276.
- Darrow, K., & Bowers, M. D. (1997). Phenological and population variation in iridoid glycosides of *Plantago lanceolata* (Plantaginaceae). *Biochemical Systematics and Ecology*, 25(1), 1-11.
- Dietz, M., Machill, S., Hoffmann, H. C., & Schmidtke, K. (2013). Inhibitory effects of *Plantago lanceolata* L. on soil N mineralization. *Plant and Soil*, 368(1-2), 445-458.
- Dunwiddie, P. W., & Rogers, D. L. (2016). Rare species and aliens: reconsidering non-native plants in the management of natural areas. *Restoration Ecology*.
- Dunwiddie, P. W., & Bakker, J. D. (2011). The future of restoration and management of prairie-oak ecosystems in the Pacific Northwest. *Northwest Science*, 85(2), 83-92.
- Fajer, E. D., Bowers, M. D., & Bazzaz, F. A. (1992). The effect of nutrients and enriched CO₂ environments on production of carbon-based allelochemicals in *Plantago*: a test of the carbon/nutrient balance hypothesis. *The American Naturalist*, 140(4), 707-723.
- Fisher, L., Bakker, J. D., & Dunwiddie, P. W. (2015). An Assessment of Seed Production and Viability of Putative *Castilleja levisecta* × *C. hispida* Hybrids. Report for the Center for Natural Lands Management.
- Fitzpatrick, G. S. (2004). Techniques for restoring native plant communities in upland and wetland prairies in the midwest and west coast regions of North America. *Report prepared for City of Eugene—Parks and Open Space Division, Eugene, Oregon*.
- Gibson, D. J. (2009). *Grasses and grassland ecology*. Oxford University Press.
- Haan, N. L., Bakker, J. D., & Bowers, M. D. (2017). Hemiparasites can transmit indirect effects from their host plants to herbivores. *Ecology*.
- Hamman, S. T., Dunwiddie, P. W., Nuckols, J. L., & McKinley, M. (2011). Fire as a restoration tool in Pacific Northwest prairies and oak woodlands: challenges, successes, and future directions. *Northwest Science*, 85(2), 317-328.
- Hill, K. C., Bakker, J. D., & Dunwiddie, P. W. (2017). Prescribed fire in grassland butterfly habitat: targeting weather and fuel conditions to reduce soil temperatures and burn severity. *Fire Ecology*, 13(3), 24-41.
- Hobbs, R. J. (2007). Setting effective and realistic restoration goals: key directions for research. *Restoration ecology*, 15(2), 354-357.

- Hunter, M. D. (2016). *The Phytochemical Landscape: Linking Trophic Interactions and Nutrient Dynamics*. Princeton University Press.
- Inouye, H., & Uesato, S. (1986). Biosynthesis of iridoids and secoiridoids. In *Fortschritte der Chemie Organischer Naturstoffe/Progress in the Chemistry of Organic Natural Products* (pp. 169-236). Springer Vienna.
- Jaeger, B. C., Edwards, L. J., Das, K., & Sen, P. K. (2017). An R² statistic for fixed effects in the generalized linear mixed model. *Journal of Applied Statistics*, 44(6), 1086-1105.
- Jamieson, M. A., Quintero, C., & Blumenthal, D. M. (2013). Interactive effects of simulated nitrogen deposition and altered precipitation patterns on plant allelochemical concentrations. *Journal of Chemical Ecology*, 39(9), 1204-1208.
- Jensen, S. R. (1992). Systematic implications of the distribution of iridoids and other chemical compounds in the Loganiaceae and other families of the Asteridae. *Annals of the Missouri Botanical Garden*, 284-302.
- Kaye, T. N., & Blakeley-Smith, M. (2008). An Evaluation of the Potential for Hybridization Between *Castilleja levisecta* and *C. hispida*. Unpublished report. Institute for Applied Ecology, Corvallis, OR.
- Keefover-Ring, K., Rubert-Nason, K. F., Bennett, A. E., & Lindroth, R. L. (2015). Growth and chemical responses of trembling aspen to simulated browsing and ungulate saliva. *Journal of Plant Ecology*, 9(4), 474-484.
- Krueger, J. J., Bois, S. T., Kaye, T. N., Steeck, D. M., & Taylor, T. H. (2014). Practical guidelines for wetland prairie restoration in the Willamette Valley, Oregon; field tested methods and techniques. *Guide produced by: Lane Council of Governments, Institute for Applied Ecology, and the City of Eugene, OR*, 1-96.
- Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. (2016). lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software*, 82(13), 1-26.
- Läärä, E. (2009). Statistics: reasoning on uncertainty, and the insignificance of testing null. In *Annales Zoologici Fennici* (Vol. 46, No. 2, pp. 138-157).
- Lindstrom, M. J., & Bates, D. M. (1988). Newton—Raphson and EM algorithms for linear mixed-effects models for repeated-measures data. *Journal of the American Statistical Association*, 83(404), 1014-1022.
- Matyssek, R. (2012). Conclusions and perspectives. In *Growth and Defence in Plants* (pp. 453-457). Springer Berlin Heidelberg.
- McCauley, A., Jones, C., & Jacobsen, J. (2009). Soil pH and organic matter. *Nutrient management module*, 8, 1-12.

- Mead, E. W., & Stermitz, F. R. (1993). Content of iridoid glycosides in different parts of *Castilleja integra*. *Phytochemistry*, 32(5), 1155-1158.
- Miller, J. O. (2016). *Soil PH and Nutrient Availability*. UME. FS-1054.
- Prudic, K. L., Oliver, J. C., & Bowers, M. D. (2005). Soil nutrient effects on oviposition preference, larval performance, and chemical defense of a specialist insect herbivore. *Oecologia*, 143(4), 578-587.
- Pyke, D. A., Brooks, M. L., & D'Antonio, C. (2010). Fire as a restoration tool: a decision framework for predicting the control or enhancement of plants using fire. *Restoration ecology*, 18(3), 274-284.
- Severns, P. M., & Warren, A. D. (2008). Selectively eliminating and conserving exotic plants to save an endangered butterfly from local extinction. *Animal Conservation*, 11(6), 476-483.
- Stanley, A. G., Kaye, T. N., & Dunwiddie, P. W. (2010). *Regional strategies for restoring invaded prairies*. final technical report. Institute for Applied Ecology, Corvallis, OR and The Nature Conservancy, Seattle, WA. <http://appliedeco.org/reports/default-page#rareplant-species-research> (accessed 3 May 2012).
- Stinson, D. W. (2005). Status Report for the Mazama Pocket Gopher, Streaked Horned Lark, and Taylor's Checkerspot. Washington Department of Fish and Wildlife.
- Storm, L., & Shebitz, D. (2006). Evaluating the purpose, extent, and ecological restoration applications of indigenous burning practices in southwestern Washington. *Ecological Restoration*, 24(4), 256-268.
- United States. Dept. of Fish and Wildlife Service. Golden paintbrush (*Castilleja levisecta*) listing status. Environmental Conservation Online System. Web. 4 December 2017.
- Wan, S., Hui, D., & Luo, Y. (2001). Fire effects on nitrogen pools and dynamics in terrestrial ecosystems: A meta-analysis. *Ecological Applications*, 11(5), 1349-1365.
- White, R. P., Murray, S., Rohweder, M., Prince, S. D., & Thompson, K. M. (2000). *Grassland ecosystems* (p. 81). Washington, DC: World Resources Institute.
- Yamane, H., Konno, K., Sabelis, M., Takabayashi, J., Sassa, T., & Oikawa, H. (2010). Chemical defence and toxins of plants.

Chapter Four: Synthesis and opportunities for future research

The findings of the studies I presented in Chapters Two and Three are synthesized here to provide context and suggestions for future investigations of biochemical aspects of ecological restoration. In this chapter, I also propose some experimental methods for identifying the mechanisms underlying iridoid glycoside patterns that emerged in my two studies, or for expanding our knowledge of them.

I observed striking phenological variation in both *C. levisecta* and *P. lanceolata* foliar iridoid glycoside concentrations, such as large increases in total iridoid glycosides in the late growing season in *C. levisecta* macfadienoside concentrations. Because iridoid glycoside responses to environmental factors are more pronounced at some phenological stages than others, it is important that future research investigate other experimental factors in the context of multiple phenological stages (Darrow and Bowers, 1997).

Simulated herbivory affected only some constituent iridoid glycoside patterns in both species. In *C. levisecta*, herbivory simulation only had a significant effect on methyl shanzhiside concentrations, and was related to late phenology decreases in concentrations in 2016, with a gradual rise over the course of the early to mid-growing season in 2017. In *P. lanceolata*, herbivory simulation treatment had a significant effect on total iridoid glycoside concentrations, aucubin abundance, and catalpol concentrations.

My findings are for the most part consistent with what is known in existing research. Stamp and Bowers found that mechanical damage to *Plantago lanceolata* leaves resulted in lowered catalpol to aucubin abundances later in the growing season (1994). Future research on how

herbivory differently affects constituent iridoid glycosides in both of my research species could provide important insight into how precursor molecules are allocated in plant iridoid glycoside pathways.

Perhaps the most interesting results of my research are those related to iridoid glycoside responses to prescribed burning, since prescribed burning is a commonly used tactic in restored grasslands for enhancing both species diversity and vegetation heterogeneity (Hill et al., 2017), and for undermining dominance of invasive species (Agee, 1996). Prescribed fire had a significant effect on some constituent iridoid glycoside concentrations in both *C. levisecta* (Chapter Two) and *P. lanceolata* (Chapter Three).

For *C. levisecta*, the effect was most significant on plots that had higher quantities of historical burns. Iridoid glycoside concentrations were negatively related to quantity of burns. However, these patterns were perhaps mitigated by phenological effects in 2017, when samples were collected in the early growing season. Quantity of historical prescribed fires proved to be an important factor in this study. Increased fire frequency has been observed to decrease the inorganic soil nitrogen and cumulative net nitrogen mineralization crucial to plant nutrient acquisition and metabolism, which could be a mechanism influencing secondary chemistry in *C. levisecta* (Blair, 1997). Frequent fires could influence iridoid glycosides directly through long-term ecological legacies in soil nutrient dynamics, or more indirectly by increasing plant community diversity (Gibson, 2009; Mraja et al., 2011). Quantity of historical burns was not tested in *P. lanceolata*, but a recent autumn burn had significant lowering effects on catalpol and aucubin abundance in this species.

Additional research on the mechanisms underlying indirect effects of fire on iridoid glycoside concentrations is needed. Future research could be carried out at multiple prairie sites that have experienced prescribed burning regimes, in order to provide a large scale regional assessment of whether the patterns observed in my study hold true across more variable habitat. Investigating landscape level iridoid glycoside responses to prescribed burning regimes would shed light on how powerful fire history is in determining iridoid glycoside concentrations in comparison to other variable environmental factors across large landscapes. As an additional tool of reintroducing indigenous people's historical prescribed burning regimes, phytochemical assessments could be used to monitor various proposed burn frequencies based on ethnographic information (Storm, 2006).

In this study, fast-acting lime application had no significant effect on any iridoid glycoside concentrations, but this should not rule it out for further research, as lime is an important aid to plant nutrient acquisition in acidic soils (McCauley et al., 2009). Future research could investigate iridoid glycoside responses to two years of lime applications, allowing more time for soil to assimilate the lime.

Soil nutrient dynamics and plant genotype may be important biological mechanisms responsible for some of the unaccounted for variation in iridoid glycoside concentrations. Plant age and genotype influence iridoid glycoside patterns in Plantaginaceae and may in Orobanchaceae as well (Bowers, 1992; Fuchs and Bowers, 2004). Future research could identify the mechanisms underlying spatial, age, and genotypic differences through common garden experiments with *C. levisecta* and *P. lanceolata* seeds gathered from different spatial units known to be factors influencing iridoid glycoside concentrations.

In conclusion, my results confirm that methods used in the restoration and maintenance of degraded ecosystems have the potential to create important chemical legacies that ought to be monitored alongside other response variables in long-term restoration projects. More research on genotypic and landscape level patterns of iridoid glycoside concentrations will provide powerful insight for long-term restoration chemical ecology goals. Based on my findings, land managers, conservation biologists, and ethnobotanists should collaborate to create prescribed burning regimes and other land management protocols that not only take into account historical precedent (Storm and Shebitz, 2006) and direct survival of Taylor's checkerspot larvae (Hill et al., 2017), but that also aim to achieve ideal phytochemical concentrations for maximizing recovery success of both threatened plants (*C. levisecta*), and endangered insects (*Euphydryas editha taylori*).

Works Cited

- Agee, J. K. (1996). Achieving conservation biology objectives with fire in the Pacific Northwest. *Weed Technology*, 10(2), 417-421.
- Bowers, M. D., Collinge, S. K., Gamble, S. E., & Schmitt, J. (1992). Effects of genotype, habitat, and seasonal variation on iridoid glycoside content of *Plantago lanceolata* (Plantaginaceae) and the implications for insect herbivores. *Oecologia*, 91(2), 201-207.
- Darrow, K., & Bowers, M. D. (1997). Phenological and population variation in iridoid glycosides of *Plantago lanceolata* (Plantaginaceae). *Biochemical Systematics and Ecology*, 25(1), 1-11.
- Fuchs, A., & Bowers, M. D. (2004). Patterns of iridoid glycoside production and induction in *Plantago lanceolata* and the importance of plant age. *Journal of chemical ecology*, 30(9), 1723-1741.
- Hill, K. C., Bakker, J. D., & Dunwiddie, P. W. (2017). Prescribed fire in grassland butterfly habitat: targeting weather and fuel conditions to reduce soil temperatures and burn severity. *Fire Ecology*, 13(3), 24-41.
- McCauley, A., Jones, C., & Jacobsen, J. (2009). Soil pH and organic matter. *Nutrient management module*, 8, 1-12.
- Stamp, N. E., & Bowers, M. D. (1994). Effects of cages, plant age and mechanical clipping on plantain chemistry. *Oecologia*, 99(1-2), 66-71.
- Storm, L., & Shebitz, D. (2006). Evaluating the purpose, extent, and ecological restoration applications of indigenous burning practices in southwestern Washington. *Ecological Restoration*, 24(4), 256-268.

Appendices

Appendix 1: Photos of example Experimental Study plots and plant markers



Example plot containing *Plantago lanceolata* quadrat pair (Chapter 3), Glacial Heritage Preserve, 2016



Castilleja levisecta experimental quadrat (Chapter 3), Glacial Heritage Preserve, 2016



Deer enclosure around *Castilleja levisecta* experimental plot (Chapter 3), Glacial Heritage Preserve, 2016



Castilleja levisecta plant marked for sampling on limed quadrat (Chapter 3), Glacial Heritage Preserve, 2017



Plantago lanceolata plant marked for sampling on limed quadrat (Chapter 3), Glacial Heritage Preserve, 2017

Appendix 2: R code for analyses

Observational Study (Chapter Two)

```
## PACKAGES ----
library(lmeans)
library(labdsv)
library(lme4)
library(lmerTest)

#create a backup of original dataframes
Raw.original <- Raw

## Correcting and subsetting raw data ----
#Create a new column with time since burn
Raw$TimeSinceBurn <- (Raw$SampleYear - Raw$LastBrnYr)

#Changing variable classes
Raw$Burns.f <- as.factor(Raw$Burns)
Raw$TimeSinceBurn.f <- as.factor(Raw$TimeSinceBurn)
Raw$SampleYear <- as.factor(Raw$SampleYear)

#replacing empty cells with zero values
Raw$Aucubin_mg[is.na(Raw$Aucubin_mg)] <- 0
Raw$Catalpol_mg[is.na(Raw$Catalpol_mg)] <- 0
Raw$Macfadienoside_mg[is.na(Raw$Macfadienoside_mg)] <- 0
Raw$Unknown_mg[is.na(Raw$Unknown_mg)]<-0

#delete samples that were missing.
Raw <- Raw[! is.na(Raw$Weight), ] #drops 2

#converting iridoid glycoside mg to percent
Raw$Auc <- (100*Raw$Aucubin_mg/Raw$Weight)
Raw$Cat <- (100*Raw$Catalpol_mg/Raw$Weight)
Raw$Mac <- (100*Raw$Macfadienoside_mg/Raw$Weight)
Raw$UNK <- (100*Raw$Unknown_mg/Raw$Weight)

#CREATE A NEW COLUMN WITH TOTAL IG DATA
Raw$TotalIG <- (Raw$Auc + Raw$Cat + Raw$Mac + Raw$UNK)
length(Raw$TotalIG[Raw$TotalIG == 0]) # 6 plants with no IGs at all, some coin envelopes were
empty, or samples are being rerun
Raw <- Raw[! Raw$TotalIG == 0, ] #drops 6

#Create a new column with macfadienoside relative abundance
Raw$MacAbund <- (Raw$Mac/Raw$TotalIG)

Raw$MacAbund[is.na(Raw$MacAbund)] <- 0 #set to zero for plants with no IGs detected

#check foliage color data
Raw$FoliagePrct <- Raw$PrctBrwn + Raw$PrctGrn + Raw$PrctPrp
length(Raw$FoliagePrct[Raw$FoliagePrct != 100 & ! is.na(Raw$FoliagePrct)])
#all are correct

#subset observational data
ObsCALE16 <- with(Raw, Raw[Species == "CALE" & Obs_Exp == "0" & SampleYear == "2016", ])
ObsCALE17 <- with(Raw, Raw[Species == "CALE" & Obs_Exp == "0" & SampleYear == "2017", ])
ObsCALEComb <- with(Raw, Raw[Species == "CALE" & Obs_Exp == "0", ])

##Testing models----

#Total IGs
m.TotIG <- stepAIC(object = lm(TotalIG ~ 1, data = ObsCALEComb),
  scope = list(upper = ~ SampleYear * Burns * TimeSinceBurn + Plot,
    lower = ~ 1),
  direction = "both", k = 2)
anova(m.TotIG) # SampleYear, Burns; SampleYear:Burns (marginal)
summary(m.TotIG)

#Aucubin
m.Auc <- stepAIC(object = lm(Auc ~ 1, data = ObsCALEComb),
  scope = list(upper = ~ SampleYear * Burns * TimeSinceBurn + Plot,
    lower = ~ 1),
  direction = "both", k = 2)
anova(m.Auc) # none

#Catalpol (2017 only)
m.Cat <- stepAIC(object = lm(Cat ~ 1, data = ObsCALEComb[ObsCALEComb$SampleYear == "2017",]),
```

```

        scope = list(upper = ~ Burns * TimeSinceBurn + Plot,
                    lower = ~ 1),
        direction = "both", k = 2)
anova(m.Cat) # none

#Macfadienoside
m.Mac <- stepAIC(object = lm(Mac ~ 1, data = ObsCALEComb),
                scope = list(upper = ~ SampleYear * Burns * TimeSinceBurn + Plot,
                            lower = ~ 1),
                direction = "both", k = 2)
anova(m.Mac) # SampleYear; Burns (marginal), SampleYear:Burns (marginal)
summary(m.Mac)

#Unknown - methyl shanzhiside
m.UNK <- stepAIC(object = lm(UNK ~ 1, data = ObsCALEComb),
                scope = list(upper = ~ SampleYear * Burns * TimeSinceBurn + Plot,
                            lower = ~ 1),
                direction = "both", k = 2)
anova(m.UNK) # SampleYear; Burns (marginal)
summary(m.UNK)

#Macfadienoside (relative abundance)
m.MacAbund <- stepAIC(object = lm(MacAbund ~ 1, data = ObsCALEComb),
                    scope = list(upper = ~ SampleYear * Burns * TimeSinceBurn + Plot,
                                lower = ~ 1),
                    direction = "both", k = 2)
anova(m.MacAbund) # SampleYear; SampleYear:TimeSinceBurn (marginal)
summary(m.MacAbund)

#IGs by individual plant variables: flowering stems, height, and pigmentation (% purple)
m.TotIG.plant <- stepAIC(object = lm(TotalIG ~ Burns, data = ObsCALEComb[ObsCALEComb$SampleYear == "2017",]),
                        scope = list(upper = ~ Burns * TimeSinceBurn * (FlwrStems + Height +
                                    PrcntPrp) + Plot,
                                    lower = ~ 1),
                        direction = "both", k = 2)
anova(m.TotIG.plant) # Burns (marginal)
summary(m.TotIG.plant)

m.Auc.plant <- stepAIC(object = lm(Auc ~ Burns, data = ObsCALEComb[ObsCALEComb$SampleYear == "2017",]),
                    scope = list(upper = ~ Burns * TimeSinceBurn * (FlwrStems + Height +
                                PrcntPrp) + Plot,
                                lower = ~ 1),
                    direction = "both", k = 2)
anova(m.Auc.plant) # Burns (marginal); height (marginal)
summary(m.Auc.plant)

m.Cat.plant <- stepAIC(object = lm(Cat ~ Burns, data = ObsCALEComb[ObsCALEComb$SampleYear == "2017",]),
                    scope = list(upper = ~ Burns * TimeSinceBurn * (FlwrStems + Height +
                                PrcntPrp) + Plot,
                                lower = ~ 1),
                    direction = "both", k = 2)
anova(m.Cat.plant) # none

m.Mac.plant <- stepAIC(object = lm(Mac ~ Burns, data = ObsCALEComb[ObsCALEComb$SampleYear == "2017",]),
                    scope = list(upper = ~ Burns * TimeSinceBurn * (FlwrStems + Height +
                                PrcntPrp) + Plot,
                                lower = ~ 1),
                    direction = "both", k = 2)
anova(m.Mac.plant) # none

m.UNK.plant <- stepAIC(object = lm(UNK ~ Burns, data = ObsCALEComb[ObsCALEComb$SampleYear == "2017",]),
                    scope = list(upper = ~ Burns * TimeSinceBurn * (FlwrStems + Height +
                                PrcntPrp) + Plot,
                                lower = ~ 1),
                    direction = "both", k = 2)
anova(m.UNK.plant) # Burns, TimeSinceBurn (marginal)
summary(m.UNK.plant)

m.MacAbund.plant <- stepAIC(object = lm(MacAbund ~ TimeSinceBurn, data =
ObsCALEComb[ObsCALEComb$SampleYear == "2017",]),
                    scope = list(upper = ~ Burns * TimeSinceBurn * (FlwrStems + Height +
                                PrcntPrp) + Plot,
                                lower = ~ TimeSinceBurn),
                    direction = "both", k = 2)
anova(m.MacAbund.plant) # TimeSinceBurn (marginal), Height (marginal)
summary(m.MacAbund.plant)

```

Experimental Study (Chapter Three)

```
## PACKAGES ----
library(ggplot2)
library(lsmmeans)
library(labdsv)
library(lme4)
library(lmerTest)
library(LMERConvenienceFunctions)
library(MuMIn)
library(plyr)
library(stringr)
library(tidyr)
library(r2glmm)

## Correcting and subsetting raw data ----
#Create a new column with time since burn
Raw$TimeSinceBurn <- Raw$SampleYear - Raw$LastBrnYr

#Changing variable classes
Raw$Burns.f <- as.factor(Raw$Burns)
Raw$TimeSinceBurn.f <- as.factor(Raw$TimeSinceBurn)
Raw$SampleYear <- as.factor(Raw$SampleYear)

#replacing empty cells with zero values
Raw$Aucubin_mg[is.na(Raw$Aucubin_mg)] <- 0
Raw$Catalpol_mg[is.na(Raw$Catalpol_mg)] <- 0
Raw$Macfadienoside_mg[is.na(Raw$Macfadienoside_mg)] <- 0
Raw$Unknown_mg[is.na(Raw$Unknown_mg)]<-0

#delete samples that were missing.
Raw <- Raw[! is.na(Raw$Weight), ] #drops 2

#converting iridoid glycoside mg to percent
Raw$Auc <- (100*Raw$Aucubin_mg/Raw$Weight)
Raw$Cat <- (100*Raw$Catalpol_mg/Raw$Weight)
Raw$Mac <- (100*Raw$Macfadienoside_mg/Raw$Weight)
Raw$UNK <- (100*Raw$Unknown_mg/Raw$Weight)

#CREATE A NEW COLUMN WITH TOTAL IG DATA
Raw$TotalIG <- (Raw$Auc + Raw$Cat + Raw$Mac + Raw$UNK)
length(Raw$TotalIG[Raw$TotalIG == 0]) # 6 plants with no IGs at all
Raw <- Raw[Raw$TotalIG > 0 , ] #drop plants without IGs

#Create a new column with macfadienoside relative abundance
Raw$MacAbund <- (Raw$Mac/Raw$TotalIG)

#check foliage color data
Raw$FoliagePrct <- Raw$PrctBrwn + Raw$PrctGrn + Raw$PrctPrp
length(Raw$FoliagePrct[Raw$FoliagePrct != 100 & ! is.na(Raw$FoliagePrct)]) #all plants have
foliage color data that is NA or sums to 100%

#Create descriptors of sampling relative to herbivory treatment
SampleDate_labels <- dply(Raw, ~(Obs_Exp, Species, SampleDate), summarize, N = length(TotalIG))
SampleDate_labels$TreatDate <- with(SampleDate_labels,
                                     ifelse(Species == "CALE", ifelse(SampleDate %in%
c("6/1/2016", "5/2/2017"), "Early",
                                     ifelse(SampleDate %in%
c("6/14/2016", "5/24/2017"), "Late", "Pre")),
                                     ifelse(SampleDate %in% c("6/29/2016", "5/9/2017"),
"Early", ifelse(SampleDate == "5/30/2017", "Late", "Pre"))))
Raw <- merge(x = Raw, y = SampleDate_labels)
Raw$TreatDate.ord <- factor(Raw$TreatDate, ordered = TRUE, levels = c("Pre", "Early", "Late"))

#extending explanatory variables to other year
Raw$Burned2016[Raw$Burned2016 == ""] <- "N"
Raw$Lime[Raw$Lime == ""] <- "N"
Raw$PriorHerbivDeer[Raw$PriorHerbivDeer == ""] <- "N"

#Changing Y's and N's to Yes and No
Raw$Lime<-revalue(Raw$Lime, c("Y"="Yes", "N"="No"))
Raw$Burned2016<-revalue(Raw$Burned2016, c("Y"="Yes", "N"="No"))
Raw$PriorHerbivDeer<-revalue(Raw$PriorHerbivDeer, c("Y"="Yes", "N"="No"))
Raw$HerbivTrt<-revalue(Raw$HerbivTrt, c("Y"="Yes", "N"="No"))
```

```

#Adjust experimental array IDs so that there are 12 total
Raw$Array <- str_replace_all(Raw$Array, "[AB]", "")

#subset experimental data
ExpCALE16 <- with(Raw, Raw[Species == "CALE" & Obs_Exp == "E" & SampleYear == "2016", ])
ExpCALE17 <- with(Raw, Raw[Species == "CALE" & Obs_Exp == "E" & SampleYear == "2017", ])
ExpCALEComb <- with(Raw, Raw[Species == "CALE" & Obs_Exp == "E", ])

ExpPLLA16 <- with(Raw, Raw[Species == "PLLA" & Obs_Exp == "E" & SampleYear == "2016", ])
ExpPLLA17 <- with(Raw, Raw[Species == "PLLA" & Obs_Exp == "E" & SampleYear == "2017", ])
ExpPLLAComb <- with(Raw, Raw[Species == "PLLA" & Obs_Exp == "E", ])

#Create a new column with PLLA Aucubin relative abundance
ExpPLLA16$AucAbund <- (ExpPLLA16$Auc/ExpPLLA16$TotalIG)
ExpPLLA17$AucAbund <- (ExpPLLA17$Auc/ExpPLLA17$TotalIG)
ExpPLLAComb$AucAbund <- (ExpPLLAComb$Auc/ExpPLLAComb$TotalIG)

#### Testing models ----

## CALE
# Run all six sets of models and then look at CALE.res object

CALE.res <- c()

#Total IGS
mFull <- lmer(TotalIG ~ SampleYear * TreatDate * HerbivTrt + (1 | Array / Plot / PlantID), data =
ExpCALEComb)
summary(m.TotIG <- bfFixeFLMER_F.fnc(model = mFull, method = "AIC", threshold = 2))
CALE.res <- rbind(CALE.res, data.frame(pamer.fnc(m.TotIG), Response = "TotIG"))
CALE.res
#Sample year, SampleYear:TreatDate; TreatDate(Marginal)

#Aucubin
mFull <- lmer(Auc ~ SampleYear * TreatDate * HerbivTrt + (1 | Array / Plot / PlantID), data =
ExpCALEComb)
summary(m.Auc <- bfFixeFLMER_F.fnc(model = mFull, method = "AIC", threshold = 2))
CALE.res <- rbind(CALE.res, data.frame(pamer.fnc(m.Auc), Response = "Auc"))
CALE.res
#SampleYear1; SampleYear:TreatDate1

#Catalpol
mFull <- lmer(Cat ~ SampleYear * TreatDate * HerbivTrt + (1 | Array / Plot / PlantID), data =
ExpCALEComb)
summary(m.Cat <- bfFixeFLMER_F.fnc(model = mFull, method = "AIC", threshold = 2))
CALE.res <- rbind(CALE.res, data.frame(pamer.fnc(m.Cat), Response = "Cat"))
CALE.res
#SampleYear2; SampleYear:

#Macfadienoside
mFull <- lmer(Mac ~ SampleYear * TreatDate * HerbivTrt + (1 | Array / Plot / PlantID), data =
ExpCALEComb)
summary(m.Mac <- bfFixeFLMER_F.fnc(model = mFull, method = "AIC", threshold = 2))
CALE.res <- rbind(CALE.res, data.frame(pamer.fnc(m.Mac), Response = "Mac"))
CALE.res
#SampleYear3, SampleYear:TreatDate3; TreatDate3(Marginal)

#Methyl shanzhiside
mFull <- lmer(UNK ~ SampleYear * TreatDate * HerbivTrt + (1 | Array / Plot / PlantID), data =
ExpCALEComb)
summary(m.UNK <- bfFixeFLMER_F.fnc(model = mFull, method = "AIC", threshold = 2))
CALE.res <- rbind(CALE.res, data.frame(pamer.fnc(m.UNK), Response = "UNK"))
CALE.res
#TreatDate4, SampleYear:TreatDate4, SampleYear:HerbivTrt, SampleYear:TreatDate:HerbivTrt

#Macfadienoside relative abundance
mFull <- lmer(MacAbund ~ SampleYear * TreatDate * HerbivTrt + (1 | Array / Plot / PlantID), data =
ExpCALEComb)
summary(m.MacAbund <- bfFixeFLMER_F.fnc(model = mFull, method = "AIC", threshold = 2))
CALE.res <- rbind(CALE.res, data.frame(pamer.fnc(m.MacAbund), Response = "MacAbund"))
CALE.res
#Sample year, treat date

##look at results and conduct pairwise comparisons (add others by changing the 'test.offs'
argument)
CALE.res[CALE.res$lower.p.val <= 0.05, ]

#Explore R^2 values for each model
library(r2glmm)
r2beta(m.TotIG, partial = "FALSE")
r2beta(m.Auc, partial = "FALSE")
r2beta(m.Cat, partial = "FALSE")

```

```

r2beta(m.Mac, partial = "FALSE")
r2beta(m.UNK, partial = "FALSE")
r2beta(m.MacAbund, partial = "FALSE")

#TotIG: SampleYear, SampleYear:TreatDate
diffsmeans(m.TotIG, test.effs = "SampleYear:TreatDate")
#Auc: SampleYear, SampleYear:TreatDate
diffsmeans(m.Auc, test.effs = "SampleYear:TreatDate")
#Cat: TreatDate, SampleYear:TreatDate, SampleYear:TreatDate:HerbivTrt
diffsmeans(m.Cat, test.effs = "SampleYear:TreatDate")
#Mac: SampleYear, SampleYear:TreatDate
diffsmeans(m.Mac, test.effs = "SampleYear:TreatDate")
#UNK: TreatDate, SampleYear:TreatDate, SampleYear:HerbivTrt, TreatDate:HerbivTrt,
SampleYear:TreatDate:HerbivTrt
diffsmeans(m.UNK, test.effs = "SampleYear:TreatDate")
diffsmeans(m.UNK, test.effs = "TreatDate:HerbivTrt")
#MacAbund: SampleYear, TreatDate
diffsmeans(m.MacAbund, test.effs = "TreatDate")

## PLLA
# Run all four sets of models and then look at PLLA.res object

PLLA.res <- c()

#Total IGS
mFull <- lmer(TotalIG ~ SampleYear * TreatDate * HerbivTrt + (1 | Array / Plot / PlantID), data =
ExpPLLAComb)
summary(m.TotIG.p <- bfixefLMER_F.fnc(model = mFull, method = "AIC", threshold = 2))
PLLA.res <- rbind(PLLA.res, data.frame(pamer.fnc(m.TotIG.p), Response = "TotIG"))
PLLA.res
# SampleYear:HerbivTrt

#Aucubin
mFull <- lmer(Auc ~ SampleYear * TreatDate * HerbivTrt + (1 | Array / Plot / PlantID), data =
ExpPLLAComb)
summary(m.Auc.p <- bfixefLMER_F.fnc(model = mFull, method = "AIC", threshold = 2))
PLLA.res <- rbind(PLLA.res, data.frame(pamer.fnc(m.Auc.p), Response = "Auc"))
PLLA.res
# SampleYear1; SampleYear:TreatDate1

#Catalpol
mFull <- lmer(Cat ~ SampleYear * TreatDate * HerbivTrt + (1 | Array / Plot / PlantID), data =
ExpPLLAComb)
summary(m.Cat.p <- bfixefLMER_F.fnc(model = mFull, method = "AIC", threshold = 2))
PLLA.res <- rbind(PLLA.res, data.frame(pamer.fnc(m.Cat.p), Response = "Cat"))
PLLA.res
# SampleYear:TreatDate:HerbivTrt1

#Relative abundance of Aucubin
ExpPLLAComb$AucAbund <- with(ExpPLLAComb, Auc / TotalIG)
mFull <- lmer(AucAbund ~ SampleYear * TreatDate * HerbivTrt + (1 | Array / Plot / PlantID), data =
ExpPLLAComb)
summary(m.AucAbund.p <- bfixefLMER_F.fnc(model = mFull, method = "AIC", threshold = 2))
PLLA.res <- rbind(PLLA.res, data.frame(pamer.fnc(m.AucAbund.p), Response = "AucAbund"))
PLLA.res
# SampleYear3, SampleYear:TreatDate3, TreatDate:HerbivTrt2

PLLA.res[PLLA.res$lower.p.val <= 0.05 , ]

#TotIG: SampleYear, SampleYear:TreatDate
diffsmeans(m.TotIG.p, test.effs = "SampleYear:TreatDate")
#Auc: SampleYear, SampleYear:TreatDate
diffsmeans(m.Auc.p, test.effs = "SampleYear:TreatDate")
#Cat: TreatDate, SampleYear:TreatDate, SampleYear:TreatDate:HerbivTrt
diffsmeans(m.Cat.p, test.effs = "SampleYear:TreatDate")
#AucAbund: SampleYear, TreatDate
diffsmeans(m.AucAbund.p, test.effs = "SampleYear:TreatDate")

r2beta(m.TotIG.p, partial = "FALSE")
r2beta(m.Auc.p, partial = "FALSE")
r2beta(m.Cat.p, partial = "FALSE")
r2beta(m.AucAbund.p, partial = "FALSE")

#### test whether other terms are significant

## CALE - TotalIG

anova(m.TotIG, update(m.TotIG, ~ . + Lime*SampleYear*TreatDate))
#P = 0.9375, so no improvement by adding Lime to the model
## CALE - Auc

```

```

anova(m.Auc, update(m.Auc, ~ . + Lime*SampleYear*TreatDate))
#P = 0.3013, so no improvement by adding Lime to the model

anova(lmer(Auc ~ TreatDate + Burned2016 + (1 | Array / Plot / PlantID), data = ExpCALE17))
#P = 0.8065

## CALE - Cat
anova(m.Cat, update(m.Cat, ~ . + Lime*SampleYear*TreatDate))
#P = 0.5058, so no improvement by adding Lime to the model

anova(lmer(Cat ~ TreatDate + Burned2016 + (1 | Array / Plot / PlantID), data = ExpCALE17))
#P = 0.1678

## CALE - Mac
anova(m.Mac, update(m.Mac, ~ . + Lime*SampleYear*TreatDate))
#P = 0.9498, so no improvement by adding Lime to the model

anova(lmer(Mac ~ TreatDate + Burned2016 + (1 | Array / Plot / PlantID), data = ExpCALE17))
#P = 0.1388

## CALE - UNK
anova(m.UNK, update(m.UNK, ~ . + Lime*SampleYear*TreatDate))
#P = 0.8795, so no improvement by adding Lime to the model

anova(lmer(UNK ~ TreatDate * HerbivTrt + Burned2016 + (1 | Array / Plot / PlantID), data =
ExpCALE17))
#P = 0.1982

anova(m.MacAbund, update(m.MacAbund, ~ . + Lime*SampleYear*TreatDate))
#P = 0.8437, so no improvement by adding Lime to the model

anova(lmer(MacAbund ~ TreatDate + Burned2016 + (1 | Array / Plot / PlantID), data = ExpCALE17))
#P = 0.3989

## PLLA - TotalIG
anova(m.TotIG.p, update(m.TotIG.p, ~ . + Lime*SampleYear*TreatDate))
#P = 0.6875, so no improvement by adding Lime to the model

anova(lmer(TotalIG ~ TreatDate * HerbivTrt + Burned2016 + (1 | Array / PlantID), data =
ExpPLLA17))
#P = 0.0023 ***, SIGNIFICANT
r2beta(lmer(TotalIG ~ TreatDate * HerbivTrt + Burned2016 + (1 | Array / PlantID), data =
ExpPLLA17)), partial = "FALSE")

## PLLA - Auc
anova(m.Auc.p, update(m.Auc.p, ~ . + Lime*SampleYear*TreatDate))
#P = 0.4259, so no improvement by adding Lime to the model

anova(lmer(Auc ~ TreatDate + Burned2016 + (1 | Array / PlantID), data = ExpPLLA17))
#P = 0.0301 ***, SIGNIFICANT
r2beta(lmer(Auc ~ TreatDate + Burned2016 + (1 | Array / PlantID), data = ExpPLLA17)), partial =
"FALSE")

## PLLA - Cat
anova(m.Cat.p, update(m.Cat.p, ~ . + Lime*SampleYear*TreatDate))
#P = 0.9516, so no improvement by adding Lime to the model

anova(lmer(Cat ~ TreatDate * HerbivTrt + Burned2016 + (1 | Array / PlantID), data = ExpPLLA17))
#P = 0.0030 ***, SIGNIFICANT
r2beta(lmer(Cat ~ TreatDate * HerbivTrt + Burned2016 + (1 | Array / PlantID), data =
ExpPLLA17)), partial = "FALSE")

#AUCUBIN ABUNDANCE
anova(m.AucAbund.p, update(m.AucAbund.p, ~ . + Lime*SampleYear*TreatDate))
#P = 0.3417, so no improvement by adding Lime to the model

anova(lmer(AucAbund ~ TreatDate * HerbivTrt + Burned2016 + (1 | Array / PlantID), data =
ExpPLLA17))
#P = 0.5029 - no difference; both IGs responded similarly to burning

```

Appendix 3: Raw data

Year	SampleID	Weight	Auc_mg	Cat_mg	Mac_mg	UNK_mg	PBGArea	PBGAmour	AreaUNK	SampleDate	Species	Obs_Exp	PlantID	Burned2016	Lime	Array	Plot	Burns	LastBmYr	Height	%Green	%Purp	%Bwn	HerbivTrt	Mildew	PriorHerbivDeer	PriorHerbivInsect	FlwStems
2016	1	50.84	0.067	0.081	1.249	0.049	142.108	0.502	13.934	5/25/2016	CALE	E	C1A1				C1A	C1		44.3	80	19	1	N		Y	Y	1
2016	2	38.71	0.026		0.447	0.049	63.732	0.502	6.193	5/25/2016	CALE	E	C1A2				C1A	C1		14	80	19.5	0.5	N		Y	Y	1
2016	3	38.54	0.017		0.472	0.038	128.556	0.502	7.779	5/25/2016	CALE	E	C1A3				C1A	C1	48	32.1	90	1	N		Y	Y	5	
2016	4	14.75	0.01	0.021	0.297	0.01	133.159	0.502	2.543	5/25/2016	CALE	E	C1A4				C1A	C1		40	96	1	3	N		Y	Y	7
2016	5	45.85	0.062		1.019	0.14	125.117	0.502	34.983	5/25/2016	CALE	E	C1B1				C1B	C1		42	38	60	2	Y		Y	Y	11
2016	6	50.35	0.054		0.94	0.127	111.153	0.502	28.053	5/25/2016	CALE	E	C1B2				C1B	C1		49.5	41	55	4	N		Y	Y	12
2016	7	52.02	0.049		1.175	0.062	129.45	0.502	15.966	5/25/2016	CALE	E	C1B3				C1B	C1		43	2	96	2	Y		Y	Y	14
2016	8	27.76	0.046		0.518	0.125	111.928	0.502	27.956	5/25/2016	CALE	E	C1B4				C1B	C1		44.2	39.5	60	0.5	N		Y	Y	7
2016	17	21.42	0.016		0.382	0.017	125.119	0.502	4.287	5/25/2016	CALE	E	C7A1				C7A	C7		11.5	9	90	1	Y		Y	Y	2
2016	18	55.67	0.035		0.524	0.024	102.464	0.502	4.83	5/25/2016	CALE	E	C7A2				C7A	C7		33.2	3	97	0	N		Y	Y	8
2016	19	19.25	0.037		0.408	0.016	121.8	0.502	3.796	5/25/2016	CALE	E	C7A3				C7A	C7		26.8	2	96	2	N		Y	Y	2
2016	20	45.37	0.034		0.525	0.032	102.334	0.502	6.587	5/25/2016	CALE	E	C7A4				C7A	C7		40.1	60	37.5	2.5	N		Y	Y	14
2016	21	41.27	0.054	0.029	1.344	0.024	117.95	0.502	5.611	5/25/2016	CALE	E	C7B1				C7B	C7		29.9	70	27	3	Y		Y	Y	7
2016	22	30.22	0.033		0.797	0.018	102.374	0.502	4.201	5/25/2016	CALE	E	C7B2				C7B	C7		24.5	3	96	1	N		Y	Y	3
2016	23	23.2	0.023		0.447	0.043	135.981	0.502	11.679	5/25/2016	CALE	E	C7B3				C7B	C7		25	38	60	2	Y		Y	Y	2
2016	24	14.98	0.023	0.019	0.444	0.027	134.071	0.502	7.079	5/25/2016	CALE	E	C7B4				C7B	C7		24.1	50	49	1	N		Y	Y	2
2016	25	29.04	0.035		0.681	0.036	117.264	0.502	8.405	5/25/2016	CALE	E	C2A1				C2A	C2		25.5	2	97	1	Y		Y	Y	2
2016	26	53.75	0.074		1.48	0.065	129.866	0.502	16.707	5/25/2016	CALE	E	C2A2				C2A	C2		33	16	80	4	N		Y	Y	7
2016	27	41.02	0.017		0.921	0.028	131.833	0.502	7.271	5/25/2016	CALE	E	C2A3				C2A	C2		30.4	0	96	4	Y		Y	Y	2
2016	28	7.37	0.067		0.162	0.006	133.788	0.502	1.48	5/25/2016	CALE	E	C2A4				C2A	C2		15.1	30	70	0	N		Y	Y	0
2016	29	32.62	0.031		0.591	0.022	124.687	0.502	9.305	5/25/2016	CALE	E	C2B1				C2B	C2		33.1	96	0	4	Y		Y	Y	3
2016	30	30.73	0.046	0.022	0.834	0.101	195.354	0.502	39.229	5/25/2016	CALE	E	C2B2				C2B	C2		22.9	49	50	1	N		Y	Y	1
2016	31	37.34	0.052		1.809	0.129	140.828	0.502	36.212	5/25/2016	CALE	E	C2B3				C2B	C2		21.3	92	4	4	Y		Y	Y	8
2016	32	50.73	0.049		1.234	0.056	126.586	0.502	14.136	5/25/2016	CALE	E	C2B4				C2B	C2		41.2	92	0	8	N		Y	Y	7
2016	33	40.51	0.017		0.757	0.025	125.916	0.502	12.825	5/25/2016	CALE	E	C3A1				C3A	C3		85	33	85	11	Y		Y	Y	3
2016	34	27.27			0.693	0.023	136.273	0.502	15.719	5/25/2016	CALE	E	C3A2				C3A	C3		30.2	92	3	5	N		Y	Y	6
2016	35	13.47	0.01		0.28	0.016	333.16	0.502	10.525	5/25/2016	CALE	E	C3A3				C3A	C3		27.2	92	3	5	Y		Y	Y	5
2016	36	17.67	0.01		0.492	0.011	275.195	0.502	5.832	5/25/2016	CALE	E	C3A4				C3A	C3		33.6	48	48	4	N		Y	Y	5
2016	37	7.62	0.017		0.453	0.021	253.038	0.502	10.625	5/25/2016	CALE	E	C3B1				C3B	C3		32.6	42	55	3	Y		Y	Y	6
2016	38	42.87	0.042	0.032	0.783	0.043	394.671	0.502	33.966	5/25/2016	CALE	E	C3B2				C3B	C3		26.9	47	50	3	N		Y	Y	1
2016	39	50.1	0.054		0.9	0.055	111.083	0.502	34.252	5/25/2016	CALE	E	C3B3				C3B	C3		29.2	60	37	3	Y		Y	Y	2
2016	40	35.07	0.032		0.615	0.032	167.216	0.502	10.516	5/25/2016	CALE	E	C3B4				C3B	C3		25.2	97	0	3	N		Y	Y	4
2016	41	36.93	0.032		0.654	0.037	102.177	0.502	7.585	5/25/2016	CALE	E	C9A1				C9A	C9		33.1	97	2	1	Y		Y	Y	6
2016	42	24.37	0.034	0.025	0.401	0.065	143.717	0.502	18.742	5/25/2016	CALE	E	C9A2				C9A	C9		30.7	99	0	1	N		Y	Y	6
2016	43	11.09	0.012		0.134	0.013	147.341	0.502	3.848	5/25/2016	CALE	E	C9A3				C9A	C9		47.1	99	0	1	Y		Y	Y	7
2016	44	20.64			0.613	0.013	122.916	0.502	3.562	5/25/2016	CALE	E	C9A4				C9A	C9		34.3	85	3	1	N		Y	Y	3
2016	45	46.68	0.054		0.992	0.092	208.923	0.502	38.151	5/25/2016	CALE	E	C9B1				C9B	C9		39.6	96	3	1	N		Y	Y	3
2016	46	55.46	0.095		1.421	0.132	152.629	0.502	40.062	5/25/2016	CALE	E	C9B2				C9B	C9		31.8	92	2	6	N		Y	Y	3
2016	47	42.1	0.024		0.536	0.03	145.363	0.502	8.824	5/25/2016	CALE	E	C9B3				C9B	C9		32.9	89	8	3	Y		Y	Y	9
2016	48	51.15	0.079		1.219	0.14	164.249	0.502	45.701	5/25/2016	CALE	E	C9B4				C9B	C9		41.9	99	0	1	N		Y	Y	10
2016	49	54.19	0.039	0.033	1.088	0.016	110.477	0.502	3.565	5/25/2016	CALE	E	C4A1				C4A	C4		30	69	30	1	Y		Y	Y	4
2016	50	38.51	0.017		1.091	0.047	95.718	0.502	9.029	5/25/2016	CALE	E	C4A2				C4A	C4		43	54	45	4	N		Y	Y	4
2016	51	49.93	0.049		1.308	0.05	90.077	0.502	9.057	5/25/2016	CALE	E	C4A3				C4A	C4		32.5	54	45	1	Y		Y	Y	11
2016	52	32.35	0.024		0.82	0.028	111.037	0.502	6.119	5/25/2016	CALE	E	C4A4				C4A	C4		36.9	77	20	3	N		Y	Y	9
2016	53	54.31	0.047		1.238	0.091	112.575	0.502	20.351	5/25/2016	CALE	E	C4B1				C4B	C4		44.2	87	10	3	N		Y	Y	20
2016	54	55.45	0.057		1.479	0.154	109.943	0.502	33.709	5/25/2016	CALE	E	C4B2				C4B	C4		39.4	91	5	4	N		Y	Y	9
2016	55	31.85	0.029	0.023	0.836	0.045	124.992	0.502	11.207	5/25/2016	CALE	E	C4B3				C4B	C4		35.1	73	25	2	Y		Y	Y	4
2016	56	38.51	0.017		0.618	0.018	125.978	0.502	12.715	5/25/2016	CALE	E	C4B4				C4B	C4		40.3	24	45	4	N		Y	Y	3
2016	57	10.58	0.012	0.037	0.195	0.016	129.487	0.502	4.017	5/25/2016	CALE	E	C10A1				C10A	C10		33.4	91	7	2	Y		Y	Y	14
2016	58	54.34	0.068		1.247	0.152	112.312	0.502	34.012	5/25/2016	CALE	E	C10A2				C10A	C10		30	47	50	3	N		Y	Y	11
2016	59	17.08	0.014	0.039	0.267	0.02	124.089	0.502	4.959	5/25/2016	CALE	E	C10A3				C10A	C10		29.4	10	88	2	Y		Y	Y	3
2016	60	9.93			0.283	0.007	107.759	0.502	1.411	5/25/2016	CALE	E	C10A4				C10A	C10		34.2	56	40	4	N		Y	Y	19
2016	61	55.25	0.025		1.011	0.113	122.916	0.502	4.101	5/25/2016	CALE	E	C10B1				C10B	C10		32.9	88	10	2	Y		Y	Y	5
2016	62	17.29	0.02	0.05	0.372	0.01	124.079	0.502	2.507	5/25/2016	CALE	E	C10B2				C10B	C10		33	88	10	2	Y		Y	Y	5
2016	63	22.54	0.018	0.042	0.444	0.024	106.27	0.502	4.997	5/25/2016	CALE	E	C10B3				C10B	C10		24.8	48	50	2	N		Y	Y	6
2016	64	23.94	0.033	0.015	0.537	0.024	91.35	0.502	4.279	5/25/2016	CALE	E	C10B4				C10B	C10		31.1	98	1	1	N				

2016	146	21.96	0.021	0.514	0.027	104.264	0.502	5.691	6/1/2016	CALE	E	C4A2	C4A	C4		43	54	45	1	N	Y	N	
2016	147	50.78		0.031	1.411	0.042	82.693	0.502	6.9	6/1/2016	CALE	E	C4A3	C4A	C4		32.5	54	45	1	Y	N	N
2016	148	35.08	0.022		0.794	0.03	87.12	0.502	5.134	6/1/2016	CALE	E	C4A4	C4A	C4		36.9	77	20	3	N	Y	N
2016	149	51.04	0.004		1.341	0.077	104.923	0.502	6.189	6/1/2016	CALE	E	C4A5	C4A	C4		44.2	87	20	3	Y	N	N
2016	150	50.26	0.041		1.632	0.176	60.86	0.502	21.386	6/1/2016	CALE	E	C4B2	C4B	C4		39.4	91	5	4	N	N	N
2016	151	21.46	0.012		0.516	0.046	68.894	0.502	6.268	6/1/2016	CALE	E	C4B3	C4B	C4		35.1	73	25	2	Y	N	N
2016	152	39.14	0.028		1.015	0.108	104.429	0.502	22.548	6/1/2016	CALE	E	C4B4	C4B	C4		40.3	71	25	4	N	Y	N
2016	153	30.75	0.054		0.441	0.008	68.679	0.502	1.073	6/1/2016	CALE	E	C10A1	C10A	C10		33.4	91	7	2	Y	N	N
2016	154	29.54	0.028	0.028	0.417	0.01	57.407	0.502	1.103	6/1/2016	CALE	E	C10A2	C10A	C10		30	47	50	3	N	Y	N
2016	155	6.78	0.008		0.139	0.023	136.6	0.502	6.183	6/1/2016	CALE	E	C10A3	C10A	C10		29.4	109	88	2	Y	N	N
2016	156	50.34	0.041	0.025	1.22	0.144	96.149	0.502	27.6	6/1/2016	CALE	E	C10A4	C10A	C10		34.2	56	40	4	N	N	N
2016	157	50.21	0.182		0.41	0.05	40.661	0.502	4.048	6/1/2016	CALE	E	C10B1	C10B	C10		42.5	93	3	4	N	Y	N
2016	158	38.24	0.027		0.927	0.031	114.298	0.502	7.025	6/1/2016	CALE	E	C10B2	C10B	C10		33	88	10	2	N	Y	Y
2016	159	25.5	0.017	0.026	0.533	0.029	85.851	0.502	5.01	6/1/2016	CALE	E	C10B3	C10B	C10		24.8	48	50	2	Y	N	Y
2016	160	32.29	0.041	0.036	0.409	0.019	52.356	0.502	1.949	6/1/2016	CALE	E	C10B4	C10B	C10		31.1	98	1	1	N	Y	N
2016	161	28.25	0.029		0.568	0.021	74.945	0.502	3.168	6/1/2016	CALE	E	C8A1	C8A	C8		32	30	68	2	Y	N	N
2016	162	48.37	0.026		0.523	0.028	96.941	0.502	5.464	6/1/2016	CALE	E	C8A2	C8A	C8		36	66	30	4	N	N	N
2016	163	33.99	0.011	0.044	0.73	0.042	81.498	0.502	6.782	6/1/2016	CALE	E	C8A3	C8A	C8		30.1	76	20	4	Y	N	N
2016	164	7.81	0.014		0.262	0.011	111.431	0.502	2.428	6/1/2016	CALE	E	C8A4	C8A	C8		25.9	75	20	5	N	N	N
2016	165	18.51	0.033		0.352	0.015	85.725	0.502	2.521	6/1/2016	CALE	E	C8B1	C8B	C8		30.5	91	5	4	Y	N	N
2016	166	19.24	0.011		0.352	0.022	104.866	0.502	4.54	6/1/2016	CALE	E	C8B2	C8B	C8		28.5	87	10	3	N	N	N
2016	167	31.63	0.018	0.02	0.312	0.014	107.108	0.502	3.049	6/1/2016	CALE	E	C8B3	C8B	C8		27.5	10	88	2	N	N	N
2016	168	53.71	0.045	0.038	0.633	0.01	75.343	0.502	1.509	6/1/2016	CALE	E	C8B4	C8B	C8		30.3	50	47	3	Y	N	N
2016	169	33.17	0.033	0.053	1.063	0.016	57.433	0.502	1.863	6/1/2016	CALE	E	C5A1	C5A	C5		27.2	74	25	1	Y	N	N
2016	170	22.46	0.02		0.563	0.011	97.609	0.502	2.146	6/1/2016	CALE	E	C5A2	C5A	C5		27.8	67	30	3	N	N	N
2016	171	24.65	0.035		0.805	0.007	105.465	0.502	1.659	6/1/2016	CALE	E	C5A3	C5A	C5		2.8	94	2	4	Y	N	N
2016	172	49.41	0.164	0.088	0.614	0.022	47.053	0.502	2.016	6/1/2016	CALE	E	C5A4	C5A	C5		38.5	25	72	3	N	N	N
2016	173	17.51	0.025		0.245	0.017	73.899	0.502	2.484	6/1/2016	CALE	E	C5B1	C5B	C5		23	25	71	4	Y	N	N
2016	174	26.24	0.028		0.924	0.02	94.025	0.502	3.747	6/1/2016	CALE	E	C5B2	C5B	C5		36.4	30	68	2	N	N	N
2016	175	47.56	0.025	0.044	0.522	0.023	56.751	0.502	2.627	6/1/2016	CALE	E	C5B3	C5B	C5		33.5	1	95	4	Y	N	N
2016	176	36.06	0.066	0.047	0.445	0.021	49.909	0.502	2.062	6/1/2016	CALE	E	C5B4	C5B	C5		28.5	1	95	4	N	N	N
2016	177	11.08	0.011		0.172	0	61.243	0.502	0	6/1/2016	CALE	E	C11A1	C11A	C11		27.1	2	95	3	N	Y	N
2016	178	19.09	0.013		0.139	0	53.142	0.502	0	6/1/2016	CALE	E	C11A2	C11A	C11		19.2	0	97	3	N	N	N
2016	179	56.22	0.081	0.034	0.401	0.017	48.87	0.502	1.654	6/1/2016	CALE	E	C11A3	C11A	C11		28.5	87	10	3	Y	N	N
2016	180	55.69	0.03		0.623	0.013	49.765	0.502	1.332	6/1/2016	CALE	E	C11A4	C11A	C11		25.8	91	5	4	N	N	N
2016	181	12.96	0.009	0.016	0.218	0	112.401	0.502	0	6/1/2016	CALE	E	C11B1	C11B	C11		27.4	25	74	1	Y	N	N
2016	182	21.01	0.012		0.127	0	60.362	0.502	0	6/1/2016	CALE	E	C11B2	C11B	C11		22.3	10	87	3	N	Y	N
2016	183	42.6	0.012	0.027	0.497	0.017	67.046	0.502	2.334	6/1/2016	CALE	E	C11B3	C11B	C11		25.9	15	82	3	Y	N	N
2016	184	51.57	0.029	0.029	0.475	0.017	57.72	0.502	1.94	6/1/2016	CALE	E	C11B4	C11B	C11		24.3	10	86	4	N	N	N
2016	185	52.33	0.036		1.207	0.019	50.383	0.502	1.898	6/1/2016	CALE	E	C12A1	C12A	C12		27.5	30	68	2	Y	N	N
2016	186	19.18	0.024		0.502	0.008	97.381	0.502	1.541	6/1/2016	CALE	E	C12A2	C12A	C12		29	15	83	2	N	N	N
2016	187	27.49	0.023	0.036	0.454	0.009	92.701	0.502	1.609	6/1/2016	CALE	E	C12A3	C12A	C12		31.1	15	82	3	Y	N	N
2016	188	10.74	0.012		0.194	0	117.259	0.502	0	6/1/2016	CALE	E	C12A4	C12A	C12		27.1	2	95	3	N	Y	N
2016	189	22.53	0.014	0.03	0.612	0.005	113.935	0.502	1.227	6/1/2016	CALE	E	C12B1	C12B	C12		26.2	92	5	3	Y	N	N
2016	190	39.44	0.031	0.041	0.977	0.005	105.937	0.502	1.133	6/1/2016	CALE	E	C12B2	C12B	C12		30.2	93	4	3	N	N	N
2016	191	23.38	0.024	0.044	0.567	0.009	121.298	0.502	2.276	6/1/2016	CALE	E	C12B3	C12B	C12		19.5	97	2	1	Y	N	N
2016	192	13.2	0.015	0.017	0.365	0	96.664	0.502	0	6/1/2016	CALE	E	C12B4	C12B	C12		23.4	15	81	4	N	N	N
2016	193	53.58	0.018	0.043	1.57	0	108.891	0.502	3	6/1/2016	CALE	E	C6A1	C6A	C6		34	25	72	3	Y	N	N
2016	194	32.01	0.024		0.802	0.031	117.519	0.502	7.346	6/1/2016	CALE	E	C6A2	C6A	C6		39.1	47	50	3	N	N	N
2016	195	29.96	0.027	0.05	0.667	0.018	102.615	0.502	3.718	6/1/2016	CALE	E	C6A3	C6A	C6		40.5	25	72	3	Y	N	N
2016	196	62.77	0.06		1.422	0.129	75.127	0.502	19.371	6/1/2016	CALE	E	C6A4	C6A	C6		37.6	87	10	3	N	N	N
2016	197	35.46	0.03		0.926	0.045	106.862	0.502	9.531	6/1/2016	CALE	E	C6B1	C6B	C6		38.6	66	30	4	Y	N	N
2016	198	36.85	0.029	0.045	0.77	0.027	81.858	0.502	4.386	6/1/2016	CALE	E	C6B2	C6B	C6		42.1	72	25	3	Y	N	N
2016	199	37.29	0.041	0.056	0.775	0.028	93.75	0.502	4.611	6/1/2016	CALE	E	C6B3	C6B	C6		48	77	60	0	1	N	N
2016	200	20.97	0.028		0.216	0.027	65.487	0.502	3.562	6/1/2016	CALE	E	C6B4	C6B	C6		43.2	99	0	1	N	N	N
2016	201	39.12	0.049		1.65	0.062	66.962	0.5	8.249	6/9/2016	CALE	O				2009	09	6	A	4	2015	Y	Y
2016	202	32.5	0.037		0.999	0.041	111.118	0.5	9.08	6/9/2016	CALE	O				2009	09	6	A	4	2015	Y	Y
2016	203	41.61	0.035		1.157	0.027	106.702	0.5	5.779	6/9/2016	CALE	O				2009	09	6	A	4	2015	Y	Y
2016	204	52.9	0.021		0.685	0.038	74.363	0.5	5.686	6/9/2016	CALE	O				2009	09	6	A	4	2015	Y	Y
2016	205	49.08	0.011		0.446	0.015	106.852	0.5	3.09	6/9/2016	CALE	O				2009	09	6	A	4	2015	Y	Y
2016	206	50.36	0.048		1.855	0.089	79.296	0.5	14.1	6/9/2016	CALE	O				2009	09	6	E	3	2014	Y	Y
2016	207	52.08	0.021		0.915	0.043	131.96	0.5	11.22	6/9/2016	CALE	O				2009	09	6	E	3	2014	Y	Y
2016	208	52.88	0.037		1.488	0.047	101.475	0.5	9.521	6/9/2016	CALE	O				2009	09	6	E	3	2014	Y	Y
2016	209	51.44	0.006		0.568	0.01	148.778	0.5	2.898	6/9/2016	CALE	O				2009	09	6	E	3	2014	Y	Y
2016	210	35.75	0.035		0.935	0.024	105.279	0.5	3.														

2016	418	52.76	2.992	1.712	6/22/2016	PLLA	E	P4B2	P4B	P4	26.8	93	0	7	N	N	N	Y
2016	419	53.89	3.277	2.514	6/22/2016	PLLA	E	P5A1	P5A	P5	30.7	95	0	10	N	Y	N	N
2016	420	56.08	3.162	1.842	6/22/2016	PLLA	E	P5A2	P5A	P5	15.6	90	0	5	N	N	N	Y
2016	421	47.25	1.626	2.285	6/22/2016	PLLA	E	P5B1	P5B	P5	22.9	97	0	3	Y	N	N	Y
2016	422	51.11	1.651	1.824	6/22/2016	PLLA	E	P5B2	P5B	P5	27	95	0	5	N	N	N	Y
2016	423	48.82	3.498	1.816	6/22/2016	PLLA	E	P12A1	P12A	P12	18.2	90	0	10	Y	N	N	Y
2016	424	54.43	3.194	5.407	6/22/2016	PLLA	E	P12A2	P12A	P12	13	92	0	8	N	N	N	N
2016	425	50.51	1.657	3.614	6/22/2016	PLLA	E	P12B1	P12B	P12	25	95	0	5	N	N	N	Y
2016	426	55.21	2.989	3.189	6/22/2016	PLLA	E	P12B2	P12B	P12	20	97	0	3	N	N	N	Y
2016	427	50.37	2.026	3.359	6/22/2016	PLLA	E	P6A1	P6A	P6	19.9	93	0	7	Y	N	N	Y
2016	428	57.8	1.081	3.327	6/22/2016	PLLA	E	P6A2	P6A	P6	19.4	95	0	5	N	N	N	Y
2016	429	58.99	4.75	4.561	6/22/2016	PLLA	E	P6B1	P6B	P6	20.4	95	0	5	Y	N	N	N
2016	430	52.94	1.319	2.045	6/22/2016	PLLA	E	P6B2	P6B	P6	21.7	93	0	7	N	N	N	Y
2016	431	58.82	4.551	4.835	6/29/2016	PLLA	E	P1A3	P1A	P1	19.7	98	0	2	N	N	N	Y
2016	432	52.29	1.774	2.109	6/29/2016	PLLA	E	P1A1	P1A	P1	14	90	0	5	Y	N	N	Y
2016	433	53.19	3.664	3.145	6/29/2016	PLLA	E	P1A2	P1A	P1	9.3	99	0	1	N	N	N	N
2016	434	52.8	1.248	3.732	6/29/2016	PLLA	E	P1B3	P1B	P1	12.5	97	0	2	N	N	N	Y
2016	435	57.58	2.062	5.612	6/29/2016	PLLA	E	P1B2	P1B	P1	12	98	0	3	N	N	N	Y
2016	436	54.73	3.615	2.456	6/29/2016	PLLA	E	P1B1	P1B	P1	13.1	98	0	2	Y	N	N	Y
2016	437	59.08	2.55	2.894	6/29/2016	PLLA	E	P9A3	P9A	P9	13.7	96	0	4	N	N	N	Y
2016	438	56.04	2.136	2.784	6/29/2016	PLLA	E	P9A2	P9A	P9	19.6	99	0	1	N	N	N	N
2016	439	58.78	3.117	6.486	6/29/2016	PLLA	E	P9A1	P9A	P9	13	99	0	1	Y	N	N	N
2016	440	52.93	1.93	1.406	6/29/2016	PLLA	E	P9B3	P9B	P9	19.4				N	N	N	N
2016	441	52.36	1.749	5.121	6/29/2016	PLLA	E	P9B1	P9B	P9	17.1	98	0	2	Y	N	N	N
2016	442	53.74	3.361	1.509	6/29/2016	PLLA	E	P9B2	P9B	P9	22.9	98	0	2	N	N	N	Y
2016	443	56.16	1.911	2.616	6/29/2016	PLLA	E	P7A3	P7A	P7	19.3	97	0	9	5	N	N	N
2016	444	50.04	4.222	3.734	6/29/2016	PLLA	E	P7A2	P7A	P7	15.2	90	0	10	N	N	N	N
2016	445	56.6	3.753	4.834	6/29/2016	PLLA	E	P7A1	P7A	P7	15.5	90	0	10	Y	N	N	N
2016	446	52.81	0.481	1.407	6/29/2016	PLLA	E	P7B3	P7B	P7	16.6	70	0	3	N	N	N	N
2016	447	53.15	3.082	3.461	6/29/2016	PLLA	E	P7B1	P7B	P7	19.6	95	0	5	Y	N	N	Y
2016	448	58.74	0.643	1.695	6/29/2016	PLLA	E	P7B2	P7B	P7	20.5	95	0	5	N	N	N	Y
2016	449	50.3	1.591	4.416	6/29/2016	PLLA	E	P8A3	P8A	P8	22.5	97	0	3	N	N	N	Y
2016	450	50.42	1.254	2.699	6/29/2016	PLLA	E	P8A2	P8A	P8	16.8	90	0	10	N	N	N	N
2016	451	53.71	2.221	2.8	6/29/2016	PLLA	E	P8A1	P8A	P8	18.7	95	0	5	Y	N	N	N
2016	452	55.57	1.09	1.743	6/29/2016	PLLA	E	P8B3	P8B	P8	17.2	95	0	5	N	N	N	N
2016	453	51.92	1.086	0.964	6/29/2016	PLLA	E	P8B2	P8B	P8	15.8	80	0	20	N	N	N	N
2016	454	47.49	1.233	2.616	6/29/2016	PLLA	E	P8B1	P8B	P8	14.4	95	0	5	Y	N	N	Y
2016	455	50.56	0.625	0.928	6/29/2016	PLLA	E	P2A3	P2A	P2	16	92	0	8	N	N	N	N
2016	456	55.46	4.816	5.374	6/29/2016	PLLA	E	P2A2	P2A	P2	19.1	95	0	5	N	N	N	N
2016	457	49.87	1.841	3.767	6/29/2016	PLLA	E	P2A1	P2A	P2	15.3	95	0	5	Y	N	N	Y
2016	458	47.99	2.191	4.676	6/29/2016	PLLA	E	P2B3	P2B	P2	16	97	0	3	N	N	N	Y
2016	459	51.04	3.305	2.216	6/29/2016	PLLA	E	P2B1	P2B	P2	33.2	80	0	20	Y	N	N	Y
2016	460	46.26	1.415	4.416	6/29/2016	PLLA	E	P2B2	P2B	P2	17.82	92	0	3	N	N	N	Y
2016	461	53.55	2.227	2.027	6/29/2016	PLLA	E	P10A3	P10A	P10	30.5	95	0	5	N	Y	N	N
2016	462	48.85	0.726	0.61	6/29/2016	PLLA	E	P10A2	P10A	P10	42.3	70	0	30	Y	N	N	Y
2016	463	54.75	3.143	1.411	6/29/2016	PLLA	E	P10A1	P10A	P10	41	95	0	5	Y	N	N	Y
2016	464	54.64	1.891	1.197	6/29/2016	PLLA	E	P10B3	P10B	P10	22	95	0	5	N	N	N	N
2016	465	30.26	2.132	2.631	6/29/2016	PLLA	E	P10B2	P10B	P10	16.3	90	0	10	Y	N	N	Y
2016	466	53.79	1.459	2.826	6/29/2016	PLLA	E	P10B1	P10B	P10	16.5	95	0	5	Y	N	N	Y
2016	467	48.14	1.913	3.032	6/29/2016	PLLA	E	P3A3	P3A	P3	22.4	90	0	10	N	N	N	Y
2016	468	49.47	3.782	1.404	6/29/2016	PLLA	E	P3A1	P3A	P3	19	80	0	20	Y	N	N	Y
2016	469	46.83	3.29	1.885	6/29/2016	PLLA	E	P3A2	P3A	P3	22.1	95	0	5	N	N	N	Y
2016	470	52.57	3.138	2.469	6/29/2016	PLLA	E	P3B3	P3B	P3	16.3	95	0	5	N	N	N	N
2016	471	47.74	1.233	3.642	6/29/2016	PLLA	E	P3B2	P3B	P3	14.9	95	0	5	Y	N	N	Y
2016	472	44.29	2.297	3.614	6/29/2016	PLLA	E	P3B1	P3B	P3	17.1	98	0	2	Y	N	N	N
2016	473	47.33	2.151	3.545	6/29/2016	PLLA	E	P11A3	P11A	P11	21.8	96	0	4	N	N	N	N
2016	474	51.99	2.023	2.363	6/29/2016	PLLA	E	P11A2	P11A	P11	19.4	80	0	20	N	N	N	N
2016	475	45.63	2.292	1.287	6/29/2016	PLLA	E	P11A1	P11A	P11	20.5	80	0	20	Y	N	N	Y
2016	476	58.07	1.98	3.408	6/29/2016	PLLA	E	P11B3	P11B	P11	16.1	97	0	3	N	N	N	Y
2016	477	50.02	1.118	4.689	6/29/2016	PLLA	E	P11B2	P11B	P11	14.4	95	0	5	N	N	N	Y
2016	478	51.68	0.678	2.581	6/29/2016	PLLA	E	P11B1	P11B	P11	24	97	0	3	Y	N	N	Y
2016	479	46.05	1.243	0.521	6/29/2016	PLLA	E	P4A3	P4A	P4	32.4	97	0	3	N	N	N	N
2016	480	49.12	2.94	0.567	6/29/2016	PLLA	E	P4A2	P4A	P4	32.4	95	0	5	N	N	N	N
2016	481	49.31	1.733	0.505	6/29/2016	PLLA	E	P4A1	P4A	P4	31.7	95	0	5	Y	N	N	Y
2016	482	56.38	3.311	2.616	6/29/2016	PLLA	E	P4B3	P4B	P4	24.7	97	0	3	N	N	N	Y
2016	483	53.9	1.427	2.165	6/29/2016	PLLA	E	P4B1	P4B	P4	28.7	90	0	10	Y	N	N	N
2016	484	56.67	2.727	1.409	6/29/2016	PLLA	E	P4B2	P4B	P4	26.8	93	0	7	N	N	N	Y
2016	485	62.74	1.891	3.077	6/29/2016	PLLA	E	P5A3	P5A	P5	18.3	97	0	3	N	N	N	Y
2016	486	47.55	1.064	0.855	6/29/2016	PLLA	E	P5A1	P5A	P5	30.7	95	0	5	Y	N	N	N
2016	487	58.54	5.426	3.835	6/29/2016	PLLA	E	P5A2	P5A	P5	15.6	90	0	10	Y	N	N	Y
2016	488	62.79	2.631	1.536	6/29/2016	PLLA	E	P5B3	P5B	P5	23.2	97	0	3	Y	N	N	Y
2016	489	52.97	1.112	1.441	6/29/2016	PLLA	E	P5B1	P5B	P5	23.2	97	0	3	Y	N	N	Y
2016	490	56.45	1.389	2.734	6/29/2016	PLLA	E	P5B2	P5B	P5	27	95	0	5	N	Y	N	Y
2016	491	44.24	1.517	1.979	6/29/2016	PLLA	E	P12A3	P12A	P12	16	97	0	3	N	N	N	Y
2016	492	54.5	3.504	4.411	6/29/2016	PLLA	E	P12A2	P12A	P12	13	92	0	8	N	N	N	Y
2016	493	51.65	1.615	2.616	6/29/2016	PLLA	E	P12A1	P12A	P12	20.1	97	0	10	Y	N	N	Y
2016	494	50.62	1.78	2.284	6/29/2016	PLLA	E	P12B3	P12B	P12	20.1	97	0	3	N	N	N	Y
2016	495	49.62	3.068	3.412	6/29/2016	PLLA	E	P12B2	P12B	P12	20	97	0	3	N	N	N	Y
2016	496	53.11	1.718	3.212	6/29/2016	PLLA	E	P12B1	P12B	P12	25	95	0	5	Y	N	N	Y
2016	497	51.08	4.385	1.89	6/29/2016	PLLA	E	P6A3	P6A	P6	19.7	90	0	10	N	N	N	Y
2016	498	41.86	1.6	2.104	6/29/2016	PLLA	E	P6A2	P6A	P6	19.4	95	0	5	N	N	N	Y
2016	499	44.62	1.026	2.026	6/29/2016	PLLA	E	P6A1	P6A	P6	14.9	95	0	5	N	N	N	Y
2016	500	50.18	2.423	4.77	6/29/2016	PLLA	E	P6B3	P6B	P6	16	85	0	15	N	N	N	Y
2016	501	52.8	1.759															

2017-17-119	18.92	0.031	0	0.169	0.038488	108.611	0.5008	8.347	5/2/2017	CALE	E	C4B1	Y	Y	C4	C4B	2016	Y	N	N
2017-17-120	30.4	0.02	0	0.225	0.036874	128.316	0.5008	9.448	5/2/2017	CALE	E	C4B2	Y	Y	C4	C4B	2016	N	N	N
2017-17-121	13.12	0.02	0	0.112	0	90.255	0.5008	0	5/2/2017	CALE	E	C10A1	Y	N	C10	C10A	2016	Y	N	Y
2017-17-122	26.07	0	0	0.226	0.034994	97.4	0.5008	6.806	5/2/2017	CALE	E	C10A2	Y	N	C10	C10A	2016	Y	N	N
2017-17-123	13.02	0.016	0	0.119	0.016534	110.972	0.5008	3.686	5/2/2017	CALE	E	C10B1	Y	Y	C10	C10B	2016	Y	N	Y
2017-17-124	34.14	0.026	0	0.235	0.016994	122.093	0.5008	4.143	5/2/2017	CALE	E	C10B2	Y	Y	C10	C10B	2016	N	N	N
2017-17-125	30.3	0.053	0.05	0.241	0.074164	107.833	0.5008	15.969	5/2/2017	CALE	E	C8A1	Y	N	C8	C8A	2016	Y	N	N
2017-17-126	59.11	0.03	0.031	0.209	0.034215	114.035	0.5008	7.791	5/2/2017	CALE	E	C8A2	Y	N	C8	C8A	2016	N	N	N
2017-17-127	19.59	0.033	0	0.161	0.059858	125.297	0.5008	14.976	5/2/2017	CALE	E	C8B1	Y	Y	C8	C8B	2016	Y	N	Y
2017-17-128	23.52	0.02	0	0.142	0.031834	111.869	0.5008	7.111	5/2/2017	CALE	E	C8B2	Y	Y	C8	C8B	2016	N	N	N
2017-17-129	13.49	0.014	0	0.113	0	123.361	0.5008	0	5/2/2017	CALE	E	C5A1	Y	Y	C5	C5A	2016	Y	N	Y
2017-17-130	21.24	0.02	0	0.142	0.024254	88.023	0.5008	4.263	5/2/2017	CALE	E	C5A2	Y	Y	C5	C5A	2016	N	N	N
2017-17-131	13.88	0.016	0	0	0	73.647	0.5008	0	5/2/2017	CALE	E	C5B1	Y	N	C5	C5B	2016	Y	N	Y
2017-17-132	12.38	0	0	0.127	0	58.297	0.5008	0	5/2/2017	CALE	E	C5B2	Y	N	C5	C5B	2016	N	N	N
2017-17-133	42.65	0.067	0	0.416	0.086781	95.386	0.5008	16.529	5/2/2017	CALE	E	C11A1	Y	Y	C11	C11A	2016	Y	N	Y
2017-17-138	13.19	0.037	0	0.074	0	84.11	0.5008	0	5/2/2017	CALE	E	C12A2	N	N	C12	C12A	2016	N	N	Y
2017-17-139	11.91	0.031	0	0.057	0	121.978	0.5008	0	5/2/2017	CALE	E	C12B1	N	Y	C12	C12B	2016	Y	N	N
2017-17-140	16.88	0	0	0.102	0	95.674	0.5008	0	5/2/2017	CALE	E	C12B2	N	Y	C12	C12B	2016	N	N	N
2017-17-141	30.92	0.065	0	0.225	0.016565	115.219	0.5008	3.811	5/2/2017	CALE	E	C6A1	N	N	C6	C6A	2016	N	N	N
2017-17-142	47.67	0.026	0	0.269	0.041983	107.285	0.5008	8.994	5/2/2017	CALE	E	C6A2	N	N	C6	C6A	2016	N	N	N
2017-17-143	29.75	0.053	0	0	0	0	0.5008	0	5/2/2017	CALE	E	C6B1	N	Y	C6	C6B	2016	Y	N	Y
2017-17-145	56.63	5.124	3.889						5/9/2017	PLLA	E	P1A1	N	Y	P1	P1A	2016	Y	N	N
2017-17-146	58.57	1.925	4.658						5/9/2017	PLLA	E	P1A2	N	Y	P1	P1A	2016	N	N	Y
2017-17-147	57.2	4.338	3.087						5/9/2017	PLLA	E	P1B1	N	N	P1	P1B	2016	Y	N	Y
2017-17-148	45.45	5.592	1.072						5/9/2017	PLLA	E	P1B2	N	N	P1	P1B	2016	N	N	Y
2017-17-149	47.91	1.445	1.142						5/9/2017	PLLA	E	P9A1	Y	Y	P9	P9A	2016	Y	N	Y
2017-17-150	51.24	7.245	3.016						5/9/2017	PLLA	E	P9A2	Y	Y	P9	P9A	2016	N	N	Y
2017-17-151	52	4.106	3.61						5/9/2017	PLLA	E	P9B1	Y	N	P9	P9B	2016	Y	N	Y
2017-17-152	36.04	3.629	2.427						5/9/2017	PLLA	E	P9B2	Y	N	P9	P9B	2016	N	N	Y
2017-17-153	27.79	2.93	1.879						5/9/2017	PLLA	E	P7A1	Y	Y	P7	P7A	2016	Y	N	Y
2017-17-154	45.6	3.565	1.703						5/9/2017	PLLA	E	P7A2	Y	Y	P7	P7A	2016	N	N	Y
2017-17-155	54.67	6.068	3.526						5/9/2017	PLLA	E	P7B1	Y	Y	P7	P7B	2016	Y	N	Y
2017-17-156	13.4	0.956	1.215						5/9/2017	PLLA	E	P7B2	Y	N	P7	P7B	2016	N	N	Y
2017-17-157	22.54	0.937	1.636						5/9/2017	PLLA	E	P8A1	N	N	P8	P8A	2016	Y	N	Y
2017-17-158	49.68	3.872	1.549						5/9/2017	PLLA	E	P8A2	Y	N	P8	P8A	2016	N	N	Y
2017-17-159	49.96	4.148	2.662						5/9/2017	PLLA	E	P8B1	Y	Y	P8	P8B	2016	Y	N	Y
2017-17-160	46.76	0.936	1.526						5/9/2017	PLLA	E	P8B2	Y	Y	P8	P8B	2016	Y	N	Y
2017-17-161	44.92	2.676	1.5						5/9/2017	PLLA	E	P2A1	Y	N	P2	P2A	2016	Y	N	Y
2017-17-162	46.83	2.26	2.273						5/9/2017	PLLA	E	P2A2	Y	N	P2	P2A	2016	N	N	Y
2017-17-163	33.45	1.22	1.222						5/9/2017	PLLA	E	P2B1	Y	Y	P2	P2B	2016	Y	N	Y
2017-17-164	48.83	2.971	1.438						5/9/2017	PLLA	E	P2B2	Y	Y	P2	P2B	2016	N	N	Y
2017-17-165	41.59	3.274	1.389						5/9/2017	PLLA	E	P10A1	Y	N	P10	P10A	2016	Y	N	Y
2017-17-166	48.45	1.051							5/9/2017	PLLA	E	P10A2	Y	N	P10	P10A	2016	Y	N	Y
2017-17-167	58.75	4.44	1.876						5/9/2017	PLLA	E	P10B1	Y	Y	P10	P10B	2016	Y	N	Y
2017-17-168	26.23	1.467	1.549						5/9/2017	PLLA	E	P10B2	Y	Y	P10	P10B	2016	N	N	Y
2017-17-169	24.29	2.939	1.098						5/9/2017	PLLA	E	P3A1	Y	Y	P3	P3A	2016	Y	N	Y
2017-17-170	36.15	1.768	3.381						5/9/2017	PLLA	E	P3A2	Y	Y	P3	P3A	2016	N	N	Y
2017-17-171	51.03	4.217	2.785						5/9/2017	PLLA	E	P3B1	Y	Y	P3	P3B	2016	Y	N	Y
2017-17-172	56.15	1.179							5/9/2017	PLLA	E	P3B2	Y	Y	P3	P3B	2016	Y	N	Y
2017-17-173	44.84	2.827	1.752						5/9/2017	PLLA	E	P11A1	Y	Y	P11	P11A	2016	Y	N	Y
2017-17-174	3.77	0.303	0.055						5/9/2017	PLLA	E	P11A2	Y	Y	P11	P11A	2016	N	N	Y
2017-17-175	45.96	2.396	4.133						5/9/2017	PLLA	E	P11B1	Y	N	P11	P11B	2016	Y	N	Y
2017-17-176	63.96	1.726	4.312						5/9/2017	PLLA	E	P11B2	Y	N	P11	P11B	2016	N	N	Y
2017-17-177	50.62	4.262	0.927						5/9/2017	PLLA	E	P4A1	Y	Y	P4	P4A	2016	Y	N	Y
2017-17-178	55.45	4.143	1.275						5/9/2017	PLLA	E	P4A2	Y	N	P4	P4A	2016	N	N	Y
2017-17-179	62.49	4.615	1.468						5/9/2017	PLLA	E	P4B1	Y	Y	P4	P4B	2016	Y	N	Y
2017-17-180	38.28	1.258	1.52						5/9/2017	PLLA	E	P4B2	Y	Y	P4	P4B	2016	N	N	Y
2017-17-181	29.19	2.584	1.032						5/9/2017	PLLA	E	P5A1	Y	Y	P5	P5A	2016	Y	N	Y
2017-17-182	51.98	2.483	2.938						5/9/2017	PLLA	E	P5A2	Y	Y	P5	P5A	2016	Y	N	Y
2017-17-183	47.07	1.65	1.944						5/9/2017	PLLA	E	P5B1	Y	Y	P5	P5B	2016	Y	N	Y
2017-17-184	41.24	1.834	1.894						5/9/2017	PLLA	E	P5B2	Y	N	P5	P5B	2016	N	N	Y
2017-17-185	49.09	3.794	1.598						5/9/2017	PLLA	E	P12A1	N	N	P12	P12A	2016	N	N	Y
2017-17-186	45.82	2.424	4.839						5/9/2017	PLLA	E	P12A2	N	N	P12	P12A	2016	N	N	Y
2017-17-187	65.17	2.663	8.085						5/9/2017	PLLA	E	P12B1	N	Y	P12	P12B	2016	Y	N	Y
2017-17-188	59.27	4.966							5/9/2017	PLLA	E	P12B2	N	Y	P12	P12B	2016	Y	N	Y
2017-17-189	45.76	4.213	3.536						5/9/2017	PLLA	E	P6A1	N	N	P6	P6A	2016	Y	N	Y
2017-17-190	38.34	3.637	2.361						5/9/2017	PLLA	E	P6A2	N	N	P6	P6A	2016	Y	N	Y
2017-17-191	54	5.942	2.891						5/9/2017	PLLA	E	P6B1	N	Y	P6	P6B	2016	Y	N	Y
2017-17-193	36.7	0.047	0.078	0.232	0.045228	87.06	0.49988	7.877	5/24/2017	CALE	E	C1A1	N	Y	C1	C1A	2016	Y	N	N
2017-17-194	48.81	0.029	0.037	0.296	0.037875	90.309	0.49988	6.888	5/24/2017	CALE	E	C1A2	N	C1A	C1A	2016	N	N	N	
2017-17-195	30.77	0.024	0	0.186	0.030829	110.447	0.49988	24.81	5/24/2017	CALE	E	C1B1	N	N	C1	C1B	2016	Y	N	N
2017-17-196	50.41	0.011	0.032	0.486	0.050538	133.759	0.49988	13.523	5/24/2017	CALE	E	C1B2	N	N	C1	C1B	2016	N	N	N
2017-17-197	32.58	0.021	0	0.152	0.031988	69.697	0.49988	4.46	5/24/2017	CALE	E	C7A1	Y	Y	C7	C7A	2016	Y	N	Y
2017-17-198	45.16	0.027	0	0.287	0.029856	131.014	0.49988	7.825	5/24/2017	CALE	E	C7A2	Y	Y	C7	C7A	2016	N	N	Y
2017-17-199	37.57	0.043	0.032	0.217	0.095651	101.883	0.49988	19.495	5/24/2017	CALE	E	C7B1	Y	N	C7	C7B	2016	Y	N	Y
2017-17-200	52.28	0.0																		

2017 17-271	51.1	1.869	7.287	5/30/2017	PLLA	E	P11B1	Y	N	P11	P11B	2016	Y	N	N
2017 17-272	58.27	2.794	5.511	5/30/2017	PLLA	E	P11B2	Y	N	P11	P11B	2016	N	N	Y
2017 17-273	46.15	4.566	0.9	5/30/2017	PLLA	E	P4A1	Y	N	P4	P4A	2016	Y	N	N
2017 17-274	53.58	2.72	0.581	5/30/2017	PLLA	E	P4B2	Y	N	P4	P4A	2016	N	N	N
2017 17-275	49.1	3.37	1.371	5/30/2017	PLLA	E	P4B1	Y	Y	P4	P4B	2016	Y	N	Y
2017 17-276	42.19	2.065	1.618	5/30/2017	PLLA	E	P4B2	Y	Y	P4	P4B	2016	N	N	N
2017 17-277	50.69	1.8	1.648	5/30/2017	PLLA	E	P5A1	Y	Y	P5	P5A	2016	Y	N	Y
2017 17-278	51.14	3.108	3.883	5/30/2017	PLLA	E	P5A2	Y	Y	P5	P5A	2016	N	N	Y
2017 17-279	60.47	2.878	1.906	5/30/2017	PLLA	E	P5B1	Y	N	P5	P5B	2016	Y	Y	N
2017 17-280	60.98	4.062	3.846	5/30/2017	PLLA	E	P5B2	Y	N	P5	P5B	2016	N	N	N
2017 17-281	50.88	4.398	1.515	5/30/2017	PLLA	E	P12A1	N	N	P12	P12A		N	N	Y
2017 17-282	34.35	3.529	4.513	5/30/2017	PLLA	E	P12A2	N	N	P12	P12A		N	N	Y
2017 17-283	48.25	1.847	2.077	5/30/2017	PLLA	E	P12B1	N	Y	P12	P12B		Y	N	Y
2017 17-284	53.41	1.865	1.002	5/30/2017	PLLA	E	P12B2	N	Y	P12	P12B		N	Y	Y
2017 17-285	44.43	6.571	6.373	5/30/2017	PLLA	E	P6A1	N	N	P6	P6A		Y	N	Y
2017 17-286	44.66	5.791	2.203	5/30/2017	PLLA	E	P6A2	N	N	P6	P6A		N	Y	Y
2017 17-287	58.68	4.515	4.655	5/30/2017	PLLA	E	P6B1	N	Y	P6	P6B		Y	N	Y