EVALUATING THE EFFECTS OF CLIMATE CHANGE AND PATHOGENS ON POLLINATOR HEALTH USING PLANT FUNCTIONAL TRAITS AND LONGITUDINAL MONITORING

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Biological Sciences

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 2017

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank my committee members including my co-advisors Drs. Laura Burkle and Michelle Flenniken, along with Dr. Justin Runyon. Their dedication to this project, and investment in my future has set an incredible example I one day wish to emulate. I would like to thank my family including Dr. Robb Glenny, Anne Glenny, Elle Glenny, and Colin Glenny and especially Dr. William Robb Glenny for always prioritizing education above all else. Additionally, special thanks to my friends and colleagues from Gonzaga University that cultivated my interest in science a long time ago and constantly motivate me to “save the bees”. Finally, I would not be able to complete this work without the unending support of the Montana State Ecology Department with special thanks to the administrative staff, fellow graduate students, and professors. Finally, thanks to anyone that believes education is a worth while pursuit.

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ABSTRACT

Pollinators are essential for the maintenance of biodiversity, ecosystem function, and economic productivity. In particular, bee pollinators are required for plant reproduction and pollination of agricultural crops. However, land use change, climate change, pathogens, pesticide exposure, among other factors likely act alone and in combination to negatively impact bee pollinators and the services they provide. Further resolution of the effects of these stressors, both individually and combined, on bee pollinators is important to understand the global decline of pollinator health.

Abiotic conditions associated with climate change may alter plant traits important for pollinator attraction leading to in shifts in plant-pollinator communities. Floral visual and chemical traits were measured in four species of forbs subjected to elevated or ambient concentrations of carbon dioxide, and decreased or normal water availability in a fully factorial crossed design. Treated plants were observed for pollinator visitation rates and community composition to better understand the mechanisms by which climate change can influence pollinator attraction. Results indicate that changes in both visual and chemical cues of plants will alter plant-pollinator interactions. Furthermore, plant functional trait responses to climate change increase competition for pollinators in forbs with overlapping flower types, while facilitating pollinator visitation to forbs with dissimilar flower types.

Pathogens contribute to annual honey bee colony losses and the declining populations of some wild bee species. Bee pathogens, including viruses, fungi, microparasites and ectoparasites, can vary across geographic location and season. To examine the impact of pathogens on honey bee colony health, using colony size as a proxy for health, we longitudinally monitored pathogen prevalence and abundance of pathogens in honey bee colonies involved in California almond pollination. Individual honey bee associated pathogens varied throughout the one year monitoring period, but Deformed wing virus in parallel with increasing levels of *Varroa destructor* mite infestation predominated shifts in honey bee pathogen profiles by the end of the sampling period. Our results indicate that bee populations experience multiple concurrent threats operating at multiple scales to affect pollinator health. Continued investigation into factors affecting pollinator health both independently and in concert are needed to develop strategies mitigating declines in pollination services.

CHAPTER ONE

INTRODUCTION

Pollinators play a vital role in the maintenance of biodiversity, ecosystem function, and economic productivity (Klein et al. 2007, Gallai et al. 2009, Ollerton et al. 2011, Garibaldi et al. 2013). It is estimated that between 60-90% of plants globally require animal pollination (Ollerton et al. 2011), the value of which is estimated to be $118 billion annually (Gallai et al. 2009). In particular, bee pollinators are essential for the reproductive success of plants and the production of agricultural crops. However, the negative effects of the urbanization of wild lands, pesticide exposure, pathogens, and climate change, among other factors, on plant-pollinator interactions threaten the services provided by pollinators (Biesmeijer et al. 2006, Potts et al. 2010, Graystock et al. 2015). Although causes of pollinator population decline are often studied in isolation, multiple factors likely act directly and in combination to influence pollinator populations (Schweiger et al. 2010). Therefore, to better understand the impact of multiple factors on pollinator health, we used an experimental approach to evaluate the effects of climate change on plant traits important for pollinator attraction, and a longitudinal monitoring project relating pathogen communities to colony health status in honey bee colonies involved in California almond pollination.

Anthropogenic climate change is anticipated to have broad-scale effects on plant-pollinator interactions. Components of climate change can result in

spatial and temporal mismatches of plants and pollinators (Memmott et al. 2007, Tylianakis et al. 2008, Hegland et al. 2009, Scaven and Rafferty 2013), and the alteration of traits important for pollinators to locate floral rewards (Carroll et al. 2001). Flowers are complex structures that display multimodal signals relying on pollinator's visual, olfactory, tactile, and gustatory senses to guide pollinators to floral rewards (Junker and Parachnowitsch 2015). While studies have traditionally focused on the role of plant visual traits in pollinator attraction, flowers emit information-rich chemicals known as volatile organic compounds (VOCs), which mediate cross-trophic interactions (i.e., defense against herbivores, or attract pollinators) (Kessler and Baldwin 2001, Raguso 2008, Schiestl 2015). However, the propensity for VOCs to change in response to environmental conditions can alter signals conveyed by VOCs (Yuan et al. 2009, Penuelas and Staudt 2010, Farre-Armengol et al. 2013, Farre-Armengol et al. 2014). Therefore, studying changes in VOCs alongside plant visual traits is necessary to develop a holistic view of the roles of flower traits important for pollinator attraction.

To date, few studies have evaluated the potential for components of climate change to alter floral scent and other floral traits important for pollinator attraction (Burkle and Runyon 2016). This has been difficult largely because of the multifaceted nature of climate change. As a result of anthropogenic climate change, water availability is anticipated to decrease throughout the western United States, and carbon dioxide (CO_2) concentration is anticipated to increase

concurrently (Pederson et al. 2010, Dai 2013, Pachauri et al. 2014, Cook et al. 2015). Therefore, it is important to consider the non-additive effects of simultaneous changes in components of climate change on plant traits important for pollinator attraction (Hoover et al. 2012). To investigate this question, we measured plant visual and chemical traits of four species of forbs subjected to elevated or ambient concentrations of CO_2 and reduced or normal levels of water availability in a two-by-two fully factorial design. Additionally, each plant individual was observed for pollinator visitation, and pollinators were identified to species to investigate shifts in plant-pollinator interactions as a result of climate change.

Pathogens are another threat implicated in the global decline of pollinator health (Potts et al. 2010). Bees are often infected by pathogens including viruses, fungi, bacteria, trypanosomatids, and are frequently parasitized by mites. (Schmid-Hempel 2001, Cox-Foster et al. 2007, Cameron et al. 2011, Evans and Schwarz 2011, Levitt et al. 2013, McMahon et al. 2015) and are associated with reduced honey bee and bumble bee health (Cox-Foster et al. 2007, Vanengelsdorp et al. 2008, Cameron et al. 2011, Daughenbaugh et al. 2015, McMenamin and Genersch 2015, Cameron et al. 2016, Cavigli et al. 2016). Pathogens (particularly RNA viruses) can spread between and within bee species through the sharing of floral resources, raising concern for the future health of both Apidae and other non-Apidae species (Singh et al. 2011, Graystock et al. 2015, McMahon et al. 2015). To better understand bee-pathogen

relationships, we utilized honey bees as a tractable model and anticipate that our findings will be relevant beyond this single species.

Recently, increases in mean annual colony loss increased from historic rates of 12%, to unsustainable levels of 33% in the U.S., raising concern for honey bee health. Multiple factors including agrochemical exposure, climate change, management practices, co-infections, bee genetics, and queen performance interact to influence pathogen abundance in honey bees (McMenamin et al. 2016). However, the majority of studies monitoring honey bee pathogens in the U.S. have been conducted at the apiary level with the primary goal of detecting exotic pathogens and establishing a baseline understanding of honey bee colony health (Lee et al. 2015, Seitz et al. 2016, Traynor et al. 2016). To examine the relationship between honey bee colony health, using colony population size as a proxy for colony health, and pathogen prevalence and abundance, we longitudinally monitored honey bee colonies involved in California almond pollination. California almond pollination is the largest managed pollination event in the world requiring over 1.6 million commercially managed honey bee colonies in the U.S. and thus accounts for considerable economic production. Results from this study will help identify factors with the potential to influence specific pathogens, and suites of pathogens that can affect honey bee colony health.

Results from these studies help to elucidate the impact of threats to bee pollinators from the landscape to the cellular level. The studies presented herein

highlight the benefit of approaches with broad-scale applicability, including *in situ* field experiments, gas chromatography/mass spectrometry (GC/MS), colony monitoring, and molecular based pathogen diagnostics, to comprehensively investigate the nature of threats to pollinator health. Results indicate that climate change can alter plant traits important for pollinator attraction, leading to shifts in plant-pollinator interactions. Additionally, pathogen composition is seasonal in honey bee colonies, and is likely influenced by increasing mite abundance. These studies employ a framework incorporating multidisciplinary techniques to resolve aspects of the decline in pollinator health in order to benefit efforts needed to improve pollinator conservation strategies.

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CHAPTER TWO

THE INTERACTIVE EFFECTS OF DROUGHT AND CARBON DIOXIDE FERTILIZATION ON PLANT-POLLINATOR INTERACTIONS THROUGH PLANT VISUAL TRAITS AND VOLATILE ORGANIC COMPOUNDS

Abstract

Climate change can alter species interactions essential for maintaining biodiversity and ecosystem function, like pollination. For example, climate change can affect floral traits important for pollinator attraction, but the extent to which altered floral traits increase or decrease pollinator visitation to individual forb species is unknown. To examine the potential effects of climate change on plant-pollinator interactions, floral visual and floral volatile organic compounds (VOCs) were measured in four species of forbs subjected to decreased or normal water availability, and elevated or ambient concentrations of carbon dioxide in a fully factorial design, and were observed for pollinator visitation. Additionally, pollinator visitation was observed to forbs in both single-species and multi-species forb assemblages to investigate the degree to which climate change affects pollinator visitation to forb species in different plant community contexts. Results revealed species-specific effects of drought and CO_2 fertilization on floral visual and VOC traits. While drought reduced floral visual traits in all forb species measured, drought increased total VOC emissions in three forb species relative to control treated plants. Additionally, CO_2 fertilization increased floral visual

traits, but had inconsistent effects on total VOC emissions and composition relative to control treated plants. These results indicate that drought will negatively affect floral visual traits but positively affect floral scent, while CO_2 fertilization will positively affect floral visual traits with mixed effects on floral scent. Pollinator visitation was linked to changes in floral visual traits in drought treated plants for one species within a single-species assemblage. An interactive effect of drought and CO_2 was rarely observed, potentially indicating CO_2 fertilization buffers plant traits against the effects of drought. To examine the effects of climate change in different plant community contexts, pollinator visitation was observed to multi-species assemblages of the four forb species. Within multi-species assemblages, pollinator visitation was reduced to drought–treated plants in two forb species while pollinator visitation increased to CO_2-fertilized plants to one forb species when compared to single-species assemblages, suggesting that drought increases competition for pollinators and CO_2 fertilization facilitates pollination to forbs in a more diverse plant community context.

Introduction

Mutualistic interactions between plants and pollinators are essential for the maintenance of biodiversity and ecosystem function (Bascompte et al. 2006). However, climate change can alter plant traits and cues important for pollinator attraction, leading to shifts in plant-pollinator interactions (Memmott et al. 2007,

Tylianakis et al. 2008, Schweiger et al. 2010, Hoover et al. 2012, Scaven and Rafferty 2013, Burkle and Runyon 2016). Shifts in plant-pollinator interactions can occur as a result of altered plant phenological responses (Fitter and Fitter 2002, Hegland et al. 2009, Forrest et al. 2010), pollinator physiology (Bartomeus et al. 2011, Scaven and Rafferty 2013), the spatial distribution of plants and pollinators (Hegland et al. 2009, Burkle et al. 2013), and floral reward quality (Galen 1999, Carroll et al. 2001). Consequently, multiple potential mechanisms leading to shifts in plant-pollinator interactions at finer scales, can have cascading effects with the potential to shape overall plant-pollinator interaction networks (Memmott et al. 2007, Burkle et al. 2013).

The compatibility of plant and pollinator traits can be used to predict community-wide plant-pollinator interactions, and are important to study in order to predict the responses of plant-pollinator networks to climate change (reviewed in Vasquez et al 2009(Rezende et al. 2007). For example, interaction patterns in a plant-pollinator network were predicted successfully using the length of a flower’s nectar tube compared to pollinator tongue length, demonstrating how trait matching between plants and pollinators is important for the maintenance of an intact plant-pollinator community (Stang et al. 2009). However, altered floral traits or shifts in flower community composition, can lead to discrepancies in traits fundamental for plant-pollinator interactions resulting in novel plant-pollinator community interactions (Miller-Struttman et al. 2015). Therefore, the effects of climate change on individual- and community-level floral traits

important for mediating plant-pollinator interactions, has the potential to affect the structure and function of pollinator communities.

Floral trait diversity reduces plant competition for pollinators and can facilitate pollination to other forb species (Moeller, 2004, Ghazoul, 2006). For example, competition for pollinators increased in *Ipomopsis aggregata* when co-occuring with a competitor, *Castilleja linariaefolia,* which has overlapping floral traits (i.e. similar phenologies and flower morphologies) than when *I. aggregata* occurred alone (Caruso 2000). However, facilitation of pollination is thought to occur as a result of floral displays of different forbs that collectively attract pollinators shared across plant species (Moeller, 2004; Ghazoul, 2006). Consequently, forbs blooming in low population densities that otherwise would not receive floral visits can be located by pollinators using large floral displays of co-occuring forb species (Moeller, 2004; Ghazoul, 2006). Therefore, the community composition of co-occurring forb species is important to consider when testing the role of plant traits on pollinator visitation.

Flowers have evolved complex structures that utilize multiple pollinator sensory abilities and influence pollinator attraction. Flower characteristics, such as the number of flowers produced, morphology, and pigmentation, utilize pollinator visual senses, while floral rewards such as nectar and pollen rely on pollinator gustatory senses to attract pollinators (Junker and Parachnowitsch 2015). While research to date has primarily focused on the role of plant visual traits and floral rewards in mediating pollinator visitation, chemical cues known as

volatile organic compounds (VOCs) play important roles in modifying pollinator foraging behavior. Consequently, incorporating the roles of both chemical and visual traits in pollinator attraction is important to develop a more holistic approach to studying how plant traits mediate plant-pollinator interactions (Raguso 2008).

Plant VOCs are airborne chemicals emitted from plant tissues that serve a variety of ecological functions. VOCs are important for both plant defense (De Moraes et al. 2001, Kessler et al. 2008) and for facilitating mutualistic interactions between plants and pollinators (Schiestl 2015). In particular, pollinator response to floral VOCs can increase pollinator fidelity to flowers (Schiestl and Peakall 2005, Dudareva et al. 2006, Raguso 2008, Xiao et al. 2012), increase pollinator foraging efficiency (Howell and Alarcon 2007, McFrederick et al. 2009, Fuentes et al. 2016), and drive the diversification of floral traits (Gervasi and Schiestl 2017). As a result, floral VOCs can enhance or deter pollinator visitation to flowers, pollinator resource partitioning, and flower specialization, thereby serving as an important trait structuring plant-pollinator community interactions (Junker and Bluthgen 2010, Junker et al. 2010).

The composition of floral VOCs can change in response to abiotic conditions, and in turn, lead to shifts in the composition of pollinators visiting plants (Burkle and Runyon, 2016). The relative composition of floral VOC blends can be influenced by water availability, CO_2 concentration, temperature, light intensity, and humidity via changes in the abundances of individual compounds

(Penuelas and Staudt 2010, Farre-Armengol et al. 2013, Farre-Armengol et al. 2014). As a result, novel blends of compounds can form in response to changes in the environment, with community-level implications for pollinator visitation (Junker et al. 2010, Schweiger et al. 2010, Hoover et al. 2012, Burkle and Runyon 2016, Larue et al. 2016). Therefore, the influence of abiotic conditions on individual compounds and VOC bouquets are important to understand the mechanisms behind shifts in plant-pollinator interactions in response to components of climate change.

Water availability and atmospheric CO_2 concentration are two components of climate change anticipated to affect plant visual and chemical traits important for pollinator attraction. Increases in atmospheric CO_2 concentration (Pachauri et al. 2014) resulting in CO_2 fertilization of plants can increase plant visual traits, resulting in more flowers, fruits, seeds, and primary productivity (Jablonski et al. 2002, Poorter and Navas 2003), and increase the efficiency of plant resource use (Korner C., 2009). However, the effects of CO_2 fertilization on plant chemical traits have varied among studies, most of which have been conducted on leaf volatiles in wind-pollinated plants. For example, elevated CO_2 concentration was observed to decrease monoterpene emissions from the evergreen oak species *Quercus ilex* (Loreto et al. 2001), but increase monoterpene emissions in conifer species (Constable et al. 1999). Additionally, reduced precipitation as a result of climate change is expected to result in drought conditions for plants (Pederson et al. 2010, Dai 2013, Cook et al. 2015). Drought can reduce floral traits, such as

flower size and nectar production, which are important for pollinator visitation (Carroll et al., 2001). However, the compound-specific effects of drought on VOCs contribute to uncertainty surrounding the role of water availability in altering plant chemical traits (Bertin and Staudt 1996, Ormeno et al. 2007, Lavoir et al. 2009, Loreto and Schnitzler 2010, Penuelas and Staudt 2010). Consequently, the effects of water availability and CO_2 enrichment on floral volatiles important for pollinator attraction remain understudied.

The multifaceted nature of climate change makes it increasingly important to consider the combinatorial effects of multiple abiotic factors on floral traits. Experiments evaluating the responses of floral traits to single abiotic factors may misrepresent the implications of climate change for plant-pollinator interactions because of potential non-independent, and combinatorial effects of changes in abiotic conditions on plant traits. Therefore, studying the combinatorial effects of components of climate change on floral traits is important for developing a realistic understanding of the potential impacts of climate change. While there is evidence for non-additive effects of multiple components of climate change on plant-pollinator interactions (Hoover et al. 2012, Burkle and Runyon 2016), no other study has investigated the combinatorial effects of carbon dioxide concentration and water availability on visual and chemical floral traits important for pollinator attraction. To address these important knowledge gaps, we investigated the interactive effect of water availability and CO_2 concentration on floral visual and chemical traits, rates of pollinator visitation, and shifts in

pollinator community composition in three native forbs (*Heterotheca villosa, Phacelia hastata,* and *Campanula rotundifolia*), and one invasive forb (*Potentilla recta*) by subjecting plants to normal or decreased water availability and ambient or increased levels of CO_2 concentration in an experimental, fully-crossed, factorial design. Additionally, we observed the degree to which the effects of drought and CO_2 fertilization resulted in competitive or facilitative effects on pollinator visitation to individual forb species by comparing visitation rates of pollinators to individual forb species within a single-species assemblages and multi-species assemblages.

The goal of this study was to answer three main questions evaluating the effects of climate change on plant-pollinator interactions. Firstly, 1.) what is the effect of drought, CO_2 fertilization, and the interaction of drought and CO_2 fertilization on floral visual traits and VOCs across forb species? Evidence from previous studies indicated drought reduced floral traits important for pollinator attraction likely as a result of reduced plant water status (Carroll, 2001). Therefore, we expected drought to negatively effect floral visual traits. Additionally, VOC emissions have been observed to increase as stress a response to drought (Ormeno et al. 2007, Penulas and Staudt, 2010). Therefore, we anticipate drought to have a positive effect on VOC emissions. Additionally, CO_2 fertilization is anticipated to increase resource use efficiency, and rates of photosynthesis (Ainsworth and Rogers, 2007). Therefore, we expect the positive effects of increasing CO_2 concentration to buffer floral visual traits and VOCs

against the negative effects of drought. Secondly, 2.) what affect will changes in floral visual traits and VOCs have on visiting pollinator communities across forb species? Floral visual traits and VOCs positively affected by CO_2 fertilization and drought will result in increased pollinator visitation rates, while floral visual traits and VOCs negatively affected by drought and CO_2 fertilization will result in decreased pollinator visitation rates (Burkle and Runyon, 2016). Additionally, multi-species plant assemblages can increase competitive, or facilitative plant-pollinator interactions depending on the co-occurring forb species within the assemblage (Ghazoul, 2006, Moeller, 2004, Caruso, 2009). Therefore, we asked 3.) if the patterns of pollinator visitation in response to climate treatment are plant community context dependent by comparing pollinator visitation patterns within climate treatments in a single-species plant assemblage and a multi-species plant assemblage?

Results from this study demonstrate that drought and CO_2 fertilization alter plant visual and chemical traits important for pollinator attraction, but surprisingly, rarely resulted in interactive effects. Drought and CO_2 treatments influenced floral VOCs. In contrast to our hypotheses, neither drought nor CO_2 treatments strongly influenced pollinator visitation rate or pollinator community composition. Pollinator visitation rates decreased to forb species negatively affected by drought in the multi-species assemblage compared to the single-species assemblage demonstrating evidence that drought increases competition for pollinators in a plant community context. Additionally, pollinator visitation

increased to forb species positively affected by CO_2 fertilization in the multi-species assemblage compared to the single-species assemblage demonstrating evidence that CO_2 fertilization increases facilitation of pollination in a plant community context. As a result, the functional responses of plant species to drought or CO_2 fertilization outlines a paradigm for categorizing species of forbs into plant functional types based on the species-specific responses to components of climate change.

Methods

Source and Materials for Plants

Six individuals of each of three species of native forbs (*Campanula rotundifolia* L., harebell; *Heterotheca villosa* (Pursh) Shinners, hairy false goldenaster; and *Phacelia hastata* Douglas ex Lehm., silver leaf phacelia) and one invasive forb (*Potentilla recta* L., sulphur cinquefoil) were grown from seeds collected locally from the Mt. Ellis site (see "pollinator visitation" below). Seeds were planted in the fall and allowed to grow and establish for 5-6 weeks in a greenhouse. Plants were then transplanted into cone-tainers (6.5 cm wide by 25 cm tall) with Sunshine Mix #1 soil (Sun Gro Horticulture, Agawam, MA, USA) with 1-teaspoon (5 ml) of Osmocote fertilizer per pot (The Scotts Company, Marysville, OH, USA). Transplanted plants were vernalized in a climate-controlled chamber at approximately 4°C with a 12 h photoperiod for 100-130 days. Plants were removed from the cold room and returned to the greenhouse

with a day/night temperature regime of 26°C/15°C and a 16 h photoperiod with metal halide lamps providing supplemental light. Plants began flowering within 4-6 weeks of being transferred into the greenhouse.

Drought and CO_2 Treatments

Once each forb species began flowering, we subjected plant individuals to 25 days of experimentally manipulated watering regimes (drought vs. normal watering) and CO_2 concentrations (ambient vs. CO_2 fertilization) in a factorial design within growth chambers (Model: PGC-6L, Percival Scientific, Perry, IA). Treatment combinations were as follows: 1.) normal watering + ambient CO_2 (control), 2.) drought + ambient CO_2, 3.) normal watering + CO_2 fertilization, 4.) drought + CO_2 fertilization (n = 5-6 individual plants of each forb species per treatment). CO_2 concentrations inside plant growth chambers were set to reflect current approximate atmospheric concentrations of CO_2 (400 ppm) for the ambient CO_2 treatment, or the predicted atmospheric concentration for the year 2100 (800 ppm) for the CO_2 fertilization treatment (Pachauri et al. 2014). In order to simulate drought, water was withheld from plants until the first signs of wilting were observed. After wilting, plants were watered until water drained freely through the holes in the bottom of the cone-tainer. Wilting cycles were repeated for 25 days prior to measuring plant traits and pollinator field observations (Huberty and Denno 2004). Pulsed drought treatments are thought to mimic natural conditions better than continuous drought (Huberty and Denno 2004), and plants subjected to continuous drought did not produce flowers (*unpublished*

data). The time it took each species to wilt varied with species: *C. rotundifolia* and *H. villosa* wilted after about 3 days, *P. hastata* wilted in about 2 days, and *P. recta* wilted within 1 day after water was withheld.

Measuring Physical and VOC Plant Traits

Floral traits important for pollinator attraction were measured including plant size, flower size, floral display (e.g., number of open flowers at the time of measurement), and VOC emission rate (see "Volatile collection" below). Plant size, flower size, and VOC emission rate were measured the day before the first day of field observations, while floral display was counted at the beginning of each day of field observations.

Plant size was estimated as plant height (in cm; the distance from the surface of the soil to the tallest part of the plant) for each individual of *C. rotundifolia*, *P. recta*, and *H. villosa*. Plant size was estimated as plant area (in cm^2) for each individual of *P. hastata* since area is a better measure of plant size for this species due to its prostrate, sprawling growth form. Plant area was estimated for *P. hastata* as the widest part of the plant multiplied by the width 90° to the widest part of the plant (length x width).

To estimate flower size, up to five flowers (when available) were randomly measured from each individual using digital calipers (Mitutoyo, Takatsu-ku, Kawasaki, Japan). Petal area was estimated for bell-shaped flowers (*C. rotundifolia* and *P. hastata*) by measuring the flower's width (r) and depth (h) (in mm), and calculating the surface area of a cone ($A = \pi r(r + \sqrt{h^2 + r^2})$). Petal

area was estimated for flat flowers (*H. villosa* and *P. recta*) by measuring the diameter of the flower (*d*) (in mm) and calculating the area of a circle ($A = \pi \frac{1}{2} d^2$). For *H. villosa*, flower heads (capitula) were measured rather than individual flowers.

Floral display was determined for *C. rotundifolia*, and *P. recta* by counting the number of open flowers on each individual at the end of the growth chamber period; for *H. villosa* the number of flower heads were counted. Floral display was estimated for *P. hastata* by multiplying the number of open flowers per branch by the number of branches on the individual at the end of the growth chamber period.

Volatile Collection and Analysis

Volatiles were collected by enclosing flowers in 950 mL, clear polyethylene cups with clear dome lids (Dart Container Corporation, Mason, MI, USA) and by pulling air for 1-hour (0.5 L/min) through volatile traps containing 30 mg of the adsorbent HayeSep-Q (Restek, Bellefonte, Pennsylvania) using portable volatile collection systems (Volatile Assay Systems, Rensselaer, New York) and AirLite air sampling pumps (SKC Inc., Eighty Four, PA). Volatiles were collected once from each of the six replicates of each species/treatment combination prior to field observations. We intended to include only one flower within each volatile collection system per sample to exclude as much green tissue as possible. However, due to the available number and size of flowers, and plant architecture, the number of flowers from which volatiles were sampled

simultaneously per individual varied for *P. hastata* (5-30), and *P. recta* (1-2), but was always one flower or flower head for *H. villosa* and *C. rotundifolia*. Volatile emissions were standardized by total petal area within the cup, and are reported as an emission rate per floral area (ng/mm^2/h).

Volatiles were eluted from traps with 150 µL of dichloromethane, and 500 ng of *n*-nonyl-acetate was added as an internal standard. Samples were analyzed using an Agilent 7890A gas chromatograph (GC) coupled with a 5975C mass spectrometer and separated on a HP-1ms column (30 m × 0.25 mm inside diameter, 0.25 µm film thickness); helium was used as the carrier gas. The GC oven was maintained at 35 °C for 3 min and then increased by 5° C/min to 125 °C, then 25° C/min to 250 °C. Quantifications were made relative to the internal standard using ChemStation software (Agilent Technologies, Wilmington, DE, USA). Identification of compounds were made using NIST 08 Mass Spectral Search Program (National Institute of Standards and Technology, Gaithersburg, MD, USA) and identifications of compounds were confirmed by comparing mass spectra and retention times with commercial standards, when available.

Pollinator Visitation

To investigate how climate change might affect pollinator attraction and community visitation through changes in plant traits, treated plants were transported to a diverse native meadow community five miles southeast of Bozeman, Montana, USA (45.627181, -110.960287) at the base of Mt. Ellis. Typical to this region, plants begin blooming after snowmelt (May-June) and

continue through August. The four focal species included in this study are found in this meadow, and the timing of field observations was near peak-bloom in the field system.

To determine if treatments affected pollinator visitation within species, six individual plants of the same species and treatment combination were grouped into a single-species observation unit (ca. 1m^2 area) and observed for pollinator visitation (N = 16 total observation units). Each observation unit was separated by at least 8 meters at the field site and was observed for 1-hour during peak pollinator activity on each of two separate days (04June2016 and 06June2016), and 0.5-hour on a third day (07June16) (2.5 total observation hours per unit, 40 total person-hours). Pollinator observations were stopped after three days to ensure trends in pollinator visitation reflected the plants response to experimental treatments instead of confounding variables associated with plant damage from transportation or field observations. We hand netted all floral visitors contacting the reproductive parts of flowers. Additionally, we recorded the number of visitors not captured to calculate total visitation rate. Pollinators (including bees and flies) were identified to species or morphospecies for community analysis.

To determine the potential for climate change treatments to have facilitative or competitive effects on pollinator visitation in a plant community context, three individuals of each species of forb subjected to the same treatment combination were grouped to create a multi-species assemblage of 12 individuals (c. 1m^2 area) (N = 8 total assemblages). Each multi-species

assemblage was observed for 1-hour during peak pollinator activity on two separate days (6/4/16 and 6/6/16), and 0.5-hour on a third day (6/7/16) (2.5 total observation hours per assemblage, 20 total person-hours) using methods similar to the single-species observation units as explained above.

Effects of Drought and CO_2 Fertilization on Plant Traits and Pollinator Visitation

Separate two-way Multivariate Analysis of Co-Variance (MANCOVA) tests were performed for each forb species to test for the main and combinatorial effects of carbon dioxide and water treatments on plant visual traits (plant size, flower size, number of flowers), and pollinator visitation rates (visits per flower and visits per plant) within the single-species observation units. Significant MANCOVAs ($\alpha < 0.05$) were followed by two-way Analysis of Variance (ANOVA) tests, to test for the main and combinatorial effects of carbon dioxide and drought on each trait for each forb species. Significant ANOVAs ($\alpha < 0.05$) were followed by a Tukey's honest significant difference (HSD) *post-hoc* test to identify pairwise differences in plant traits between growth treatments.

Effects of Drought and CO_2 Fertilization on Floral VOCs

Separate two-way ANOVA tests for each forb species were performed to test for the combinatorial effects of the drought and CO_2 fertilization treatments on floral VOC emission rates. A permutational analysis of variance (PERMANOVA) was used for each forb species to test for the main and combinatorial effects of carbon dioxide and water treatments on volatile

composition. Significant PERMANOVAs ($\alpha < 0.05$) were followed by a similarity-percentage test (SIMPER) to identify specific volatile compounds that contributed the most to differences in floral VOC between treatments. For each forb species, we evaluated differences among treatments in floral VOC variability using homogeneity of dispersions tests ('betadisper' function in the vegan package), followed by ANOVAs to compare the mean distance-to-centroid of floral VOCs among treatments for each forb species.

Using Standardized Mean Effect Sizes to Directly Compare Changes in Pollinator Visitation Rates with Plant Traits in Different Plant Community Contexts

The strength and direction of changes in pollinator visitation rates in response to changes in plant traits was estimated by calculating mean effect sizes to compare the magnitude of the effect of climate treatments on plant traits to pollinator visitation rates. Standardized mean effect sizes (Hedges *g* to account for small sample sizes) with 95% confidence intervals for each response variable (i.e., plant size, floral display, flower size, VOC emission rate, visitation per plant in single-species assemblages, visitation per flower in single-species assemblages, visitation per plant in multi-species assemblages, and visitation per flower in multi-species assemblages) were calculated by contrasting a traits' mean value in treatment groups to control groups (normal watering + ambient) and dividing by a pooled standard deviation using bias-corrected bootstrapping. Significant differences are indicated by 95% confidence intervals that do not overlap zero. Pollinator visitation was thought to be linked to changes in floral

cues if pollinator visitation rates shifted by a similar magnitude as floral traits within the same forb species and climate change treatment.

The ability of climate change to influence pollinator visitation in different plant community contexts was evaluated by estimating the strength of shifts in pollinator visitation to focal plant species in the single- and multi-species forb assemblages within the same climate change treatment. Evidence of competition for pollinators was indicated by a reduction in pollinator visitation rates to the same forb species in the same climate treatments in the multi-species assemblage than the single-species assemblage. Conversely, evidence for facilitation of pollination was indicated by an increase in pollinator visitation rates to the same forb species in the same climate treatments in the multi-species assemblage than the single-species assemblage.

Combinatorial Effects of Drought and CO_2 Fertilization on Visiting Pollinator Community Composition in Different Plant Community Contexts

To test for the ability of drought and CO_2 fertilization to shift visiting pollinator communities in different plant community contexts we used a PERMANOVA ('adonis' function in the 'R' package Vegan) to evaluate if pollinator communities visiting observational units subjected to treatment combinations were different depending on the plant community context. Therefore, we included water availability, CO_2 concentration, and forb assemblage as main and interactive effects in a PERMANOVA model. Additionally, to evaluate differences in pollinator community variability among

treatments across forb species, we compared the mean distance-to-centroid of pollinator communities between treatments for each forb species using separate homogeneity of variances tests (‘betadisper’ function in the ‘R’ package Vegan) followed by ANOVAs. Significant ANOVAs ($\alpha < 0.05$) were followed by a Tukey’s honest significant difference (HSD) *post-hoc* tests to identify pairwise differences in pollinator community dispersion between forb species.

Statistical Software Used

All analyses were performed in the statistical software, ‘R’ (Version 3.1.2: Smooth Sidewalk) (Team 2014). Multivariate analyses, and NMDS plots were created using the Vegan package (Oksanen et al. 2007). Effect sizes were calculated using the bootES package in ‘R’ (Kirby and Gerlanc 2013).

Results

Overall, floral visual traits were negatively affected by drought and positively affected by CO_2 fertilization, while floral chemical traits were positively affected by both drought and CO_2 fertilization. However these effects were forb species-specific (summarized in Figure 1).

	Plant traits			Floral VOCs			Pollinator attraction	
	Plant height	Flower size	Floral display	Total VOCs per flower area	VOC composition	VOC dispersion	Pollinator visits per flower	Pollinator visits per plant
Campanula rotundifolia	↓	—	—	↑ drought ↓ CO2	*	—	—	—
Heterotheca villosa	—	↓ drought ↑ CO2	—	—	—	—	—	—
Phacelia hastata	↑	↓	↑	↑	*	—	—	—
Potentilla recta	↑	↓	↓	↑	*	—	—	↓

change driven by drought; change driven by CO2 enrichment; ↑ increased; ↓ decreased; — no effect

Figure 1: Summarized effects of carbon dioxide enrichment and drought on plant traits and pollinator visitation. The treatment effects of carbon dioxide enrichment (green) and drought (brown) on plant traits and pollinator visitation of *Campanula rotundifolia*, *Heterotheca villosa*, *Phacelia hastata*, and *Potentilla recta* are indicated by the direction of arrows and asterisks. Boxes with both brown and green indicate a combinatorial effect of carbon dioxide and drought. Arrows pointing up designate responses (top row) increased within species (vertical row), and arrows pointing down designate responses decreased within species. Asterisks designate changes in composition.

Campanula rotundifolia

Contrary to our hypothesis, floral visual traits were not affected by drought or CO_2 fertilization (whole model MANOVA, $F_{9,54}$ = 1.63, P = 0.13). Plants subjected to drought emitted 98.4% more volatiles per flower area than plants in the watered control treatments (Table 1, Figure 2M). Pollinator visitation rates were not different between treatment and control groups in single-species (Table 2), or multi-species (Table 3) plant assemblages.

There were main and combinatorial effects of drought and CO_2 fertilization on VOC composition (Table 2). Specifically, drought-treated plants (Figure 3b, PERMANOVA, $F_{1,21}$ = 3.37, P = 0.009), CO_2 fertilized plants (Figure 3a, PERMANOVA, $F_{1,21}$ = 3.43, P = 0.013), and plants in the combination treatment (i.e., drought + CO_2 fertilization) (PERMANOVA, $F_{1,21}$ = 8.49 P = 0.001) emitted different compositions of floral VOCs than control plants. Monoterpenes hypothesized to be important for pollinator foraging ability primarily accounted for the most dissimilarity in floral VOCs between treatments. For example, 6-methyl-5-hepten-2-one, β-pinene, and benzaldehyde contributed the most towards the dissimilarity in floral VOCs and were emitted at 49.6%, 147.1%, and 96.2% greater rates, respectively, from drought-treated plants compared to watered controls. Similarly, 6-methyl-5-hepten-2-one, β-pinene, and β-myrcene contributed the most towards the dissimilarity of floral VOCs between CO_2 treatments and were emitted at 71.8%, 254.9%, and 23.8% greater rates,

respectively, from plants grown under ambient CO_2 concentration than plants in the CO_2 fertilization treatment.

Pollinator visitation rates were not affected by climate treatments in a single- or multi-species assemblage, which was surprising based on the effects of drought and CO_2 fertilization on floral visual and chemical traits. Pollinator visitation rates did not shift in response to the negative effects of drought on plant visual traits, or the positive effects of drought or of the combination treatment on plant chemical traits compared to the control treatment. (Figure 4a).

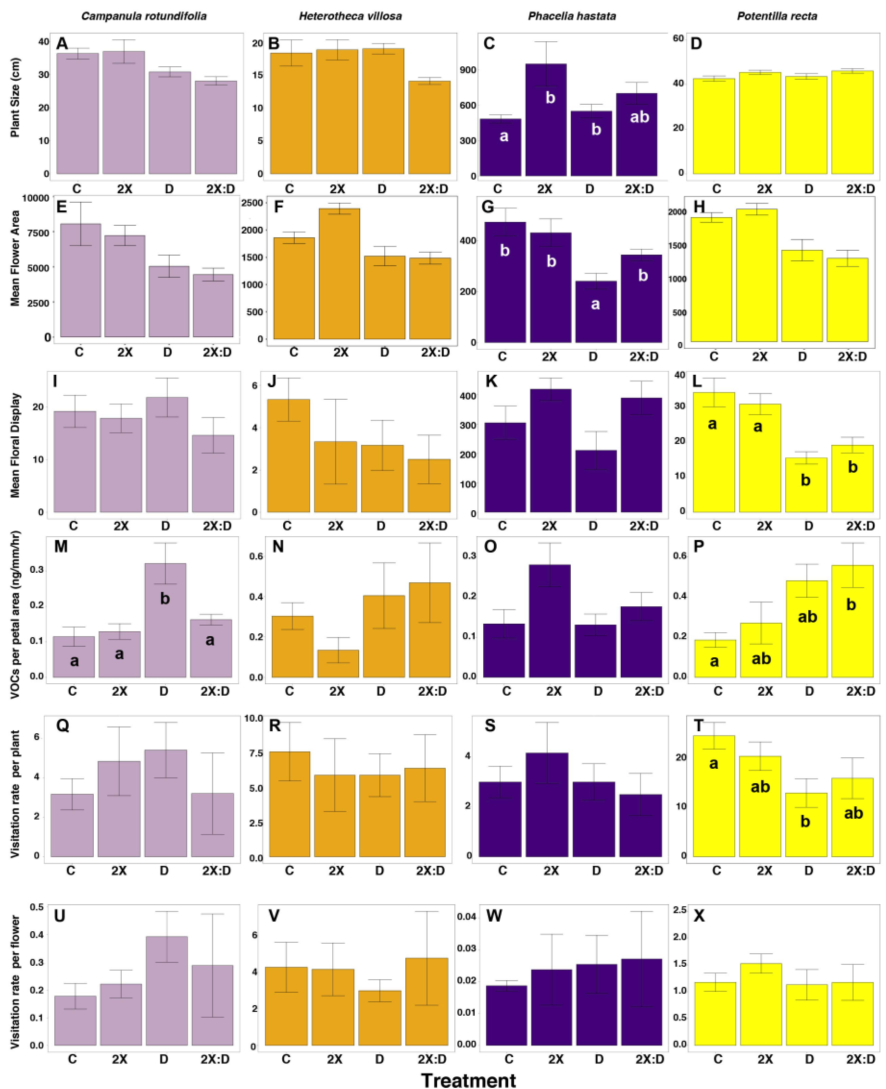

Figure 2: Mean values of floral traits and pollinator visitation rates by climate treatment. The height of bars represent the mean (±1 SE) value of plant visual traits (panels A-L), chemical traits (panels M-P), and pollinator visitation rates (panels Q-X) in response to climate treatments for *Campanula rotundifolia* (light purple), *Heterotheca villosa* (orange), *Phacelia hastata* (deep purple), and *Potentilla recta* (yellow). Lowercase letters indicate significant differences in means between treatments using Tukey HSD tests following a two-way ANOVA ($P < 0.05$). C = normal water : ambient CO_2, 2X = normal water : CO_2 fertilization, D = drought : normal CO_2, 2X:D = drought : CO_2 fertilization.

Table 1: Two-way ANOVA results testing for differences in mean floral traits (plant size, flower size, floral display) in response to the main and combinatorial effects of water availability and carbon dioxide treatments (CO_2) for each of the four forb species. Bolded text indicates significant differences in means following a significant whole model MANOVA (α < 0.05).

	Plant Size*			Flower Size#			Floral Display		
Source	df	F	*P*	df	*F*	*P*	df	*F*	*P*
Campanula rotundifolia									
whole model	3,18	3.35	**0.04**	3,18	2.79	0.07	3,18	0.80	0.57
drought	1,18	9.37	**0.01**	1,18	7.87	0.01	1,18	0.01	0.93
CO_2	1,18	0.15	0.70	1,18	0.49	0.49	1,18	1.57	0.23
drought x CO_2	1,18	0.51	0.48	1,18	0.01	0.91	1,18	0.84	0.37
Heterotheca villosa									
whole model	3,20	3.00	**0.05**	3,16	7.32	**<0.001**	3,20	0.76	0.53
drought	1,20	2.30	0.14	1,16	5.34	**0.04**	1,20	1.15	0.30
CO_2	1,20	2.70	0.12	1,16	13.97	**<0.001**	1,20	0.91	0.35
drought x CO_2	1,20	4.01	0.06	1,16	2.69	0.12	1,20	0.23	0.64
Phacelia hastata									
whole model	3,20	3.51	**0.03**	3,20	5.71	**0.01**	3,20	2.91	0.06
drought	1,20	0.68	0.42	1,20	13.74	**<0.001**	1,20	1.28	0.27
CO_2	1,20	7.79	**0.01**	1,21	0.52	0.48	1,20	7.11	0.01
drought x CO_2	1,20	2.04	0.17	1,22	2.87	0.11	1,20	0.34	0.57
Potentilla recta									
whole model	3,20	1.97	0.15	3,20	9.06	**<0.001**	3,20	9.43	**<0.001**
drought	1,20	0.49	0.49	1,20	26.25	**<0.001**	1,20	26.85	**<0.001**
CO_2	1,20	5.40	0.03	1,20	0.00	0.95	1,20	0.00	0.96
drought x CO_2	1,20	0.02	0.89	1,20	0.93	0.35	1,20	1.43	0.25

* Plant size was measured as height for all forbs except *P. hastata* where area was measured

#Flower size was estimated using the equation for area of a circle for *P. recta* and *H. villosa*, and area of a cone for *P. hastata.*

Heterotheca villosa

As expected, floral visual traits were affected by drought and CO_2 treatments (whole model MANOVA, $F_{9,48} = 2.60$, $P = 0.020$). Specifically, drought-treated plants produced flowers that were 18.31% smaller compared to well-watered control plants (Table 1, Figure 2F). Plants in the CO_2 fertilization

treatment produced flowers that were 10.1% larger (Table 1, Figure 2F). We did not detect any combinatorial effects of drought and CO_2 on visual traits (Table 1). Pollinator visitation rates were not different between treatment and control groups in single-species (Table 2), or multi-species (Table 3) plant assemblages.

Contrary to our hypothesis, VOCs emission rate and floral VOCs composition were not affected by the main or combinatorial effects of drought or CO_2 treatments (Table 2).

Pollinator visitation rates were affected by climate treatments, but only in a more diverse plant community context. Pollinator visitation rate per plant in drought treated plants decreased by 240% in the multi-species treatment when compared to the single-species treatment, potentially indicating that drought increases competition for pollinators in *H. villosa* in a plant community context (Figure 4b).

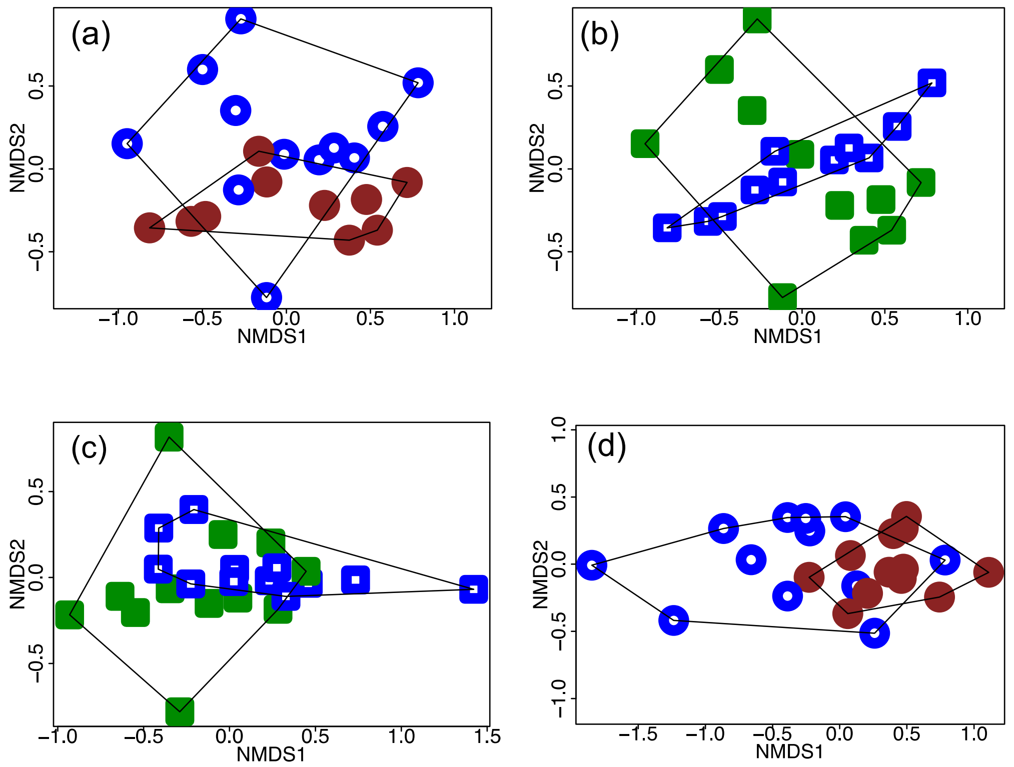

Figure 3: Treatment effects on floral volatile composition**.** Drought (red circles) affected the composition of floral VOCs of (a) *C. rotundifolia* and (d) *P. recta* compared to watered controls (blue circles). CO_2 fertilization (green squares) affected floral VOCs of (b) *Campanula rotundifolia*, and (c) *Phacelia hastata* when compared to ambient CO_2 (blue squares).

Table 2: Two-way ANOVA results testing for differences in mean floral chemical traits (volatile emission rates ($ng/mm^2/hr$)) and pollinator visitation rates within single-species assemblages (visits/plant and visits/flower) in response to the main and combinatorial effects of drought and CO_2 treatments for each of the four forb species. Bolded text indicates significant differences in means following a significant whole model MANOVA ($\alpha < 0.05$).

	Total Volatiles[%] ($ng/mm^2/hr$)			Per-Plant Visitation Rate			Per-Flower Visitation Rate		
Source	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Campanula rotundifolia									
whole model	3,18	5.04	**0.01**	3,18	0.54	0.66	3,18	0.38	0.77
drought	1,18	6.56	**0.02**	1,18	0.04	0.85	1,18	0.73	0.41
CO_2	1,18	1.19	0.29	1,18	0.00	0.95	1,18	0.00	0.93
drought x CO_2	1,18	7.35	**0.01**	1,18	1.57	0.23	1,18	0.39	0.54
Heterotheca villosa									
whole model	3,17	1.36	0.29	3,20	0.13	0.94	3,19	0.56	0.65
drought	1,17	2.72	0.11	1,20	0.07	0.79	1,19	1.05	0.32
CO_2	1,17	0.58	0.46	1,20	0.07	0.79	1,19	0.00	0.97
drought x CO_2	1,17	0.80	0.38	1,20	0.24	0.63	1,19	0.64	0.44
Phacelia hastata									
whole model	3,20	3.19	**0.05**	3,20	0.64	0.60	3,20	0.18	0.91
drought	1,20	1.85	0.19	1,20	0.88	0.36	1,20	0.00	0.94
CO_2	1,20	6.02	**0.02**	1,20	0.14	0.71	1,20	0.01	0.92
drought x CO_2	1,20	1.68	0.21	1,20	0.88	0.39	1,20	0.52	0.48
Potentilla recta									
whole model	3,20	3.89	**0.02**	3,20	2.51	0.09	3,20	0.51	0.68
drought	1,20	10.84	**<0.001**	1,20	6.27	**0.02**	1,20	1.27	0.27
CO_2	1,20	0.84	0.37	1,20	0.22	0.86	1,20	0.06	0.62
drought x CO_2	1,20	0.00	0.97	1,20	1.85	0.28	1,20	0.02	0.90

[%] Standardized by flower petal area

Phacelia hastata

As expected, floral visual traits were affected by drought and CO_2 treatments (whole model MANOVA, $F_{9,60} = 3.19$, $P = 0.003$). Consistent with our hypothesis, drought-treated plants produced flowers with 35.3% smaller volumes

than plants in the control treatment (Table 1, Figure 2G). CO_2 fertilized plants grew 37.4% larger (Table 1, Figure 2G) and produced 33.0% more flowers (Table 1, figure 2K) than plants in the control treatment. However, we did not detect any combinatorial effects of drought and CO_2 treatments on visual traits (Table 1). Within the single-species treatment, pollinator visitation rates were not different between treatment and control groups (Table 2). Within the multi-species assemblage, pollinator visitation per plant was 4.6% greater to plants in the combination treatment than controls (Table 3).

CO_2 fertilized plants emitted 42.1% more total volatiles (Table 1, Figure 2O) compared to plants in the control treatment. CO_2 fertilization affected the composition of floral VOCs (Figure 3c, PERMANOVA: $F_{1,23}$ =2.47, P = 0.030). However, neither drought (PERMANOVA: $F_{1,23}$ = 1.33, P = 0.210), nor the combination of drought and CO_2 enrichment affected floral VOCs (PERMANOVA: $F_{1,23}$ = 0.90, P = 0.450). A combination of monoterpenes and GLVs (green leaf volatiles) primarily accounted for differences in floral VOCs between CO_2 treatments. Specifically, linalool, limonene, and (*Z*)-3-hexenyl-acetate contributed the most towards the dissimilarity in floral VOCs and were emitted at 75.1%, 271.8%, and 42.0% greater rates in the CO_2 fertilized plants than the ambient CO_2 plants, respectively.

Pollinator visitation rates were affected by climate change treatments as expected, however only in the multi-species assemblage. Pollinator visitation rates per plant increased in the multi-species assemblage by a similar magnitude

as plant size and VOC emissions in CO_2 fertilized plants (Figure 4c). Pollinator visitation rate per plant was 63.6% greater to CO_2 fertilized plants in the multi-species assemblage than pollinator visitation rates per plant to CO_2 fertilized plants in the single-species assemblage (Figure 4c). Additionally, pollinator visitation rates increased to drought-treated plants in the multi-species assemblage despite decreased flower volume (Figure 4c). These results suggest that pollinator visitation to *P. hastata* will increase to CO_2 fertilized plants in a multi-species context, potentially indicating facilitation of pollination to *P. hastata* in a plant community context.

Potentilla recta

As expected, floral traits were affected by drought and CO_2 treatments (whole model MANOVA, $F_{9,60}$ = 3.78, P = 0.005). Consistent with our hypothesis, plants receiving the drought treatment produced 46.7% fewer flowers (Table 1, Figure 2L) and 17.9% smaller flowers (Table 1, Figure 2H) compared to control plants. However, we did not find evidence for a combinatorial effect of drought and CO_2 on visual plant traits (Table 1). Within the single-species assemblage, pollinator visitation per plant was 32.6% lower to drought-treated *P. recta* plants compared to control plants (Table 2). Within the multi-species assemblage, pollinator visitation per plant was 60.2% lower to drought-treated plants compared to well-watered controls (Table 3).

In-line with expectations, drought positively affected floral chemical traits. Plants subjected to drought emitted 129% more volatiles per flower area than

plants in the watered control treatments (Table 1, Figure 2P). Drought-treated plants emitted a different relative composition of floral VOCs compared to well-watered control plants (figure 3d, PERMANOVA, $F_{1,23}$ = 6.45, P = 0.001). Two green leaf volatiles and an irregular terpene contributed the most towards the dissimilarity of floral VOCs between drought treatments. (*Z*)-3-hexenyl acetate, 6-methyl-5-hepten-2-one, and (*Z*)-3-hexenol were emitted at 106%, 29.8%, and 61.1% greater rates, respectively, in drought treated plants compared to control plants. We did not find evidence for a combinatorial effect of drought and CO_2 on chemical traits (Table 1).

Pollinator visitation rates were affected by climate treatments, but only in a more diverse plant community context. Pollinator visitation rates per plant decreased to drought-treated plants by a similar magnitude as reductions in flower area and floral display within both the single- and multi-species assemblages (Figure 4d). However, the negative effects of drought reduced pollinator visitation rates per plant by 35%-40% more than the positive effect of drought on VOC emissions (Figure 4d). Reductions in both pollinator visitation and plant visual traits were of similar magnitude, indicating that pollinator visitation may be more strongly linked to changes in visual traits than chemical traits for *P. recta*. Additionally, in drought treated plants, pollinator visitation per plant decreased by 17.9% more in the multi-species assemblage compared to the single-species assemblage, suggesting that drought increases competition for pollinators within a more diverse plant community context.

Pollinator visitation rates per plant decreased in the combination treatment but by a lesser degree than plants treated with the main effect of drought (Figure 4d). Pollinator visitation rates to plants in the combination treatment decreased by 22.3% and 17.3% less than pollinator visitation rates to plants affected by the main effects of drought in the single- and multi-species assemblages, respectively (Figure 4d), indicating that while drought is still the main driver of effects on pollinator visitation rates, CO_2 may partially buffer these negative effects.

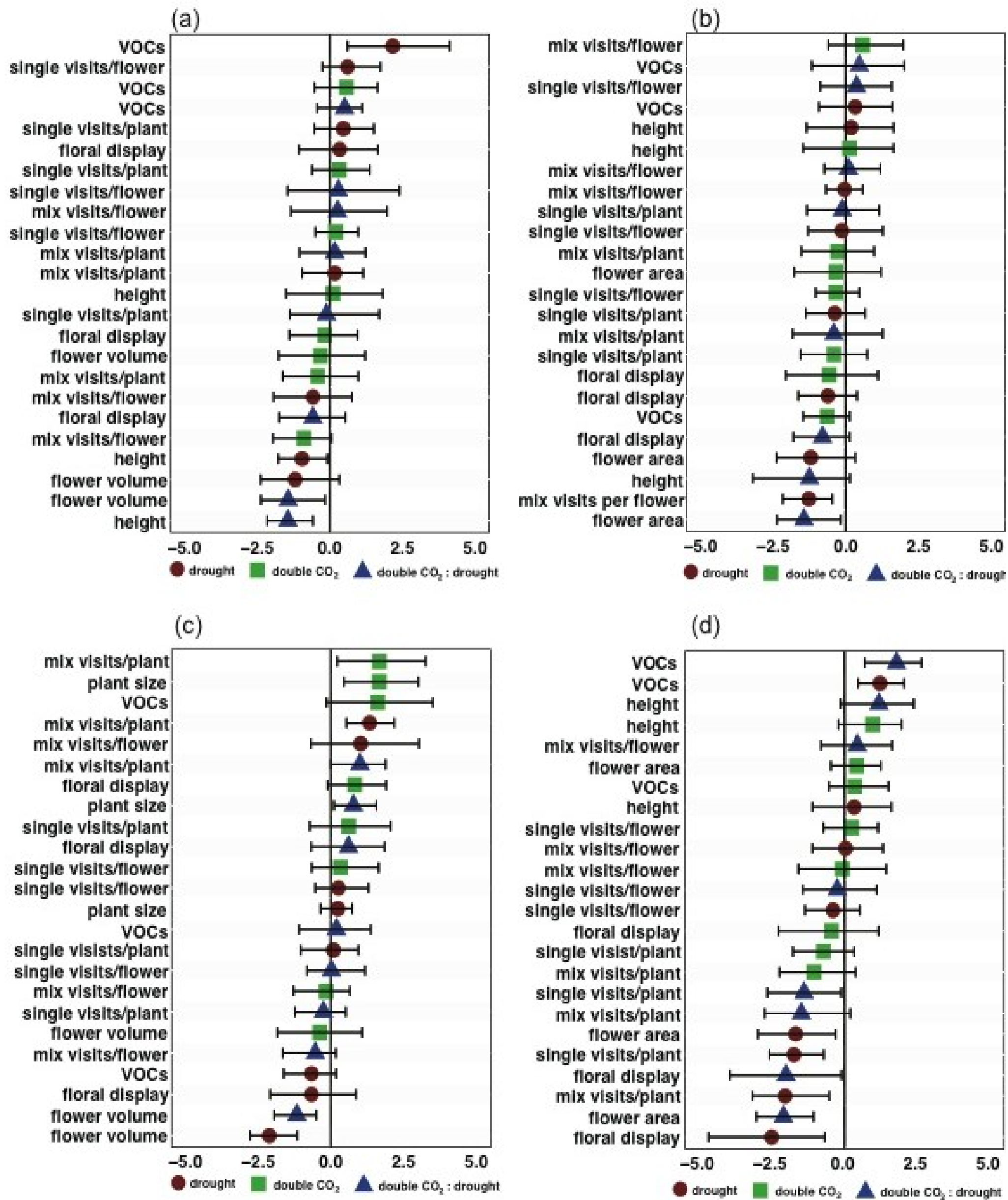

Figure 4: Standardized effect sizes of all response variables. Mean effect sizes (Hedges *g*) of plant and pollinator responses in drought (brown, circle), CO_2 fertilization (green square), and drought + CO2 fertilization (blue, triangle) treatments, relative to the control treatment (normal watering + ambient CO_2) for a) *C. rotundifolia*, b) *H. villosa*, c) *P. hastata*, and d) *P. recta*. Floral display, flower area, and plant size were measured as visual traits, while total VOC emissions were measured as chemical traits. Pollinator visitation rates were measured per-flower and per-plant in both the single- and multi-species plant assemblages. Mean effect sizes are sorted from most negative to most positive and are surrounded by bias-corrected 95% confidence intervals. Differences were significant when 95% confidence intervals did not overlap zero.

Pollinator Communities

Thirty-six species of pollinators (30 bees, six flies) totaling 408 specimens (95.3% bees, 4.7% flies) were collected from single-species observation units (Supporting Table S5). Within the single-species assemblage, there were no effects of drought (PERMANOVA, $F_{1,15}$ = 0.31, P = 0.950), CO_2 fertilization (PERMANOVA, $F_{1,15}$ = 0.42, P = 0.910), or the combination between drought and CO_2 fertilization (PERMANOVA, $F_{1,15}$ = 1.12, P = 0.390) on the composition of visiting pollinators across all forb species. However, the dispersion of pollinator communities visiting observation units across treatments within individual forb species was different among forb species ('betadisper', ANOVA, $F_{3,12}$ = 5.70, P = 0.012). Specifically, pollinator communities visiting *P. recta* observation units were less variable than pollinator communities visiting *P. hastata* observation units (Tukey HSD, 95% CI = -0.22 to -0.39) (Figure 5), indicating that pollinator communities visiting *P. recta* were more consistent across treatments than pollinator communities visiting *P. hastata.*

We sampled 35 species of pollinators (31 bees, 4 flies) in the multi-species assemblages, totaling 418 specimens (96.9% bees, 3.1% flies) (Supporting Table S6). Within the multi-species assemblage, there were no effects of drought (PERMANOVA, $F_{1,15}$ = 0.46, P = 0.870), CO_2 fertilization (PERMANOVA, $F_{1,15}$ = 0.45, P = 0.900), or the combination of drought and CO_2 fertilization (PERMANOVA, $F_{1,15}$ = 0.67, P = 0.710) on the composition of visiting pollinators across all forb species. Additionally, there were no differences in the

dispersion of pollinator communities visiting observation units across treatments among forb species. (ANOVA, $F_{3,12}$ = 0.061, P = 0.980).

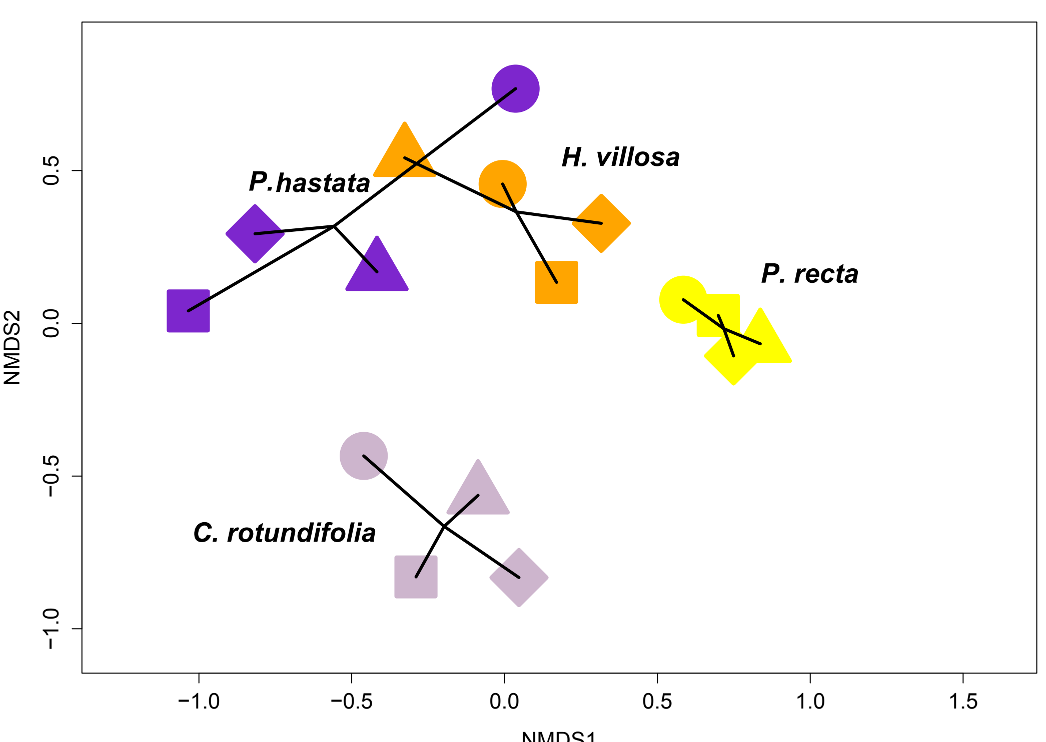

Figure 5: Visiting pollinator community composition varied among the four forb species (light purple = *Campanula rotundifolia,* orange = *Heterotheca villosa,* dark purple = *Phacelia hastata,* and yellow = *Potentilla recta*). Treatments did not significantly influence the visiting pollinator communities (control = diamond, drought = circle, CO_2 fertilization = triangle, CO_2 fertilization + drought = square). Pollinator communities were more variable between treatment combinations visiting *P. hastata* when compared to *P. recta*.

Table 3: Two-way ANOVA results testing for differences in mean pollinator visitation rates within multi-species assemblages (visits/plant and visits/flower) in response to the main and combinatorial effects of drought and CO_2 fertilization for each of the four forb species. Bolded text indicates significant differences in means following a significant whole model MANOVA (α < 0.05).

	Per-Plant Visitation Rate			Per-Flower Visitation Rate		
Source	*df*	*F*	*P*	df	*F*	*P*
Potentilla recta						
Whole model	3,20	4.84	**0.011**	3,20	0.34	0.80
Drought	1,20	9.91	**0.005**	1,20	0.49	0.49
CO2	1,20	0.38	0.54	1,20	0.18	0.68
Drought x CO2	1,20	4.22	0.053	1,20	0.37	0.55
Campanula rotundifolia						
Whole model	3,18	0.47	0.71	3,20	1.69	0.21
Drought	1,18	0.84	0.37	1,20	0.49	0.49
CO2	1,18	0.31	0.58	1,20	0.18	0.68
Drought x CO2	1,18	0.26	0.62	1,20	0.37	0.55
Heterotheca villosa						
Whole model	3,20	1.94	0.16	3,20	0.53	0.66
Drought	1,20	3.16	0.09	1,20	0.44	0.52
CO2	1,20	0.58	0.45	1,20	0.84	0.37
Drought x CO2	1,20	2.07	0.17	1,20	0.32	0.57
Phacelia hastata						
Whole model	3,20	3.33	**0.04**	3,20	2.88	0.06
Drought	1,20	0.71	0.41	1,20	0.67	0.42
CO2	1,20	2.86	0.11	1,20	4.8	0.04
Drought x CO2	1,20	6.43	**0.02**	1,20	3.14	0.09

Discussion

Our findings provide evidence that components of climate change can lead to shifts in plant-pollinator interactions as a result of altered floral traits in plant-pollinator communities. Drought and CO_2 fertilization influenced floral traits important for pollinator visitation in all four forb species tested, although the responses of floral traits were species-specific. Generally, across forb species,

drought reduced floral visual traits and increased floral VOC emissions. Additionally, CO_2 fertilization increased floral visual traits and had mixed effects on floral VOCs. Moreover, in two forb species, floral visual traits and VOCs were both consistently affected by the same climate treatments (i.e., drought or CO_2 fertilization), suggesting that the response of a plant species to changes in abiotic conditions can be used as a criteria for classifying plant species into plant functional types (Lavorel et al. 1997). Despite frequent main effects of drought and CO_2 fertilization on floral traits, the combination of drought and CO_2 was only observed to affect VOC composition in one forb species (*C. rotundifolia*). Furthermore, pollinator visitation rates in the multi-species assemblage decreased to drought-treated plants for two forb species relative to the single-species assemblage, while pollinator visitation rates in the multi-species assemblage increased to CO_2-fertilized plants for one forb species relative to the single-species assemblage, indicating the effects of components of climate change can increase competition for pollinators or facilitate pollination depending on the plant community context.

Drought affected floral visual traits and VOCs more consistently than CO_2 fertilization across forb species, suggesting that water limitation, as opposed to CO_2 fertilization will predominately affect floral traits. Drought decreased the values of floral visual traits important for pollinator attraction. Flower size appeared to be the most susceptible trait to the effects of drought, given that flower size was reduced by drought in three of the four forb species tested in this

study. Responses of other floral traits to drought were not consistent across forb species. For example, drought-treated plants were shorter than control plants but only in *C. rotundifolia*, and drought-treated plants produced fewer flowers than control plants but only in *P. recta*. Given that floral visual traits are a strong determinant of pollinator attraction (Nilsson 1988, Aspi et al. 2003, Hegland and Totland 2005, Hegland et al. 2009), it was unsurprising that pollinator visitation rates were reduced by a similar magnitude as reductions in the values of flower size and floral display for *P. recta*. Therefore, drought-tolerant plants that maintain consistent floral displays despite reduced water availability may be more capable than non-drought tolerant plants at attracting pollinators in future climate scenarios.

Despite the widespread, negative effects of drought on floral visual traits, the effects of drought on pollinator visitation were weak across forb species. This could partially be explained by the increased emissions of floral VOCs in drought-treated plants. For example, floral VOC emissions increased in drought-treated *C. rotundifolia*, and *P. recta* plants. Increases in VOC emissions in response to drought was somewhat expected, because plants have been observed to increase VOC emissions as a defense response against herbivores, pathogens, and environmental stress such as drought (Yuan et al. 2009). Increases in olfactory cues used in plant-pollinator communication could have offset the potential responses of pollinators to reductions in floral visual traits. This observation, along with the predicted decrease in water availability throughout

the Western US, could indicate that plants and pollinators will increasingly rely on olfactory cues for plant-pollinator communication in the future.

Novel blends of compounds are formed via biosynthetic pathway specific and forb species-specific responses to abiotic conditions. Additionally, the functional role of the compounds that contribute to changes in VOC blends can serve different purposes in pollinator attraction. For instance, monoterpenes, which are typically associated with increased pollinator fidelity to flowers, accounted for the greatest change in VOC composition in *C. rotundifolia* (Schiestl and Roubik 2003, Theis 2006, Byers et al. 2014). Given the likely role of monoterpenes in pollinator attraction, increased monoterpene emissions in *C. rotundifolia* in response to drought may have stabilized pollinator visitation despite the negative impact of drought on floral visual traits. By contrast, GLVs accounted for the most change in VOC composition in *P. recta* and are associated with defensive functions important for deterring herbivores, attracting predators of herbivores, and attracting ovipositing insects (Dicke and van Loon 2000, De Moraes et al. 2001, Kessler and Baldwin 2001). While little is known about the role of GLVs in pollinator attraction, obligate pollinators have still been observed to visit flowers despite the emissions of compounds known to be repellent to herbivores (Junker and Bluthgen 2010). If this is the case, GLVs could serve multiple beneficial functions by increasing attraction of beneficial pollinators while filtering ineffective pollinators, and further contributing to the

specialization and partitioning of pollinator visitors to plants (Junker et al. 2015, Junker and Bluthingen 2010).

Based on the traits measured in this experiment, the mechanisms behind the inconsistent effect of CO_2 fertilization on floral traits across species remains unclear. For instance, floral traits were positively affected by CO_2 fertilization in *P. hastata*, while floral traits responded variably to CO_2 fertilization and were primarily affected by drought across other forb species. The positive effects of CO_2 fertilization on *P. hastata* floral traits could indicate CO_2 is a resource more limited than water, soil, or light for *P. hastata*. For instance, plants often experience the positive effects of elevated $CO_{2,}$ which can support a variety of plant functions, when other resources such as light, water, and soil nutrients are not in limiting quantities (Korner C, 2006). As a result, without a clear understanding of the nutrient demands of the plant, it is unclear if extra carbon uptake will support reproductive, structural, defensive, or non-reproductive functions. (Korner 2006). This was demonstrated in a meta-analysis measuring the effects of increases in CO_2 concentration on traits in non-crop plant species, which indicated that reproductive allocation (i.e., floral display and seed/fruit set) decreased relative to increases in structural allocation (i.e., total plant mass) (Jablonski et al. 2002), suggesting that plant traits other than those involved with pollinator attraction can be positively affected by elevated CO_2 concentration.

Little is known about the effects of increased CO_2 concentration on floral VOCs. Studies primarily investigating the effects of CO_2 fertilization on VOC

production and composition have largely focused on vegetative or leaf volatiles in wind-pollinated plants. To our knowledge, no other studies have investigated the effects of CO_2 concentration on floral VOC emissions. Total floral VOC emissions decreased in CO_2 fertilized *C. rotundifolia* plants compared to controls, which is consistent with other studies of evergreen oak (*Quercus ilex*) that demonstrated decreased terpene synthase activity as a likely mechanism (Loreto et al. 2001, Scholefield et al. 2004). Conversely, total floral VOC emissions increased in *P. hastata* plants treated with CO_2 fertilization compared to controls. While the mechanisms behind changes in floral VOC emissions in response to CO_2 fertilization remain unknown, other studies cited increased leaf area as a contributory factor to increased VOCs (Penuelas and Llusia 1997, Constable et al. 1999), potentially indicating VOC emissions are linked with processes influencing plant growth. Therefore, it is important to study the specific mechanisms by which CO_2 fertilization affects floral VOCs to predict the potential shifts of chemical signals affecting pollinator foraging ability in response to climate change.

There are several non-mutually exclusive explanations for observing weak combinatorial effects between drought and CO_2 fertilization on floral traits. First, CO_2 fertilization can increase plant water use efficiency and potentially buffer plants against the effects of drought. Plants grown under increased CO_2 concentrations have been observed to maintain normal photosynthetic capacity in water-limited environments through reductions in stomatal conductance

leading to increased use efficiency of water (Ainsworth and Rogers 2007), light (Korner C, 2006) and soil nitrogen (Korner C, 2006). Secondly, CO_2 fertilization and drought could have had combinatorial effects on other plant traits not measured in this experiment, such as belowground biomass or nonstructural carbohydrates in leaf tissues (Korner 2006). Thirdly, the relatively short-term exposure of plants to elevated CO_2 conditions in growth chambers could have restricted the ability of CO_2 fertilization treatments to affect plants. For example, photosynthetic capacity within plants could have acclimated to elevated CO_2 concentrations quickly, and inconsistencies in treatment applications as a result of growth chamber microclimates could have down-regulated carbon gain in plants and restricted the ability of CO_2 fertilization treatments to affect plants (Ainsworth and Rogers 2007, Leakey et al. 2009). Future experiments may consider measuring a suite of plant functional traits using long-term field experiments to increase our understanding of the combinatorial effects of drought and CO_2 fertilization on plants.

Climate change treatments did not result in shifts in pollinator community composition across forb species regardless of the influence of drought and CO_2 fertilization on floral visual traits and floral VOCs. This is contrary to a previous study using the same forb species in the same meadow system, which observed that visiting pollinator community shifts were driven primarily by drought (Burkle and Runyon 2016). The main difference between these two studies was the use of growth chambers to administer treatments to plants in the current study, as

opposed to the use of a greenhouse as in (Burkle and Runyon 2016). Growth chamber effects, such as lower light intensity and/or more humid microclimates, could have retained soil moisture, resulting in less frequent and less severe drought pulses in plants grown in growth chambers compared to plants grown in a greenhouse (Ainsworth and Rogers 2007, Leakey et al. 2009). Therefore, it is possible that the current study did not replicate the severity of drought required to produce shifts in pollinator communities like those observed in (Burkle and Runyon, 2016). Additionally, inter- and intra-annual variation in pollinator communities (Price et al. 2005, Olesen et al. 2008, Petanidou et al. 2008) could have contributed to inconsistent pollinator communities sampled between the two studies. Therefore, studies in which observations of pollinator visitation to manipulated plants that span the duration of the flowering season for multiple years may help clarify this problem.

While neither drought or CO_2 fertilization affected pollinator communities across forb species, the species-specific responses of forbs to climate change treatments have the potential to shift visiting pollinator communities within individual forb species. For example, pollinator communities visiting *P. recta* observation units were consistent regardless of the treatment combination they received. The consistency of pollinator communities to *P. recta* observation units was surprising because drought altered floral visual traits and VOCs in *P. recta* likely important for pollinator visitation. These unexpected results could imply that *P. recta* is robust to the effects of climate change because it attracts nearly

identical suites of pollinators regardless of the effects of drought on floral traits. Conversely, pollinator communities visiting *P. hastata* observation units were variable across treatment combinations. This result was expected because CO_2 fertilization altered floral visual traits and VOCs in *P. hastata* likely important for pollinator visitation. Although CO_2 fertilization altered floral traits and pollinator communities of *P. hastata*, it remains unclear how the generally positive effect of CO_2 fertilization on *P. hastata* floral traits will influence pollination (Biesmeijer et al. 2006). Future studies should aim to measure pollination and reproduction from forbs visited by different pollinator communities to determine if treated plants attract a functionally equivalent pollinator community compared to control plants.

The effects of climate change on floral traits can increase competition or facilitation of pollinator attraction to forbs depending on the plant community context. Pollinator visitation rates decreased to drought treated *P. recta* and *H. villosa* plants in the multi-species assemblage when compared to the single-species assemblage, potentially indicating that drought increases competition for pollinators to these forb species in a plant community context. Plant reproduction is often pollen limited, which can lead to strong selection on the diversification of floral traits and reduces competition among plant species for pollinators (Ashman et al. 2004). As a result, it is thought that competition for pollinators increases among co-occurring plants with high levels of overlap of floral traits (Caruso 2000, Ghazoul 2006). Increased competition for pollinators as a result of floral

trait overlap is supported by the observation that pollinator visitation was reduced to *P. recta* and *H. villosa,* which both display flat, yellow flowers that were reduced in size in response to drought. Conversely, facilitation of pollination as a result of less floral trait overlap is supported by the observation that pollinator visitation increased to *P. hastata* plants, which display a bell-shaped, purple flower that was positively affected when fertilized by CO_2 in the multi-species assemblage. This result is consistent with other studies that documented increased pollinator visitation to rare plants with low floral densities when co-occurring with other large plant populations (Moeller 2004, Ghazoul 2006). Within our study system, *P. recta* is an invasive species that blooms in large, high density populations, which produces floral displays attractive to pollinators, potentially contributing to the facilitative effect such as those observed to *P. hastata* by guiding pollinators from a far distance to visit rare plants with low floral densities. (Herron-Sweet et al. 2016). However, it has also been observed that invasive species can outcompete other forb species for pollinators or have neutral effects on pollinator visitation to native plants (Munoz and Cavieres 2008, Flanagan et al. 2010, Chung et al. 2014) such as those demonstrated in *H. villosa* or neutral effects on pollinator visitation such as those demonstrated in *C. rotundifolia* within the multi-species assemblage. Addition and removal studies manipulating the presence and absence of forb species in multi-species assemblages will be better equipped to evaluate facilitative, competative, or neutral effects of other forb species on plant-pollinator interactions.

Habitats with high floral trait diversity will be important for supporting intact plant-pollinator networks essential for the maintenance of ecosystem biodiversity and function (Bascompte et al. 2006, Biesmeijer et al. 2006, Burkle et al. 2013). Preserving functionally equivalent pollinator communities in response to disturbances posed by climate change will mitigate subsequent parallel declines in plant biodiversity critical for maintaining more robust and resilient ecosystems (Tilman 1999). High floral trait diversity may help minimize the negative consequences of trait mismatches likely to occur as a result of climate change. Here we provided evidence from an *in situ* field experiment demonstrating that components of climate change have species-specific effects on plant visual traits and VOCs important for pollinator attraction, which in turn can influence pollinator visitation depending on the plant community context. While incorporating floral VOCs into traditional measures of pollinator attraction adds an additional level of complexity for understanding the responses of plant-pollinator interactions to climate change, the importance of VOCs in conjunction with floral visual traits to plant-insect communication highlights the holistic approach required to study the effects of climate change on plant-pollinator trait mismatch. Additionally, approaches linking the responses of individuals to community level processes will be vital to predict ecological responses to climate change across scales in ecology (Gornish and Tylianakis 2013). Given that floral traits have the ability to mediate pollinator attraction at difference scales, studying the responses of floral traits to climate change will help elucidate the specific mechanisms contributing

to the worldwide decline in pollinators (Potts et al. 2010, Burkle et al. 2013, Kerr et al. 2015).

Acknowledgements

We thank members of the Burkle lab including E.Reese, Dr. C. Delphia, M. Simanonok, A. Slominski, L. Heil, S. Durney, and Michele Rockwell for expert advice and data collection. Additionally, thanks to Dr. Michelle Flenniken and Maureen Kessler for manuscript edits. This work was supported by funding from the US Forest Service-Rocky Mountain Research Station, Montana State University, and the Vice-President of Research Grant at Montana State University.

Supporting Tables

Supporting Table S1: Volatile compounds released by *Campanula rotundifolia* flowers (mean ± SE) subjected to drought, CO_2 fertilization, and drought + CO_2 fertilization.

Compounds	ID[1]	Floral emission rates (ng/hr/flower), n=6			
		Control	**CO_2 fertilization**	**Drought**	**Drought + CO_2 fertilization**
Total volatiles		128 ± 27.5	123.1 ± 23.1	295 ± 78	129 ± 8.6
Nitrogen containing compounds					
Indole	NIST	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.6	0.0 ± 0.0
1-(3-amino-4methoxyphenyl)ethanone	NIST	0.0 ± 0.0	0.5 ± 0.4	0.4 ± 0.1	0.5 ± 0.5
GLVs[2]					
2-Hexenal	NIST	0.0 ± 0.0	0.0 ± 0.0	5.0 ± 2.7	0.0 ± 0.0
(*Z*)-3-Hexenol	SS	0.0 ± 0.0	2.1 ± 1.5	0.4 ± 0.1	0.0 ± 0.0
(*Z*)-3-Hexenyl acetate	SS	0.0 ± 0.0	0.0 ± 0.0	2.5 ± 1.5	1.8 ± 1.8
Benzenoids					
Benzyl alcohol	NIST	6.4 ± 1.0	5.4 ± 0.9	6.2 ± 1.1	8.9 ± 1.5
Benzaldehyde	NIST	14.7 ± 3.4	7.8 ± 3.5	14.4 ± 3.6	19.4 ± 1.2
1-2-4-Trimethoxybenzene	NIST	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.1	0.0 ± 0.0
Monoterpenes					
α-Pinene	SS	4.6 ± 0.36	3.9 ± 0.8	7.1 ± 2.7	3.3 ± 0.5
Camphene	SS	0.8 ± 0.2	2.3 ± 0.6	1.5 ± 0.5	5.7 ± 1.8
6-methyl-5-hepten-2-one	SS	23.5 ± 10.4	64.1 ± 16.9	109.3 ± 33.8	6.4 ± 0.4
β-Pinene	SS	48.1 ± 13.3	14.4 ± 9.3	109.4 ± 33.8	23.8 ± 8.0
β-Myrcene	SS	20.1 ± 4.0	6.5 ± 4.9	13.5 ± 5.2	14.3 ± 3.9
p-Cymene	SS	4.1 ± 1.1	4.4 ± 0.8	1.9 ± 0.7	10.1 ± 2.1
Eucalyptol (1,8-Cineole)	SS	0.0 ± 0.0	0.0 ± 0.0	2.1 ± 1.2	8.4 ± 0.9
Limonene	SS	1.6 ± 0.9	3.9 ± 1.3	5.3 ± 3.3	5.2 ± 0.9
D-Camphor	SS	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.5	1.6 ± 0.5
Bornyl acetate	SS	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
MT8	NIST	2.2 ± 0.5	0.0 ± 0.0	2.2 ± 0.6	3.4 ± 0.9
Irregular terpenes					
(*E*)-Geranylacetone	NIST	1.8 ± 1.0	3.0 ± 0.9	7.1 ± 2.6	6.5 ± 2.0

[1]Identification (ID) of compounds based upon comparison of retention time and mass spectra with synthetic standards (SS) or comparison of mass spectra using

Supporting Table S1 Continued

NIST 08 Mass Spectral Search Program; compound names are given if match probability greater than 50%.
[2]Green Leaf Volatiles (C6 compounds)
[3]BZN = unidentified benzenoid
[4]MT = unidentified monoterpene
[5]ST = unidentified sesquiterpene

Supporting Table S2: Volatile compounds released by *Heterotheca villosa* flowers (mean ± SE) subjected to drought, CO_2 fertilization, and drought + CO_2 fertilization.

Compounds	ID[1]	Floral emission rates (ng/hr/flower), n=6			
		Control	Drought	CO_2 fertilization	Drought + CO_2 fertilization
Total volatiles		618.9 ± 141	615 ± 236	706 ± 326	382 ± 123
GLVs[2]					
3-Hexenal	SS	11.8 ± 4.0	1.9 ± 0.4	0.01 ± 0.01	1.7 ± 0.6
Hexanal		15.5 ± 2.7	10.6 ± 5.3	2.5 ± 0.4	4.5 ± 1.6
(*Z*)-3-Hexenol	SS	48.7 ± 29.5	74.3 ± 36.8	4.5 ± 2.7	7.6 ± 7.4
(*Z*)-3-Hexenyl acetate	SS	33.9 ± 24.8	22.2 ± 8.9	6.6 ± 1.8	5.4 ± 2.0
Benzenoids					
Benzaldehyde	SS	119.7 ± 29.7	78.1 ± 22.6	46.7 ± 23.5	127.7 ± 46.5
Benzyl alcohol	SS	61.5 ± 28.5	32.6 ± 11.0	17.2 ± 7.9	39.1 ± 17.7
Monoterpenes					
α-Pinene	SS	11.6 ± 5.5	6.3 ± 3.9	40.9 ± 29.6	11.9 ± 11.9
Camphene	SS	6.9 ± 1.6	3.0 ± 0.9	12.7 ± 8.4	5.5 ± 2.8
6-methyl-5-hepten-2-one	SS	77.9 ± 23.7	65.1 ± 39.0	23.5 ± 14.7	142.8 ± 81.9
β-Pinene	SS	0.0 ± 0.0	12.9 ± 8.3	43.7 ± 26.3	13.4 ± 13.4
3-Carene	SS	0.0 ± 0.0	2.9 ± 1.0	3.4 ± 1.4	4.7 ± 1.0
p-Cymene	SS	10.3 ± 3.2	21.2 ± 14.2	19.5 ± 8.9	25.2 ± 14.1
Eucalyptol (1,8-Cineole)	SS	75.9 ± 49.9	123.1 ± 110.6	39.7 ± 29.7	31.5 ± 17.2
Limonene	SS	4.3 ± 1.3	5.8 ± 1.3	7.4 ± 2.4	6.2 ± 1.5
(*E*)-β-Ocimene	SS	7.9 ± 1.4	11.8 ± 5.2	4.7 ± 1.2	6.3 ± 1.9
λ-Terpinene	SS	2.7 ± 0.5	2.7 ± 1.0	3.8 ± 1.6	4.3 ± 1.2
[3]MT2	NIST	27.2 ± 24.1	70.3 ± 62.6	41.9 ± 26.2	115.1 ± 85.6
β-Thujone	SS	0.4 ± 0.1	0.3 ± 0.2	1.3 ± 0.6	1.2 ± 0.9
MT3	NIST	29.4 ± 27.4	29.4 ± 23.4	34.6 ± 22.2	141.5 ± 110.3
D-Camphor	SS	12.9 ± 10.4	0.03 ± 0.03	0.0 ± 0.0	0.0 ± 0.0
α-Terpineol	SS	6.7 ± 3.5	6.4 ± 5.1	1.6 ± 1.2	2.8 ± 1.6
MT8	NIST	3.4 ± 0.3	1.6 ± 0.6	1.6 ± 1.6	1.7 ± 0.5
MT9	NIST	0.5 ± 0.18	0.7 ± 0.2	0.0 ± 0.0	1.6 ± 0.9
Sesquiterpenes					
[4]ST1	NIST	2.7 ± 1.8	1.7 ± 0.8	0.0 ± 0.0	1.2 ± 0.6
ST2	NIST	20.5 ± 14.7	21.8 ± 10.7	12.5 ± 11.2	1.5 ± 0.8
ST3	NIST	26.4 ± 20.9	8.0 ± 3.9	11.3 ± 10.5	1.2 ± 0.3

[1]Identification (ID) of compounds based upon comparison of retention time and mass spectra with synthetic standards (SS) or comparison of mass spectra using NIST 08 Mass Spectral Search Program; compound names are given if match probability greater than 50%.
[2]Green Leaf Volatiles (C6 compounds)
[3]MT = unidentified monoterpene
[4]ST = unidentified sesquiterpene

Supporting Table S3: Volatile compounds released by *Phacelia hastata* flowers (mean ± SE) subjected to drought, CO_2 fertilization, and drought + CO_2 fertilization.

Compounds	ID[1]	Floral emission rates (ng/hr/flower), n=6			
		Control	**Drought**	**CO_2 fertilization**	**Drought + CO_2 fertilization**
Total volatiles		19.8 ± 4.6	35.0 ± 8.2	28.8 ± 5.5	43.4 ± 9.4
Aliphatics					
2-Heptanone	NIST	0.0 ± 0.0	0.04 ± 0.01	0.0 ± 0.0	0.14 ± 0.04
2-Nonanone	NIST	0.48 ± 0.18	0.41 ± 0.14	1.76 ± 0.85	0.72 ± 0.25
GLVs[2]					
3-methyl-3-butenol	NIST	0.0 ± 0.0	0.02 ± 0.02	0.0 ± 0.0	0.0 ± 0.0
3-Hexenal	SS	0.05 ± 0.01	0.1 ± 0.02	0.02 ± 0.02	0.15 ± 0.03
Hexanal	SS	0.70 ± 0.25	0.14 ± 0.02	1.06 ± 0.28	0.27 ± 0.07
2-Hexenal	NIST	2.51 ± 1.99	0.38 ± 0.06	6.10 ± 5.26	0.40 ± 0.14
(*Z*)-3-Hexenol	SS	0.09 ± 0.02	0.07 ± 0.02	0.12 ± 0.02	0.0 ± 0.0
(*Z*)-3-Hexenyl acetate	SS	3.82 ± 2.11	0.24 ± 0.12	2.51 ± 1.02	2.77 ± 1.32
Cyclohexane	NIST	0.12 ± 0.04	0.14 ± 0.03	0.52 ± 0.28	0.28 ± 0.18
Benzenoids					
Benzaldehyde	SS	1.63 ± 0.22	1.26 ± 0.15	2.82 ± 0.71	1.68 ± 0.29
Benzyl alcohol	SS	0.70 ± 0.11	0.59 ± 0.11	0.76 ± 0.20	0.43 ± 0.09
Monoterpenes					
MT1[3]	NIST	0.0 ± 0.0	0.01 ± 0.01	0.0 ± 0.0	0.0 ± 0.0
α-Pinene	SS	1.72 ± 0.29	1.34 ± 0.15	3.0 ± 0.80	1.85 ± 0.38
Camphene	SS	0.32 ± 0.07	0.47 ± 0.17	0.43 ± 0.09	0.74 ± 0.27
6-methyl-5-hepten-2-one	SS	2.20 ± 0.71	1.67 ± 0.49	2.20 ± 0.73	1.32 ± 0.32
β-Pinene	SS	1.71 ± 0.41	0.99 ± 0.16	4.83 ± 1.30	2.18 ± 0.51
β-Myrcene	SS	1.16 ± 0.33	0.60 ± 0.11	3.34 ± 0.94	1.32 ± 0.34
3-Carene	SS	0.23 ± 0.07	0.29 ± 0.06	0.64 ± 0.15	0.45 ± 0.17
p-Cymene	SS	0.34 ± 0.17	0.27 ± 0.08	0.67 ± 0.15	0.57 ± 0.12
Eucalyptol (1,8-Cineole)	SS	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.54 ± 0.65

Supporting Table S3 Continued

Limonene	SS	1.19 ± 0.47	0.99 ± 0.29	5.09 ± 2.3	3.57 ± 0.79
(*E*)-β-Ocimene	SS	0.00 ± 0.00	0.02 ± 0.02	0.18 ± 0.18	0.00 ± 0.00
λ-Terpinene	SS	0.00 ± 0.00	0.02 ± 0.02	0.24 ± 0.24	0.00 ± 0.00
MT2	NIST	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
Linalool	SS	4.21 ± 0.99	3.26 ± 0.42	10.39 ± 3.28	5.55 ± 1.28
D-Camphor	SS	0.00 ± 0.00	0.002 ± 0.002	0.00 ± 0.00	0.00 ± 0.00
MT6	NIST	0.00 ± 0.00	0.016 ± 0.016	0.00 ± 0.00	0.00 ± 0.00
Sesquiterpenes					
β -Bourbonene	NIST	0.15 ± 0.06	0.08 ± 0.02	0.53 ± 0.13	0.21 ± 0.08

[1]Identification (ID) of compounds based upon comparison of retention time and mass spectra with synthetic standards (SS) or comparison of mass spectra using NIST 08 Mass Spectral Search Program; compound names are given if match probability greater than 50%.
[2]Green Leaf Volatiles (C6 compounds)
[3]MT = unidentified monoterpene
[4]ST = unidentified sesquiterpene

Supporting Table S4: Volatile compounds released by *Potentilla recta* flowers (mean ± SE) subjected to drought, CO_2 fertilization, and drought + CO_2 fertilization.

Compounds	ID[1]	Floral emission rates (ng/hr/flower), n=6			
		Control	Drought	CO_2 fertilization	Drought + CO_2 fertilization
Total volatiles		314 ± 56	560 ± 64.2	454 ± 152	595 ± 64
GLVs[2]					
3-Hexenal	SS	22.4 ± 10.1	65.2 ± 16.7	34.7 ± 17.8	19.4 ± 5.3
(*Z*)-3-Hexenol	SS	33.9 ± 15.4	137.5 ± 22.9	107.3 ± 54.3	89.2 ± 22.9
(*Z*)-3-Hexenyl acetate	SS	59.4 ± 25.9	159.3 ± 28.5	97.8 ± 44.5	162.9 ± 42.7
Benzenoids					
Benzaldehyde	SS	20.1 ± 2.4	25.7 ± 1.2	9.4 ± 3.3	22.3 ± 0.8
Acetophenone	NIST	0.0 ± 0.0	2.8 ± 0.4	2.6 ± 0.5	3.5 ± 0.4
Benzophenone	NIST	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 0.5	2.7 ± 0.8
Monoterpenes					
α-Pinene	SS	16.1 ± 1.0	19.7 ± 1.1	19.4 ± 0.9	16.2 ± 0.6
Camphene	SS	0.0 ± 0.0	3.7 ± 0.6	5.8 ± 0.8	4.6 ± 1.3
6-methyl-5-hepten-2-one	NIST	123.7 ± 22.1	115.7 ± 18.8	133.3 ± 50.1	214.5 ± 41.1
β-Myrcene	SS	25.3 ± 5.1	18.3 ± 2.3	9.9 ± 6.4	28.6 ± 5.9
3-Carene	SS	3.9 ± 0.7	2.2 ± 0.2	4.1 ± 0.7	5.1 ± 0.9
p-Cymene	SS	3.7 ± 0.4	4.7 ± 0.5	5.2 ± 1.0	4.7 ± 0.7
[3]MT2	NIST	0.0 ± 0.0	0.9 ± 0.9	0.0 ± 0.0	7.9 ± 3.5
Limonene	SS	0.0 ± 0.0	0.0 ± 0.0	3.2 ± 0.6	3.5 ± 0.6
(E)-β-Ocimene	SS	0.0 ± 0.0	0.0 ± 0.0	8.1 ± 0.9	4.71 ± 0.9
D-Camphor	SS	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.3	0.0 ± 0.0
α-Terpineol	SS	1.0 ± 0.3	1.0 ± .04	1.0 ± 0.3	1.3 ± 0.5
MT9	NIST	4.1 ± 1.1	4.4 ± 0.7	8.2 ± 2.0	7.6 ± 1.4
Sesquiterpenes					
β-Caryophyllene	SS	1.1 ± 1.1	0.0 ± 0.0	3.3 ± 1.8	0.0 ± 0.0
Caryophyllene oxide	NIST	1.17 ± 1.17	0.0 ± 0.0	3.9 ± 1.4	0.0 ± 0.0
[4]ST3	NIST	0.0 ± 0.0	0.0 ± 0.0	2.9 ± 0.7	0.0 ± 0.0
Irregular terpenes					
(*E*)-Geranylacetone	NIST	7.7 ± 2.8	20.5 ± 4.3	20.6 ± 14.6	14.3 ± 4.2

[1]Identification (ID) of compounds based upon comparison of retention time and mass spectra with synthetic standards (SS) or comparison of mass spectra using NIST 08 Mass Spectral Search Program; compound names are given if match probability greater than 50%.
[2]Green Leaf Volatiles (C6 compounds)
[3]MT = unidentified monoterpene
[4]ST = unidentified sesquiterpene

Supporting Table S5: Summary of pollinators observed visiting each of the treatment plant species in single-species treatments. C is watered control, D is drought, 2X is CO_2 fertilization, and 2X:D is the crossed treatment.

Pollinator Species	**Plant Species**	*Campanula rotundifolia*				*Heterotheca villosa*				*Phacelia hastata*				*Potentilla recta*			
		D	C	2X:D	2X	D	C	2X:D	2X	D	C	2X:D	2X	D	C	2X:D	2X
Andrena	*nigracerulea*	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0
anthophora	*sp1*	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cerantina	*neomexicana*	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
Halictus	*confusus*	0	0	0	0	0	2	0	0	1	0	0	0	1	6	4	6
Halictus	*ligatus*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Hylaeus	*wootoni*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Lasioglossum (Dialictus)	*albipenne*	1	1	1	2	9	7	6	3	5	1	0	3	25	22	28	32
Lasioglossum (Dialictus)	*albohirtum*	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Lasioglossum (Dialictus)	*brunneiventre*	3	0	0	1	4	10	10	6	0	3	3	1	3	5	6	4
Lasioglossum (Dialictus)	*emberellum*	0	0	0	0	0	0	1	0	0	0	0	0	0	2	1	0
Lasioglossum (Dialictus)	*laevissimum*	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1
Lasioglossum (Dialictus)	*sp1*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Lasioglossum (Dialictus)	*nevadensis*	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Lasioglossum (Dialictus)	*sp3*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Lasioglossum (Dialictus)	*occidentalis*	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Lasioglossum (Dialictus)	*pavonium*	0	1	0	0	0	0	0	0	0	0	0	0	0	2	1	1
Lasioglossum (Dialictus)	*perpunctatum*	0	2	0	1	0	0	2	0	1	0	0	1	1	4	3	3
Lasioglossum (Dialictus)	*piradmirandam*	0	1	1	0	0	1	1	0	0	0	0	0	0	3	2	3
Lasioglossum (Dialictus)	*rubicundus*	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1
Lasioglossum (Dialictus)	*ruidosense*	3	2	1	4	1	1	0	1	1	0	0	1	3	1	2	0
Lasioglossum (Dialictus)	*succinipenne*	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Lasioglossum (Dialictus)	*tenax*	5	5	3	2	0	2	3	0	0	0	1	1	1	5	4	3

Supporting Table S5 Continued

Lasioglossum (Dialictus)	*versans*	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
Lasioglossum (Evylaeus)	*evy1*	0	0	1	0	0	0	0	0	0	1	0	0	0	4	1	2
Lasioglossum (Evylaeus)	*evy2*	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1
Lasioglossum (Evylaeus)	*evy3*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lasioglossum (Evylaeus)	*evy4*	0	1	0	1	0	2	0	0	0	0	0	0	2	4	0	1
Lasioglossum (Evylaeus)	*evy5*	0	0	1	1	0	0	1	0	0	0	0	0	2	2	6	2
Lasioglossum (Evylaeus)	*evy7*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Syrphid	*sp1*	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	2
Syrphid	*sp2*	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0
Syrphid	*sp3*	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Syrphid	*sp4*	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
Tripartitus	*tripartitus*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
	cano1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
muscidae	*sp1*	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	1
		1	**1**	**1**	**1**	**1**	**3**	**2**	**1**					**4**	**6**	**6**	**7**
	Total	**5**	**5**	**0**	**7**	**4**	**1**	**4**	**4**	**8**	**7**	**4**	**8**	**1**	**9**	**1**	**0**

Supporting Table S6 Summary of pollinators observed visiting each of the treatment plant species within the multi-species treatment. C is watered control, D is drought, 2X is CO_2 fertilization, and 2X:D is the crossed treatment.

	Plant Species	*Campanula rotundifolia*				*Heterotheca villosa*				*Phacelia hastata*				*Potentilla recta*			
Pollinator Species		D	C	2X:D	2X	D	C	2X:D	2X	D	C	2X:D	2X	D	C	2X:D	2X
Andrena	*craetagi*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Andrena	*nigracerulea*	0	0	0	0	2	1	1	0	0	0	0	0	1	1	0	3
Cerantina	*nanula*	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2
Cerantina	*neomexicana*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Halictus	*confusus*	3	0	1	1	1	2	0	4	0	0	0	0	3	9	6	8
Halictus	*ligatus*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Halictus	*tripartitus*	0	0	1	0	0	0	0	0	0	0	0	0	4	1	1	1
Hoplitis	*sp1*	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Lasioglossum (Dialictus)	*albipenne*	1	3	1	3	3	3	1	3	3	1	1	3	48	39	33	50
Lasioglossum (Dialictus)	*brunneiventre*	1	3	0	0	3	1	3	4	1	0	1	0	7	3	0	3
Lasioglossum (Dialictus)	*emberellum*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Lasioglossum (Dialictus)	*incompletum*	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Lasioglossum (Dialictus)	*lass2*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Lasioglossum (Dialictus)	*nevadensis*	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1
Lasioglossum (Dialictus)	*paradisiea*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Lasioglossum (Dialictus)	*pavonium*	0	0	1	0	0	0	2	1	0	0	0	0	1	1	0	2
Lasioglossum (Dialictus)	*pentsemonis*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Lasioglossum (Dialictus)	*perpunctatum*	1	1	2	1	0	0	0	0	0	0	1	0	0	0	3	2
Lasioglossum (Dialictus)	*piradmirandam*	0	0	0	1	0	0	0	0	0	0	0	0	6	0	0	2
Lasioglossum (Dialictus)	*prasinogaster*	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	2
Lasioglossum (Dialictus)	*pusilla*	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Lasioglossum (Dialictus)	*rubicundus*	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	2
Lasioglossum (Dialictus)	*ruidosense*	0	0	0	0	0	0	0	0	0	0	0	0	3	1	1	2
Lasioglossum (Dialictus)	*tenax*	3	1	2	1	0	1	0	0	0	0	0	0	7	9	3	2
Lasioglossum (Evylaeus)	*evy1*	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0
Lasioglossum (Evylaeus)	*evy2*	0	0	0	0	0	0	0	0	0	0	0	1	3	2	1	1
Lasioglossum (Evylaeus)	*evy4*	0	0	1	0	0	0	0	0	0	0	0	0	2	0	0	1
Lasioglossum (Evylaeus)	*evy5*	3	1	1	0	0	0	1	0	0	0	1	0	0	2	1	4

Supporting Table S6 Continued

Lasioglossum (Evylaeus)	*evy6*	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Lasioglossum (Evylaeus)	*evy8*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0
Muscidae	*musc1*	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1
Nomada	*sp1*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Syrphid	*sp1*	0	0	0	0	0	1	0	0	0	0	0	0	1	2	0	3
Syrphid	*sp2*	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0
Syrphid	*sp4*	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
	Total	**14**	**9**	**12**	**8**	**9**	**10**	**8**	**14**	**4**	**2**	**7**	**6**	**89**	**78**	**55**	**93**

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CHAPTER THREE

FACTORS INFLUENCING HONEY BEE (*APIS MELLIFERA*) COLONY HEALTH AND PATHOGEN COMPOSITION IN MIGRATORY BEEKEEPING OPERATIONS INVOLVED IN CALIFORNIA ALMOND POLLINATION

Abstract

Honey bees are important pollinators of agricultural crops. Pathogens and other factors have been implicated in high annual losses of honey bee colonies in North America and some European countries. To further investigate the relationship of multiple factors, including pathogen prevalence and abundance, and colony health we monitored commercially managed migratory honey bee colonies involved in California almond pollination in 2014. At each sampling event, honey bee colony health was assessed, using colony population size as a proxy for health, and the prevalence and abundance of seven honey bee pathogens was evaluated using PCR and quantitative PCR, respectively. In this sample cohort, pathogen prevalence did not correlate with colony health, but did correlate with the date of sampling. In general, pathogen prevalence (i.e., the number of specific pathogens harbored within a colony) was lower early in the year (January – March) and was greater later in the year, with peak prevalence occurring in June, coinciding with peak colony populations. Pathogen abundance, using the number of RNA transcripts of each pathogen as a proxy for pathogen abundance, varied in individual colonies and total pathogen abundance

was not correlated with colony health over the course of the study. Results from multiple linear regression analysis indicate that the abundance of each pathogen was most associated with date of sampling and influenced by beekeeping operation, colony strength rating, and mite infestation level. We determined that Deformed wing virus (DWV) abundance was positively associated with the percentage of *Varroa destructor* mite infestation, whereas Lake Sinai virus 2 (LSV2) was not associated with the percentage of mite infestation. Colonies that exceeded the 3% threshold for mite infestation by the end of the study were more likely to die in only one of the two beekeeping operations, indicating additional factors affected colony longevity. Data from this and other temporal observational cohort studies that monitor individual honey bee colonies and precisely account for sampling date will lead to a better understanding of the influence of pathogens on colony mortality and the effects of abiotic factors (e.g., season/date, weather events, and agrochemical exposure) on these associations.

Introduction

Honey bees (*Apis mellifera*) are the primary insect pollinators of agricultural crops, including fruits, nuts, and vegetables, which have an approximate annual value of $17 - 18 billion in the United States (Gallai et al. 2009, Calderone 2012). California almond production is the most renowned example of the role of honey bee pollination services in the US. Every year, over

60% of the 2.5 million commercially managed honey bee colonies in the United States are transported to the Central Valley of California to pollinate the almond crop, which accounts for 80% of the global almond supply and was valued at $1.7 billion in 2014 (2014). Honey bee colonies are important for the pollination of plants in both agricultural and non-agricultural landscapes (Ollerton et al. 2011, Garibaldi et al. 2013). Thus, high annual honey bee colony losses in the US, which have averaged ~ 33% annually since 2006 and increased from historic levels of approximately 12%, are concerning to stakeholders, including beekeepers, growers, and scientists (Lee et al. 2015, Traynor et al. 2016). Furthermore, from 2009-2014 annual honey bee colony mortality approached 50% for some US commercial beekeeping operations (Traynor et al. 2016). Although annual losses of honey bee colonies in the US have been high, beekeeping operations have mitigated these losses by splitting colonies (i.e., producing two colonies from one colony) more frequently to compensate for increased colony losses and meet pollination demands. Therefore, while the total number of commercially managed colonies has remained constant since the late 1990s (~2.5 million) (NASS 2016), US beekeeping operations are experiencing unsustainable annual colony losses.

There are multiple factors that impact colony health, including pathogens (i.e., mites, viruses, bacteria, and fungi), queen failure, colony genetics, weather, nutrition, management practices (i.e., transportation, treatment regimes), and agrochemical exposure (Robinson et al. 2006, Vanengelsdorp et al. 2008,

vanEngelsdorp et al. 2009, Danka et al. 2012, Vanengelsdorp et al. 2012, Mordecai et al. 2016, Ryabov et al. 2016, Simone-Finstrom et al. 2016, Smart et al. 2016, Traynor et al. 2016). Honey bee colony health is typically evaluated by estimating colony population size (Runckel et al. 2011, Sagili and Burgett 2011, COLOSS 2013, Daughenbaugh et al. 2015, Cavigli et al. 2016). Colonies are comprised of ~35,000 sterile female workers, hundreds of males (drones), and a single reproductive queen that can lay approximately 1,000 eggs per day during peak colony building (Langstroth 2016). While no single factor has been deemed responsible for above average annual honey bee colony losses, pathogens are major contributing factors (Tentcheva et al. 2004, Cox-Foster et al. 2007, vanEngelsdorp et al. 2009, Genersch et al. 2010, Nguyen et al. 2010, Evans and Schwarz 2011, Runckel et al. 2011, Cornman et al. 2012, Vanengelsdorp et al. 2012, Ravoet et al. 2013, Chen et al. 2014, Lee et al. 2015, Cavigli et al. 2016, Seitz et al. 2016, Traynor et al. 2016).

Honey bees host a diversity of pathogens, the majority of which are single-stranded RNA viruses (Evans and Schwarz 2011, Brutscher et al. 2016). Honey bee infecting viruses include Acute bee paralysis virus (ABPV), Black queen cell virus (BQCV), Deformed wing virus (DWV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), Sacbrood virus (SBV), Chronic bee paralysis virus (CBPV) (Chen and Siede 2007, de Miranda et al. 2010, Genersch and Aubert 2010, Brutscher et al. 2016, McMenamin et al. 2016) and the Lake Sinai viruses (Daughenbaugh et al. 2015). In addition to viruses, pathogens of honey bees

include eukaryotes such as the trypanosomatid *Lotmaria passim* (formerly *Crithidia mellificae* strain sf (Runckel et al. 2014, Schwarz et al. 2015)), the microsporidial pathogen *Nosema ceranae* (Cornman et al. 2009), and bacterial pathogens such as *Paenibacillus larvae* (Genersch 2010a) and *Melissococcus plutonius* (Forsgren 2010), the causative agents of American and European foul brood diseases, respectively. In addition, the ectoparasitic mite, *Varroa destructor,* contributes to decreased colony health by feeding on developing bees (brood) and facilitating virus transmission (Nazzi and Le Conte 2016). *Varroa destructor* mites are a vector of several honey bee viruses including, DWV (Ryabov et al. 2014, Mordecai et al. 2016), KBV (Chen et al. 2004, Shen et al. 2005), and IAPV (Di Prisco et al. 2011, Brutscher et al. 2016, Nazzi and Le Conte 2016). Mite parasitization of developing honey bees can result in physical deformities, reduced body weight, and/or greater DWV virus levels (Nazzi et al. 2012, Nazzi and Le Conte 2016).

Temporal monitoring studies, particularly those carried out at the colony rather than the apiary level, are essential for identifying and understanding the factors that influence honey bee colony health and pathogen prevalence and abundance. Identifying particular pathogens or suites of pathogens that are associated with colony losses is complicated because pathogen prevalence and abundance varies by season and geographic location (Tentcheva et al. 2004, Genersch et al. 2010, Runckel et al. 2011, Ravoet et al. 2013, Cavigli et al. 2016). Furthermore, there have been relatively few studies that have monitored

individual commercially managed colonies (vanEngelsdorp et al. 2009, Runckel et al. 2011, Danka et al. 2012, Cavigli et al. 2016, Traynor et al. 2016). The majority of studies in the US have monitored honey bee health at the apiary level with the main goals of detecting exotic pathogens and establishing a baseline understanding of honey bee colony health (Lee et al. 2015, Seitz et al. 2016, Simone-Finstrom et al. 2016, Smart et al. 2016, Traynor et al. 2016). Additional studies that monitor colonies located in varying geographic locations are needed to better understand the impacts of multiple factors on honey bee colony health. To address this knowledge gap, we monitored Minnesota-based commercially managed honey bee colonies involved in the 2014 California almond pollination event. The goal of this study was to investigate the association between multiple factors, including pathogen seasonality, pathogen abundance, beekeeping operation, colony population size, and level of mite infestation, on honey bee colony health, and colony losses. We determined that sampling date strongly influenced pathogen prevalence and abundance and confirmed a strong association between DWV abundance and *Varroa destructor* mite infestation level. However, the abundance of other viruses (i.e., BQCV, LSV1, and LSV2) was not associated with mite infestation. Data from this and other temporal observational cohort studies that precisely account for sampling date (i.e., day of the year, rather than season) and other factors (i.e. apiary management, abiotic conditions, etc…) will aid in a better understanding of drivers affecting pathogen

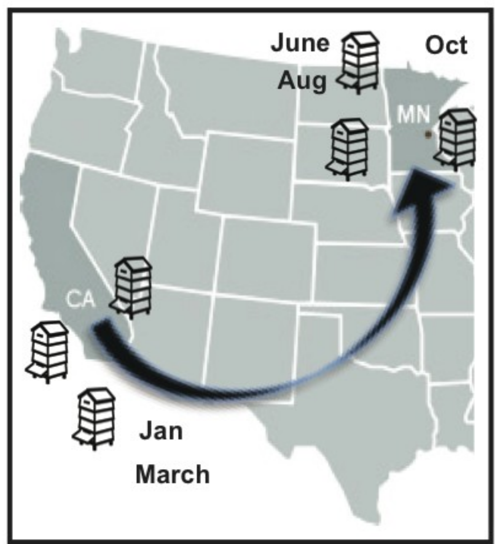
abundance. The development of management strategies used to reduce pathogen abundance may aid in decreasing annual honey bee colony losses.

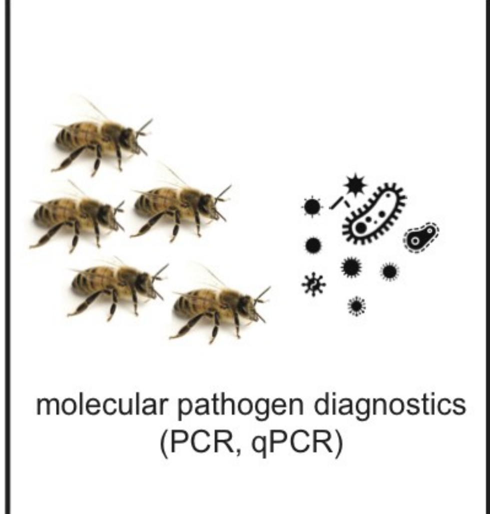
Methods

Longitudinal Monitoring and Sampling of Commercially Managed Honey Bee Colonies

Honey bee (*Apis mellifera*) colonies from two Minnesota-based commercial beekeeping operations that transport honey bee colonies to California for almond pollination were monitored for one year beginning in January 2014 to January 2015. At the onset of the study in January 2014, beekeepers identified 20 colonies that successfully overwintered. A subset of these colonies was utilized for the pathogen analysis (n = 28), of which two died and were replaced by three new colonies bringing the total unique colonies described in this dataset to 31. Each beekeeping operation administered anti-mite treatments. To reduce *Varroa destructor* mite infestation levels in 2014, Operation 1 treated colonies with amitraz on April 22 and Sept. 4, and oxalic acid on Oct. 20 and Operation 2 treated colonies with amitraz on March 15, oxalic acid of Aug. 21, amitraz on Sept. 9, and formic acid on Oct. 24. Colony health, using colony population size as a proxy for health, was estimated by counting and imaging the number of frames more than two-thirds covered with bees. Colony health was categorized as weak (< 7 frames), average (7-12 frames), or strong (> 12 frames) based on frame count (Supporting Table S2) (Runckel et al. 2011, Sagili and Burgett 2011). Samples of honey bees were obtained from

monitored colonies, up to four times each. Specific 2014 sample dates varied and were categorized into discrete sampling events in order to better compare data within and between colonies managed by two beekeeping operations. Specifically, “before almond pollination” samples were obtained from colonies located in California in January 2014 (Beekeeping Operation 1 on Jan. 27 and Beekeeping Operation 2 on Jan. 29), samples that represent the pathogens associated with honey bee colonies “during almond pollination” were obtained immediately post-almond pollination in March 2014 (Beekeeping Operation 1 on March 3 and Beekeeping Operation 2 on March 14), “after almond pollination” samples were obtained from colonies that were located in Minnesota in early (June 9) and late (Aug. 25 and Sept. 2) summer (Fig 1 and Supporting Table S2).





Sampling Event	**January**	**March**	**June**	**August**	**September**
Location	California	California	Minnesota	Minnesota	Minnesota
Beekeeper					
Operation 1	16	16	0	0	11
Operation 2	12	12	11	14	0
Total	28	28	11	14	11

Fig 1. Commercially managed honey bee colonies were longitudinally monitored before, during, and after the 2014 almond pollination season. Honey bee colonies from two Minnesota-based commercial beekeeping operations that transport their colonies to California for almond pollination were monitored from January 2014 to January 2015. At each sampling event, colony health, using colony population size as a proxy for health, was monitored and samples of live honey bees were obtained. In a subset of samples described in the table, PCR was utilized to assess pathogen prevalence and qPCR was utilized to determine the pathogen abundance.

Additionally, core samples containing pollen/bee bread, wax, and honey were obtained from a representative frame in each colony and analyzed for pesticide residues, as described in Kegley et al. 2017 (Susan Kegley 2017). The

sample cohort analyzed herein includes pathogen prevalence, abundance, and survivorship data obtained in 2014, including variable numbers of samples obtained from dead (n = 2), weak (n = 8), average (n = 34), and strong (n = 48) rated colonies (total = 92) spanning the duration of the 2014 California almond pollination season, and colony longevity data through January 2015 (Fig 1 and Supporting Table S2).

Honey Bee Samples

At each sampling event, live honey bees (~ 150 per sample) were obtained from a frame containing developing bees (brood) in the center each colony. Samples were composed of female bees of mixed age, weighted toward younger nurse bees since samples were obtained from a brood frame. Collected samples were placed on ice or dry ice in the field, stored at -20°C, transported on dry ice, and transferred to -80°C for storage prior to analysis. Five female bees from each sample were used for RNA extraction, cDNA synthesis, pathogen-specific PCR, and qPCR. The objective for pathogen screening was to identify the most prevalent pathogens associated with honey bees sampled from individual colonies at each sampling event. Based on empirical data, literature values, and practical sample handling considerations, we assayed five bees per colony per sampling event (Cavigli et al. 2016). The following equation from Pirk et al. 2013, $N = \ln(1-D) / \ln(1-P)$ (N = sample size, ln = natural logarithm, D = probability of detection, P = proportion of infected bees) predicts that with a sample size of five bees, pathogenic infections affecting 45% or more of the

individuals within a colony would be detected with 95% probability (Pirk et al. 2013); this sample size has proven sufficient for the pathogen-specific PCR detection of highly prevalent pathogens (Runckel et al. 2011, Daughenbaugh et al. 2015, Cavigli et al. 2016).

Varroa destructor Mite Infestation Level

Varroa destructor mite infestation levels for each colony were estimated using the alcohol wash technique (Lee et al. 2010). At each sampling event, 150 adult bees were obtained from a frame in the center of the brood nest and rinsed in ethanol to dislodge attached mites into a collection jar; mites were counted and reported as the number of mites per 100 bees, which equates to mite infestation percentage (Lee et al. 2010). At each sampling event the number of *Varroa destructor* mites per 100 bees was recorded. In total 92 samples were included in the analysis (Fig 1 and Supporting Table S2).

RNA Isolation

Five female bees per sample were homogenized together in water using beads (3 mm) and a TissueLyzer (Qiagen) at 30 Hz for 2 minutes. Samples were centrifuged for 12 minutes at 12,000 x g at 4°C to pellet debris, and RNA from supernatants was extracted using Trizol reagent (Life Technologies) according to the manufacturer's instructions (Runckel et al. 2011, Daughenbaugh et al. 2015, Cavigli et al. 2016).

Reverse Transcription/cDNA Synthesis

cDNA synthesis reactions were performed by incubating 2 μg of total RNA extracted from honey bee samples, Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega), and 500 ng random hexamer primers (Integrated DNA Technologies) for 1 hour at 37°C, according to the manufacturer's instructions (Runckel et al. 2011, Cavigli et al. 2016).

Polymerase Chain Reaction (PCR)

Pathogen specific PCR was performed according to standard methods using the primers listed in Supporting Table S1. Specifically, PCR was used to determine the prevalence of Deformed wing virus (DWV), Black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), Lake Sinai virus 1 (LSV1), Lake Sinai virus 2 (LSV2), *Nosema ceranae (N. ceranae*), *Lotmaria passim (L. passim*) (formerly *Crithidia mellificae* sf strain (Supporting Table S1). In brief, each PCR included 1 µl cDNA template, combined with 10 pmol each of forward and reverse pathogen specific primers, and amplified with ChoiceTaq polymerase (Denville) according to the manufacturer's instructions using the following cycling conditions: 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds, followed by final elongation at 72°C for 4 minutes. The PCR products were visualized using 2% agarose gel electrophoresis followed by fluorescence imaging. In addition, the PCR assays utilized in this study were verified by sequencing. Positive and negative controls reactions were included for all pathogen-specific PCR analyses

and exhibited the expected results. IAPV was not detected by PCR in any of the samples, thus IAPV abundance was not examined by quantitative PCR.

Quantitative Polymerase Chain Reactions (qPCR)

Quantitative PCR was used to determine the relative pathogen abundance in each sample. Specifically, qPCR was used to determine the abundance of Deformed wing virus (DWV), Black queen cell virus (BQCV), Kashmir bee virus (KBV), Lake Sinai virus 1 (LSV1), Lake Sinai virus 2 (LSV2), *Nosema ceranae (N. ceranae*), *Lotmaria passim (L. passim*) (formerly *Crithidia mellificae* sf strain (Supporting Table S1). Two micrograms of RNA from each sample was reverse transcribed with M-MLV as described above. All qPCR reactions were performed in triplicate with a CFX Connect Real Time instrument (BioRad) and the following reaction conditions: 2 µL of cDNA template in 20 µL reactions containing 1X ChoiceTaq Mastermix (Denville), 0.4 µM each forward and reverse primer, 1X SYBR Green (Life Technologies), and 3 mM $MgCl_2$. The qPCR thermo-profile consisted of a single pre-incubation 95°C (1 minute), 40 cycles of 95°C (10 seconds), 58°C (20 seconds), and 72°C (15 seconds). Plasmid standards, containing from 10^9 to 10^3 copies per reaction, were used as qPCR templates to assess primer efficiency and quantify the relative abundance of each pathogen. Positive amplification from all plasmid standard reactions indicated that qPCR detection levels were at least 1,000 copies per sample. The linear standard equations generated by plotting the crossing point (Cp) versus the $\log_{10}$ of the initial plasmid copy number for each primer set were as follows: BQCV: y = -

3.7336x+ 42.849, R^2 = 0.996; DWV: y = -3.443x + 41.277, R^2 = 0.99958; LSV1: y = -3.1994x + 38.71, R^2 = 0.982, LSV2: y = -3.8147x + 44.805, R^2 = 0.980; KBV: y = -3.4505x + 40.099, R^2 = 0.99927, *L. passim*: y = -3.4825x + 41.025, R^2 = 0.98588 and *N. ceranae*: y = -3.9656 + 42.124, R^2 = 0.9961. In addition, qPCR of a host encoded gene, *Apis m*. Rpl8, was performed using 2 μL cDNA template on each qPCR plate to ensure consistency and cDNA quality. qPCR products were analyzed by melting point analysis and 2% agarose gel electrophoresis. An estimate of the number of viral RNA copies per bee can be obtained by multiplying the reported qPCR copy number values by 25. This estimate is based on the following: typical RNA yield was approximately 50 μg per bee, each qPCR reaction was performed on cDNA generated from 2 μg ng RNA, therefore each well represents 1/25th of an individual bee. Positive amplification from all plasmid standard reactions indicated that qPCR detection levels were at least 1,000 copies per sample (Runckel et al. 2011).

Statistical Analysis of PCR Data

For this study, we use “pathogen prevalence” to refer to the sum of the number of pathogens detected by PCR out of seven total; an additional pathogen was counted (+1) when *Varroa* mite infestation surpassed 3%, in order to represent the hypothesized negative effects of pathogens on colony health. A one-way ANOVA was used to compare pathogen prevalence between colonies with differential colony health ratings as they were graded at the time of sampling (i.e., dead, strong, average, and weak), and to compare pathogen prevalence

between sampling events (i.e., before, during, after 1, after 2, and after 3 almond pollination).

Statistical Analysis of qPCR Data with Multiple Linear Regression

The association between pathogen abundance and other monitored factors, including colony strength, mite count, and beekeeping operation was evaluated for each pathogen using multiple linear regression (Smart et al. 2016). The natural logarithm (ln) was used to transform pathogen abundance (as determined by qPCR) to normalize the response variable; 1 was added to each observation since some observations had 0 total abundance. The confounding effects of seasonality on pathogen abundance was removed by using the residuals of a linear regression evaluating the relationship between pathogen abundance and the “day of year” of sampling as the response variable in the multiple linear regression models. As a result, models reported associations between the residuals of pathogen abundance after accounting for the effects of pathogen seasonality (“residual abundance”), and colony strength rating, beekeeping operation, and mite count. Most colonies were measured multiple times in the 92 samples; therefore, a random effect for individual colony was included in the models to account for repeated measures. Estimated models for each pathogen are described as:

$$\ln(y_i) = \beta_0 + \beta_1 \text{ x Operation } 2_i + \beta_2 \text{ x Frame Count}_i + \beta_3 \text{ x Mites}_i + \gamma_{ij} + \varepsilon_i$$

Where, $\ln(y_i)$ represents the residuals of pathogen abundance in response to day of year of sampling for each pathogen, β_0 is the estimate of the model intercept, β_1 is the estimate of the change in pathogen abundance of Operation 2 compared to Operation 1, β_2 is the estimate of the change in pathogen abundance for each unit increase in frame count, and β_3 is the estimate of the change in pathogen abundance for each unit increase in mite count. $\gamma_{i(j)}$ represents a random effect for colony *i* sampled *j* times, and ε_i represents the residual error from the model. Coefficients (est) of the estimated linear model describe the magnitude and direction of associations between the residual abundance of individual pathogens (i.e., DWV, BQCV, KBV, LSV1, LSV2, *L. passim*, and *N. ceranae*) and other monitored factors throughout 2014. Even though many of these factors are correlated through time and geographic space, monitoring factors influencing pathogen abundance is nonetheless important to increase our overall understanding of variables influencing colony health.

Statistical Analysis of *Varroa destructor* Mite Infestation
with Binomial Regression

A generalized linear mixed effects model with a binomial family distribution and random effect for individual colony was used to estimate the odds that a colony will surpass the threshold of 3% mite infestation, after which treatment is recommended, in response to the date of sampling. Colonies were assigned a value of "1" when mite infestation surpassed 3%, and were assigned a value of "0" when *Varroa* mite infestation was less than 3%. The odds that a colony would

surpass the 3% mite infestation threshold were plotted in response to the day of year and a best fit line was fit to the data.

Multidimensional Statistical Analysis of Pathogen Compositions

We defined the pathogen composition of a honey bee colony as the abundance of seven pathogens (DWV, BQCV, LSV1, LSV2, KBV, *L. passim*, and *N. ceranae*) relative to all other samples. We used the Bray-Curtis index to create a dissimilarity matrix using the natural logarithm (ln) of pathogen abundance of seven pathogens. The relative composition of pathogens was visualized using nonmetric Multidimensional Scaling (NMDS). Relative differences in colony pathogen compositions were visualized using the five discrete categories of sampling events; January (California, before), March (California, during), June (Minnesota, after 1), August (Minnesota, after 2), and September (Minnesota, after 3).

We used a permutational analysis of variance (PERMANOVA) to test the ability of sampling event, mite count, and colony health to explain variation within the NMDS plot (Oksanen et al. 2007). The main effects as well as an interaction between sampling event and mite count were included in the reported PERMANOVA model, because mite count is likely to affect pathogen composition dependent on the time of the year of sampling due to the reproductive phenology of *V. destructor* mites. Colony health rating was included as a main effect in a separate PERMANOVA model, but was not considered a significant predictor. Additionally, we described any differences in the variability

of pathogen composition by sampling event using an analysis of multivariate homogeneity of group dispersions (betadisper) (Oksanen et al. 2007). Furthermore, we used a similarity percentage (SIMPER) analysis to describe the percent dissimilarity explained by each pathogen in comparisons between consecutive sampling events (Oksanen et al. 2007).

Statistical Software

All statistical analysis was performed using the software “R” (R version 3.1.3, “Smooth Sidewalk”) (Team 2013). Generalized linear models were conducted using the nlme package (Pinheiro et al. 2014) . NMDS plots were generated using the labdsv package while all other multivariate analyses were performed using the vegan package (Oksanen et al. 2007, Roberts 2007).

Results

Honey Bee Colony Monitoring and Pathogen Diagnostics

Commercially managed honey bee colonies that were transported from Minnesota to California for almond pollination were monitored from January 2014 to January 2015 (Figure 1)(Susan Kegley 2017). At each sampling event, colony health, using colony population size as a proxy for health, was monitored and samples of live honey bees were obtained for pathogen diagnostics, which included estimating *Varroa destructor* mite infestation levels. The prevalence and abundance of five commonly occurring viruses (i.e., Black queen cell virus (BQCV), Deformed wing virus (DWV), Lake Sinai virus 1 (LSV1), Lake Sinai virus

2 (LSV2), and Kashmir bee virus (KBV)) and two eukaryotic pathogens (the trypanosomatid *Lotmaria passim,* formerly known as *Crithidia mellificae sf*, and the microsporidial pathogen *Nosema ceranae*), were assessed in each sample using pathogen specific PCR and qPCR, respectively (Tentcheva et al. 2004, Daughenbaugh et al. 2015, Cavigli et al. 2016). Israeli acute paralysis virus (IAPV) was only detected in one sample using PCR, thus it was not included in further analysis. In this work, we use “pathogen prevalence” to indicate the number of different pathogens detected in a sample, and “pathogen abundance” as the estimated number of relative RNA transcripts for each pathogen using RT-qPCR (Supporting Table S2). Furthermore, we use “pathogen composition” to refer to the abundance of all co-infecting pathogens in a sample relative to all other samples. Therefore, both pathogen prevalence and abundance affect the pathogen composition of each colony throughout the course of the study.

Pathogen Prevalence

To examine the relationship between pathogen prevalence and colony health, the total number of pathogens detected in each sample, using PCR results for seven pathogens (i.e. the viruses DWV, BQCV, LSV1, LSV2, KBV, *L. passim,* and *N. ceranae*), and *V. destructor* mite infestation when levels were above the recommended treatment threshold of 3% (i.e., 3 mites per 100 bees) was averaged by colony health rating (i.e., weak, average, strong, or dead) (Supporting Table S2) (Currie and Gatien 2006, Lee et al. 2010, Giacobino et al. 2015, Traynor et al. 2016). Mean pathogen prevalence varied by colony health

rating (ANOVA, $F_{3,88}$ = 3.05, *p*-value = 0.03). Specifically, samples from dead colonies averaged 6.50 (SE +/- 1.50) pathogens, weak colonies 5.25 (SE +/- 0.38) pathogens, average colonies 5.38 (SE +/- 0.18) pathogens, and strong colonies 5.95 (SE +/- 0.13) pathogens (Supporting Table S2 and Supporting Fig S1). Strong rated colonies had higher pathogen prevalence than average rated colonies (Supporting Fig S1). No other pairwise comparisons of mean pathogen prevalence between colony health ratings were statistically different.

The mean pathogen number associated with each colony varied with the sampling date (ANOVA, $F_{4,87}$ = 21.31, *p*-value < 0.001) (Supporting Table S2 and Supporting Fig S2). Specifically, colonies sampled early in the year before almond pollination, averaged 5.00 (SE +/- 0.17) pathogens, and colonies sampled in March, immediately after almond pollination and thus representing the pathogen profile of colonies during the almond bloom, averaged 5.39 (SE +/- 0.09) pathogens. Colonies sampled when they were located in Minnesota averaged 7.09 (SE +/- 0.28) pathogens in early summer (i.e., after 1, June), averaged 6.43 (SE +/- 0.13) pathogens in late summer (i.e., after 2, Aug.), and averaged 6.00 (SE +/- 0.23) pathogens at the end of summer (i.e., after 3, Sept.) (Supporting Table S2 and Supporting Fig S2). Results from Tukey's HSD *post-hoc* tests indicate that the mean pathogen prevalence was greater in sampling events later in the year, when the colonies were located in Minnesota (after 1, 2, and 3) compared to sampling events in January and March, before and during

almond pollination, respectively, when the colonies were located in California (Supporting Fig S2).

Longitudinal Monitoring of Pathogen Composition

To examine the relationships between pathogen abundance and other monitored factors (i.e., colony health, sample date, percent mite infestation, and beekeeping operation) we performed mite counts at each sampling event and utilized qPCR to quantify the relative abundance of seven pathogens (i.e. DWV, BQCV, LSV1, LSV2, and KBV, *L. passim,* and *N. ceranae*) (Supporting Table S2). Quantitative PCR values ranged from 0 to $6.7x10^{8}$ per sample (Supporting Table S2). Evidence from an overall longitudinal assessment of the abundance of common honey bee pathogens at the colony level from January to September 2014 reveal that individual colonies both within a single beekeeping operation and between two typically managed commercial beekeeping operations varies from the beginning of the monitoring period in January, until the end of the monitoring period in June (Fig 2 and Supporting Fig S3). The pathogen abundance associated with each honey bee colony monitored from January 2014 to September 2014 for each pathogen assayed for within this study is represented in (Supplemental Figs. 3-9).

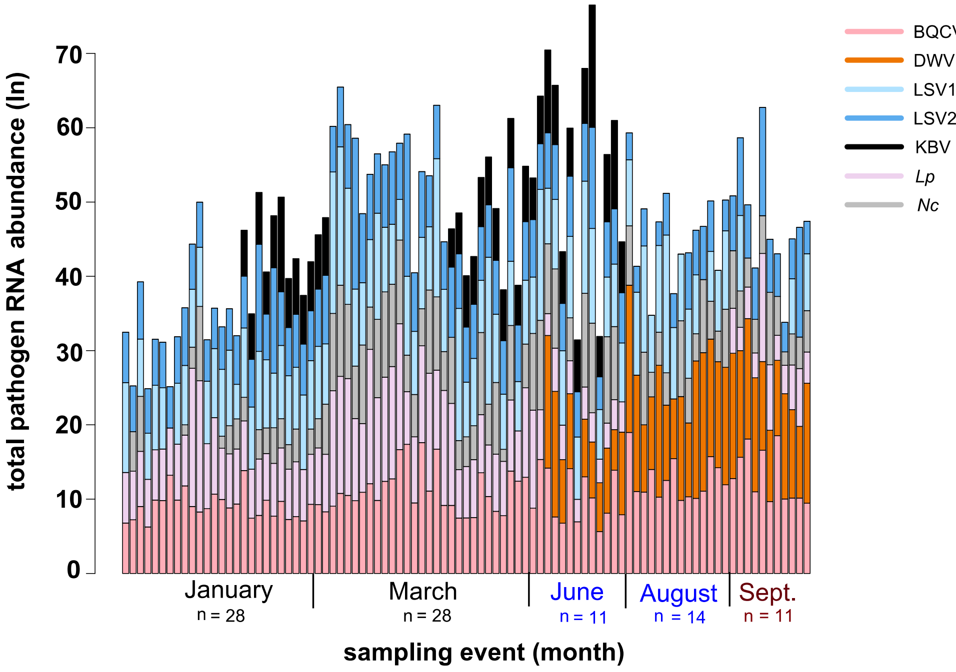


Fig 2. Longitudinal assessment of honey bee pathogen abundance at the colony level. Spatiotemporal change of the pathogen composition of individual honey bee colonies (designated by each vertical bar). In this sample cohort, the most prominent changes are attributed to the detection of DWV and reduced abundance of *L. passim* (*Lp*) in June, August, and September. Quantitative PCR was used to determine the relative pathogen RNA abundance. The natural log transformed data for each pathogen, BQCV (pink), DWV (orange), *Lp* (light pink), *Nc* (gray), LSV1 (light blue), LSV2 (dark blue), and KBV (black), is represented by the height of each segment of the vertical bar representing a sample from an individual colony (y-axis); the height of the bar represents the total pathogen abundance for each colony over the course of the study (January to September 2014, x-axis).

To investigate if weak and strong colonies had different pathogen compositions, we quantified the differences in pathogen prevalence and abundances between honey bee samples using a Bray-Curtis dissimilarity matrix.

The pathogen composition of each sample relative to all other samples were visualized using a non-metric multidimensional scaling (NMDS) plot. Each point represents the particular pathogen composition of a single sample and will appear closer to other points with similar pathogen compositions. The results of this analysis illustrated that pathogen compositions were most closely related by sampling date (Fig 3).

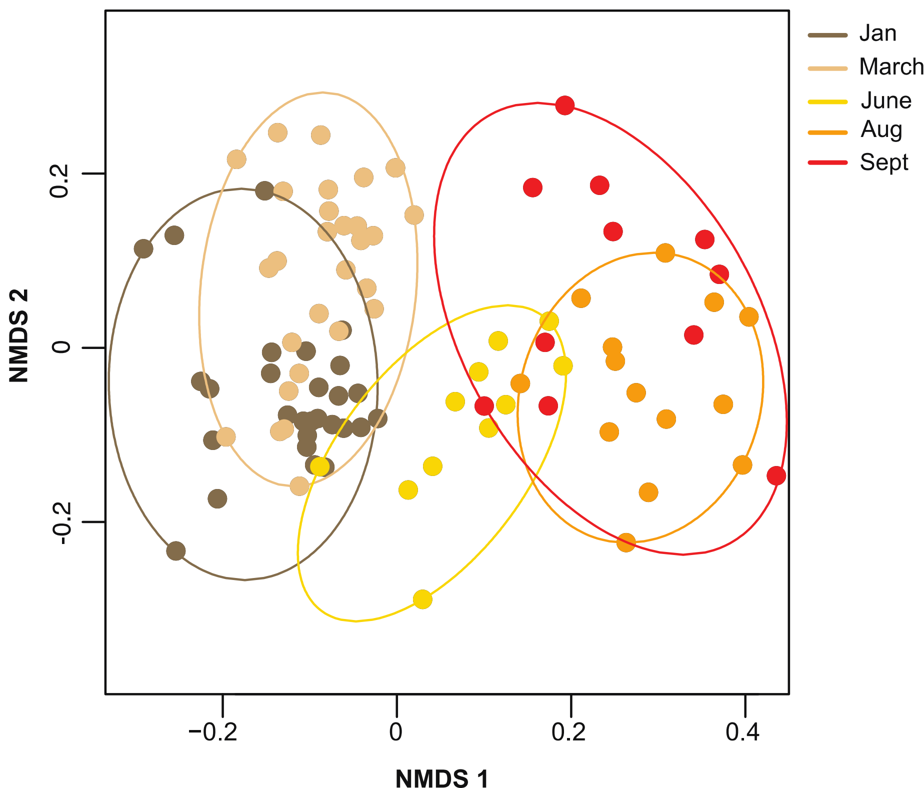


Fig 3. Relative pathogen composition of honey bee colonies visualized by sample event. Pathogen compositions of honey bee colonies form unique and defined clusters according to the month they were sampled (i.e., January - dark brown, March - light brown, June - yellow, August - orange, or September - red). The position of each point indicates the pathogen composition of each sample relative to all other samples (i.e. samples with more similar pathogen compositions are closer), calculated using a Bray-Curtis dissimilarity and plotted on a non-metric multidimensional scaling (NMDS) plot with an associated stress value of (0.197); the results from a permutational analysis of variance (PERMANOVA) in Table 2 indicated that the sampling event explained the most amount of variance in the pathogen composition.

While pathogen composition did not differ between colony health ratings (PERMANOVA, $F_{3,83}$ = 1.467, p = 0.14, R^2 = 0.018), a PERMANOVA indicated that sampling date significantly explained the most variation in pathogen composition (Table 1, R^2 = 0.61, p < 0.001), followed by levels of *Varroa*

destructor mite infestation (Table 2, $R^2 = 0.01$, $p < 0.001$), indicating pathogen composition varies seasonally and with changing levels of mite abundance (Oksanen et al. 2007).

Table 1: Analysis of variance of pathogen composition. The relative pathogen composition of honey bee samples, based on all pathogen abundances, is primarily explained by the date the sample was obtained (or "day of year") and level of *Varroa* mite infestation. Results from a permutational analysis of variance (PERMANOVA) indicate that the day of year ($R^2 = 0.61$), followed by the percent mite infestation ($R^2 = 0.01$) explained the most variation in the relative pathogen composition of all honey bee samples; factors contributing significantly to the relative pathogen composition are indicated by *p*-values $\leq$ to 0.05 in bold

Source	**d.f.**	***F*-Statistic**	$\mathbf{R^2}$	***p*-value**
day of year	4	34.93	0.61	**<0.01**
mite infestation (%)	1	2.69	0.01	**0.05**
sampling event x *mite infestation (%)*	4	0.81	0.01	0.63
residuals	83	-	0.36	
Total	92	-	1.00	

To identify the specific pathogens that contributed the most to the changes in pathogen composition between sampling events, a SIMPER analysis was used to calculate the contribution of each pathogen to the dissimilarity of pathogen compositions between consecutive sampling events (Table 2) (Oksanen et al. 2007). The most difference in pathogen composition of honey bee colonies between each consecutive sampling event was explained by a different pathogen, highlighting the dynamic nature of honey bee colony pathogen composition. Furthermore, DWV contributed the most to the differences between pathogen compositions of honey bee colonies when the

mean difference in *V. destructor* mite infestation was greatest (Table 3), potentially underscoring the role of high levels of *Varroa* in the transmission of DWV.

Table 2: Similarity percentage analysis of the relative pathogen composition between consecutive sampling events. The greatest differences in honey bee colony pathogen composition was explained by changes in abundance of a different pathogen between consecutive sampling events. The percent difference attributed to each pathogen between consecutive sampling events was calculated using a similarity percentage (SIMPER) analysis of a Bray-Curtis dissimilarity matrix. The cumulative difference of the top three pathogens in comparisons between consecutive sampling events is reported as a percentage. Furthermore, the difference in percent mite infestation was calculated in comparisons between consecutive sampling events. When the change in mean mite infestation was greatest, DWV accounted for the most difference in honey bee colony pathogen composition.

Sampling Event	***Pathogen 1***	***Pathogen 2***	***Pathogen 3***	***Cumulative Dissimilarity (%)***	***Δ mean mite infestation (%)***
Jan-Mar	*Nc*	*Lp*	LSV1	60.0	0.02
Mar-Jun	DWV	*Lp*	KBV	58.0	6.19
Jun-Aug	KBV	DWV	*Nc*	57.0	3.01
Aug-Sept	LSV1	*Lp*	LSV2	63.0	-5.09

Results from a multivariate homogeneity of group dispersions analysis (betadisper) indicated that the dispersion of pathogen composition among sampling events did not change ($F_{4,87}$ = 0.27, *p*-value = 0.90) (Oksanen et al. 2007). Together, this analysis indicated that the composition of honey bee associated pathogens is most influenced by sampling date and the level of mite infestation.

<u>*Varroa destructor*</u>

V. destructor mites are a major contributor to honey bee colony mortality (Currie and Gatien 2006, vanEngelsdorp et al. 2009). In total, 21 colonies in this sample cohort died by the end of the sampling period in January 2015. From January 2014-January 2015, 22 of the monitored colonies surpassed the 3% mite infestation threshold, and of those colonies that died, 40.9% (9/22) of colonies surpassed the 3% mite infestation threshold by the end of the sampling period in January 2015. However, the percentage of colonies that had at some point in the study surpassed the 3% mite infestation threshold and died differed by beekeeping operation; Beekeeping Operation 1 experienced 22.2% loss (i.e., 2 of 9 colonies with > 3% mite infestation) and Beekeeping Operation 2 experienced 53.8% loss (i.e., 7 of 13 colonies with > 3% mite infestation) (Supporting Table S2). It is notable that both beekeeping operations had numerous colonies with high levels of mite infestation, despite application of several anti-mite treatments including amitraz, oxalic acid, and formic acid over the course of the sampling period (see Methods).

V. destructor mite infestation increased over the course of this study. Specifically, honey bee samples from colonies early in the year, when the colonies were located in California, harbored a mean mite infestation of 0.45% (mean = 0.45, SE +/- 0.09, n = 56) (Supporting Table S2). Whereas, honey bee samples obtained from colonies later in the year, when the colonies were located in Minnesota after almond pollination (i.e. after1, after2, and after3), harbored a

7.17% mean mite infestation (mean = 7.17, SE+/- 1.21, n = 36) (Supporting Table S2). To evaluate the risk *Varroa* mites pose to colonies throughout the sampling period, results from a generalized linear mixed effects model (GLMM) with a binomial family distribution demonstrate that the odds of a colony exceeding the recommended treatment threshold level of 3% *Varroa* mite infestation is $e^{.26}$ (1.3 ± SE 1.07) times higher with each increase in the day of the year (log-odds of surpassing threshold, est = 0.26) (Fig 4 and Table 3). These results indicate that honey bee colonies within this sample cohort frequently accumulated higher levels of mites towards the end of the pathogen monitoring period (Fig 4), consistent with the typical mite population growth pattern that tracks the seasonal colony buildup and decline over the course of a year.

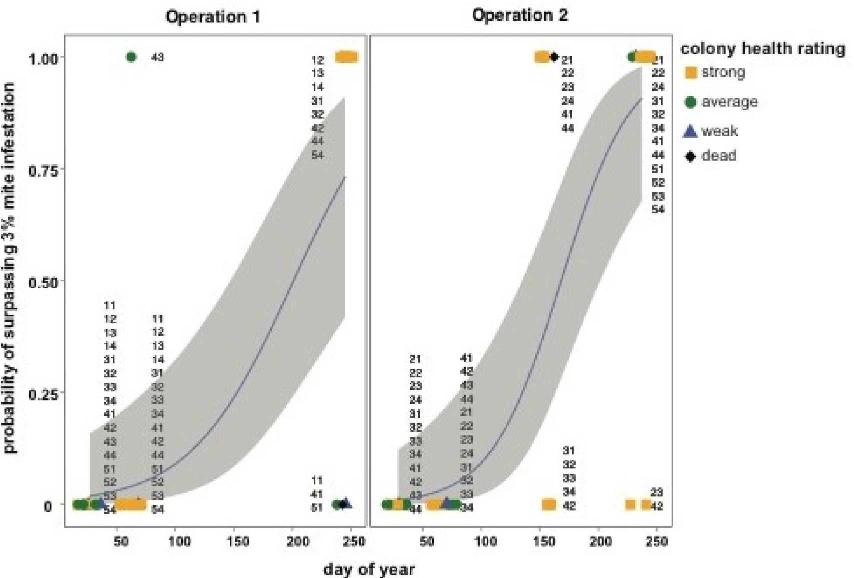


Fig 4. The probability of honey bee colonies accumulating *Varroa destructor* mite densities above the threshold recommended for treatment is greater later in the year. Honey bee colonies have a high probability of accumulating levels of *Varroa destructor* mites that surpass the threshold for recommended treatment (i.e., 3%) by the end of the pathogen monitoring period in both beekeeping operations. Results from a generalized linear mixed effects model with a binomial family distribution indicate that by the end of the sampling period, colonies had a 62.5% chance of crossing the recommended treatment threshold. Since mite count data differed between beekeeping operations, binomial regression results were plotted independently. Mite count data obtained from honey bee samples collected from colonies rated dead (black diamonds), weak (blue triangles), average (green circle), and strong (yellow square) are shown with their unique colony identifier numbers, with the first digit identifying the pallet and the second digit identifying individual colony. A best-fit line (blue) with odds-estimates surrounded by upper and lower standard error estimates (gray) depicts the odds of a colony surpassing the recommended treatment threshold (y-axis), which increases with the day of the year (x-axis).

Residual Pathogen Abundance

Individual pathogen abundance was consistently affected by the sample date (“day of year”), (Supplemental figures 3-9), therefore we further examined the relationship between pathogen abundance and other factors (e.g., beekeeping operation, colony health, and mite infestation) after removing the confounding effects of sample date on pathogen abundance. In order to remove the confounding effects of sample date on pathogen abundance, we examined the relationship between the residuals of a linear regression of pathogen abundance in response to “day of year” (i.e., residual abundance), and used residual abundance as the response variable in multiple linear regression to evaluate associations with residual abundance and beekeping operation, mite infestation percentage, and colony health, which was represented in the models as frame count (Table 3).

Table 3: Pathogen abundance model summaries.
Multiple linear regression models were utilized to identify associations between colony-level residual pathogen abundance and the factors monitored in this study (i.e., colony health, percent mite infestation, and beekeeping operation) throughout the monitoring period from January to September 2014. Residual pathogen abundance was used as the response variable in the models to remove the confounding effect of seasonality on pathogen abundance in order to investigate associations between residual pathogen abundance and other covariates. Associations between the residual abundance of each pathogen assayed in this sample cohort and other covariates are denoted by the magnitude and direction of the estimate listed in the table. Operation 2 estimates the residual pathogen abundance in comparison to Operation 1, frame count was used as a continuous variable as an estimate of colony health, and mite infestation (%) represents the number of mites counted per 100 bees. The standard error, t-value, and p-value for each estimate of coefficients (est.) within the final model are provided; *p*-values <0.05 are indicative of significant associations (bold).

BQCV	***estimate***	***SE (+/-)***	***t-value***	***p-value***
intercept	0.47	0.93	0.50	0.612
operation 2	-1.51	0.68	-2.23	**0.03**
frame count	0.01	0.07	0.16	0.87
mite infestation (%)	0.06	0.06	1.06	0.29
DWV	***estimate***	***SE (+/-)***	***t-value***	***p-value***
intercept	0.42	0.77	0.55	0.59
operation 2	-0.37	0.52	-0.71	0.48
frame count	-0.09	0.06	-1.49	0.15
mite infestation (%)	0.23	0.05	4.84	**<0.001**
LSV1	***estimate***	***SE (+/-)***	***t-value***	***p-value***
intercept	-1.52	1.38	-1.10	0.27
operation 2	2.27	0.95	2.38	**0.02**
frame count	0.03	0.10	-0.33	0.74
mite infestation (%)	-0.02	0.08	-0.23	0.82
LSV2	***estimate***	***SE (+/-)***	***t-value***	***p-value***
intercept	-0.54	0.98	-0.54	0.59
operation 2	-0.41	0.68	-0.61	0.55
frame count	0.09	0.08	1.19	0.24
mite infestation (%)	-0.07	0.06	-1.14	0.26
KBV	***estimate***	***SE (+/-)***	***t-value***	***p-value***
intercept	-1.59	0.82	1.94	0.06
operation 2	5.73	0.56	-1.95	**<0.001**

Table 3 Continued

frame count	-0.11	0.06	-1.74	0.09
mite infestation (%)	-0.09	0.05	-1.91	0.06
L. passim	***estimate***	***SE (+/-)***	***t-value***	***p-value***
intercept	-0.53	1.02	-0.52	0.61
operation 2	-3.54	0.70	-5.07	**<0.001**
frame count	0.23	0.08	2.94	**0.005**
mite infestation (%)	-0.01	0.06	-0.19	0.84
N. ceranae	***estimate***	***SE (+/-)***	***t-value***	***p-value***
intercept	-1.92	1.22	-1.57	0.12
operation 2	0.20	0.83	0.24	0.81
frame count	0.18	0.09	1.99	**0.05**
mite infestation (%)	-0.06	0.07	-0.75	0.46
total abundance	***estimate***	***SE (+/-)***	***t-value***	***p-value***
intercept	-0.33	1.02	-0.33	0.75
operation 2	-1.49	0.73	-2.05	**0.05**
frame count	0.08	0.08	1.00	0.32
mite infestation (%)	0.10	0.06	1.64	0.11
odds surpassing Varroa threshold	***estimate***	***SE (+/-)***	***t-value***	***p-value***
Intercept	-51.02	14.35	-3.56	**<0.001**
day of year	0.26	0.07	3.66	**<0.001**

Concurrent with increased *V. destructor* mite levels were increased Deformed wing virus (DWV) prevalence and abundance (Figs 2, and 5). All the honey bee samples in this sample cohort (n = 92) were tested for the presence of DWV using PCR (Supporting Table S2). Interestingly, none of the samples collected in January and March in California (n = 56) were DWV positive, whereas 96.4% of samples collected in Minnesota (n = 36) were positive for DWV. Quantitative PCR was utilized to determine the relative DWV abundance in each sample (n = 92), which in general increased over the course of the monitoring period (Fig 5 and Supporting Table S2). Multiple linear regression was used to investigate associations between residual DWV abundance with the predictor of interest, frame count (i.e., colony strength), while also accounting for beekeeping operation and level of mite infestation, finding that median residual DWV abundance increased by $e^{0.23}$ (126%) for each unit increase in mite infestation after accounting for beekeeping operation and frame count (Table 3).

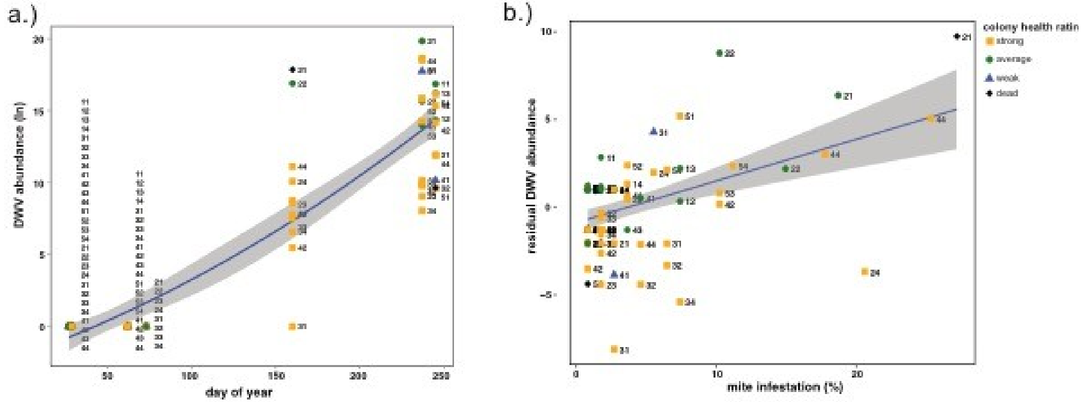


Fig 5. DWV abundance was greatest in honey bee samples with higher mite infestation levels, obtained later in the year (June through September). A.) The natural log transformed values of the relative DWV RNA abundance as determined by qPCR (y-axis) analysis of honey bee samples obtained from colonies with varying colony health ratings (i.e., dead (black diamonds), weak (blue triangles), average (green circle), and strong (yellow square)); the date of sample collection (i.e. day of year, x-axis). The best-fit line (blue), surrounded by upper and lower standard error estimates (gray), indicates that DWV abundance increased at an exponential rate across the longitudinal monitoring period. Unique colony identifier numbers, with the first digit identifying the pallet and the second digit identifying individual colony, label each point on the graph and illustrate changes in the pathogen abundance individual colonies throughout the sampling period. B.) The residual abundance of the natural log transformed values of DWV RNA abundance (y-axis) increases as the level of mite infestation (number of mites counted per 100 bees, x-axis) increases.

KBV was only detected in Operation 2, and as a result residual abundance was estimated in the model to be greater within Operation 2 when compared to Operation 1, while also accounting for frame count and mite infestation percentage (Table 3). While not significant, evidence was found for a negative association between residual KBV abundance and levels of mite infestation

(mites, est = -0.09, SE = 0.06, *p*-value = 0.06) (Table 3). Correlations between KBV, DWV, and mites are interesting, but an expected result since *V. destructor* mites serve as vectors of both DWV and KBV (Chen et al. 2004, Shen et al. 2005, Nazzi and Le Conte 2016).

The prevalence and abundance patterns of other viruses did not consistently associate with the levels of *Varroa destructor* mite infestation. LSV2 is a member of the recently discovered and described Lake Sinai virus group, which is prevalent, abundant, and globally distributed (Runckel et al. 2011, Granberg et al. 2013, Ravoet et al. 2013, Cepero et al. 2014, Daughenbaugh et al. 2015, Cavigli et al. 2016). Consistent with other studies, LSV2 abundance was higher in weak colonies, as compared to healthy colonies with greater bee populations in this sample cohort (Cornman et al. 2012, Daughenbaugh et al. 2015). While we did not find evidence for an association between LSV2 abundance and frame count (used as an estimate of colony health), when categorizing colonies into health ratings based on frame count within models, the median residual LSV2 abundance was $e^{7.39}$ (161970.60%) greater in “dead” colonies compared to “weak” rated colonies (t-test, $t\text{-value}_{56} = 3.02$, $p = 0.004$), while also accounting for the effect of beekeeping operation and mites. However, samples were only able to be collected from two of the 22 colonies that died throughout the entire monitoring period. Despite the close phylogenetic relationship of LSV1 and LSV2 (Daughenbaugh et al. 2015), changes in LSV1 abundance were associated with monitored factors that were different from those

that correlated with LSV2 abundance. Although LSV1 was similarly widespread, testing positive in 86.9% of samples (80/92) (Supporting Table S2), median residual LSV1 abundance was $e^{2.27}$ (967.94%) times greater in operation 2 compared to operation 1 when also accounting for frame count and mite infestation (Operation 2, est = 2.27, SE ± 0.95, p = 0.02) (Table 3) (Supporting Fig S6)

Unexpectedly, the residual abundance of *L. passim* and *N. ceranae* increased with colony health, using colony population size as a proxy for health (Table 3). The median residual abundance of *L. passim* increased by $e^{0.23}$ (125%) with each unit increase in frames counted, and the median residual abundance of *N. ceranae* increased by $e^{0.18}$ (119.72%) in response to each unit increase in frames counted when also accounting for the effects of beekeeping operation, and mites (Table 3).

In a similar method, changes in colony health status between time points was investigated by regressing a colonies' change in frame count between sampling events (Δ-Frame Count) in response to pathogen abundance, after accounting for change in frame count explained by "day of year" (Supporting Fig. S10). A negative correlation was observed between KBV pathogen abundance and frame count independent of the day of year of sampling, however no other pathogen correlated with changes in frame count independent of the day of year of sampling (Supporting Fig. S10).

Beekeeping Operation

The abundance of each pathogen associated with samples obtained from monitored honey bee colonies managed by two commercial beekeeping operations was plotted as function of time (i.e., day of the year) (Supporting Figs S3-S9). Together these data illustrate the similarities and differences in pathogen loads over the course of this study, and between colonies managed by different beekeeping operations, which encompasses numerous confounding variables including precise geographic location, intensities of pathogen exposures, honey bee colony genetics, the availability and quality of bee forage, and specific management practices, all of which may impact colony health and pathogen prevalence and abundance. In this sample cohort, we found evidence supporting differences in the abundances of BQCV, LSV1, KBV, and *L. passim,* and the total pathogen abundance between beekeeping operations (Table 3). However, a large gap in data between March and August for each beekeeping operation within this sample cohort cannot identify apiary management as a causative factor contributing to pathogen abundance. Regardless, some evidence was found to support pathogen abundances differing between beekeeping operations, highlighting that apiary management should be considered in future studies as a factor contributing to pathogen abundance in honey bee colonies, and thus reflected in study design.

Pairwise Interactions Between Pathogens

Honey bee colonies are infected by a suite of pathogens that fluctuate in prevalence and abundance over the course of a year (vanEngelsdorp et al. 2009, Cornman et al. 2012, Ravoet et al. 2013, McMenamin and Genersch 2015, Cavigli et al. 2016). To investigate pairwise interactions between the pathogens monitored in this study, we generated a correlation matrix illustrating the correlation coefficients (R-values) that quantify the strength and direction of the relationship between pathogen abundance data (Fig 6). The strongest positive correlation, observed in this sample cohort, was between *V. destructor* mites and DWV (R = 0.70, *p*-value < 0.001). While DWV and *L. passim* (R = -0.65, *p*-value < 0.001), followed by *V. destructor* mites and *L. passim* (R = -0.49, *p*-value < 0.001) demonstrated the strongest negative correlation (Fig 6).

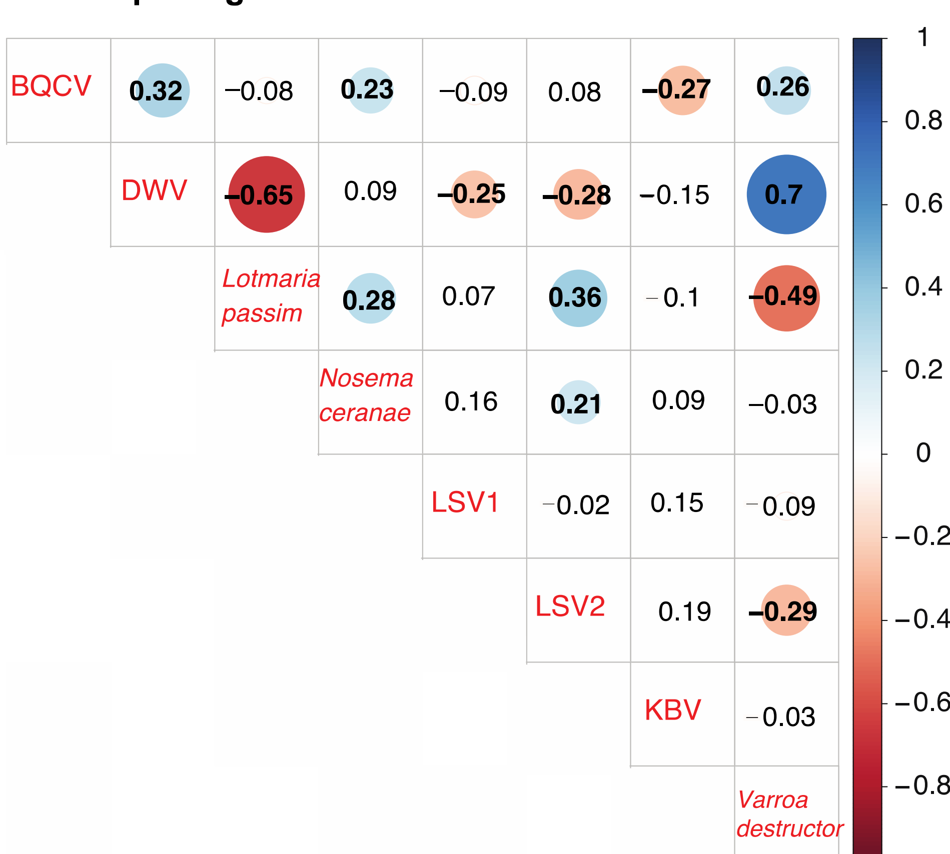


Fig 6. Pathogen co-infection correlation matrix. The abundance of pathogens in co-infected colonies was analyzed by calculating the correlation coefficients for each pair-wise comparison, which are listed in each cell. Correlation coefficients (reported as *r* values) quantify the strength and direction of the changes in pathogen abundance between pairs of pathogens. The shaded red circles represent negative correlations and blue circles represent positive correlations, darker hues and larger circles indicate stronger correlations, and bold numbers indicate significant correlations (p-value<0.05). DWV and *Varroa destructor* mites had the strongest positive correlation, while DWV and *L. passim* displayed the strongest negative correlation

Discussion

Honey bees are important pollinators of numerous agricultural crops, which are valued globally at $153 billion annually (Calderone 2012). In conjunction with an increase in the worldwide demand for pollinators, the percentage of annual honey bee colony losses has also increased. Particularly in the US, average annual overwinter losses of honey bee colonies has averaged 33%, since 2006, and can surpass 50% annually (Lee et al. 2015, Traynor et al. 2016). Several studies have demonstrated that weak, diseased, and/or Colony Collapse Disorder (CCD)-affected honey bee colonies have a greater pathogen burden than healthier and/or surviving colonies in the US (Cox-Foster et al. 2007, vanEngelsdorp et al. 2009, Li et al. 2011, Cornman et al. 2012, Chen et al. 2014, Daughenbaugh et al. 2015), Canada (van der Zee et al. 2012), Austria (Berenyi et al. 2006), Denmark (Nielsen et al. 2008), Luxembourg (Clermont et al. 2014), and Belgium (Ravoet et al. 2013), but few of these studies have temporally monitored individual honey bee colonies. Instead, most studies have been performed monitoring projects at the apiary level and/or are based on voluntarily submitted samples and survey data. Monitoring pathogen prevalence and abundance across a spatiotemporal gradient and developing an understanding of the typical pathogen prevalence is important to understanding the role of pathogens on honey bee colony health.

To further investigate the impact of pathogens on honey bee colony health and the factors that influence pathogen prevalence and abundance, we

monitored Minnesota-based honey bee colonies involved in California almond pollination. Over the course of nine months, colony health and the prevalence and abundance of a subset of pathogens, including viruses (i.e., BQCV, DWV, LSV1, LSV2, and KBV), the trypanosomatid *L. passim*, the microsporidia *N. ceranae*, and *V. destructor* mites, were monitored.

V. destructor mites are a major contributor to honey bee colony losses (Currie and Gatien 2006, vanEngelsdorp et al. 2009, Genersch 2010b, Genersch et al. 2010, Nazzi and Le Conte 2016). Similar to previous work, our results indicate that the seasonal abundance of *V. destructor* mite infestation impacts pathogen composition, specifically DWV (Ryabov et al. 2014, Mordecai et al. 2016, Traynor et al. 2016). *Varroa* mite infestation increased over the course of this study, and consequently the majority of colonies surpassed the 3% mite infestation threshold considered harmful to colony health (Currie and Gatien 2006, vanEngelsdorp et al. 2009, Giacobino et al. 2015, Traynor et al. 2016). Much of this increase is likely attributable to the coupled phenologies of mite and honey bee reproduction. Mites reproduce in cells containing developing honey bee larvae. As a result, *V. destructor* mite reproduction increases as honey bee colonies build up brood in March-June (Nazzi and Le Conte 2016). While bee populations decline towards the end of the summer in temperate climates, *V. destructor* mite infestation levels generally increase. The combination of the ability of *V. destructor* mites to vector viruses such as DWV, KBV and IAPV, and their close association with fluctuations in honey bee colony populations

underscore the importance of the need to monitor all of these biotic factors to better understand their independent and synergistic impacts on colony health.

The ability of *V. destructor* to vector DWV may explain the sudden detection of DWV in June and rapid accumulation in honey bee colonies through September (Fig 2). Pathogens with the ability to be vectored by *V. destructor* mite increased throughout the year in response to changes in *V. destructor* infestation, predominately influencing pathogen communities (Traynor et al. 2016). For example, when changes in mite infestation levels between consecutive sampling events were greatest, DWV accounted for the most difference in pathogen composition. These results are in agreement with previous studies demonstrating that *V. destructor* supports DWV replication (Shen et al. 2005, Nazzi and Le Conte 2016) and vectors KBV (Chen et al. 2004). Additionally, data from this sample cohort indicated that levels of KBV abundance is also associated with levels of *V. destructor* mite infestation, though the dynamics of KBV abundance differed from DWV. KBV increased to a maximum level in mid-summer after which levels decreased, indicating that abundance varied primarily in response to the day of year. While the exact role of mites in KBV infection dynamics has not been as extensively investigated, positive correlations between KBV abundance and mite infestation levels suggest an association between mites and KBV abundance (Chen et al. 2004, Shen et al. 2005, Nazzi and Le Conte 2016).

In contrast, several other pathogens, including some viruses, were either not associated with or negatively associated with changes in percent *V. destructor* mite infestation. For example, the abundances of Black queen cell virus, Lake Sinai virus 1, Lake Sinai virus 2, *L. passim*, *N. ceranae* were not associated with changes in *V. destructor* mite infestation levels. Furthermore, there was no evidence to suggest a significant correlation between Lake Sinai virus 2 abundance and increased mite levels. This relationship is interesting given that research to date suggests that mites may support LSV2 virus replication and in turn serve as active vectors of LSV2, though the importance of this transmission route, as compared to other routes of virus transmission requires further investigation (Ravoet et al. 2013, Daughenbaugh et al. 2015). Together, the data herein demonstrate that the association between pathogen levels, including some viruses, and mite infestation levels is not universally positively correlated (Traynor et al. 2016). Therefore, reducing mite levels in honey bee colonies (i.e., via acarcide treatment or establishing a broodless period), which is typically advised to reduce virus abundance, may not result in the reduction of all pathogens, including some viruses.
(Ravoet et al. 2013, Daughenbaugh et al. 2015)

Though *V. destructor* mite infestation levels influenced colony pathogen composition, high percentages of *V. destructor* mite infestation (i.e., ranging from 4 – 28%) did not correlate with weak colony health ratings within samples obtained from 2014. This result was unexpected based on the large body of

literature demonstrating that poor colony health and colony losses correlate with high mite levels (i.e., over the 2-3%) (Currie and Gatien 2006, vanEngelsdorp et al. 2009, Genersch 2010b, Giacobino et al. 2015, Traynor et al. 2016). However, 40.09% of the 22 colonies that surpassed the 3% mite threshold in 2014, died in 2015 (2 colonies from Operation 1, and 7 colonies from Operation 2) (Supporting Table S2). Despite beekeeper applications of amitraz, oxalic acid, and formic acid throughout the monitoring period, colonies were still likely to accumulate harmful levels of mites. Thus, results from our small observation cohort study, combined with previous studies suggest that *V.* destructor mites infestation negatively influences colony health, though examining the percent mite infestation alone, particularly at lower levels of infestation, is likely insufficient to understand the impact on colony health. The synergistic impacts of mite infestation and virus infection are best studied for DWV strains (i.e., DWV-A and DWV-B) and recent studies suggest that viruses with enhanced virulence to honey bees may be preferentially transmitted by *V. destructor* mites (Martin et al. 2012, Ryabov et al. 2014, McMahon et al. 2016, Nazzi and Le Conte 2016).

Counterintuitively, the abundances of *L. passim* and *N. ceranae* were greater in strong rated colonies, rather than in weak rated colonies. These results differ from previous studies demonstrating that *N. ceranae* influences co-infections of key pathogens to colony health decline in the US (vanEngelsdorp et al. 2009, Cornman et al. 2012, Nazzi et al. 2012), or as the causative agent of colony loss in Spain (Martin-Hernandez et al. 2007, Higes et al. 2008). In fact,

within our cohort, *N. ceranae* abundance did not strongly correlate with the abundance of any pathogen included in this study (Cornman et al. 2012, Nazzi et al. 2012). Inconsistent results between studies can be mitigated by analyzing data from multiple sample cohorts to more fully understand the role of pathogens in colony health.

In the observational sample cohort described herein, levels of LSV2 were higher in colonies that died (when analyzed as a categorical variable), indicating a potential association between LSV2 and colony loss, as previously shown (Cornman et al. 2012, Daughenbaugh et al. 2015). However, the number of colonies that died within the pathogen monitoring period of this study and from which we obtained honey bee samples was small (n = 2), and a universal association between LSV2 and poor colony health has not been observed (Cavigli et al. 2016) warranting further investigation of the factors affecting LSV2 prevalence and abundance and the effects of LSV2, and other members of the Lake Sinai virus group, on honey bee colony health.

The abundance of BQCV, LSV1, KBV, *L. passim* and total pathogen abundance were different between beekeeping operations and results were presented independently when evidence was found for beekeeper as a contributory factor to pathogen abundance. Importantly, sampling inconsistencies for each beekeeping operation should restrict inferences from these findings, however these results still provide some evidence that apiary management can be a contributing factor to pathogen abundance and should be considered in

future experimental design. Black queen cell virus, *L. passim*, and total pathogen abundance were more abundant in samples collected from colonies managed by beekeeping Operation 1, as compared to beekeeping Operation 2, while LSV1 and KBV was less abundant in the samples from beekeeping Operation 1, as compared to beekeeping Operation 2. The specific beekeeping operation affected pathogen abundances within other US cohorts (Cavigli et al. 2016); however, this was not observed in colonies monitored as part of the German Bee Monitoring Project (Genersch et al. 2010). Additionally, in other studies, apiaries affected by CCD had more weak colonies with higher pathogen prevalence within closer proximity to other weak colonies with high pathogen prevalence (vanEngelsdorp et al. 2009). Future investigations aimed at better understanding the mechanisms and dynamics of pathogen transmission between colonies (e.g., via mites, foraging/pollen, robbing, beekeeper mediated) are important to developing strategies to reduce inter-colony transmission.

Temporal fluctuations in pathogen prevalence and abundance in this study and other studies (Tentcheva et al. 2004, Genersch et al. 2010, Runckel et al. 2011, Cavigli et al. 2016, Traynor et al. 2016) underscore the importance of monitoring projects to evaluate the effects of pathogens and other factors at the colony level. The majority of the variation in pathogen composition (61%) was explained by sampling event, potentially describing the seasonality associated with honey bee pathogens (Tentcheva et al. 2004, Genersch et al. 2010, Runckel et al. 2011, Cavigli et al. 2016, Traynor et al. 2016). Therefore, it is important to

consider the day of year as a confounding variable affecting pathogen abundance and composition, in order to appropriately evaluate the effects of other factors affecting pathogen abundance and colony health. While general trends in pathogen prevalence and abundance are important to form a baseline understanding of pathogen dynamics, the rapid onset of individual pathogens, such as DWV, which within this cohort predominates pathogen compositions after March, could signal times and locations when colonies become subject to specific threats, which if detected in a timely fashion could perhaps be mitigated.

Commercially managed, migratory honey bees are faced with by a diverse set of factors that potentially affect colony health. In addition to pathogens, other factors including seasonal variation in colony size, foraging requirements, nutrition, and pesticide exposure all impact colony health (Chen and Siede 2007, vanEngelsdorp et al. 2009, Degrandi-Hoffman and Chen 2010, Singh et al. 2010, McMenamin et al. 2016). Therefore, temporal monitoring projects that emphasize robust sampling designs with large sample sizes, colonies consistently sampled at a fine-scaled daily instead of coarse seasonal resolution and multiple monitored factors can fully describe both general and acute threats to honey bee colonies throughout the year and in the context of pollination of specific crops. Additionally, the synergistic effects of co-infecting honey bee pathogens requires a multidimensional approach to study the effect of honey bee pathogen prevalence and abundance on colony health, as numerous pathogens co-infect honey bees (Chen and Siede 2007, McMenamin and Genersch 2015), and can

influence the prevalence and abundance of other pathogens (Cornman et al. 2012, Nazzi et al. 2012, Ravoet et al. 2013, Traynor et al. 2016). The combination of sampling individual honey bee colonies on specific days of the year, across multiple sites, and employment of statistical methods aimed at integrating and interpreting multifactorial data sets will further our understanding of the factors with the greatest influence on colony losses and may result in the development of strategies to limit future losses.

Acknowledgements

This research was supported by the National Honey Board and in addition the Flenniken Laboratory is supported by the United States Department of Agriculture National Institute of Food and Agriculture, Agriculture and Food Research Initiative (USDA-NIFA-AFRI) Program, Montana Department of Agriculture Specialty Crop Block Grant Program, the National Institutes of Health IDeA Program COBRE grant GM110732, National Science Foundation EPSCoR NSF-IIA-1443108, Hatch Multistate Funding (NC-1173), Project *Apis m.*, the Montana State Beekeepers Association, Montana State University, and the Montana State University Agricultural Experiment Station. We would like to thank members of the Flenniken laboratory (Laura Brutscher and Alex McMenamin) and the Pesticide Research Institute (Geoffrey W. Marcy) for reviewing this manuscript prior to publication and Dr. Kezia Manlove (Washington State University USGS/USDA) for statistical consultation.

Supporting Tables

Supporting Table S1: Primers used in this study

Genome / Gene Name	NCBI # GI #	Primer Name	Sequence (5'-3')	Product Size (bp)	Reference
Ribosomal protein L8 (*Apis m.*)	XM_393671.5 GI:571556074	Rpl8Fw Rpl8Rev	TGGATGTTCAACAGGGTTCATA CTGGTGGTGGACGTATTGATAA	121	Evans et al. (2006) Insect Mol Bio
Lake Sinai virus 1 (LSV1)	HQ871931 GI:335057596	qLSV1-F-2569 qLSV1-R-2743**	AGAGGTTGCACGGCAGCATG GGGACGCAGCACGATGCTCA	174	Runckel, Flenniken (2011) PLoS One
Lake Sinai virus 2 (LSV2)	HQ888865 GI:335057589	qLSV2-F-1722 qLSV2-R-1947**	CGTGCTGAGGCCACGGTTGT GCGGTGTCGATCTCGCGGAC	225	Runckel, Flenniken (2011) PLoS One
Black queen cell virus (BQCV)	AF183905 GI:8100530	qBQCVorf2F_6664 qBQCVorf2R_6805	TCCTCAAATCTGGAGCGAAC GTATTCGCTGGCCGTAAAAC	141	Runckel, Flenniken (2011) PLoS One
Deformed wing virus (DWV)	AY292384.1 GI:31540603	DWVfw1165 DWVrev1338	CTTACTCTGCCGTCGCCCA CCGTTAGGAACTCATTATCGCG	173	Chen et a. (2005) J Invert Path
Israeli acute paralysis virus (IAPV)	NC_009025.1 GI:126010924	IAPV_F_7762 IAPV_R_7876	GCAGCTATTTTTGGCTGGTC CCAATGTACGCTCATATCG	114	Daughenbaugh, et al. (2015) Viruses
Kashmir bee virus (KBV)	NC_004807.1 GI:30793779	KBV_F_4470 KBV_R_4581	TCGACAAGGACATGATCGAG GAGCCACAAATGGCTTCTTC	111	Stoltz et al. (1995) J Apicult Res
Lotmaria passim / (Crithidia mellificae)	PRJNA78249 AHIJ01002555 Contig 777	qCrFw1 qCrRev1	TCCACTCTGCAAACGATGAC GGGCCGAATGGAAAAGATAC	153	Runckel, Flenniken (2011) PLoS One
P. larvae	PRJNA30269	PL2-Fw PL2-Rev	CGGGAGACGCCAGGTTAG TTCTTCCTTGGCAACAGAGC	380	Marinez et al 2010; Marinez et al 2011
M. plutonius	PRJDA73165	MelissoF MelissoR	CAGCTAGTCGGTTTGGTTCC TTGGCTGTAGATAGAATTGACAAT	796	Roetschi et al 2007; Roetschi et al 2008
N. ceranae	DQ673615.1 GI:110293152	N ceranae F-4186 N ceranae R-4435	CGGATAAAAGAGTCCGTTACC TGAGCAGGGTTCTAGGGAT	249	Chen et al. (2008) J Inv Path

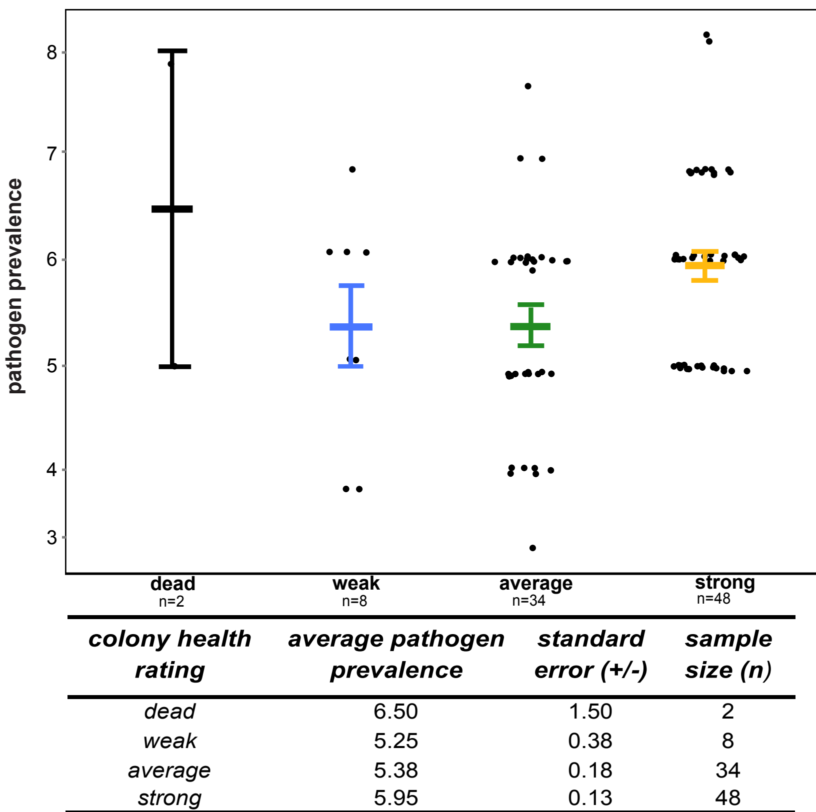
Supporting Table S2. Pathogen prevalence by colony health rating. Pathogen prevalence varied significantly between strong and average colony health ratings. Honey bee samples obtained from dead, weak, average, and strong colonies were tested for the presence of seven pathogens, including five viruses (i.e., DWV, BQCV, LSV1, LSV2, KBV), *L. passim*, and *N. ceranae* using PCR, and for *Varroa destructor* mites, which were counted as a pathogen when infestation levels were above the recommended treatment threshold of 3%. Total pathogen prevalence was determined by summing the number of pathogens detected in each sample. Honey bee colony population size was used as a proxy for colony health by counting the number of frames more than 2/3 covered with bees (i.e. weak < 7 frames, average = 7-12 frames, strong >12 frames covered with bees). Included in the table are the mean number of pathogens per colony strength rating, the standard error estimate of the mean, and the number of colonies per colony health rating within this cohort. This sample cohort was comprised of 52.2% strong, 37% average, 8.6% weak, and 2.2% dead colonies.

colony health rating	***mean pathogen prevalence***	***standard error (+/-)***	***sample size (n)***
dead	6.50	1.50	2
weak	5.25	0.38	8
average	5.38	0.18	34
strong	5.95	0.13	48

Supporting Table S3. Pathogen prevalence by sampling event**.**
Pathogen prevalence varied significantly by sampling event. Honey bee samples were obtained from two Minnesota based, commercially managed honey bee colonies, twice when colonies were located in California before and immediately after almond pollination and thus reflective of the pathogen prevalence during the almond bloom, and three times after the almond pollination event when colonies were located in Minnesota. Pathogen prevalence was measured by totaling the number of pathogens detected via PCR in each sample. Included are the average number of pathogens per sampling event, the standard error estimate of the mean, and the number of colonies per colony health rating within this cohort.

sampling event	***location***	***mean pathogen prevalence***	***standard error (+/-)***	***sample size (n)***
before	California	5.00	0.17	28
during	California	5.39	0.09	28
after 1	Minnesota	7.09	0.28	11
after 2	Minnesota	6.43	0.13	14
after 3	Minnesota	6.00	0.23	11

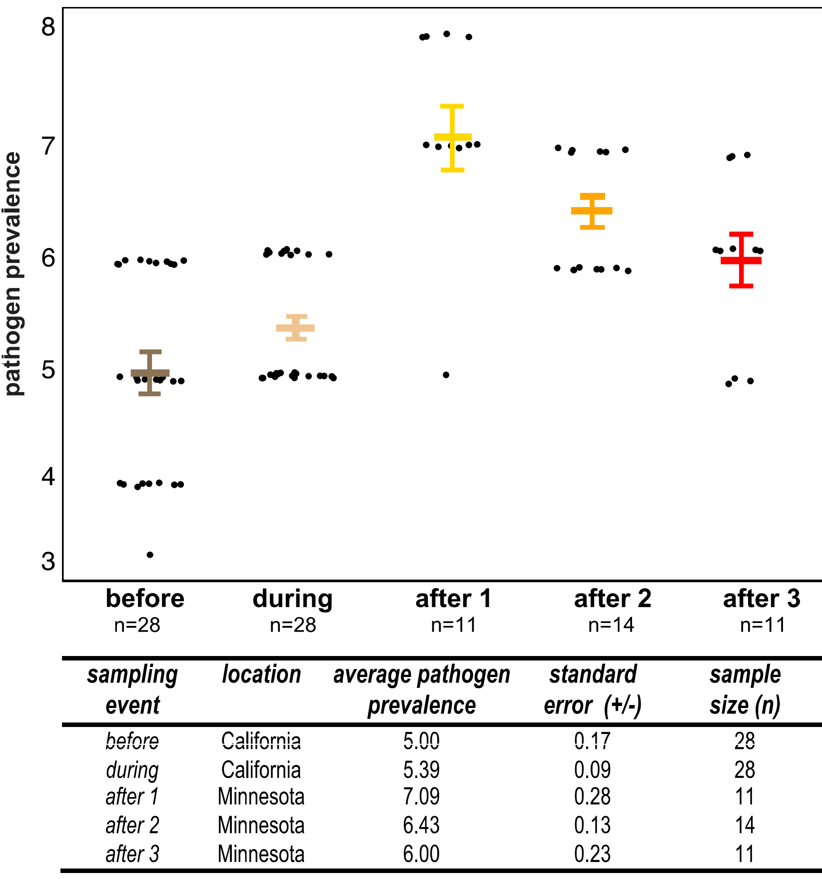
Supporting Figures



colony health rating	*average pathogen prevalence*	*standard error (+/-)*	*sample size (n)*
dead	6.50	1.50	2
weak	5.25	0.38	8
average	5.38	0.18	34
strong	5.95	0.13	48

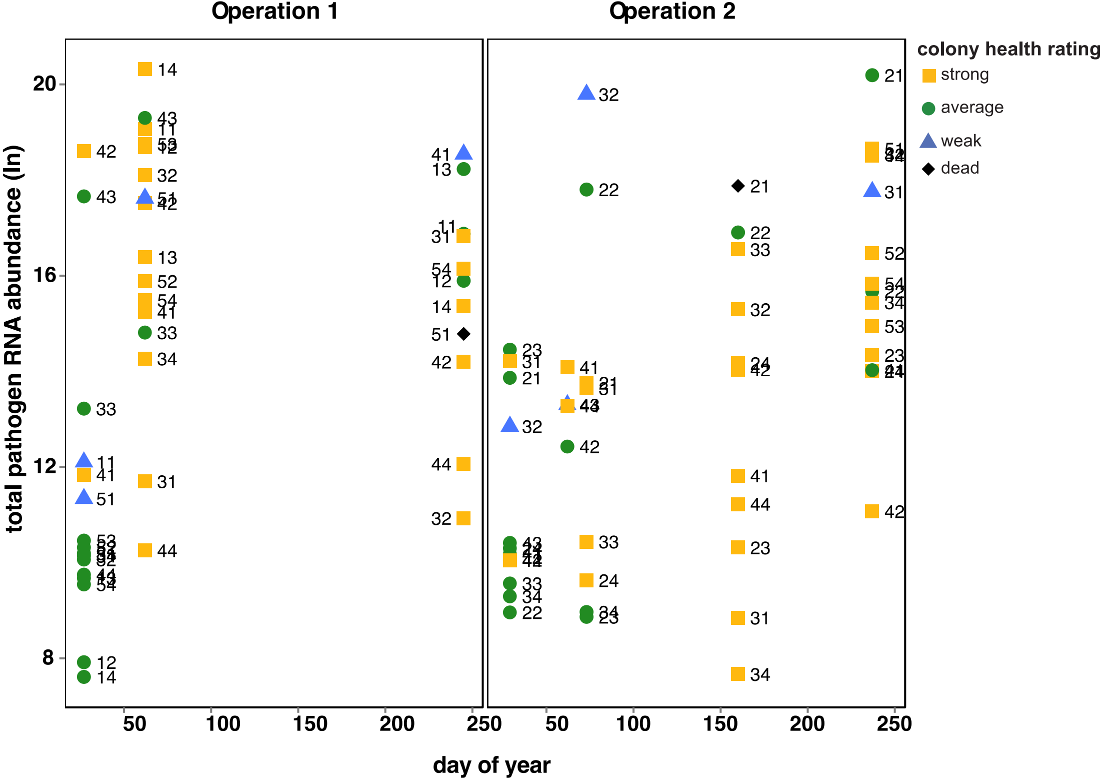
Supporting Fig S1. Analysis of mean pathogen prevalence by colony health rating.

Mean pathogen prevalence is higher in samples from strong rated colonies, than samples from average rated colonies. The number of pathogens detected by PCR, and mites (when infestation percentage surpassed 3 mites per 100 bees) were summed for each sample and averaged (y-axis) for each colony health rating (x-axis). Colony health was rated using colony size as a proxy, where colonies with >7 frames covered with bees were rated weak (n=8, blue), 7-12 frames were rated average (n=34), <12 frames were rated strong (n=48, gold), or 0 frames were rated dead (n=2, black). Points represent raw data, and center bars represent the mean colony pathogen prevalence, which are surrounded by the upper and lower standard error estimates. Significant differences between mean pathogen prevalence of colony health ratings was calculated using Tukey's HSD *post-hoc* test (p-value < 0.05).



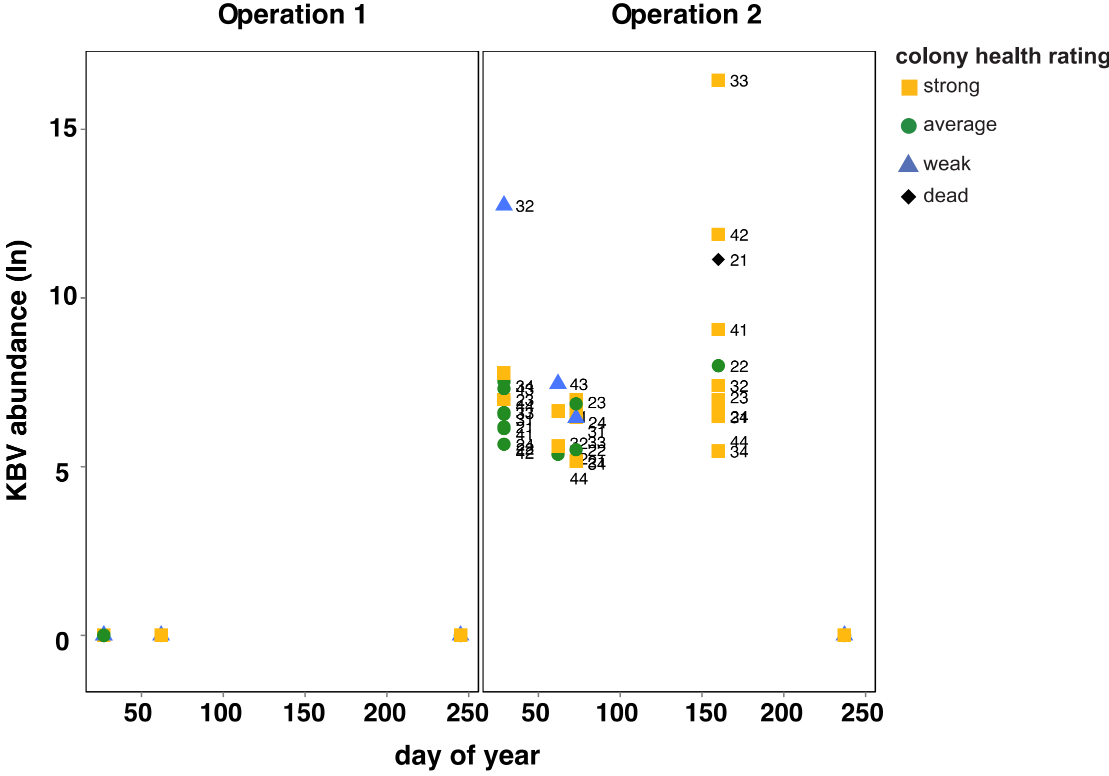
sampling event	*location*	*average pathogen prevalence*	*standard error (+/-)*	*sample size (n)*
before	California	5.00	0.17	28
during	California	5.39	0.09	28
after 1	Minnesota	7.09	0.28	11
after 2	Minnesota	6.43	0.13	14
after 3	Minnesota	6.00	0.23	11

Supporting Fig S2. Analysis of mean pathogen prevalence by sampling event. Colonies acquired the highest mean number of pathogens by the first sampling event after almond pollination.
The number of pathogens detected by PCR, and mites (when infestation percentage surpassed 3 mites per 100 bees) were summed in each sample and averaged (y-axis) for each sampling event (x-axis). Sampling events were conducted once before (n=28, dark brown), once during (n=28, light brown), and three times after California almond pollination (n= 11 yellow, n=14 orange, and n=11 red). Points represent raw data, and center bars represent the mean pathogen prevalence, which are surrounded by the upper and lower standard error estimates. Significant differences between the mean colony-level pathogen prevalence between sampling events were calculated using Tukey's HSD *post-hoc* test (*p*-value < 0.05). The mean pathogen prevalence was greater in sampling events carried out after almond pollination when the colonies were located in Minnesota (after 1, 2, and 3) compared to sampling events before and during almond pollination when the colonies were located in California (with the exception of the pairwise comparison between during and after 3).

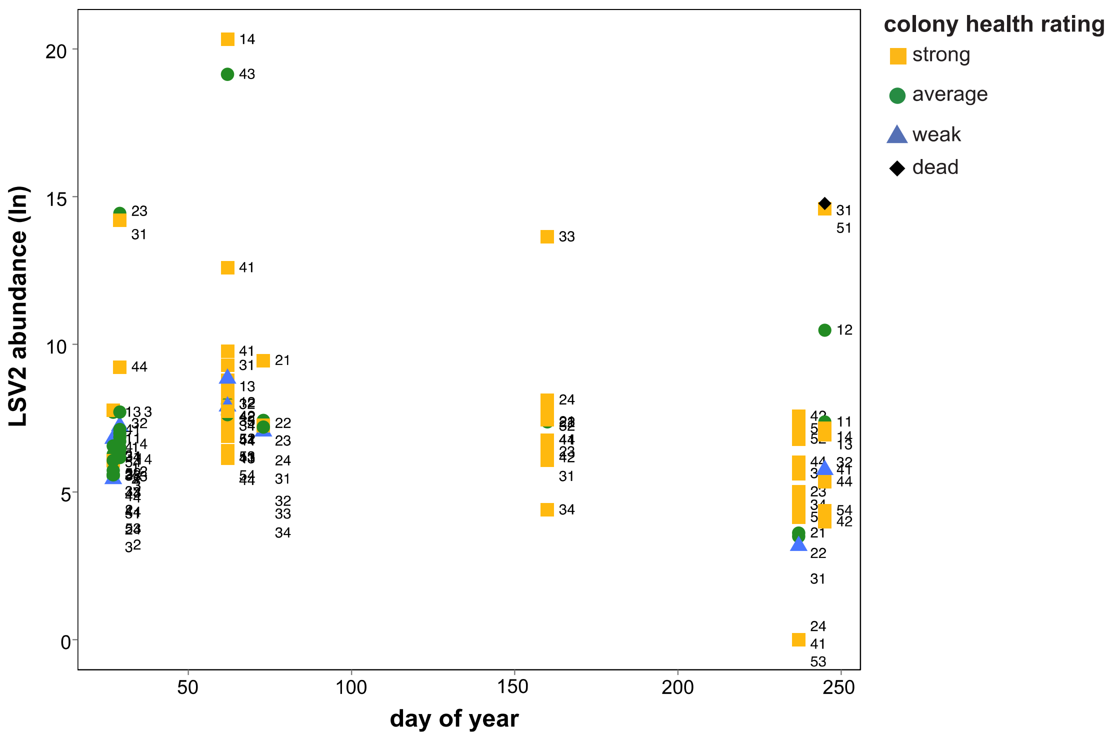


Supporting Fig S3. Changes in total pathogen abundance in honey bee colonies throughout the sampling period.

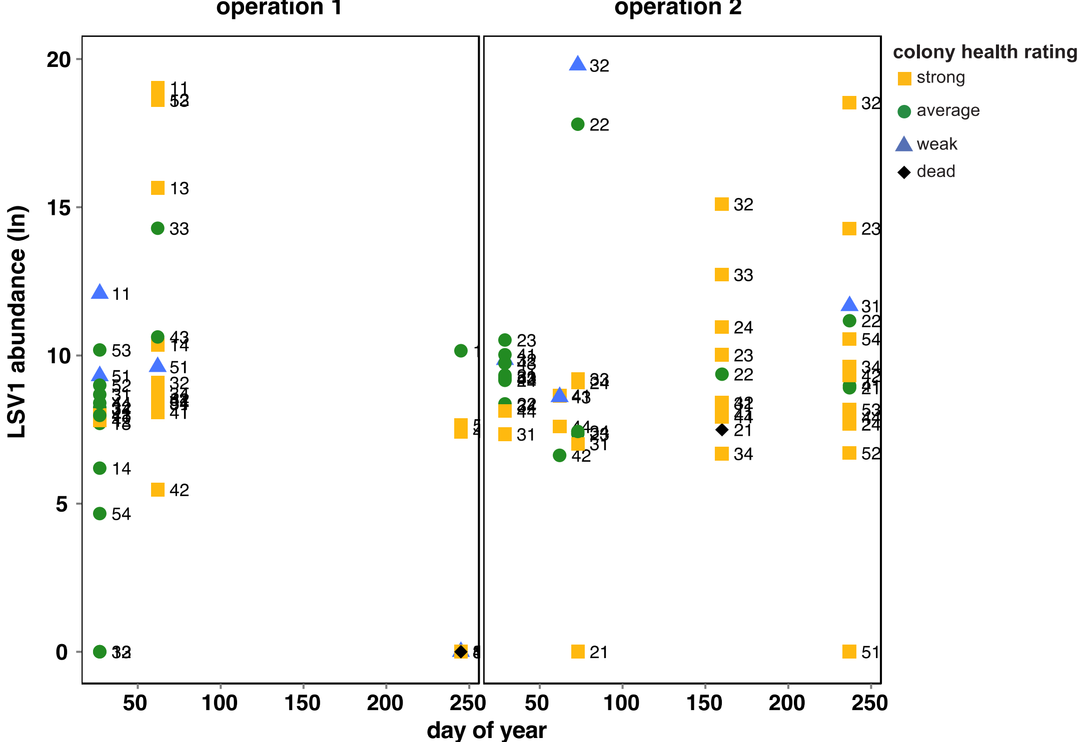
Total pathogen abundance changed throughout the monitoring period, and was different between beekeepers. The natural log transformed values of individual pathogens measured by qPCR were summed (y-axis) and plotted in response to the date of sample collection (i.e. day of year, x-axis). Live honey bee samples were collected from colonies rated dead (black diamonds), weak (blue triangles), average (green circle), and strong (yellow square)). Unique colony identifier numbers are used to label points, to identify changes in individual colonies throughout the sampling period with the first digit identifying the palate of the sample, and the second digit identifying the sample within the palate**.**



Supporting Fig S4. Changes in KBV abundance in honey bee colonies throughout the sampling period.
KBV abundance varied across the monitoring period in beekeeping operation 2, but was never detected in colonies from beekeeping operation 1. The natural log transformed values of the relative KBV RNA abundance as determined by qPCR (y-axis) in honey bee samples (y-axis) were plotted in response to the date of sample collection (i.e. day of year, x-axis). Live honey bee samples were collected from colonies with varying colony health ratings (i.e., dead (black diamonds), weak (blue triangles), average (green circle), and strong (yellow square)). KBV abundance in colonies were plotted separately by beekeeping operation to indicate the effect of beekeeper on KBV abundance. Unique colony identifier numbers, with the first digit identifying the pallet and the second digit identifying individual colony, label each point on the graph and illustrate changes in the pathogen abundance individual colonies throughout the sampling period.

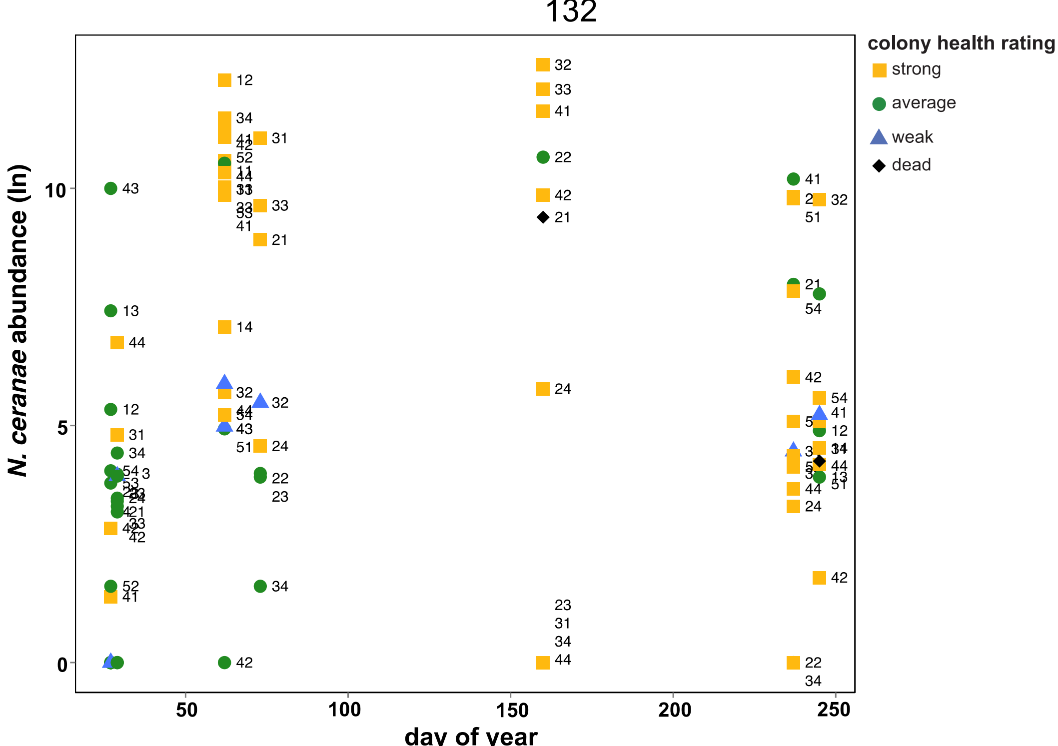


Supporting Fig S5. Changes in LSV2 abundance in honey bee colonies throughout the sampling period.
LSV2 abundance was weakly correlated with date of sampling, but was greater in dead colonies. The natural log transformed values of the relative LSV2 RNA abundance as determined by qPCR (y-axis in honey bee samples (y-axis) were plotted in response to the date of sample collection (i.e. day of year, x-axis). Live honey bee samples were collected from colonies with varying colony health ratings (i.e., dead (black diamonds), weak (blue triangles), average (green circle), and strong (yellow square)). Unique colony identifier numbers, with the first digit identifying the pallet and the second digit identifying individual colony, label each point on the graph and illustrate changes in the pathogen abundance individual colonies throughout the sampling period.

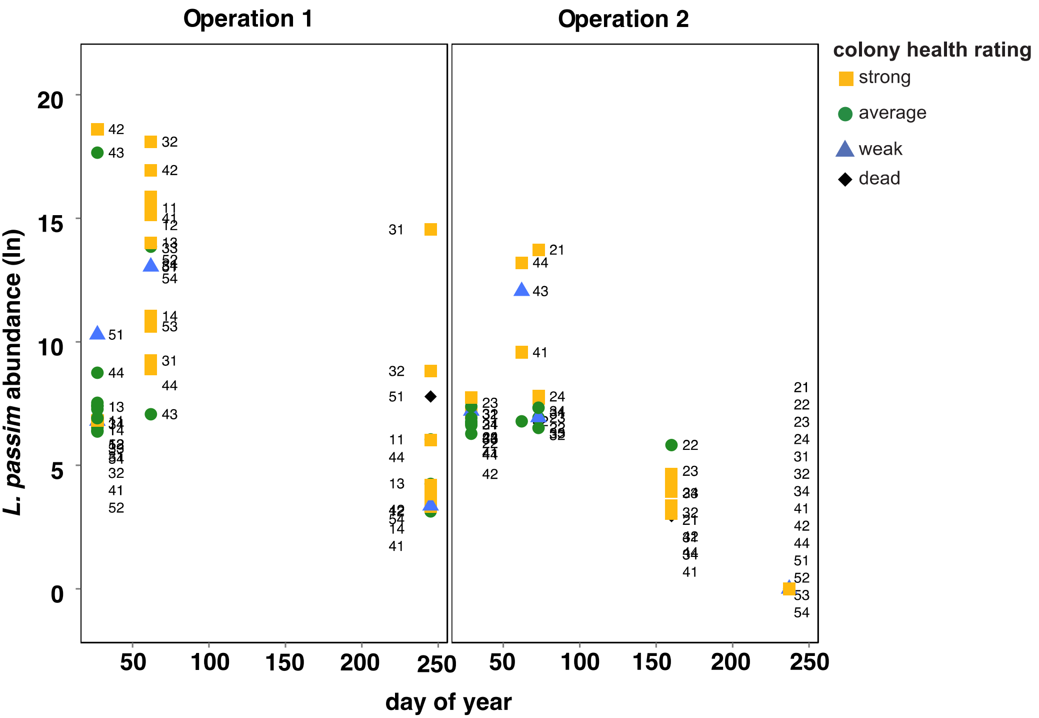


Supporting Fig S6. Changes in LSV1 abundance in honey bee colonies throughout the sampling period.

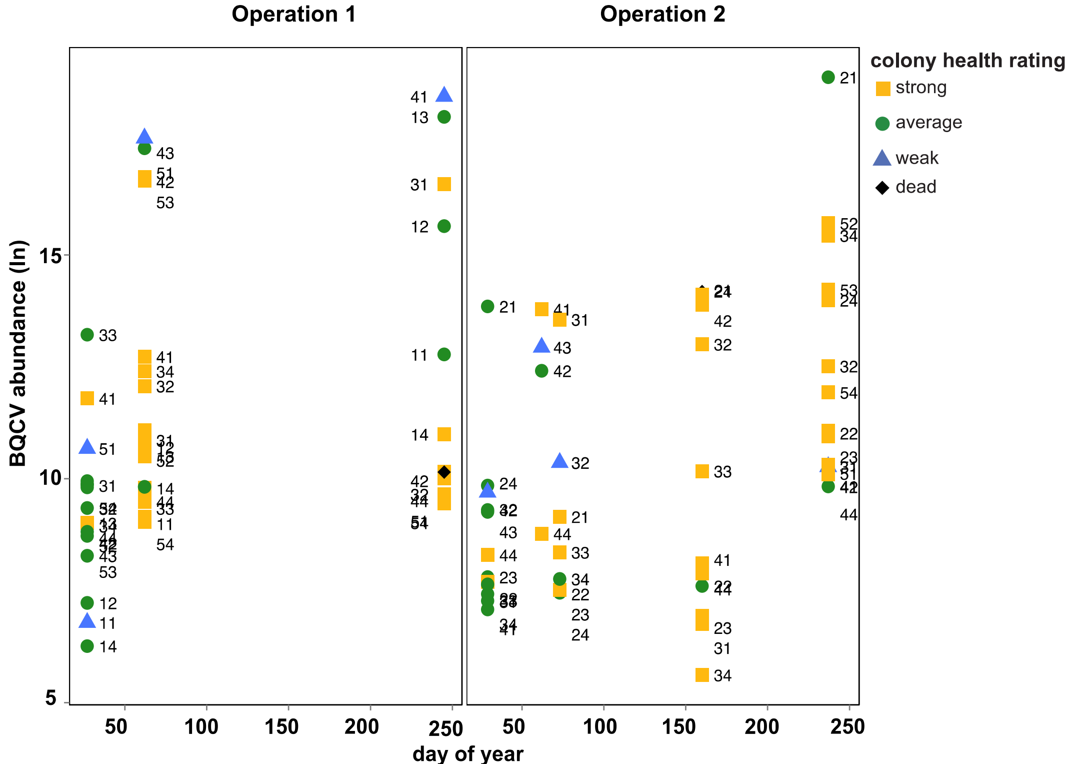
LSV1 abundance changed throughout the monitoring period, and was different between beekeeping operations. The natural log transformed values of the relative LSV1 RNA abundance as determined by qPCR (y-axis in honey bee samples (y-axis) were plotted in response to the date of sample collection (i.e. day of year, x-axis). Live honey bee samples were collected from colonies with varying colony health ratings (i.e., dead (black diamonds), weak (blue triangles), average (green circle), and strong (yellow square)). Unique colony identifier numbers, with the first digit identifying the pallet and the second digit identifying individual colony, label each point on the graph and illustrate changes in the pathogen abundance individual colonies throughout the sampling period.



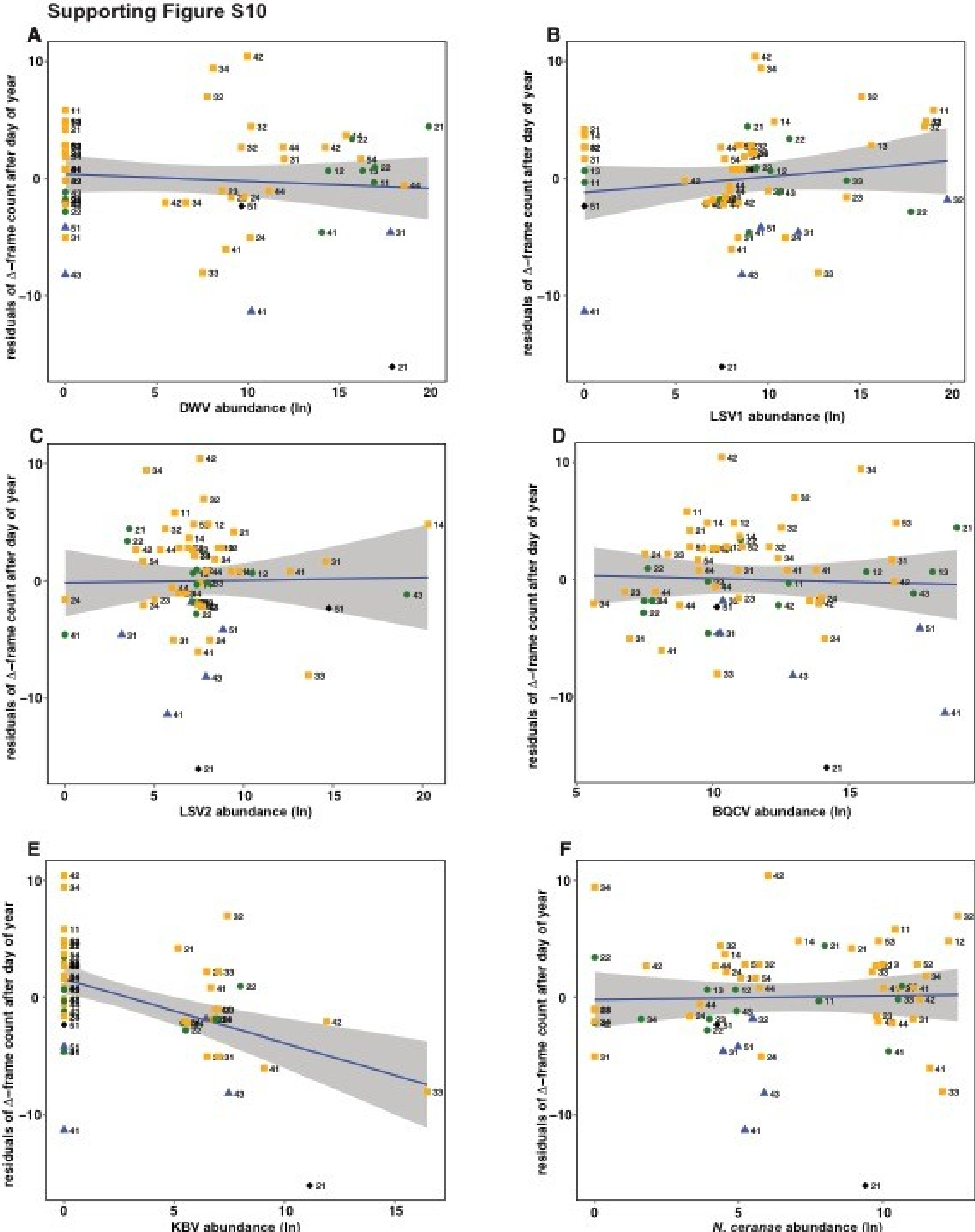
Supporting Fig S7. Changes in *N. ceranae* abundance in honey bee colonies throughout the sampling period.
N. ceranae abundance changed throughout the monitoring period to a maximum, and was predicted to be greater in strong rated colonies, compared to weak rated colonies. The natural log transformed values of the relative N. ceranae RNA abundance as determined by qPCR in honey bee samples (y-axis) were plotted in response to the date of sample collection (i.e. day of year, x-axis). Live honey bee samples were collected from colonies with varying colony health ratings (i.e., dead (black diamonds), weak (blue triangles), average (green circle), and strong (yellow square)). Unique colony identifier numbers, with the first digit identifying the pallet and the second digit identifying individual colony, label each point on the graph and illustrate changes in the pathogen abundance individual colonies throughout the sampling period**.**



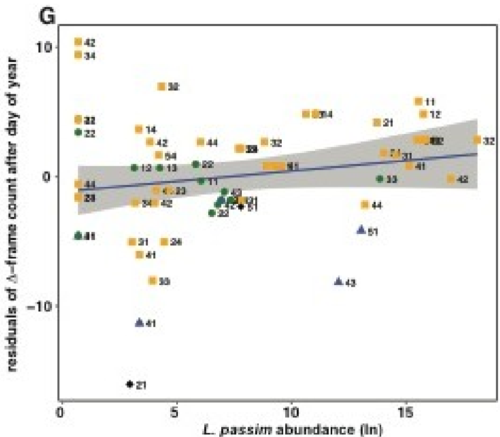
Supporting Fig S8. Changes in *L. passim* abundance in honey bee colonies throughout the sampling period.
L. passim abundance was predicted to change throughout the monitoring period, but the abundance was greater in beekeeping operation 1, than beekeeping operation 2. The natural log transformed values of the relative *L. passim* RNA abundance as determined by qPCR in honey bee samples (y-axis) were plotted in response to the date of sample collection (i.e. day of year, x-axis). Live honey bee samples were collected from colonies with varying colony health ratings (i.e., dead (black diamonds), weak (blue triangles), average (green circle), and strong (yellow square)). *L. passim* abundance in colonies were plotted separately by beekeeping operation to indicate the effect of beekeeper on *L. passim* abundance. Unique colony identifier numbers, with the first digit identifying the pallet and the second digit identifying individual colony, label each point on the graph and illustrate changes in the pathogen abundance individual colonies throughout the sampling period**.**



Supporting Fig S9. Changes in BQCV abundance in honey bee colonies throughout the sampling period.
BQCV abundance changed throughout the monitoring period, and levels of abundance were higher in beekeeping operation 2, than beekeeping operation 1. The natural log transformed values of the relative BQCV RNA abundance as determined by qPCR in honey bee samples (y-axis) were plotted in response to the date of sample collection (i.e. day of year, x-axis). Live honey bee samples were collected from colonies with varying colony health ratings (i.e., dead (black diamonds), weak (blue triangles), average (green circle), and strong (yellow square)). BQCV abundances in colonies were plotted separately by beekeeping operation to indicate the effect of beekeeper on BQCV abundance. Unique colony identifier numbers, with the first digit identifying the pallet and the second digit identifying individual colony, label each point on the graph and illustrate changes in the pathogen abundance individual colonies throughout the sampling period.


Supporting Figure S10
A
B
C
D
E
F
residuals of Δ−frame count after day of year
DWV abundance (ln)
LSV1 abundance (ln)
LSV2 abundance (ln)
BQCV abundance (ln)
KBV abundance (ln)
N. ceranae abundance (ln)

Supporting Figure S10 Continued



Supporting Figure S10. Correlations between changes in frame count and pathogen abundance, after accounting for sampling date.
The residuals of the change in frame count (used as a proxy for changes in colony health rating), after accounting for the sampling date (i.e., “day of year”) (y-axis) were plotted relative to the natural log transformed values of the relative pathogen RNA abundance (x-axis) for (A) DWV (B) LSV1, (C) LSV2, (D) BQCV, (E) KBV, (F) *N. ceranae*, and (G*) L. passim*. Live honey bee samples were collected from colonies with varying colony health ratings (i.e., dead (black diamonds), weak (blue triangles), average (green circle), and strong (yellow square)). The best-fit line (blue) surrounded by upper and lower standard error estimates (gray) indicates trends in the changes in frame count relative to pathogen abundance. After accounting for changes in frame counts throughout time, the only significant result was a negative relationship between KBV abundance and changes in frame count. Unique colony identifier numbers, with the first digit identifying the pallet and the second digit identifying individual colony, label each point on the graph.

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CHAPTER FOUR

CONCLUSION

Elucidating the effects of climate change and pathogens as threats to insect pollinator health will be important for informing conservation strategies aimed at maintaining biodiversity, ecosystem function, and economic productivity. The negative impact of anthropogenic effects on services provided by bee pollinators threatens habitat quality and agricultural sustainability. Therefore, investigating the ways in which climate change and pathogens contribute to declining pollinator health will be important for developing effective conservation strategies.

Climate change can affect plant visual and chemical traits important for pollinator attraction. Results from our study indicated that drought and CO_2 fertilization alter pollinator visitation to some plant species by a similar magnitude as changes in floral visual and chemical traits. Furthermore, the effects of drought on flowers with overlapping floral types in a plant community may increase competition for pollinators, while the effects of CO_2 fertilization on flowers in a plant community with dissimilar flower types may facilitate pollination. As a result, conservation strategies aimed at enhancing ecosystems by increasing the diversity of floral resources will ensure the most robust habitat to support pollinator conservation into the future (Biesmeijer et al. 2006, Burkle et al. 2013, Tilman, 1999).

Bee pollinator pathogens also threaten insect pollinator populations, and are associated with decreased honey bee health. Results from our study monitoring pathogen community composition of honey bee colonies involved in California almond pollination indicated that pathogen composition varies seasonally, potentially as a result of increased mite infestation levels. Shifts in pathogen composition throughout the year highlight the importance of repeated sampling to encompass the annual variation of bee pathogens. Therefore, longitudinal monitoring projects encompassing the seasonal variation of pathogen prevalence and abundance are essential to understand pollinator-pathogen relationships.

Increased understanding of the independent effects of climate change and bee-pathogen relationships on pollinator health will inform the non-independent pathways through which pollinator populations are negatively affected. Integrating multidisciplinary methods will be essential for the future of pollinator conservation. For example, climate change could directly increase the virulence, distribution, and transmission pathways of pathogens infecting bees, but studies in these areas are lacking. Therefore, adapting methods with broad-scale applicability to diverse fields of biology are imperative to understand threats to pollinator populations from the global to cellular scale. Prioritizing the integration of methods informing processes that threaten biodiversity, ecosystem function, and economic production are essential to develop solutions to globally relevant issues in the future.

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APPENDIX A

SOUTHWEST MONTANA BEE PATHOGEN SURVEY

Purpose

Bees are infected by a diversity of pathogens including fungi, trypanosomatids, RNA viruses, and ecoparasites, a majority of which are taxa specific. However, RNA viruses are bee pathogens most capable of inter- and intra-specific transmission (Genersch et al. 2006, Singh et al. 2011, Furst et al. 2014, Ravoet et al. 2014, Graystock et al. 2015, McMahon et al. 2015). For example, Black queen cell virus (BQCV), Sacbrood virus (SBV), and Deformed wing virus (DWV) have been detected in honey bee collected pollen pellets (Singh et al. 2011, Mazzei et al. 2014). Additionally, transmission between bee species of the same strain of honey bee infecting Israeli acute paralysis virus (IAPV) was detected in foraging bumble bees in a greenhouse setting (Singh et al. 2011). Therefore, the detection of RNA viruses in pollen pellets collected by bees, and the transmission of viruses between bee species demonstrated through bee foraging activities highlights the sharing of floral resources as a likely source of interspecific bee virus transmission (Singh et al. 2011, Mazzei et al. 2014, McMahon et al. 2015). However, research investigating the origins and mechanisms of pollinator pathogen transmission has primarily been conducted in non-natural settings (i.e., greenhouse). Therefore, to increase our understanding of bee pathogen transmission in natural settings, bumble bees and honey bees were caught in the summer of 2015 and screened for bee pathogens using the primers listed in Table 1 to determine if bumble bee populations in Southwest Montana carry similar viruses to those detected in local honey bee populations,

The Lake Sinai viruses (LSVs) are a recently discovered and globally distributed group of viruses (Runckel et al. 2011, Ravoet et al. 2013) that can infect a diversity of bee taxa. LSVs have been detected in honey bee (*Apis mellifera*) samples in the US, Belgium, and Spain (Runckel et al. 2011, Ravoet et al. 2013), *Bombus atratus* in South America (Gamboa et al. 2015), *Bombus lapidarius* (red-tailed bumble bee), *Bombus pascuorum* (common carder bee), and *Bombus pratorum (*Early-nesting bumble bee) in Europe (Parmentier et al. 2016), along with the solitary bee species *Osmia cornuta, Andrena vaga, Andrena ventralis,* and *Osmia bicornis* in Europe (Ravoet et al. 2014), and recently in a diversity of bees in the US (Dolezal et al. 2016), but the prevalence of LSVs in wild bee species of North America still remains somewhat unknown. Therefore, to determine if Lake Sinai virus 1 (LSV1) and Lake Sinai virus 2 (LSV2) are circulating in wild bumble bee populations in Southwest Montana, we caught specimens and tested for these viruses using virus specific polymerase chain reaction (PCR).

Results

In the summer of 2015, 13 individuals (representing six species) of actively foraging bumble bees were collected from a combination of wildlands at the base of Mt. Ellis, and in urban settings on Wallace avenue in Bozeman, Montana and stored at -80 °C prior to analysis. Half of a bumble bee split bilaterally with a razor blade was used for RNA isolation, cDNA synthesis, and pathogen specific PCR. Bumble bee tissue was homogenized in sterile water

using a metal bead (3mm) and TissueLyzer (Qiagen, Hilden, GER) and RNA was isolated according to the manufactures' instructions. Pathogen specific PCR was used to assay cDNA from specimens for seven different bee pathogens (Appendix A, Table 1). Several bee viruses, including BQCV, DWV, SBV, KBV, and LSV2 were detected (Appendix A, Table 2). LSV2 product amplified from bumble bee species *Bombus griseocollis* and *Bombus flavifrons* was confirmed to be the strain currently circulating in Montana using Sanger Sequencing Sanger (Appendix A, Fig. 1). In total, 1,498 base-pairs of PCR product amplified from bumble bees share a high sequence identity to the open reading frame 1 (ORF1), RNA dependent RNA polymerase (RdRp), and capsid regions of the genome of the currently circulating strain of LSV2 among honey bees in Montana (Appendix A, Table 2).

These results indicate that pathogens typically studied in honey bees can be detected in wild bee populations in Southwest, Montana. Additionally, these results confirm for the first time that LSV2 is present within bumble bee populations in North America. Future work should prioritize amplifying the complete LSV2 genome found within bumble bees of Southwest, Montana, and comparing sequence identities to that of the currently circulating strain found within honey bees. Additionally, assaying for the negative strand replicative intermediate of LSV2 in bumblebees is necessary to confirm that LSV2 is replicating in bumble bee populations (Daughenbaugh et al. 2015).

Furthermore, comprehensive pathogen screening of 34 unidentified bumble bee and honey bee specimens collected in the summer of 2016 will continue the survey of wild bee populations for the prevalence of pollinator pathogens. Sequencing PCR amplified product will confirm if bumble bees are infected by the currently circulating strain of pollinator viruses, or if bumble bee specific variants exist. Additionally, assaying for the negative strand replicative intermediate of honey bee pathogens detected in bumble bees is required to confirm pathogens are replicating in bumble bee populations (Daughenbaugh et al. 2015). Surveying bumble bee populations for pollinator pathogens will increase our understanding of the ability of pollinator pathogens to infect a diversity of hosts, and the susceptibility of individual pollinator species to virus pathogens (McMahon et al. 2015).

Tables

Table 1: Primers used in this study

Genome / Gene Name	NCBI # GI #	Primer Name	Sequence (5'-3')	Product Size (bp)	Reference
Ribosomal protein L8 (*Apis m.*)	XM_393671.5 GI:571556074	Rpl8Fw Rpl8Rev	TGGATGTTCAACAGGGTTCATA CTGGTGGTGGACGTATTGATAA	121	Evans et al. (2006) Insect Mol Bio
Lake Sinai virus 1 (LSV1)	HQ871931 GI:335057596	qLSV1-F-2569 qLSV1-R-2743**	AGAGGTTGCACGGCAGCATG GGGACGCAGCACGATGCTCA	174	Runckel, Flenniken (2011) PLoS One
Lake Sinai virus 2 (LSV2)	HQ888865 GI:335057589	qLSV2-F-1722 qLSV2-R-1947**	CGTGCTGAGGCCACGGTTGT GCGGTGTCGATCTCGCGGAC	225	Runckel, Flenniken (2011) PLoS One
Lake Sinai virus 2-RdRp		qLSV2-F-1722** LSV1-4-R-2239	CGTGCTGAGGCCACGGTTGT CCTCCCACCGCGTGTGC	517	This work
Lake Sinai virus 2-ORF1		LSV2-1440F qLSV2-R-1947**	CGAAAAGCGCTTCGTCAGTTAT ACGGC GCGGTGTCGATCTCGCGGAC	507	This work
Lake Sinai virus 2-Capsid		LSV2-F-3685 LSV1&2U-R-4714	ATGAATCCACCAACTACGACTA CGACTACG AGGGACGACGGAGCACAATT	976	This work
Bumble bee Elongation Factor 1A	AF492955	EEF1A-F EE1A-R	AGAATGGACAAACCCGTGAG CACAAATGCTACCGCAACAG	187	Hornakova et al. (2010) Analytical Biochemistry
black queen cell virus (BQCV)	AF183905 GI:8100530	qBQCVorf2F_6664 qBQCVorf2R_6805	TCCTCAAATCTGGAGCGAAC GTATTCGCTGGCCGTAAAAC	141	Runckel, Flenniken (2011) PLoS One
deformed wing virus (DWV)	AY292384.1 GI:31540603	DWVfw1165 DWVrev1338	CTTACTCTGCCGTCGCCCA CCGTTAGGAACTCATTATCGCG	173	Chen et a. (2005) J Invert Path
sacbrood virus (SBV)	AF092924.1 GI:4416206	SBV_F2_5120 SBV_R2_5243	AATGTCACCCACGAGTGTTG GCGATGCAACCATACAACTG	123	Daughenbaugh, et al. (2015) Viruses
Israeli acute paralysis virus (IAPV)	NC_009025.1 GI:126010924	IAPV_F_7762 IAPV_R_7876	GCAGCTATTTTTGGCTGGTC CCAATGTACGCTCATATCG	114	Daughenbaugh, et al. (2015) Viruses
Kashmir bee virus (KBV)	NC_004807.1 GI:30793779	KBV_F_4470 KBV_R_4581	TCGACAAGGACATGATCGAG GAGCCACAAATGGCTTCTTC	111	Stoltz et al. (1995) J Apicult Res
N. ceranae	DQ673615.1 GI:110293152	N ceranae F-4186 N ceranae R-4435	CGGATAAAAGAGTCCGTTACC TGAGCAGGGTTCTAGGGAT	249	Chen et al. (2008) J Inv Path

Table 2: Prevalence of bee viruses detected in bumble bees caught in Southwest Montana. Several bee viruses were detected in individual bumble bees including six different species.

Species	Number of individuals	LSV2	BQCV	DWV	SBV	KBV	Total Detections
rufocinctus	1	0	1	1	1	0	3
bifarius	1	0	1	0	1	1	3
mixtus	5	0	2	0	1	0	3
centralis	2	0	2	0	2	0	4
griseocollis	2	1	0	0	0	0	1
flavifrons	2	1	2	0	1	0	4
Bombus spp.	13	2	8	1	6	1	18

Table 3: Sequence analysis of bumble bee infecting LSV2.
Sanger Sequencing confirmed a high degree of nucleotide identity between the LSV PCR product amplified from bumble bees and the LSV2 PCR products amplified from honey bees. These results indicate that the same LSV2 strain is currently circulating in both bumble bee and honey bee populations in Southwestern, Montana.

Region Sequenced	**Length (Bp)**	**Sequence Identity (%)**
ORF1	568	93.5
RdRp	113	94.1
Capsid	817	94.1

Figures

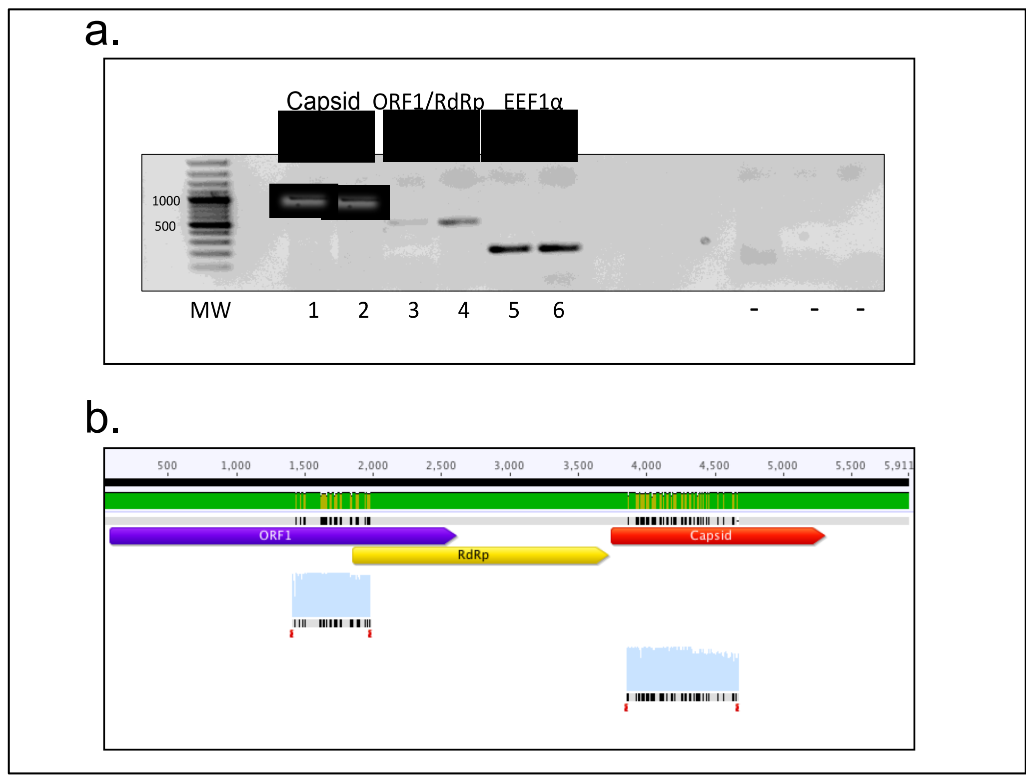

Figure 1: Confirmation of LSV2 presence in North American bumble bees using gel electrophoresis and Sanger sequencing. a.) Product amplified from bumble bees from the capsid, ORF1, and RdRp specific regions of LSV2 visualized on a 1.5% agarose gel. Elongation Factor 1-Alpha (EEF1α) was used as an internal control to ensure cDNA quality. b.) Amplified product was aligned to the ORF1, RdRp, and capsid regions (bottom) of the currently circulating strain of LSV2 in honey bees in Southwestern, Montana.

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