Running head: Soil shapes floral chemistry, bee health

From plant fungi to bee parasites: mycorrhizae and soil nutrients shape floral chemistry and bee pathogens

Julie K. Davis[1,2*], Luis A. Aguirre[3], Nicholas A. Barber[4], Phil C. Stevenson[5,6], and Lynn S. Adler[3]

[1] Stockbridge School of Agriculture, University of Massachusetts, Amherst, MA 01003, USA

[2] Department of Entomology, Cornell University, Ithaca, NY 14850, USA

[3] Department of Biology, University of Massachusetts, Amherst, MA 01003, USA

[4] Department of Biological Sciences, San Diego State University, San Diego, CA 92182, USA

[5] Jodrell Laboratory, Royal Botanic Gardens, Kew, Surrey TW9 3AB, UK

[6] Natural Resources Institute, University of Greenwich, Chatham, Kent ME4 4TB, UK

*corresponding author: jd982@cornell.edu, ph 413-387-3124

Abstract

Bee populations have experienced declines in recent years, due in part to increased disease incidence. Multiple factors influence bee-pathogen interactions, including nectar and pollen quality and secondary metabolites. However, we lack an understanding of how plant interactions with their environment shape bee diet quality. We examined how plant interactions with the belowground environment alter floral rewards and, in turn, bee-pathogen interactions. Soil-dwelling mycorrhizal fungi are considered plant mutualists, although the outcome of the relationship depends on environmental conditions such as nutrients. In a 2x2 factorial design, we asked whether mycorrhizal fungi and nutrients affect concentrations of nectar and pollen alkaloids (anabasine and nicotine) previously shown to reduce infection by the gut pathogen *Crithidia* in the native bumblebee *Bombus impatiens*. To ask how plant interactions affect this common bee pathogen, we fed pollen and nectar from our treatment plants, and from a wildflower pollen control with artificial nectar, to bees infected with *Crithidia*. Mycorrhizal fungi and fertilizer both influenced flowering phenology and floral chemistry. While we found no anabasine or nicotine in nectar, high fertilizer increased anabasine and nicotine in pollen. AMF decreased nicotine concentrations, but the reduction due to AMF was stronger in high than low-nutrient conditions. AMF and nutrients also had interactive effects on bee pathogens via changes in nectar and pollen. High fertilizer reduced *Crithidia* cell counts relative to low fertilizer in AMF plants, but increased *Crithidia* in non-AMF plants. These results did not correspond with effects of fertilizer and AMF on pollen alkaloid concentrations, suggesting that other components of pollen or nectar were affected by treatments and shaped pathogen counts. Our results indicate that soil biotic and abiotic environment can alter bee-pathogen interactions

via changes in floral rewards, and underscore the importance of integrative studies to predict disease dynamics and ecological outcomes.

Key words: *bee decline, bee parasites,* Bombus impatiens, Crithidia, *mycorrhizae, multi-trophic, trypanosome, soil, alkaloid, floral chemistry*

Introduction

Pollinators provide critical ecosystem services important for both natural ecosystem resilience and crop yield, and bees are major contributors to these services (Klein et al. 2003). Many bee species are experiencing population declines due to a range of factors, including increased disease incidence (Cameron et al. 2011, Goulson et al. 2015). Host-pathogen dynamics do not occur in isolation, and diet quality may act as a buffer against disease stressors. For instance, protein can enhance bee immunocompetence (Alaux et al. 2010). Furthermore, environmental factors, including soil conditions and species interactions, shape the quality of floral resources (Adler et al. 2006). However, we lack a comprehensive understanding of how biotic and abiotic factors, such as soil microbes and nutrients, shape bee-pathogen outcomes mediated by diet quality. Due to the importance of pollinators for diversity in natural ecosystems and yield in crop systems (Ashman et al. 2004, Klein et al. 2007), bottom-up effects of soil conditions on bees could have widespread ecological and economic applications.

Secondary metabolites in plants can provide resistance against plant antagonists but also may shape interactions with pollinators. Alkaloids in nectar can deter or attract pollinators depending on concentration and pollinator taxa (Detzel and Wink 1993, Adler 2000, Kessler et al. 2008), and nectar compounds could benefit pollinators by reducing bee pathogen load (Manson et al. 2010, Richardson et al. 2015). In particular, consuming the alkaloids nicotine and anabasine, found in Solanaceous plants, reduced infection by the gut pathogen *Crithidia* in the

bumblebee *Bombus impatiens* at ecologically-relevant concentrations (Richardson et al. 2015). However, these effects can be dose-dependent (Anthony et al. 2015), and in some studies, no effect was detected (Biller et al. 2015, Thorburn et al. 2015). Given that some bee species have declined in recent years, due in part to pathogens (Potts et al. 2010), and the largely unknown role of floral traits in pollinator-pathogen dynamics (McArt et al. 2014, Koch et al. 2017), it is critical to explore factors that influence flower-bee-pathogen interactions.

Fine scale shifts in the diverse sugars, secondary compounds, and other nutrients in nectar (Bentley and Elias 1983) may alter bee-pathogen interactions, and it is thus important to study bee-disease dynamics in the context of real nectar. Sugar concentration and composition can influence *Crithidia* growth (Conroy et al. 2016, Palmer-Young and Thursfield 2017) directly or possibly synergistically with secondary metabolites in nectar (Palmer-Young et al. 2017). Few studies have examined the effects of secondary compounds in real nectar on bee pathogens (Tiedeken et al. 2016). To address this, we tested the effect of plant growing environment on bumble bee pathogens *via* changes in floral chemistry using real nectar and pollen.

While some studies have examined the effect of nectar secondary chemistry on pollinator pathogens, comparatively few have examined pollen secondary chemistry. Pollen is the male gamete and is typically more defended than nectar with higher concentrations of secondary compounds (Cook et al. 2013, Palmer-Young et al. 2019), and therefore may have a stronger effect on bee pathogen loads. Furthermore, adequate dietary protein from pollen is important for honeybee and bumblebee immune response, and other dietary constituents, including amino acid and lipid levels, may also shape bee tolerance to infection (reviewed in Koch et al. 2017). A diet including both pollen and nectar is ecologically realistic, and because pollen may have higher

91 concentrations of secondary compounds, it is crucial to understand the combined role of these
92 diet constituents on bee pathogens.

93 Plants interact with a range of organisms that can affect nectar and pollen traits, which
94 may scale up to alter bee-pathogen interactions. For example, plants widely associate with
95 arbuscular mycorrhizal fungi (AMF), which can enhance nutrient absorption (Brundrett and
96 Tedersoo 2018) and induce systemic expression of proteins and genes involved in defense in root
97 and foliar tissues (Liu et al. 2007, Campos-Soriano et al. 2010). We therefore hypothesize that
98 AMF could alter expression of secondary chemistry in floral reward tissues *via* a systemic
99 defense response. Moreover, because plant benefit from AMF depends on the relative
100 availability of soil nitrogen and phosphorus (Johnson 2010), and soil nutrients can shape nectar
101 secondary chemistry (Adler et al. 2006), AMF and nutrients may interactively influence defense
102 expression, including in floral tissue. Therefore, AMF have the potential to affect floral traits
103 including defensive chemistry *via* both changes in nutrient uptake and other pathways involved
104 in defense. To date, no study has examined the effect of AMF on floral secondary chemistry,
105 other than floral volatiles (Becklin et al. 2011), although there is evidence that AMF can increase
106 or decrease pollinator attraction via changes in floral display (Barber and Soper Gordon 2015).

107 By examining how plant interactions with AMF and nutrients affect floral chemistry and
108 bee-pathogen dynamics, our study addresses a novel pathway by which belowground interactions
109 affect pollinator health. Specifically, we asked whether floral traits (flowering phenology, nectar
110 volume, and mean value or variation in pollen and nectar alkaloid and sugar concentrations)
111 change in response to plant nutrient or mycorrhizal status; and whether AMF and nutrient effects
112 on pollen and nectar affect pathogen levels in bumblebees infected with *Crithidia*. We
113 hypothesized that if effects of AMF on secondary metabolism depend on soil nutrients (Getman-

Pickering et al, unpublished data), AMF would increase alkaloids more under low than high nutrient conditions. Alternatively, if AMF effects on alkaloid levels are due to up-regulation of defense pathways, rather than nutrient status (Vannette and Hunter 2009), we would expect plants grown with AMF to have higher floral alkaloids regardless of fertilizer treatment. We expected plants grown with high fertilizer to have higher floral alkaloids, consistent with a previous study of nutrient effects on nectar (Adler et al. 2006). We predicted that diet from treatment groups with high alkaloid concentrations would reduce *Crithidia* cell counts. Furthermore, because variation in diet quality could affect plant-pollinator interactions (Kessler et al. 2012, Wetzel et al. 2016), we assessed mean value and variation in floral chemical traits.

Methods

Study System

We selected *Nicotiana tabacum* L. (cultivated tobacco; Solanaceae) as a model system because it is colonized by AMF (Andrade et al. 2013) and produces plentiful nectar containing the alkaloids nicotine and anabasine (Detzel and Wink 1993, Adler et al. 2006) that can reduce *Crithidia bombi* in *Bombus impatiens* (Richardson et al. 2015). *Rhizophagus irregularis* (previously *Glomus intraradices)* is a commercially available species of AMF used in agriculture and restoration. It increases alkaloid concentrations in root and leaf tissues of *N. tabacum* (Andrade et al. 2013), but its effect on floral chemistry is unknown. *Bombus impatiens* (common eastern bumblebee) is native to the eastern US (Williams et al. 2014). While *B. impatiens* is not in decline, it is related to several bee species that are in decline, associated with pathogen incidence (Cameron et al. 2011). It is therefore a useful model species to study pollinator-pathogen dynamics. *Bombus* spp. are infected by the hind gut pathogen *Crithidia bombi,* which is found in up to 80% of bees at some sites (Gillespie 2010). *Crithidia* reduces queen colony

137 founding success, size and fitness (Brown et al. 2003), and worker foraging and learning abilities
138 (Gegear et al. 2006, Otterstatter and Thomson 2006).
139
140 *Experimental Design*
141 Starting in November 2016, we grew 200 tobacco plants in the greenhouse in a 2x2
142 blocked factorial design, manipulating presence and absence of AMF, and high and low levels of
143 fertilizer. Each block contained four plants, one from each treatment group. We collected pollen
144 and nectar and analyzed nicotine and anabasine concentrations. We also collected and pooled
145 pollen and nectar from plants for laboratory bioassays with bees. We fed *Crithidia*-infected bees
146 pollen and nectar from plants in each treatment combination, and included a control group with a
147 diet of wildflower pollen blend and artificial nectar to determine whether tobacco nectar and
148 pollen in general reduced *Crithidia* counts relative to a typical laboratory experimental diet.
149 We broadcast *N. tabacum* seeds into sterilized potting soil/sand mixture and kept
150 seedlings fertilized uniformly throughout early development. When seedlings were ready for
151 transplant in April 2017, we transferred them to 10 cm bleach-sterilized pots with sterilized
152 potting soil/sand mixture. At this point, plants were randomly assigned AMF treatments (~50
153 plants/treatment) and inoculated with either 1.71 g of 500 spores/g inoculum *Rhizophagus*
154 *irregularis* (Premier Tech, Quebec, Canada) in perlite carrier, or the same AMF and perlite
155 mixture that had been autoclaved. In May, we added high (4.02 g ± 0.05 g) or low (1.005 g ± -
156 0.05 g) NPK fertilizer, as in Adler et al. (2006) to plants assigned high and low fertilizer
157 treatments (for details see Appendix S1, *Plant propagation and administering treatments*).
158 In early April, after administering AMF/fertilizer treatments, we noticed an aphid
159 outbreak in the greenhouse. We quantified (scored 0-3) and manually removed aphids on two
160 sampling dates in April, and estimated proportion of leaf area that had mold resulting from aphid

honeydew (see Appendix S1, *Aphid and mold quantification*). Because aphid incidence and mold were associated with AMF or fertilizer treatments (Appendix S1, *Aphid and mold quantification*), we included these as separate predictors in our models to account for potential effects of aphids and mold on responses. Collinearity was low in all cases (VIF < 2.0), suggesting that we could separate effects of aphids and mold from treatment effects.

Quantifying Mycorrhizal Colonization

We sampled roots after all nectar and pollen collection was complete, between August 25th-31st, 2017. Samples were taken from the center of the root ball, washed in tap water, then stained with trypan blue and mounted on microscope slides (Appendix S1, *Mycorrhizal inoculation quantification and validation*). Colonization was confirmed using a compound microscope at 400 magnification and the grid intersect method outlined in McGonigle et al. (1990). AMF treatment increased the likelihood a plant would have arbuscules by 79% (residual deviance = 795.12 on 79 df, $p < 0.001$), and quadrupled mean arbuscular colonization (residual deviance = 868.16 on 87 df, $p < 0.001$), indicating treatments were effective. Colonization was not affected by the fertilizer treatment ($p > 0.09$), but colonized plants were more likely to have aphids (Appendix S1, *Mycorrhizal inoculation, quantification and validation*).

Measuring Plant Traits

Plant and floral traits. The date of first flowering (first open flower with five dehisced anthers) was recorded for each plant, ranging from April 27th until the experiment ended on August 31st, 2017. During the last week of the experiment we measured plant height and leaf number to estimate treatment effect on size. From the first two flowers, we measured nectar volume using 50 µL glass micro-capillary tubes (Fisher Scientific, Hampton, New Hampshire, USA) and a

digital caliper and nectar sugar concentration using a refractometer. To determine treatment effect on nectar and pollen alkaloids, pollen (from the first several flowers a plant produced until minimum amount for analysis was obtained) and nectar (from the first 2-4 flowers as needed for a volume of 25 μL) were collected between 0700 and 1300 and stored at -80°C until chemical analysis. To collect pollen, we plucked dehiscing anthers from the filament, holding flowers upside-down to reduce the risk of contaminating nectar. After we removed pollen, we separated sepals and ovaries from the floral tube so that nectar remained in the floral tube. By holding the flower at an angle, we were able to prevent the nectar from contacting the site of phloem exposure. Nectar contaminated with pollen or other fluids, or that made contact with the damage site was discarded. After completing collection of pollen and nectar for chemical analysis, we collected nectar (using a 200 μL micropipetter) and pollen for the bee bioassay from subsequent flowers. For 52 plants, we analyzed a separate set of pollen from late-season sampling dates to test for correlations between early and late-season alkaloids (Appendix S1, *Pollen and Nectar Chemistry*).

Nectar and pollen alkaloids. Because some plants died or never flowered, our analysis of floral chemistry included 120 plants; 33 AMF-/high fertilizer, 27 AMF-/low fertilizer, 31 AMF+/ high fertilizer, and 28 AMF+/low fertilizer. We weighed approximately 6 mg pollen samples for extraction but unfortunately did not record exact weights. After extraction, we confirmed that pollen weights approximated this target value and did not differ between treatments (Appendix S1, *Pollen and Nectar Chemistry*). We therefore used the target value of 6 mg of pollen to calculate anabasine and nicotine in μg/mg. We also analyzed our data using post-extraction weights to calculate alkaloids/mg pollen, but chose to present results using an estimate of 6 mg due to high variability in post-extraction weights (Appendix S1, *Pollen and Nectar Chemistry*).

209

210 *Bee*-Crithidia *Bioassays*

211 *Preparing nectar and pollen*. We used nectar and pollen from treatment plants to determine how
212 AMF and fertilizer affected bee interactions with a gut pathogen. During collection, we pooled
213 nectar and pollen within plants in 1.5 mL micro-centrifuge tubes and stored in a -20°C freezer.
214 We ultimately pooled pollen and nectar samples within treatment group for use in
215 bioassays. When we pooled pollen, we separated it from anthers using a modified insect vacuum
216 (BioQuip Products, Inc. Compton, California, USA) outfitted with a 25-μm filter
217 (CellMicroSieves, BioDesign Inc. of NY, Carmel, New York, USA) to catch pollen, overlaid
218 with a piece of mesh to remove anthers.

219

220 *Treatment Diets*. We conducted week-long bioassays from early January through early February
221 2018. To assess the effect of diet from plants grown under different soil conditions on *Crithidia*-
222 infected *B. impatiens* (BioBest LTD, Leamington, Ontario, Canada), infected bees were provided
223 with pollen and nectar from one of the four treatment combinations, or a control diet. The control
224 diet contained a wildflower pollen blend collected by honeybees from an organic farm in North
225 Dakota, USA and artificial sugar water mimicking the 12:15:10 glucose:fructose:sucrose ratios
226 in *N. tabacum* nectar (Tiedge and Lohaus 2017) and the 16% overall sugar concentration
227 observed in our nectar. We note that the control diet is not intended to be interpreted as non-
228 mycorrhizal, but rather a comparison of tobacco nectar and pollen to a more standardized lab diet
229 typically used in experiments. Because preliminary trials suggested pure tobacco pollen may be
230 toxic to bees (data not shown), we mixed tobacco pollen from each treatment group in a 1:1 ratio
231 with the control wildflower pollen. To prepare pollen for bee consumption, we mixed 1 g of each
232 pollen treatment with 1 mL of distilled water, adding small amounts of water to reach the

consistency of moist paste. We stored this in a -20°C freezer until use, adding small amounts of water as needed when pollen dried out. For concentrations of anabasine and nicotine in bee diets, see Table S2. Bees received 330 μL of nectar and 0.013 ± SE 0.006 g of pollen on the first day of the experiment. Nectar was topped off to 330 µL each day. Bees received fresh pollen each day, unless they still had adequate amounts that had not dried out.

Infecting bees. Experimental worker bees were infected using inoculum made from bee colonies maintained in the laboratory that had been initially infected with *Crithidia* from wild *B. impatiens* (Stone Soup Farm, Hadley, Massachusetts, USA, 42.363911 N, -72.567747 W) in fall 2014, transferring to new colonies as needed. Following methods outlined in Richardson et al. (2015), we made inoculum from a *Crithidia*-infected colony (Appendix S1, *Inoculating bees*), and infected experimental bees with 15-20 µL of inoculum so that they received 9-12,000 *Crithidia* cells; all bees on a single day were given only one inoculum volume depending on availability, such that variation due to quantity of cells provided was part of variation due to inoculation date. Both concentrations are well within natural variation in feces (Otterstatter and Thomson 2006). Bees that did not consume inoculum were excluded from the trial. We housed experimental bees in a deli cup modified for pollen and nectar feeding (Appendix S1, *Bee containers*) in a dark incubator at 28°C. We monitored and recorded bee death daily. Surviving bees were dissected seven days after inoculation and *Crithidia* cells were assessed with the same methods used to make inoculum. We measured the marginal cell of the right forewing as an estimate of body size. Sample sizes were limited by food availability in the low-fertilizer treatments, which produced far fewer flowers (and therefore less nectar and pollen). Initial and final sample sizes (due to deaths and escapes) were: AMF+/high fertilizer (55 initial, 33 final

bees), AMF+/low fertilizer (10 initial, 3 final), AMF-/high fertilizer (60 initial, 41 final), AMF-/low fertilizer plants (14 initial, 9 final), and control diet (58 initial, 30 final).

Measuring Consumption. To measure pollen and nectar consumption, we weighed pollen and nectar dispensers with their rations before placing them in deli cups on the fourth day post-inoculation. The next day, we weighed them again and used the difference to estimate consumption. To account for evaporation, we simultaneously ran control consumption trials every time we measured consumption (10 replicates of control pollen and nectar, 10 replicates of tobacco nectar), which mimicked the experimental feeding setup without bees.

Statistical Analyses

All analyses were performed using R version 3.4.1 (R Core Team, 2019), and plots were made using ggplot2 (Wickham 2016). For all analyses we fit a set of candidate models and used the *AICcmodavg* package to perform model selection (shown in Table S3). We compared top models to one that excluded each term of interest (e. g., AMF, fertilizer), using ANOVA to test for a significant effect on the response. To assess pairwise differences between treatments, we used the emmeans package, adjusting for multiple comparisons using false discovery rate (FDR). To assess treatment effects on plant size and nectar volume, we fit linear models using AMF, fertilizer, their interaction, block, aphids and mold (VIF < 2) in the top models. We used number of leaves/cm to represent plant size (Appendix S1 *Plant Size*).

We used the *coxme* package to conduct a Cox Proportional Hazards test of treatment effect on flowering date. The global model included fertilizer, AMF, their interaction, block, mold and aphid level. Survival analysis estimates differences in the time to an event (flowering), while accounting for censored values (plants that failed to flower).

To assess plant treatment effects on pollen anabasine and nicotine concentrations (ng/mg), we performed two analyses. Overall, 21% and 14% of plants had no pollen nicotine and anabasine, respectively, so we analyzed likelihood of having the compound using a binomial model and the *MASS* package. We then fit a generalized linear model with a negative binomial distribution to test treatment effects on concentration of pollen nicotine/anabasine using all plants, including those with zero values. We added a squared term of sampling date to test for quadratic effect of sampling date, due to patterns observed in exploratory analyses. We used the *car* package to test for collinearity among multiple predictors. There was not strong collinearity between sampling date and treatment (VIF < 2 in both cases), indicating that the effect of treatment was not confounded with that of sampling date. The global models included AMF, fertilizer, their interaction, block, sampling date, squared sampling date, mold and aphid level. The top model for anabasine included AMF, fertilizer, sampling date and squared sampling date; it did not include aphids, mold, block, or the AMF by fertilizer interaction. The top model for nicotine included AMF, fertilizer, their interaction, sampling date and squared sampling date, and did not include aphids, mold or block. During data exploration, we noticed that variation in chemical concentration differed between treatments. Because inter-plant variation in pollen chemistry may be ecologically significant, we used a Levene test to evaluate whether variance differed by treatment. We evaluated the correlation between anabasine and nicotine concentrations using a Kendall rank correlation test.

To assess effects of AMF and fertilizer on *Crithidia* counts, we used two approaches. First, we analyzed treatments excluding the control diet to compare the effect of AMF, fertilizer and their interactions on pathogen load. We note that all four treatments incorporated equal ratios of tobacco to wildflower pollen; we hoped that mixing tobacco and wildflower pollen would

facilitate bee survival but note that this also means our diets are a conservative evaluation of the strength of treatment effects. We conducted this analysis using a generalized linear model with AMF, fertilizer, their interaction, colony, inoculation date, bee size (estimated as wing marginal cell length), pollen and nectar consumption, and anabasine and nicotine consumption (calculated as mg pollen consumed * concentration of each compound in diet treatment) in the global model, with a negative binomial distribution. Our top model included all covariates except inoculation date, and measures of pollen/nectar and nicotine/anabasine consumption. We used a *post hoc* chi-squared test to test residual deviance. Next, we analyzed differences in counts between all diets, treating diet as a single five-level factor. The goal of this additional analysis was to ask if tobacco diets differed from the control diet. The global models had diet, colony, inoculation date, bee size, nicotine consumption, anabasine consumption and either pollen or nectar consumption as covariates, and a negative binomial distribution. Replication was too low to include both pollen and nectar consumption as covariates in the same global model. The top model included diet treatment (5 levels), bee size and colony, but not nectar or pollen consumption. To assess differences between tobacco and control diets, we performed a *post hoc* generalized linear hypothesis test using the *multcomp* package, adjusting for multiple comparisons using FDR. For both analyses testing the effect of diet on *Crithidia* counts, we ran models excluding the diet treatment with low replication (AMF+, low fertilizer), and found that fertilizer and AMF still significantly affected *Crithidia* in similar directions (Appendix S1, *Crithidia counts*). Separately, we tested whether diet treatment affected consumption of pollen or nectar using a linear model (Appendix S1, *Consumption*). We conducted Cox Proportional Hazards tests to assess diet effect on bee survival. Because replication was too low to use the five-level factor of diet as a predictor, we excluded the control diet and tested the effects of fertilizer and AMF separately.

326

327 **Results**

328 *Treatment effects on plant size and floral traits*

329 High fertilizer increased plant size by 22% ($F_{9, 96} = 6.6$, $p < 0.001$) and likelihood of
330 flowering by a factor of 2.1 relative to low fertilizer (Figure S1; log likelihood = -713.33, $= p <$
331 0.001). AMF increased plant size by about 15% ($F_{9, 96} = 6.6$, $p = 0.002$) but reduced the
332 likelihood of flowering by a factor of -0.7 (Appendix S1, *AMF and nutrients influence flowering*
333 *phenology,* Figure S1; log likelihood = -671.7, $p < 0.001$). Aphids did not affect flowering time
334 (log likelihood = -663.75, $p = 0.094$) or size ($F_{9, 96} = 6.6$, $p = 0.094$). We concluded that AMF
335 and fertilizer treatments had no effect on nectar sugar concentration after finding no variation in
336 the first 40 plants sampled (10 per treatment combination; all plants had 16% sucrose
337 equivalents). In contrast with previous work on *N. tabacum* nectar (Adler et al. 2006), we did not
338 find anabasine or nicotine in any of our nectar samples. While this finding was unexpected, it is
339 possible that differences in abiotic factors between our study site and that of previous work
340 account for this result (Appendix S1, *Pollen and Nectar Chemistry*).

341 AMF had no effect on the likelihood of anabasine in pollen ($\chi^2 = 57.789$, N=115, $p =$
342 0.092), while plants sampled at later dates were more likely to have anabasine ($\chi^2 = 85.839$,
343 N=115, $p < 0.001$). Fertilizer had no effect on the likelihood of anabasine in pollen and was not
344 in the top model. However, high fertilizer increased anabasine concentration by 484% compared
345 to low fertilizer (Figure 1; $\chi^2 = 94.949$, N = 115, $p < 0.001$). AMF had a non-significant trend to
346 increase pollen anabasine concentration by 56% (Figure 1; $\chi^2 = 85.888$, N=115, $p = 0.072$).
347 Sampling date had a positive quadratic effect on pollen anabasine concentration ($\chi^2 = 92.165$, N
348 = 115, $p < 0.001$), such that anabasine concentrations peaked mid-season. AMF and fertilizer did

not interact, and this term was not in the top model. We found similar results using post-extraction weights, except that the effect of AMF became significant (Appendix S1, *Pollen and Nectar Chemistry*).

Neither AMF nor fertilizer affected the likelihood pollen would have nicotine ($\chi^2 > 77$, $p > 0.21$ for both), but plants that flowered later were more likely to have nicotine ($\chi^2 = 147.83$, $p < 0.001$). Plants with high fertilizer had 173% more pollen nicotine than those with low fertilizer (log likelihood = -614.884 on 111 df, $p = 0.003$). However, these effects depended on the interaction between fertilizer and AMF, such that AMF plants had reduced nicotine concentration under high, but not low fertilizer conditions (Figure 1; log likelihood = -607.242 on 110 df, $p = 0.044$). Alone, AMF did not significantly affect nicotine concentration (log likelihood = -608.183, $p = 0.082$). Sampling date had a positive quadratic effect on nicotine concentration (log likelihood= -68.624 on 109 df, $p = 0.02$), such that nicotine concentrations peaked mid-season. We found similar results using post-extraction weights, except that the interaction between AMF and fertilizer was no longer significant (Appendix S1 *Pollen and Nectar Chemistry*).

High fertilizer increased pollen anabasine ($F_{1,113} = 0.828$, $p < 0.001$) and nicotine ($F_{1,113} = 10.97$, $p = 0.001$) concentration variance compared to low fertilizer. AMF did not affect anabasine concentration variance ($F_{1,112} = 1.20$, $p = 0.276$), but marginally decreased nicotine concentration variance ($F_{1,113} = 3.68$, $p = 0.058$). Anabasine and nicotine concentration were not correlated (coefficient = 0.154, p = 0.099), and there was no correlation between early and late-season anabasine or nicotine concentrations (Appendix S1, *Pollen and Nectar Chemistry*).

Treatment effects on pathogen counts and bee survival via changes in nectar and pollen

When we excluded the control diet and tested the interaction of AMF and fertilizer on *Crithidia* counts, AMF and fertilizer interacted such that diet from plants grown without AMF and low fertilizer resulted in the lowest *Crithidia* counts (Figure 2; interaction term log likelihood= -508.07 on 81 df, $p = 0.04$). Alone, neither AMF (log likelihood = -509.63 on 82 df, $p = 0.211$), nor fertilizer (log likelihood = -510.72 on 82 df, $p = 0.104$) affected *Crithidia*. Colony significantly affected counts (log likelihood = -514.82 on 81 df, $p = 0.001$), and larger bees had lower infection (log likelihood = -508.73 on 81 df, $p = 0.028$). Pairwise comparisons revealed that for plants grown without AMF, diet from high fertilizer treatments increased *Crithidia* counts compared to low fertilizer ($p = 0.010$). In contrast, for plants grown with AMF, diet from high fertilizer treatments had no effect on *Crithidia* counts compared to those grown with low fertilizer ($p = 0.975$). For plants grown with high fertilizer, there was a trend for AMF to result in lower *Crithidia* counts than those grown without AMF ($p = 0.053$).

Diet had a significant effect on *Crithidia* counts when we included the control diet (wildflower pollen and sugar solution) in the analysis (log likelihood = -711.64 on 118 df, $p = 0.040$). When we compared the control diet to the four tobacco diets using non-orthogonal contrasts, the control did not differ from plants grown with AMF regardless of fertilizer treatment ($p > 0.46$). Of plants without AMF, diet from plants with high fertilizer did not differ from the control ($p = 0.328$), and plants grown with low fertilizer had lower *Crithidia* counts than the control ($p = 0.035$). Colony affected *Crithidia* counts (log likelihood = - 711.88, $p < 0.001$), and larger bees had lower infection (log likelihood = - 707.80, $p = 0.013$). There was no effect of AMF (Wald test = 1.18, df = 1, $p = 0.277$) or fertilizer (Wald statistic = 1.36, df = 2, $p = 0.2443$) on survival. Pollen consumption and nectar consumption did not differ between diet

394 treatments, and diet treatment was not included in the top model of nectar consumption
395 (Appendix 1, *Consumption*).
396
397 **Discussion**
398 *AMF and nutrients affect pollen and nectar secondary chemistry*
399 We found that soil nutrients and mycorrhizal fungi affect pollen alkaloid concentrations.
400 High nutrients increased pollen nicotine and anabasine concentrations (Figure 1). This is
401 consistent with previous work showing that fertilizer increased nectar secondary compounds
402 (Adler et al. 2006), but to our knowledge is the first study to examine effects of belowground
403 environment on pollen secondary chemistry. Secondary metabolite production can be costly
404 (Züst et al. 2011), and high fertilizer plants may have more resources for defense production. The
405 effect of AMF on pollen alkaloids was more subtle. No study to our knowledge has researched
406 the effect of mycorrhizal fungi on nectar or pollen secondary chemistry, although one study
407 found that higher AMF colonization was negatively correlated with floral volatile compound
408 emission rate and diversity (Becklin et al. 2011). In our study, AMF modified effects of fertilizer
409 on nicotine in pollen (Figure 1). The increased suppressive effect of AMF on nicotine under high
410 fertilizer could be due to AMF-plant competition for nitrogen, an important ingredient in alkaloid
411 synthesis (Xi et al. 2008); under high resource conditions, plants and AMF may compete for,
412 rather than equally share, resources (Walder and van der Heijden 2015). This finding supports
413 our hypothesis that the effect of AMF on alkaloids is conditional on soil nutrients. On the other
414 hand, we did not find support for the hypothesis that AMF effect on alkaloids is due to defense
415 up-regulation alone, because AMF had inconsistent effects on alkaloids across fertilizer
416 treatments (Figure 1).

AMF and nutrients affected pollen alkaloid concentrations, but we did not detect any anabasine or nicotine in nectar. Other work also found higher and more diverse secondary compounds in pollen than nectar (Cook et al. 2013, Palmer-Young et al. 2019), consistent with Optimal Defense Theory, which predicts that plants invest more defense in tissues more directly related to fitness. Since many plants face pollen theft by ineffective pollinators (Solís-Montero et al. 2015), and floral alkaloids can reduce floral larceny (Barlow et al. 2017), reduced nicotine in plants with AMF and high fertilizer suggests a potential cost of the AMF-plant mutualism that depends on nutrient availability. While this hypothesis would need to be tested in the presence of floral antagonists, it is consistent with other studies showing that AMF exists along a mutualist-parasite continuum (reviewed in Johnson 2010).

In addition to having higher mean anabasine and nicotine concentrations, plants grown with high fertilizer had more variable anabasine and nicotine concentrations than those grown with low fertilizer. Unpredictable nectar quality can alter pollinator behavior and increase outcrossing in wild *Nicotiana* species (Kessler et al. 2012). If soil conditions increase variability of floral resources, there could be important implications for plant population dynamics or crop yields. Future studies should also examine whether soil conditions affect variance of pollen macronutrients; a recent meta-analysis showed that variation in, rather than the mean, of plant nutrient traits is an important limiting factor for herbivorous insect pest performance (Wetzel et al. 2016). If belowground conditions cause variation in nutritive quality of floral rewards, and if pollinators, which can be thought of as a specialized type of herbivore, respond to nutrient variation as insect pests do, then increased variation in floral resource nutritive quality could have a detrimental effect on pollinator growth and survival.

AMF and nutrients affect bee pathogens via changes in floral rewards

We found that plant interactions with the belowground environment can affect pathogen cell counts in pollinators via changes in nectar and/or pollen. Although other work has shown that pollen and nectar quality can affect bee resistance to pathogens (Brunner et al. 2014, Richardson et al. 2015), this is the first study to demonstrate that plant interactions with their abiotic and biotic environment can shape bee-pathogen interactions. Plants have a well-established ability to structure communities. This can happen directly, such as when plant diversity drives arthropod diversity (Potts et al. 2003), or indirectly when plants act as intermediaries of species interactions (Strauss 1997). Our results demonstrate the potential for multitrophic consequences of plant-environment interactions on pathogens of pollinators.

Treatment effects on *Crithidia* counts could not be explained by changes in floral alkaloid concentrations. Because anabasine- and nicotine-containing sucrose solutions reduced *Crithidia* pathogen counts in bumblebees previously (Richardson et al. 2015) and fertilizer increased nectar anabasine concentrations in *N. tabacum* (Adler et al. 2006), we hypothesized that high fertilizer would reduce *Crithidia* counts by increasing nectar or pollen anabasine and possibly nicotine. However, pollen with low mean anabasine reduced *Crithidia* most, and pollen with high anabasine resulted in intermediate *Crithidia* counts (Figure 1, Figure 2). In one study, nectar nicotine and anabasine reduced *Crithidia* independently but not in tandem (Thorburn et al. 2015), which could explain some of our results; nicotine and anabasine could have either neutral, synergistic or antagonistic interactions at varying concentrations. However, we cannot rule out the possibility of a mechanism other than alkaloids affecting *Crithidia* counts.

Other components of pollen or nectar could act with or independent of alkaloids to reduce *Crithidia* counts. For example, excessive soil nutrients can decrease amino acid concentrations in

463 pollen, with consequences for bumblebee larval survival (Ceulemans et al. 2017). Bumblebees
464 consuming a pollen-limited diet have reduced expression of immune genes (Brunner et al. 2014),
465 which could be due to lack of protein or other pollen constituents. Alternatively, diets rich in
466 certain constituents could have promoted *Crithidia* growth by providing the pathogen with a
467 more beneficial food source. Future studies should examine ecological factors that shape pollen
468 and nectar constituents other than alkaloids, and manipulate presence and concentrations in bee
469 diet to identify mechanisms mediating belowground effects on bee-pathogen interactions.
470 In conclusion, our results demonstrate that abiotic and biotic soil components change
471 floral defensive chemistry and traits that affect bumblebee pathogens. These results suggest
472 potential novel costs of the mycorrhizae-plant mutualism *via* changes in floral reward chemistry,
473 and pose exciting directions for studying context dependency of mutualisms in communities.

474 **Acknowledgements**

475 This research took place on occupied lands of the Wbanaki Confederacy and Nipmuc nation. We
476 thank E. Stone, E. Palmer-Young, E. Amponsah, B. Joyce, G. LoCascio, R. Pasquale, D. Farman
477 and K. Urban-Mead for feedback and help with data collection, Z. Getman-Pickering, J. Thaler
478 and two reviewers for comments on the manuscript, BioBest (Ontario, Canada) for bee colonies,
479 Premier Tech Biotechnologies (Quebec, Canada) for AMF inoculum, and H. Grab, C.
480 Sutherland, the UMass Amherst Quantitative Statistics Group and Cornell Statistical Consulting
481 Unit for advice on data analysis. Funding was provided by the UMass Amherst Honors College
482 Research Fellowships, Torrey Plant Biology Fellowship, Nauset Garden Club, a PGAV
483 Pollinator Research Grant, NIH 1 R01 GM122062-01, NSF-DEB-1258096, and USDA-AFRI
484 2013-02536. Opinions, findings, conclusions or recommendations expressed in this material are
485 those of the authors and do not necessarily reflect the views of the funding agency.

References

Adler, L. S. 2000. The ecological significance of toxic nectar. Oikos 91:409–420.

Adler, L. S., M. Wink, M. Distl, and A. J. Lentz. 2006. Leaf herbivory and nutrients increase nectar alkaloids. Ecology Letters 9:960–967.

Alaux, C., F. Ducloz, D. Crauser, and Y. L. Conte. 2010. Diet effects on honeybee immunocompetence. Biology Letters.

Andrade, S. a. L., S. Malik, A. C. H. F. Sawaya, A. Bottcher, and P. Mazzafera. 2013. Association with arbuscular mycorrhizal fungi influences alkaloid synthesis and accumulation in *Catharanthus roseus* and *Nicotiana tabacum* plants. Acta Physiologiae Plantarum 35:867–880.

Anthony, W. E., E. C. Palmer-Young, A. S. Leonard, R. E. Irwin, and L. S. Adler. 2015. Testing dose-dependent effects of the nectar alkaloid anabasine on trypanosome parasite loads in adult bumblebees. PLOS ONE 10:e0142496.

Ashman, T.-L., T. M. Knight, J. A. Steets, P. Amarasekare, M. Burd, D. R. Campbell, M. R. Dudash, M. O. Johnston, R. J. Mitchell, M. T. Morgan, and W. G. Wilson. 2004. Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. Ecology 85:2408–2421.

Barber, N. A., and N. L. Soper Gordon. 2015. How do belowground organisms influence plant–pollinator interactions? Journal of Plant Ecology 8:1–11.

Barlow, S. E., G. A. Wright, C. Ma, M. Barberis, I. W. Farrell, E. C. Marr, A. Brankin, B. M. Pavlik, and P. C. Stevenson. 2017. Distasteful nectar deters floral robbery. Current Biology 27:2552-2558.e3.

Becklin, K. M., G. Gamez, B. Uelk, R. A. Raguso, and C. Galen. 2011. Soil fungal effects on floral signals, rewards, and aboveground interactions in an alpine pollination web.

American Journal of Botany 98:1299–1308.

Bentley, B., and T. S. Elias. 1983. The Biology of Nectaries. Columbia University Press.

Biller, O. M., L. S. Adler, R. E. Irwin, C. McAllister, and E. C. Palmer-Young. 2015. Possible synergistic effects of thymol and nicotine against *Crithidia bombi* parasitism in bumble bees. PLOS ONE 10:e0144668.

Brown, M. J. F., R. Schmid-Hempel, and P. Schmid-Hempel. 2003. Strong context-dependent virulence in a host-parasite system: reconciling genetic evidence with theory. Journal of Animal Ecology 72:994–1002.

Brundrett, M. C., and L. Tedersoo. 2018. Evolutionary history of mycorrhizal symbioses and global host plant diversity. New Phytologist 220:1108–1115.

Brunner, F. S., P. Schmid-Hempel, and S. M. Barribeau. 2014. Protein-poor diet reduces host-specific immune gene expression in *Bombus terrestris* . Proc. R. Soc. B 281:20140128.

Cameron, S. A., J. D. Lozier, J. P. Strange, J. B. Koch, N. Cordes, L. F. Solter, and T. L. Griswold. 2011. Patterns of widespread decline in North American bumble bees. Proceedings of the National Academy of Sciences 108:662–667.

Campos-Soriano, L., J. M. García-Garrido, and B. S. Segundo. 2010. Activation of basal defense mechanisms of rice plants by *Glomus intraradices* does not affect the arbuscular mycorrhizal symbiosis. New Phytologist 188:597–614.

Ceulemans, T., E. Hulsmans, W. V. Ende, and O. Honnay. 2017. Nutrient enrichment is associated with altered nectar and pollen chemical composition in *Succisa pratensis* Moench and increased larval mortality of its pollinator *Bombus terrestris* L. PLOS ONE 12:e0175160.

Conroy, T. J., E. C. Palmer-Young, R. E. Irwin, and L. S. Adler. 2016. Food limitation affects

533 parasite load and survival of Bombus impatiens (Hymenoptera: Apidae) infected with
534 *Crithidia* (Trypanosomatida: Trypanosomatidae). Environmental Entomology 45:1212–
535 1219.
536 Cook, D., J. S. Manson, D. R. Gardner, K. D. Welch, and R. E. Irwin. 2013. Norditerpene
537 alkaloid concentrations in tissues and floral rewards of larkspurs and impacts on
538 pollinators. Biochemical Systematics and Ecology 48:123–131.
539 Detzel, A., and M. Wink. 1993. Attraction, deterrence or intoxication of bees (*Apis mellifera*) by
540 plant allelochemicals. Chemoecology 4:8–18.
541 Gegear, R. J., M. C. Otterstatter, and J. D. Thomson. 2006. Bumble-bee foragers infected by a
542 gut parasite have an impaired ability to utilize floral information. Proceedings of the
543 Royal Society of London B: Biological Sciences 273:1073–1078.
544 Gillespie, S. 2010. Factors affecting parasite prevalence among wild bumblebees. Ecological
545 Entomology 35:737–747.
546 Goulson, D., E. Nicholls, C. Botías, and E. L. Rotheray. 2015. Bee declines driven by combined
547 stress from parasites, pesticides, and lack of flowers. Science 347:1255957.
548 Johnson, N. C. 2010. Resource stoichiometry elucidates the structure and function of arbuscular
549 mycorrhizas across scales. New Phytologist 185:631–647.
550 Kessler, D., S. Bhattacharya, C. Diezel, E. Rothe, K. Gase, M. Schöttner, and Baldwin. 2012.
551 Unpredictability of nectar nicotine promotes outcrossing by hummingbirds in *Nicotiana*
552 *attenuata*. The Plant Journal 71:529–538.
553 Kessler, D., K. Gase, and I. T. Baldwin. 2008. Field experiments with transformed plants reveal
554 the sense of floral scents. Science 321:1200–1202.
555 Klein, A., I. Steffan–Dewenter, and T. Tscharntke. 2003. Fruit set of highland coffee increases

556 with the diversity of pollinating bees. Proceedings of the Royal Society of London. Series
557 B: Biological Sciences 270:955–961.
558 Klein, A.-M., B. E. Vaissière, J. H. Cane, I. Steffan-Dewenter, S. A. Cunningham, C. Kremen,
559 and T. Tscharntke. 2007. Importance of pollinators in changing landscapes for world
560 crops. Proceedings of the Royal Society of London B: Biological Sciences 274:303–313.
561 Koch, H., M. J. Brown, and P. C. Stevenson. 2017. The role of disease in bee foraging ecology.
562 Current Opinion in Insect Science 21:60–67.
563 Liu, J., I. Maldonado-Mendoza, M. Lopez-Meyer, F. Cheung, C. D. Town, and M. J. Harrison.
564 2007. Arbuscular mycorrhizal symbiosis is accompanied by local and systemic
565 alterations in gene expression and an increase in disease resistance in the shoots. The
566 Plant Journal 50:529–544.
567 Manson, J. S., M. C. Otterstatter, and J. D. Thomson. 2010. Consumption of a nectar alkaloid
568 reduces pathogen load in bumble bees. Oecologia 162:81–89.
569 McArt, S. H., H. Koch, R. E. Irwin, and L. S. Adler. 2014. Arranging the bouquet of disease:
570 floral traits and the transmission of plant and animal pathogens. Ecology Letters 17:624–
571 636.
572 McGonigle, T. P., M. H. Miller, D. G. Evans, G. L. Fairchild, and Swan. 1990. A new method
573 which gives an objective measure of colonization of roots by vesicular—arbuscular
574 mycorrhizal fungi. New Phytologist 115:495–501.
575 Otterstatter, M. C., and J. D. Thomson. 2006. Within-host dynamics of an intestinal pathogen of
576 bumble bees. Parasitology 133:749–61.
577 Palmer-Young, E. C., I. W. Farrell, L. S. Adler, N. J. Milano, P. A. Egan, R. R. Junker, R. E.
578 Irwin, and P. C. Stevenson. 2019. Chemistry of floral rewards: intra- and interspecific

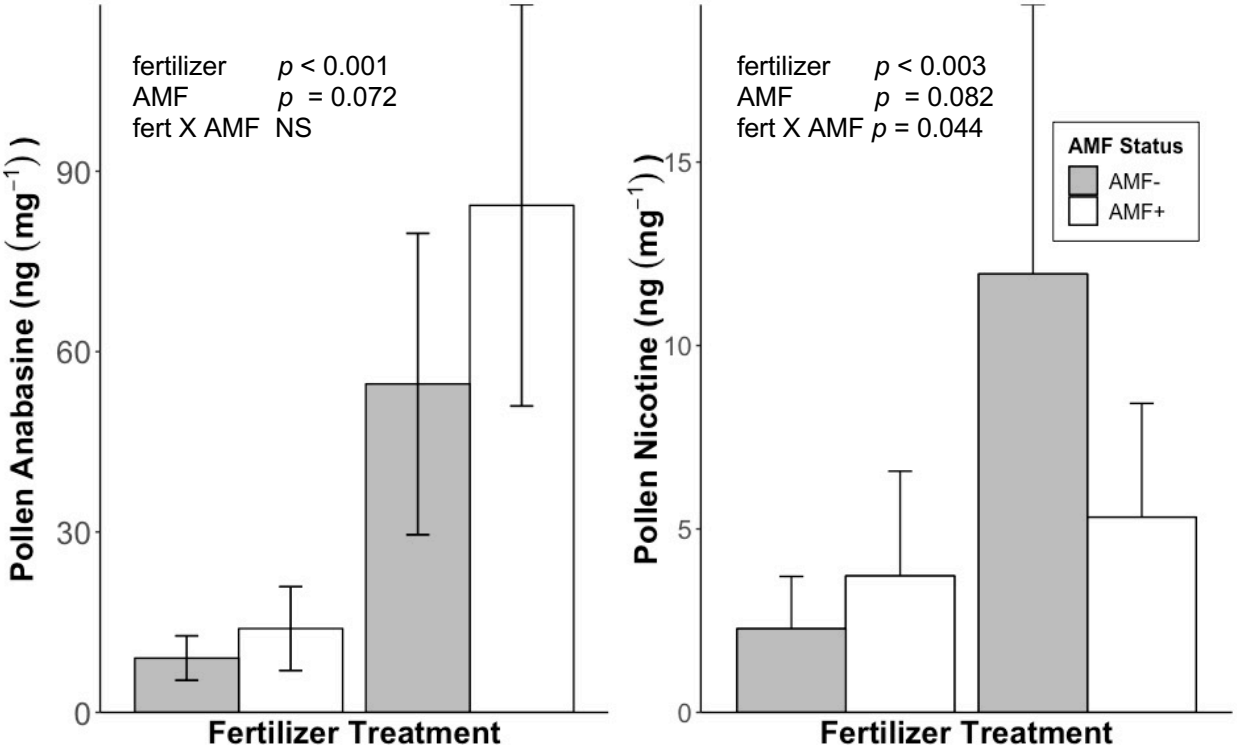
579 variability of nectar and pollen secondary metabolites across taxa. Ecological
580 Monographs 89:e01335.
581 Palmer-Young, E. C., B. M. Sadd, R. E. Irwin, and L. S. Adler. 2017. Synergistic effects of floral
582 phytochemicals against a bumble bee parasite. Ecology and Evolution 7:1836–1849.
583 Palmer-Young, E. C., and L. Thursfield. 2017. Pollen extracts and constituent sugars increase
584 growth of a trypanosomatid parasite of bumble bees. PeerJ 5:e3297.
585 Potts, S. G., J. C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger, and W. E. Kunin. 2010.
586 Global pollinator declines: trends, impacts and drivers. Trends in Ecology & Evolution
587 25:345–353.
588 Potts, S. G., B. Vulliamy, A. Dafni, G. Ne'eman, and P. Willmer. 2003. Linking bees and
589 flowers: how do floral communities structure pollinator communities? Ecology 84:2628–
590 2642.
591 R Core Team (2019). R: A language and environment for statistical computing. R Foundation for
592 statistical computing, Vienna, Austria. URL https://www.R-project.org
593 Richardson, L. L., L. S. Adler, A. S. Leonard, J. Andicoechea, K. H. Regan, W. E. Anthony, J. S.
594 Manson, and R. E. Irwin. 2015. Secondary metabolites in floral nectar reduce parasite infections
595 in bumblebees. Proceedings of the Royal Society B: Biological Sciences 282.
596 Solís-Montero, L., C. H. Vergara, and M. Vallejo-Marín. 2015. High incidence of pollen theft in
597 natural populations of a buzz-pollinated plant. Arthropod-Plant Interactions 9:599–611.
598 Strauss, S. Y. 1997. Floral characters link herbivores, pollinators and plant fitness. Ecology
599 78:1640–1645.
600 Thorburn, L. P., L. Adler, R. Irwin, and E. C. Palmer-Young. 2015. Variable effects of nicotine,
601 anabasine, and their interactions on parasitized bumble bees. F1000Research 4.

602 Tiedeken, E. J., P. A. Egan, P. C. Stevenson, G. A. Wright, M. J. F. Brown, E. F. Power, I.
603 Farrell, S. M. Matthews, and J. C. Stout. 2016. Nectar chemistry modulates the impact of
604 an invasive plant on native pollinators. Functional Ecology 30:885–893.
605 Tiedge, K., and G. Lohaus. 2017. Nectar sugars and amino acids in day- and night-flowering
606 *Nicotiana* species are more strongly shaped by pollinators' preferences than organic acids
607 and inorganic ions. PLoS ONE 12.
608 Tihelka, E. 2017. The immunological dependence of plant-feeding animals on their host's
609 medical properties may explain part of honey bee colony losses. Arthropod-Plant
610 Interactions:1–8.
611 Vannette, R. L., and M. D. Hunter. 2009. Mycorrhizal fungi as mediators of defence against
612 insect pests in agricultural systems. Agricultural and Forest Entomology 11:351–358.
613 Walder, F., and M. G. A. van der Heijden. 2015. Regulation of resource exchange in the
614 arbuscular mycorrhizal symbiosis. Nature Plants 1:15159.
615 Wetzel, W. C., H. M. Kharouba, M. Robinson, M. Holyoak, and R. Karban. 2016. Variability in
616 plant nutrients reduces insect herbivore performance. Nature 539:425–427.
617 Wickham, H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.
618 Williams, P. H., R. W. Thorp, L. L. Richardson, and S. R. Colla. 2014. Bumble bees of North
619 America: an identification guide. Princeton University Press.
620 Xi, X., C. Li, and F. Zhang. 2008. Tobacco plants can use nitrogen taken up before mechanical
621 wounding to synthesize nicotine afterwards. Plant Signaling & Behavior 3:87–90.
622 Züst, T., B. Joseph, K. K. Shimizu, D. J. Kliebenstein, and L. A. Turnbull. 2011. Using knockout
623 mutants to reveal the growth costs of defensive traits. Proceedings of the Royal Society
624 of London B: Biological Sciences 278:2598–2603

Figure Legends

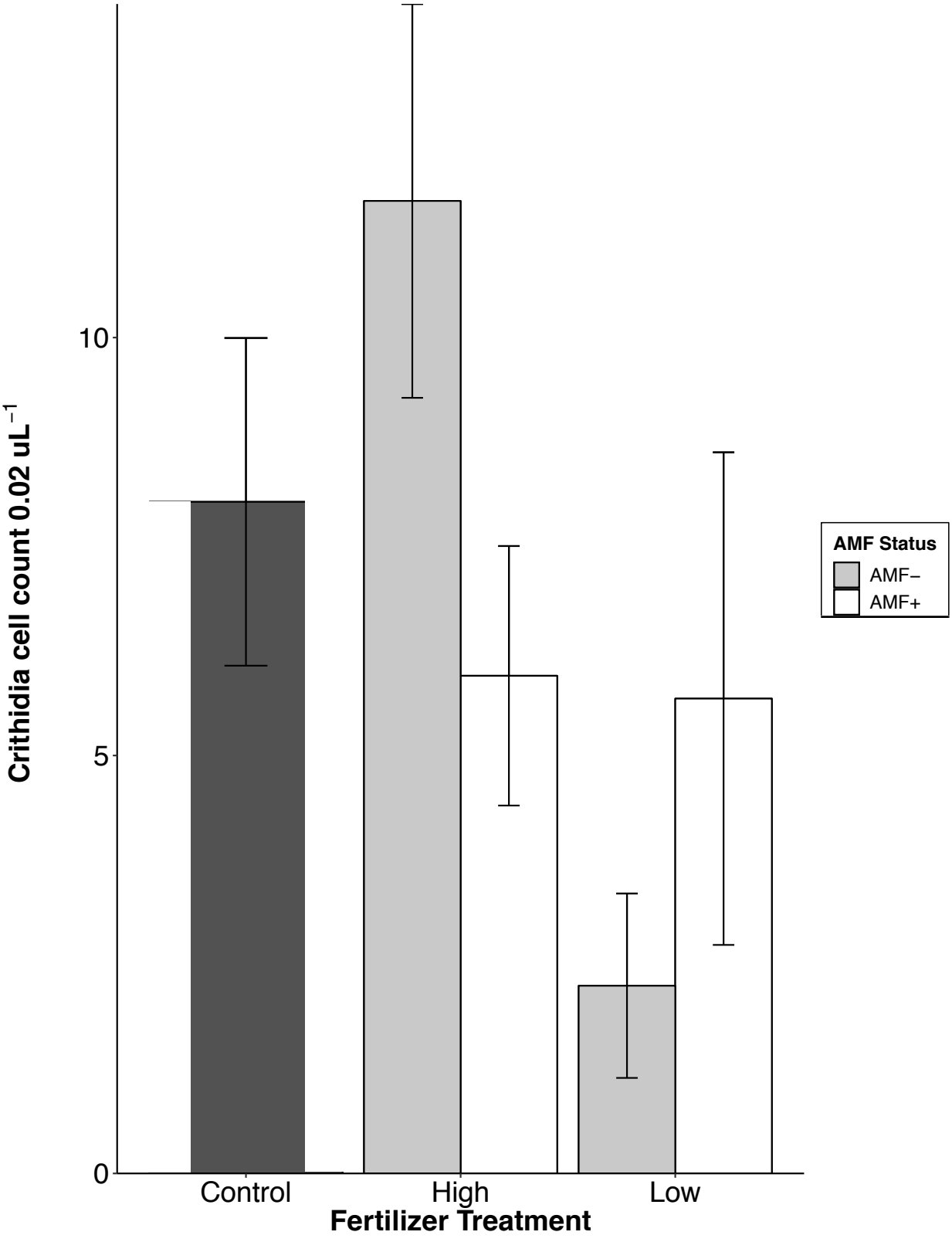
Figure 1. Effects of AMF and fertilizer treatments on pollen anabasine and nicotine concentrations. Effect of AMF and fertilizer on anabasine and nicotine concentrations. Error bars represent +/- SE. Results shown are back-transformed model estimates.

Figure 2. Effects of diet on *Crithidia* cell counts in bees. Effect of AMF and nutrients on *Crithidia* counts. Black bar shows control diet, and error bars show +/- SE. Cell counts are back-transformed estimates from the full model comparing control diet to all four diet types.



643

644 **Figure 1.**



645

646 **Figure 2**