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EFFECTS OF FLORAL PHYTOCHEMICALS ON GROWTH AND EVOLUTION OF A PARASITE OF BUMBLE BEES

A Dissertation Presented

by

EVAN C. PALMER-YOUNG

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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Organismic and Evolutionary Biology
EFFECTS OF FLORAL PHYTOCHEMICALS ON GROWTH AND EVOLUTION OF A PARASITE OF BUMBLE BEES

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To my parents, Jean and Larry
ACKNOWLEDGMENTS

This work was enabled by the kindness and support of my colleagues, friends, family, and funders.

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Finally, apologies to those whose names I never knew or have forgotten to acknowledge here.
ABSTRACT

EFFECTS OF FLORAL PHYTOCHEMICALS ON GROWTH AND EVOLUTION OF A PARASITE OF BUMBLE BEES

FEBRUARY 2018

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Directed by: Professor Lynn S. Adler

Background: Nectar and pollen are rich in phytochemicals, some of which can reduce disease in pollinators, including agriculturally important honey and bumble bees. Floral phytochemicals could influence the ecological and evolutionary relationships between plants, their pollinators, and parasites that cause pollinator disease. Antiparasitic effects of phytochemicals could be exploited to ameliorate pollinator disease and decline, and thereby sustain pollinator-dependent agricultural production. However, prior studies showed variable effects of phytochemicals on infection in live bees, where differences in bee genotype, abiotic conditions, and parasite strain could influence results.

Approach: I used cell cultures of the intestinal trypanosome parasite of bumble bees, Crithidia bombi, to (1) describe how resistance to 9 floral phytochemicals varied among 4 parasite strains, (2) describe the antiparasitic effect of phytochemical combinations, and (3) test for evolution of resistance to individual phytochemicals and a two-phytochemical blend.

Results:

(1) Resistance to floral phytochemicals: C. bombi showed striking resistance to the phenolics gallic acid, caffeic acid, and chlorogenic acid at levels beyond those found in nectar and pollen; literature searches showed that C. bombi resistance to these compounds exceeded that of
bloodstream trypanosomes by several orders of magnitude. Phytochemical resistance varied among *C. bombi* isolates, indicating that medicinal effects of phytochemicals are dependent on parasite strain. Thymol and eugenol inhibited growth at concentrations below the toxicity thresholds of bees. Inhibitory concentrations of thymol were similar to those found in *Thymus vulgaris* nectar, indicating that medicinal effects of phytochemicals on pollinator disease are ecologically relevant, and could be achieved through strategic planting of phytochemical-rich flowers.

(2) **Synergistic effects of combined phytochemicals:** Thymol and eugenol had synergistic effects against 3 of 4 *C. bombi* strains—.inhibition of parasites exposed to phytochemical combinations was stronger than predicted based on the activities of isolated phytochemicals. Synergy between phytochemicals suggests that phytochemical combinations may have greater antiparasitic potential in comparison to single phytochemicals. Synergistic phytochemical combinations in diverse floral landscapes could allow pollinators to self-medicate without toxicity, thereby ameliorating diseases that contribute to pollinator decline.

(3) **Evolution of resistance to phytochemicals:** Resistance of *C. bombi* increased under single and combined phytochemical exposure, without any associated cost of reduced growth under phytochemical-free conditions. After six weeks’ exposure, phytochemical concentrations that initially inhibited growth by >50%, and exceeded concentrations in floral nectar, had minimal effects on evolved parasite lines. Unexpectedly, a two-phytochemical combination did not impede resistance evolution compared to single compounds. These results demonstrate that repeated phytochemical exposure, which could occur in homogeneous floral landscapes or with therapeutic phytochemical treatment of managed hives, can cause rapid evolution of resistance in a pollinator parasite. Evolved resistance could diminish the antiparasitic value of phytochemical ingestion, weakening an important natural defense against infection.
**Conclusion:** These results show the potential of phytochemical-rich flowers to directly ameliorate pollinator infection, a recognized contributor to bee decline. Results also suggest benefits of diverse landscapes for pollinator health. Phytochemically complex mixtures in diverse floral landscapes could synergistically inhibit parasite growth and curtail the evolution of phytochemical resistance in parasites, thereby optimizing the medicinal effects of phytochemicals on bees. Deliberate planting of high-phytochemical crops and hedgerow species could reduce the effects of disease on bee populations, thereby benefitting agricultural production.
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CHAPTER 1

BUMBLE BEE PARASITE STRAINS VARY IN RESISTANCE TO PHYTOCHEMICALS

Authors

Evan C. Palmer-Young1*, Ben M. Sadd2, Philip C. Stevenson3, Rebecca E. Irwin4, Lynn S. Adler1

Abstract

Nectar and pollen contain diverse phytochemicals that can reduce disease in pollinators. However, prior studies showed variable effects of nectar chemicals on infection, which could reflect variable phytochemical resistance among parasite strains. Inter-strain variation in resistance could influence evolutionary interactions between plants, pollinators, and pollinator disease, but testing direct effects of phytochemicals on parasites requires elimination of variation between bees. Using cell cultures of the bumble bee parasite *Crithidia bombi*, we determined (1) growth-inhibiting effects of nine floral phytochemicals and (2) variation in phytochemical resistance among four parasite strains.

*C. bombi* growth was unaffected by naturally occurring concentrations of the known antitrypanosomal phenolics gallic acid, caffeic acid, and chlorogenic acid. However, *C. bombi* growth was inhibited by anabasine, eugenol, and thymol. Strains varied >3-fold in

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phytochemical resistance, suggesting that selection for phytochemical resistance could drive parasite evolution. Inhibitory concentrations of thymol (4.53-22.2 ppm) were similar to concentrations in Thymus vulgaris nectar (mean 5.2 ppm). Exposure of C. bombi to naturally occurring levels of phytochemicals—either within bees or during parasite transmission via flowers—could influence infection in nature. Flowers that produce antiparasitic phytochemicals, including thymol, could potentially reduce infection in Bombus populations, thereby counteracting a possible contributor to pollinator decline.

Introduction

Flowers can act as intermediaries for the transmission of plant and animal diseases 1. These diseases include infections of economically and ecologically important pollinators, many species of which are threatened by decline related to the interaction of several factors, including parasites 2–4. For example, honey bee viruses have been found on pollen grains 5,6, and bumble bee and honey bee parasites, including the internationally distributed Nosema spp. and Crithidia spp., can be spread between bee colonies and species that forage on the same plants 7. This transmission can have devastating consequences for native pollinator populations 8,9.

While flowers can act as sites of parasite transfer 10, they also provide food for pollinators. Bee diets consist of floral nectar and pollen that provide carbohydrates and proteins for bee growth and development 11. In addition to macronutrients, floral rewards also contain phytochemicals 12,13, including the major secondary compound classes alkaloids, phenolics, and terpenoids 14. Floral phytochemicals may have a variety of ecological functions, including acting as antimicrobial agents in both plants and the animals that consume them 1. For example, (E)-β-caryophyllene can protect pollen and floral tissue from infection by plant pathogens 15. Likewise, animals that consume antimicrobial phytochemicals may gain protection from their own
parasites, as shown in herbivores. In pollinators, ingestion of floral phytochemicals and certain types of honey were therapeutic for infected honey bees (Apis mellifera). Infection also stimulated collection of phytochemical-rich resins and preference for high-phytochemical nectar, indicating the potential for phytochemicals to improve pollinator health.

Many phytochemicals found in flowers have direct activity against trypanosomes. For example, gallic acid was lethal to Leishmania donovani, and thymol and eugenol inhibited growth of Trypanosoma cruzi and Crithidia fasciculata. It is therefore likely that some floral phytochemicals may inhibit trypanosome parasites of bumble bees. Crithidia bombi is an intestinal trypanosome parasite of bumble bees (Bombus spp.) that decreases queen survival and colony fitness and may exacerbate the negative effects of pesticides and nutritional stress. Crithidia bombi encounters phytochemicals throughout its life cycle, making it a relevant system for testing the effects of phytochemicals on pollinator infection.

Parasites infect new hosts via transmission at flowers and within bee hives, which contain derivatives of nectar, pollen, and other plant materials. Crithidia bombi has not been detected in floral nectar. However, within hosts, C. bombi inhabits the gut lumen, where cells have direct exposure to host-ingested nectar and pollen phytochemicals in the crop, and possibly also in the mid- and hindgut. In contrast to trypanosomes that infect the circulatory system or organs of their hosts, intestinal C. bombi lacks a physical barrier to shield it from ingested compounds, and may be exposed to phytochemical concentrations that approach those found in nectar and pollen. Hence, oral consumption of phytochemicals by bees could have strong and direct effects on parasites, and the phytochemical concentration that inhibits parasite growth in vitro may provide an estimate of the oral dose that could ameliorate infection in hosts.
Several studies have demonstrated that phytochemical ingestion by *B. impatiens* and *B. terrestris* reduces *C. bombi* infection. Five phytochemicals found in nectar—gelsemine, nicotine, anabasine, thymol, and catalpol—reduced *C. bombi* infection intensities. However, both the magnitude and direction of effects of phytochemicals on *C. bombi* varied among studies. For example, other studies found that thymol and anabasine did not affect *C. bombi* infection, and nicotine increased infection intensity. Taken together, these results suggest that phytochemicals have variable effects on *C. bombi* infection, with effects dependent on the unique combination of parasite strain, host genotype, and abiotic conditions used in each experiment. Therefore, an approach that eliminates host-related variability would help to determine the direct effects of phytochemicals on parasites, and allow comparisons of phytochemical sensitivity among parasite strains.

Both *C. bombi* strains and floral phytochemical concentrations are variable. *Crithidia bombi* populations are genetically and phenotypically diverse. Inter-strain variation could determine resistance to phytochemicals—defined here as the ability to survive, grow, and reproduce when exposed phytochemicals—as has been demonstrated within populations of other pathogenic microbes, such as quinine- and artemisinin-resistant *Plasmodium falciparum*. Like parasite strains, floral phytochemical concentrations are variable, and have dose-dependent effects on both pathogens and hosts. For example, nectar nicotine and anabasine concentrations spanned multiple orders of magnitude among related *Nicotiana* species. Within a species, nectar nicotine varied between *Nicotiana attenuata* plant populations, within populations, and across a six-fold range between flowers of a single inflorescence. Similarly, nectar concentrations of *Rhododendron ponticum* grayanotoxins varied between native and invasive populations and within patches. Testing a range of parasite strains, phytochemicals
and concentrations in a single study could identify candidate medicinal compounds and illustrate the potential effects of phytochemicals on pollinator parasites in nature.

We used a standardized, high-throughput protocol to test the direct effects of different phytochemicals against multiple parasite strains across a range of chemical concentrations. Cell culture-based methods have been used to quantify the effects of phytochemicals on insect-vectored trypanosome species such as *Leishmania donovani*, *Trypanosoma cruzi*, and *Trypanosoma brucei* that cause disease in humans and are close phylogenetic relatives of *C. bombi*. Here, we extend a previously described *C. bombi* cell culturing method to assess variation in the direct effects of nine floral phytochemicals—two alkaloids; one cyanogenic glycoside; four hydroxybenzoic, hydroxycinnamic, and phenylpropanoid phenolics; and two terpenoids—on four different *C. bombi* strains. We also searched published literature to compare phytochemical sensitivity of *C. bombi* to that of other trypanosome species, animal cells, and insects. To gauge the ecological relevance of each phytochemical's effects in culture, we combined field sampling of five plant species with literature searches to quantify phytochemical concentrations in nectar and pollen.

**Results**

**Cell culture experiments**

In comparison to other trypanosome species, *C. bombi* were remarkably resistant to common phytochemicals, with no growth inhibition at concentrations previously found to lower infection intensity in nectar fed to live bees (Table 1). Among the alkaloids, nicotine at doses of up to 1000 ppm had no effect on growth, and over 1000 ppm anabasine was required for 50% growth inhibition (EC50, Table 1, Fig. 1, Supplementary Fig. S1). None of the tested strains were susceptible to the cyanogenic glycoside, amygdalin, nor to the antitrypanosomal phenolics
caffeic acid, chlorogenic acid, and gallic acid, even at concentrations that were several orders of magnitude above the inhibitory thresholds of related pathogens (Table 1). The sesquiterpene β-caryophyllene also did not inhibit growth of any strain at concentrations up to 50 ppb. Of the nine phytochemicals tested, only three—anabasine, eugenol, and thymol—were sufficiently inhibitory to estimate dose-response curves and EC50 values (Fig. 1, Table 1, Supplementary Figs. S1-S3).

Strains varied in resistance to all three inhibitory compounds. Significant variation was found in resistance to anabasine (Fig 1A). Each strain exhibited a distinct level of resistance, which varied among strains by more than 1500 ppm. The most sensitive strain, VT1 (EC50 = 628 ppm, 95% Bayesian Credible Interval (CI): 601-659 ppm), was inhibited by one-third the anabasine concentration of the most resistant strain, 12.6 (EC50 = 2160 ppm, 95% CI: 2110-2220 ppm). The other two strains, IL13.2 (EC50 = 1030 ppm, 95% CI: 975-1080 ppm) and C1.1 (EC50 = 1440 ppm, 95% CI: 1410-1440 ppm), were intermediate in resistance.

Eugenol resistance (Fig 1B) was the most consistent across strains, with all EC50 values between 19.7 and 23.5 ppm, yet the non-overlapping 95% credible intervals (CI) still indicated statistically significant variation. The relative resistance ranks of the four strains were the same as for anabasine and eugenol: Strain VT1 (EC50 = 19.7 ppm, 95% CI: 18.9-20.4 ppm) was again the most sensitive, and strain 12.6 the most resistant (EC50 = 23.5 ppm, 95% CI: 22.1-26.2 ppm); intermediate resistance was observed in IL13.2 (EC50 = 20.5 ppm, 95% CI: 20.0-21.1 ppm) and C1.1 (EC50 = 22.0 ppm, 95% CI: 20.5-24.7 ppm).

Resistance to thymol (Fig 1C) was also variable. As was the case for the other two compounds, strain 12.6 (EC50 = 22.2 ppm, 95% CI: 22.3-21.0 ppm) was again the most resistant, with more than three times the resistance of the other three strains, which were not
significantly different from one another (VT1, EC50 = 6.26 ppm, 95% CI: 4.27-8.55 ppm; C1.1, EC50 = 4.53 ppm, 95% CI: 2.93-6.42 ppm; IL13.2, EC50 = 7.33 ppm, 95% CI: 6.10-8.62 ppm).

**Naturally occurring phytochemical concentrations**

Using published literature and field sampling, we surveyed ecologically relevant pollen, nectar, and honey concentrations of the nine phytochemicals tested against *C. bombi* (Table 2). In comparison to published values for honey, our own analyses indicated very high levels of chlorogenic acids in the pollen of the crop species *Persea americana* (avocado), *Malus domestica* (apple), and *Vaccinium corymbosum* (blueberry, both wild and cultivated; Table 2). In the three plant taxa for which we analyzed both pollen and nectar, concentrations of the chlorogenic acid 5-caffeoylquinic acid were 25- to 30-fold higher in pollen than in nectar (Wilcoxon W-test, *M. domestica*: W = 25, *P* < 0.001; *V. corymbosum* (cultivated): W = 18, *P* < 0.001; *V. corymbosum* (wild): W = 0, *P* < 0.001). Although nectar chlorogenic acid concentrations were lower than pollen concentrations, nectar concentrations were still several orders of magnitude higher than those recorded in honey, with the exception of *Leptospermum scoparium* honey (Table 2). Similarly, thymol concentrations in the nectar of *Thymus vulgaris* were over 10-fold above the highest value recorded for natural honey (Table 2), despite air-drying of samples prior to measurement (see Materials and Methods).

**Discussion**

*Crithidia bombi* was far less susceptible to the tested trihydroxybenzoic and hydroxycinnamic phenolic phytochemicals than were other, previously studied bloodstream trypanosomes. *L. donovani* and *T. brucei*, for example, were inhibited by <10 ppm of gallic acid, whereas concentrations up to 250 ppm had minimal effects on any tested strains of *C.*
Similarly, caffeic acid, which inhibited *L. donovani* and *T. brucei* at <10 ppm, had no effect on *C. bombi* strains at concentrations up to 250 ppm. Furthermore, the EC50 for chlorogenic acid against *C. bombi* was >2500 ppm, which was 100 times higher than the EC50 for *L. donovani* (EC50 7-17 ppm) and *T. brucei* (18.9 ppm). Although some variation in EC50 estimates could reflect methodological differences between our study and previous investigations, a difference of such magnitude for multiple phytochemicals provides strong evidence of comparatively high phytochemical resistance in *C. bombi*. This exceptional level of resistance may reflect the evolutionary history of *C. bombi*. In contrast to *L. donovani* and *T. brucei*, which are transmitted by blood-feeding insects and would be expected to have comparatively little direct exposure to phytochemicals, *C. bombi* may be adapted to chronic phytochemical exposure in the intestine of nectar- and pollen-consuming bumble bees. Bumble bees are generalist pollinators that consume nectar and pollen from a wide range of plant species. Both nectar and pollen contain diverse compound mixtures, to which *C. bombi* in the gut lumen would be directly exposed, particularly in the proximal parts of the gut, before phytochemicals are absorbed or metabolized by hosts or commensalists. Study of the mechanisms by which *C. bombi* withstands such high phytochemical concentrations could offer insight into the evolution of chemical resistance in medically important trypanosomes.

In addition to being less susceptible to phytochemicals than were other trypanosomes, *C. bombi* showed no growth inhibition at phytochemical concentrations exceeding those documented in honey (Table 1, Table 2). For example, for the known antitrypanosomal compound caffeic acid, *C. bombi* was not inhibited by 250 ppm (Table 1), over 9 times the maximum honey value of 26.8 ppm (Table 2, range 0.76-26.8 ppm for 14 honey types); for gallic acid, *C. bombi* was again robust to 250 ppm (Table 1), or 3 times the maximum reported
honey value of 82.5 ppm (Table 2; among 14 honey types, only oak honey exceeded 1 ppm gallic acid) 53.

There are a number of nonexclusive explanations for the insensitivity of C. bombi to phytochemicals above their natural concentration range. First, the phytochemical concentrations found in honey samples may underestimate naturally occurring concentrations. Fanning of nectar to produce honey 11, as well as prolonged storage, may evaporate volatile nectar components such as thymol, eugenol, and β-caryophyllene and could promote oxidation of phenolic compounds 54. The thymol and chlorogenic concentrations measured in our field samples (Table 1), which were orders of magnitude higher than the values for honey found in the literature, illustrate this point. Second, in natural settings, phytochemicals are encountered in complex combinations, such that total phytochemical concentrations of biologically active compounds may far exceed the concentration of any one chemical component. Pollen comprises a mixture of phytochemicals, with the sum concentration of all phenolic constituents reaching 1.3-8.2% phenolics by weight (13,000–82,000 ppm) 55. Even honey may contain up to 12,000 ppm total phenolics (range 1,600-12,000 ppm) 53. Third, in their hosts, parasites are subject to additional antimicrobial chemicals produced by the host immune system and competing gut microbiota. Multiple antimicrobial peptides produced by bees have synergistic effects with one another 56, and should be tested for synergy with floral phytochemicals as well. The Bombus gut microbiome includes species that produce ethanol and organic acids 57, which also inhibit microbial growth 56,59. Hence, the high resistance of C. bombi that we observed to single phytochemicals may be necessary to tolerate the effects of multiple phytochemicals, antimicrobial peptides, and microbiome-derived toxins acting in concert. Future experiments should explicitly address the interactive effects of multiple phytochemicals in combination.
In addition to explaining why *C. bombi* has such high resistance to individual phytochemicals under optimal conditions, the interactive effects of multiple factors may explain why low concentrations of phytochemicals were sufficient to decrease parasitism in live bees. All tested strains of *C. bombi* were resistant to phytochemicals at concentrations 100 times those previously shown to be medicinal in *B. impatiens* and *B. terrestris*. Our strains were not inhibited by up to 1000 ppm nicotine, or 500 times the 2 ppm previously found to ameliorate infection in bees. Our lowest EC50 value for anabasine (628 ppm) was still over 100-fold higher than the 5 ppm previously shown to reduce infection levels. Inhibitory concentrations of thymol, where the minimum EC50 of the four strains was 4.5 ppm, were likewise more than 20-fold the 0.2 ppm medicinal concentration in *B. impatiens*. These discrepancies far exceed the ~3-fold variation found among strains in our study, indicating that differences between *in vitro* and *in vivo* inhibitory concentrations do not merely reflect the use of different strains in our study versus previous live-bee experiments. We suggest that the low phytochemical concentrations necessary to ameliorate host infection may reflect phytochemical-induced changes in hosts, which could complement the direct effects of phytochemicals on parasites. For example, phytochemical ingestion may act indirectly on parasites by modulating the host immune response, as shown in humans and in honey bees, where a honey constituent increased expression of genes that encode antimicrobial peptides. Phytochemicals could also act as antioxidants that scavenge free radicals and reduce the deleterious effects of pathogens. Studies of live bees are needed to define how phytochemicals exert indirect effects on parasite infection via modulation of host immunity or behavior, such as induction of antimicrobial peptides or stimulation of intestinal motility that expels parasites from the gut.
Our four *C. bombi* strains varied in resistance to the three phytochemicals that inhibited growth, spanning a five-fold range for thymol and a three-fold range for anabasine. Overall, strain “12.6” exhibited both the fastest growth (Supplementary Figures S1-S4) and the highest phytochemical resistance (Figure 1). Strains with a high rate of growth might be able to form biofilms that provide protection from growth-inhibiting chemicals, or metabolize the chemicals before deleterious effects are realized. Studies that use a greater number of strains are needed to test for positive correlations between phytochemical resistance and growth rate, both in cell cultures and in live bees, where *C. bombi* exists within a diverse microbial community\(^6^4\). Alternatively, negative correlations could reflect trade-offs between resistance and growth or infectivity. Variation in phytochemical resistance among parasites could be a target and possibly a result of natural selection. At the landscape scale, regional parasite and plant sampling, combined with cell culture experiments, could establish whether parasites show evidence of adaptation to phytochemicals characteristic of their local plant community. These correlative studies could be complemented by experiments that test how parasites respond to chronic phytochemical exposure, and whether resistance can evolve over time.

Our sampling data show that thymol inhibited *C. bombi* at concentrations found in *T. vulgaris* nectar. The range of EC50 values for *C. bombi* (4.5 to 22 ppm) spanned the natural range of thymol concentrations in *T. vulgaris* nectar (5.2-8.2 ppm). Although nectar concentrations did not completely inhibit growth, 50% growth inhibition could meaningfully decrease the intensity of infection and its negative effects on bees. Also, because it is likely that some thymol was lost during sample processing, our measurements may provide a conservative estimate of thymol-mediated inhibition by *Thymus* nectar. Thymol is used prophylactically to combat *Varroa* mite infestations\(^6^5\), and inhibited *Nosema* infection in *A. mellifera*\(^1^9\) and *Crithidia* infection in *B. impatiens*\(^2^2\). Although it is possible that nectar thymol is absorbed or
metabolized by bees or their gut commensalists, or diluted through combination with nectar of other species, phytochemicals are detectable in the lumen post-ingestion, and even very low nectar concentrations (0.2 ppm) can reduce C. bombi infection intensity in B. impatiens. Because individual bumble bees generally forage from only one or several floral species, consumption of medicinally relevant amounts of thymol would seem plausible in the wild. Our study builds on prior results by reporting concentrations of thymol in floral nectar for the first time, and documenting the direct activity of this phytochemical against multiple parasite strains at naturally occurring concentrations.

Thymol and eugenol have been shown to possess broad-spectrum antimicrobial activity against bacteria, fungi, and trypanosomes. These hydrophobic compounds readily penetrate and disrupt cell and mitochondrial membranes, thereby disrupting ionic gradients and causing leakage of reactive oxygen species. Reactive oxygen species can oxidize monoterpenes and phenylpropenes like thymol and eugenol, which both contain double bonds and free hydroxyl groups. Oxidized phytochemicals can then initiate a free radical cascade that damages cell lipids and proteins, leading to disruptions of organelle function and energy production in trypanosomes. Rapidly dividing cells are especially susceptible, because they are easily penetrated during cell division. Although high phytochemical concentrations are toxic to animal intestinal cells as well as to microbes, with 25 ppm thymol and 80 ppm eugenol inducing apoptosis and necrosis within 24 h, the intestinal cells with direct phytochemical exposure may provide a renewable barrier between the gut lumen and the systemic circulation of multicellular animal hosts.

Phytochemicals such as thymol and eugenol, which display strong antimicrobial activity but are relatively benign to bees, could have high medicinal value for both wild and managed bees that have access to plants containing these compounds. In general, bees are less
susceptible than are microbes to toxic effects of essential oils \textsuperscript{70}, and can be attracted to relevant antimicrobial concentrations \textsuperscript{71}, which would increase the likelihood of voluntarily ingesting medicinally significant amounts of these phytochemicals under natural conditions. Eugenol, which has been found in over 400 plant species from 80 families \textsuperscript{72}, has been shown to stimulate bee foraging and pollen collection in bumble bees \textsuperscript{73}; 50 ppm eugenol in sugar water was attractive to honey bees \textsuperscript{74}, whereas only 19.7-23.5 ppm inhibited \textit{C. bombi} growth in our study. Similarly, the \textit{A. mellifera} 14-day LD50 for thymol exceeded 1000 ppm \textsuperscript{70}, far higher than the 4.5-22.3 ppm thymol that inhibited our \textit{C. bombi}. Future studies should test whether availability of flowers containing thymol (such as \textit{T. vulgaris}) or eugenol is sufficient to reduce bee parasitism in the field; such plant species could be recommended to gardeners and as hedgerow species in agricultural areas. Additional studies that examine correlations between plot- and landscape-level plant species composition and pollinator parasite loads will yield additional ecological insights.

Our field sampling revealed higher levels of phytochemicals in nectar and pollen compared to previous reports of the same phytochemicals in honey. For example, the 5.2-8.2 ppm nectar thymol measured in this study is more than ten times the highest reported concentration in natural honey (Table 2). For chlorogenic acid, we identified three species with pollen concentrations >400 ppm, which is 50 times the highest value previously reported for honey (Table 2). Our findings highlight large differences between the phytochemical composition of nectar and honey, and indicate the need for more comprehensive sampling of nectar and pollen, including volatile compounds such as eugenol, to establish the types and concentrations of phytochemicals to which parasites might be naturally exposed. Sampling bumble bee honey in addition to honey bee honey may also reveal differences in chemical composition due to variation in foraging preferences or post-collection processes. Future
sampling efforts will identify candidate antimicrobial phytochemicals for future testing in bees and other pollinators, and also document which floral species are sources of known antiparasitic compounds. Given the relatively unexplored nature of nectar and pollen relative to leaf phytochemistry, further sampling has significant potential to uncover new compounds of ecological and potentially medical significance.

Collectively, our experiments demonstrate the ecological and evolutionary relevance of direct effects of phytochemicals on a pollinator parasite. We show that the bumble bee parasite *C. bombi* is less susceptible to phytochemicals than are bloodstream trypanosomes, is inhibited by some nectar and pollen phytochemicals at naturally occurring concentrations, and exhibits inter-strain variation in resistance. Our results emphasize the importance of inter-strain variation and concentration-dependent responses in explaining the effects of phytochemicals on pollinator diseases, and highlight the need for additional analysis of nectar and pollen to profile the full range of phytochemicals and concentrations that occur in nature.

**Methods**

**Parasite culturing**

Parasite strains, each derived from a single *C. bombi* cell, were isolated from wild bumble bees collected near West Haven, CT, United States in 2012 (“12.6”, from *B. impatiens*, courtesy Hauke Koch); Hanover, NH, United States in 2014 (“VT1”, from *B. impatiens*, courtesy lab of Rebecca Irwin); Corsica, France in 2012 (“C1.1”, from *B. terrestris*, collected by Ben Sadd); and Normal, IL, United States in 2013 (“IL13.2”, from *B. impatiens*, collected by Ben Sadd). Strain 12.6 was isolated by diluting homogenized intestinal tracts of infected *B. impatiens* to 1 cell µL⁻¹, then adding 1 µL of the cell suspension to wells of a 96-well plate containing *Crithidia* growth medium 47 with the addition of 2% antibiotic cocktail to combat bacterial and fungal
contaminants (penicillin 6 mg mL⁻¹, kanamycin 10 mg mL⁻¹, fluorcytosin 5 mg mL⁻¹, chloramphenicol 1 mg mL⁻¹ as described ⁴⁷). The remaining strains were isolated by flow cytometry-based single cell sorting of homogenized intestinal tracts (strain VT1) or bee feces (C1.1 and IL13.2) as described previously ⁴⁷. All strains were isolated directly from wild bees with the exception of VT1, which was first used to infect laboratory colonies of B. impatiens (provided by Biobest, Leamington, ON, Canada). The cell used to initiate the parasite culture was obtained from an infected worker of one of the commercial colonies. Cultures were microscopically screened to identify samples with strong Crithidia growth and absence of bacterial or fungal contaminants, then stored at -80°C in a 2:1 ratio of cell culture:50% glycerol until several weeks before the experiments began. Thereafter, strains were incubated at 27°C and propagated weekly in 5 mL tissue culture flasks (300-500 µL cultured cells in 5 mL fresh culture medium) ⁴⁷.

**Phytochemicals for cell culture assays**

Phytochemicals were chosen to facilitate comparison with published work assessing C. bombi inhibition in B. impatiens ²²,³⁶. Additional compounds were selected based on widespread presence in flowers, nectar, honey, or pollen and documented anti-trypanosomal activities (Tables 1 and 2). We tested the effects of nine compounds: the pyridine alkaloids nicotine (Sigma-Aldrich, St. Louis, MO) and anabasine (Sigma-Aldrich), the cyanogenic glycoside amygdalin (Research Products International, Mt. Prospect, IL), the cinnamic acid caffeic acid (Indofine, Hillsborough, NJ), the cinnamic acid ester 3-caffeoylquinic acid ("chlorogenic acid", Biosynth International, Itasca, IL), the phenylpropenoid phenolic alcohol eugenol (Acros, Thermo Fisher, Franklin, MA), the trihydroxybenzoic phenolic gallic acid (Acros), the sesquiterpene β-
caryophyllene (SAFC, Milwaukee, WI), and the monoterpane alcohol thymol (Fisher Scientific, Franklin, MA).

Phytochemical treatment media were prepared by dissolving stock chemicals either directly in medium followed by sterile filtration (for the more soluble nicotine, anabasine, amygdalin, chlorogenic acid, and eugenol) or by pre-dissolving compounds in ethanol (for the less soluble caffeic acid, gallic acid, β-caryophyllene, thymol). Treatment concentrations were chosen to span the range of concentrations known to occur in plant nectar and pollen (Table 1) and/or inhibit trypanosomes (Table 2), with maximal concentrations limited by compound solubility. For experiments using dilutions prepared from an ethanol-based stock, we equalized the ethanol concentration in each treatment by adding ethanol (up to 1% by volume, depending on the phytochemical) to the treatments of lesser concentrations.

Experimental design

We conducted 9 experiments, each testing all 4 parasite strains in parallel against a single phytochemical. Cell cultures (1 mL) were transferred to fresh medium (5 mL) and allowed to grow for 48 h in tissue culture flasks. Immediately before the assay, cultures were transferred to 50 mL centrifuge tubes and centrifuged for 10 min at 10,000 g. The supernatant was removed and the cells were resuspended in 3 mL fresh medium. Cell density of the resulting suspension was calculated by counting parasite cells at 400x magnification using a Neubauer hemocytometer. Each strain was adjusted to a cell density of 1,000 cells µL⁻¹.

A separate 96-well plate was prepared for each strain, i.e., 4 plates per experiment, one for each of the four strains. Each plate contained eight replicate wells at each of six phytochemical concentrations, with each concentration assigned to columns 3-10 of a given row to minimize edge effects. To each well, 100 µL of 1,000 cells µL⁻¹ cell suspension was added to
100 µL of the phytochemical-enriched treatment medium using a multichannel pipette, resulting in a starting cell density of 500 cells µL⁻¹. The outer wells of the plate (columns 1, 2, 11, and 12, plus the remaining wells in rows A and B) were filled with 100 µL treatment medium (8 wells per concentration) and 100 µL control medium; these wells were used to control for changes in optical density (OD) unrelated to cell growth. Plates were incubated for 5 d at 27°C on a microplate shaker (250 rpm, 3 mm orbit). OD readings (630 nm) were taken at 24 h intervals, as described previously, immediately after resuspending the cells (40s, 1000 rpm, 3mm orbit) using the microplate shaker. We calculated net OD (i.e., the amount of OD resulting from parasite growth) by subtracting the average OD reading of cell-free control wells of the corresponding concentration, plate, and timepoint. For analysis of assays using the volatile phytochemicals eugenol and thymol, we excluded the replicates closest to the control wells that contained highest phytochemical concentrations (2 per treatment for eugenol, 3 per treatment for thymol). These replicates had markedly reduced growth compared to other samples in the same treatments; we attributed this growth reduction to exposure to phytochemicals that volatilized from the neighboring control wells.

**Statistical analysis of cell culture experiments**

Dose-response curves for each strain and phytochemical were computed for the three phytochemicals for which the highest tested concentration resulted in complete inhibition of growth—near-complete inhibition is necessary for accurate estimation of the concentration that inhibits growth by 50% (EC50). All statistical analysis was carried out using the open source software R v3.2.1 following methods used for antimicrobial peptides. For each sample, the growth integral (i.e., area under the curve of net OD vs. time) was calculated by fitting a model-free spline to the observed OD measurements using grofit. The relationship between
phytochemical concentration and growth integral was modeled with a Markov chain Monte
Carlo algorithm using Just Another Gibbs Sampler \textsuperscript{78} in combination with the R-package rjags \textsuperscript{79}.

We used the following model to describe the relationship between phytochemical
concentration (c) and growth integral (g):

\[
g = r - \frac{E_{\text{max}} c^h}{(c_{50})^h + c^h}
\]  

(1)

where \( r \) denotes growth in the absence of the phytochemical, \( E_{\text{max}} \) represents the maximum
effect at high concentrations, and \( c_{50} \) is the phytochemical concentration at which 50% of the
maximum effect is reached. The parameter \( h \), the Hill coefficient, indicates how steeply the
effect increases around the concentration \( c_{50} \). From this model, we derived parameter estimates
and 95% highest posterior density credible intervals (CI) of the EC\text{50} for each phytochemical. We
defined strains as having significant differences in resistance when their 95% CI’s did not
overlap. Each strain’s dose-response curve and EC\text{50} were calculated independently of the other
strains; in other words, the EC\text{50} represents the phytochemical concentration resulting in 50%
of maximal inhibition for a particular strain.

Field sampling

**Nectar and pollen collection.** Nectar and pollen were collected from agricultural and
wild species in Massachusetts and California in 2014 and 2015 (see Supplementary Table S1 for
sampling locations, dates, and cultivars). We quantified thymol in *Thymus vulgaris* nectar and
chlorogenic acids in *Malus domestica* (domestic apple), wild and cultivated *Vaccinium
corymbosum* (blueberry), *Prunus dulcis* (almond), and *Persea americana* (avocado). Up to 10
samples of each tissue were collected, typically from each of three cultivars for agricultural
species. For *Thymus vulgaris* cv. Silver, few plants were in flower at the time of collection, so it
was only possible to collect enough nectar for a single nectar sample.
Pollen samples were collected using clean forceps by pinching off anthers, avoiding as much filament as possible. We collected at least 5 mg per sample, consisting of pollen, the pollen sac, and a small amount of filament. We collected from mature, undehisced or newly dehiscing anthers only. In most species, pollen was pooled across flowers within plants, but not across plants. Nectar samples were collected using separate glass microcentrifuge tubes. Care was taken to avoid contaminating samples with pollen. Depending on the plant species, we collected nectar through the corolla opening, or by removing and gently pressing the corolla to produce nectar at the flower base. Each nectar sample contained at least 5 µL but typically 20 µL nectar, added to 80 µL EtOH to prevent spoilage. Nectar was often pooled across individual plants to obtain sufficient volumes per sample. Samples were kept on ice in the field and then stored at -20°C until lyophilization. Alcohol from *Thymus* nectar samples was evaporated at room temperature. We acknowledge that some thymol, which is volatile, may have been lost from the samples during evaporation, which we deemed necessary to prevent spoilage during shipping. As a result, our results may underestimate true nectar concentrations of this phytochemical.

**Analysis of chlorogenic acids.** Pollen samples were extracted in methanol following previously published methods. Unground pollen (5-50 mg) was sonicated for 10 min with 1 mL methanol in a 2 mL microcentrifuge tube, then incubated without shaking for an additional 24 h at room temperature. Samples were centrifuged for 5 min at 12,000 rpm, and the supernatants analyzed by liquid chromatography (LC) using High Resolution Electrospray Ionisation Mass Spectroscopy (HRESIMS). Chlorogenic acids were identified based on spectral comparisons with authentic standards in the library at Royal Botanic Gardens, Kew, UK. HRESIMS data were recorded using a Thermo LTQ-Orbitrap XL mass spectrometer coupled to a Thermo Accela LC system performing chromatographic separation of 5 µl injections on a Phenomenex Luna C18(2)
column (150 mm × 3.0 mm i.d., 3 μm particle size) with a linear mobile phase gradient of 10–100% aqueous MeOH containing 0.1% formic acid over 20 min. The column temperature was maintained at 30°C with a flow rate of 0.5 ml min⁻¹. Spectra were recorded in positive and negative modes at high resolution (30,000 FWHM (full width at half maximum)) and compared to authentic standards from the laboratory’s compound library including the three chlorogenic acid isomers: 3-caffeoylquinic acid, 4-caffeoylquinic acid and 5-caffeoylquinic acid.

Lyophilized nectar (original volume ~10 µL) was extracted in 50 µL methanol and injected directly onto an LC-MS system with a ZQ LC-MS detector on a Phenomenex Luna C18(2) column (150 × 4.0 mm i.d., 5 μm particle size) operating under gradient conditions, with A = MeOH, B = H₂O, C = 1% HCO₂H in MeCN; A = 0%, B = 90% at t = 0 min; A = 90%, B = 0% at t = 20 min; A = 90%, B = 0% at t = 30 min; A = 0%, B = 90% at t = 31 min; column temperature 30°C and flow rate of 0.5 mL min⁻¹. Aliquots (10 µL) were injected directly on to the column and components identified by comparison with pollen extracts analyzed as described above under HRESIMS. All chlorogenic acids were quantified against calibration curves of an authentic standard of 5-caffeoylquinic acid.

**Identification of chlorogenic acids.** All three chlorogenic acids have a molecular ion [M+H]⁺ with m/z = 355.1020 (calculated for C₁₆H₁₉O₉⁺ = 355.1024) and a major diagnostic fragment m/z = 163.04 (calculated for C₉H₇O₃⁺ = 163.039) from [M-quinic acid]⁺. The chlorogenic acids elute in the order 3-caffeoyl-, 5-caffeoyl- and 4-caffeoylquinic acids at 4.0 min, 5.6 min and 7.0 min respectively with the following diagnostic MS2 fragments in negative mode:

- 3-caffeoylquinic acid fragment m/z = 163
- 4-caffeoylquinic acid fragment m/z = 173
- 5-caffeoylquinic acid fragment m/z = 191.

**Statistical comparison between pollen and nectar.** Within each of the three plant types for which we measured chlorogenic acids in both pollen and nectar—*M. domestica*, wild *V.*
corymbosum, and cultivated V. corymbosum—we compared pollen and nectar 5-caffeoylquinic acid concentrations using an unpaired, two-sided Wilcoxon signed-rank test.

Analysis of thymol in Thymus vulgaris nectar. For analysis of thymol, dried nectar from a sample of known volume (~10 µL) was extracted in 250 µL of chloroform to which was added 500 ng of decyl acetate (50µL of a 10 ng µL⁻¹ solution) as an internal standard. The extract was injected directly onto an Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass spectrometer with a DB-5 fused silica capillary column (30 m length, 0.25 mm diameter, 0.25 μm film thickness) (Agilent). The column temperature was held at 50°C for 2 min, then heated to 240°C at 6°C min⁻¹. The ion source was held at 150°C, and the transfer line was held at 250°C. Thymol was identified by comparison to a thymol standard (Sigma Ltd) and quantified using the fragment ion m/z=135 relative to the Total Ion Chromatogram (TIC) for the decyl acetate internal standard. This ratio was corrected using a response factor, which was obtained by analyzing a standard sample containing equal concentrations of thymol and decyl acetate.
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Authors’ contributions

ECPY, BMS, PCS, REI and LSA conceived the study. ECPY conducted the cell culture experiments; LSA conducted the field sampling experiments. ECPY and BMS analyzed the cell culture data; PCS analyzed the field sampling data. ECPY wrote the first draft of the manuscript.
with contributions from BMS, PCS, and LSA. All authors revised the manuscript and agreed to submission.

**Additional information**

**Competing financial interests**

The authors declare no competing financial interests.

**Availability of data and materials**

The datasets supporting the conclusions of this article are available in the Zenodo repository, https://zenodo.org/record/50349. The data currently have restricted access. Data will be made freely available on Zenodo upon acceptance.

**Competing interests**

The authors declare that they have no competing interests.

**Tables**

Table 1-1. Comparison of phytochemical resistance in *Crithidia bombi*, other trypanosomes and parasites, animal cells, and insects (table continued onto next few pages)

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>EC50 (ppm)</th>
<th>Species or cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anabasine</td>
<td>628-2160</td>
<td><em>Crithidia bombi</em></td>
<td>This study</td>
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<tr>
<td></td>
<td>&gt;100</td>
<td><em>Trypanosoma cruzi</em> (epimastigote)</td>
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<tr>
<td></td>
<td>&gt;100</td>
<td><em>Spodoptera frugiperda</em> (SFS) cells</td>
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<tr>
<td></td>
<td>&gt;100</td>
<td>CHO cells (hamster ovary)</td>
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<tr>
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<td><em>Crithidia</em> (reduced infection in <em>Bombus impatiens</em>)</td>
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<td><em>Nectarinea osea</em> (sunbird feeding deterrent)</td>
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<td>Alkaloid</td>
<td>IC50 (µg/mL)</td>
<td>Target Organism</td>
<td>Reference(s)</td>
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<td><em>Leishmania donovani</em> (amastigote)</td>
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<tr>
<td></td>
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<td><em>Trypanosoma brucei rhodesiense</em> (bloodstream form)</td>
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<tr>
<td></td>
<td>&gt;30</td>
<td><em>Trypanosoma cruzi</em> (trypomastigote)</td>
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<tr>
<td></td>
<td>56</td>
<td><em>Trypanosoma cruzi</em> (trypomastigote)</td>
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<td>53.3</td>
<td>L6 rat muscle cells</td>
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<td>Human lymphocytes</td>
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<td>&gt;128</td>
<td><em>Paenibacillus larvae</em> (American foulbrood– MIC)</td>
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<td>&gt;300</td>
<td><em>Culex quinquefasciatus</em> Say (mosquito) larvae</td>
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<td></td>
<td>&gt;500 µg fly−1</td>
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<tr>
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<td>&gt;90</td>
<td><em>Trypanosoma cruzi</em> (amastigote)</td>
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<tr>
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<td>&gt;90</td>
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<td><em>Spodoptera eridania</em> larvae</td>
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<td>Eugenol</td>
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<td>93.7</td>
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<td>246</td>
<td><em>Trypanosoma cruzi</em></td>
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<td></td>
<td>93</td>
<td>HL-60 (human leukemia)</td>
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<td><em>Sarcoptes scabiei</em> mites (permethrin-sensitive)</td>
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<td>7800</td>
<td><em>Apis mellifera</em> (8 d LD50)</td>
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<td><em>Apis mellifera</em> (14 d LD50)</td>
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<tr>
<td>Compound</td>
<td>Concentration</td>
<td>Pathogen Type</td>
<td>Source</td>
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<td>Leishmania donovani (extracellular)</td>
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<td>Trypanosoma brucei brucei (procyclic form)</td>
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<tr>
<td>Trypanosoma cruzi</td>
<td>67 ppm</td>
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<tr>
<td>L6 rat muscle cells</td>
<td>14.4 ppm</td>
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<tr>
<td>Mouse macrophages</td>
<td>15.6 ppm</td>
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<tr>
<td>Culex quinquefasciatus Say (mosquito) larvae</td>
<td>&gt;300 ppm</td>
<td>89</td>
<td></td>
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<tr>
<td>Musca domestica (housefly) adults</td>
<td>&gt;500 µg fly⁻¹</td>
<td>89</td>
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<td><strong>β-caryophyllene</strong></td>
<td><strong>&gt;0.050 ppm</strong></td>
<td>This study</td>
<td></td>
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<tr>
<td>Trypanosoma brucei brucei TC221 (bloodstream form)</td>
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<tr>
<td>Trypanosoma brucei brucei Lister 427 (bloodstream form)</td>
<td>41.2 ppm</td>
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<td>Trypanosoma brucei brucei Lister 427 (procyclic form)</td>
<td>&gt;100 ppm</td>
<td>94</td>
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<tr>
<td>Pseudomonas syringae</td>
<td>0.002-0.004 ppm</td>
<td>15</td>
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<td>Heliothis virescens (cell cultures)</td>
<td>221 ppm</td>
<td>95</td>
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<tr>
<td>HL-60 (human leukemia)</td>
<td>19.31 ppm</td>
<td>44</td>
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<tr>
<td>A. mellifera (&lt;300 ppm attractive)</td>
<td>&gt;300 ppm</td>
<td>14</td>
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<tr>
<td><strong>Thymol</strong></td>
<td><strong>4.53-22.2 ppm</strong></td>
<td>This study</td>
<td></td>
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<td>Critthidias fasciculata</td>
<td>32.5 ppm</td>
<td>27</td>
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<tr>
<td>Trypanosoma brucei brucei</td>
<td>22.9 ppm</td>
<td>44</td>
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<tr>
<td>Trypanosoma cruzi (epimastigote)</td>
<td>62 ppm</td>
<td>25</td>
<td></td>
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<tr>
<td>Trypanosoma cruzi (trypomastigote)</td>
<td>53 ppm</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Paenibacillus larvae (MIC)</td>
<td>64-128 ppm</td>
<td>88</td>
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</tr>
<tr>
<td>HL-60 (human leukemia)</td>
<td>40.7 ppm</td>
<td>44</td>
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<tr>
<td>Apis mellifera (8 d LD50)</td>
<td>&gt;1000 ppm</td>
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<tr>
<td>Culex quinquefasciatus Say (mosquito) larvae</td>
<td>30 ppm</td>
<td>89</td>
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<tr>
<td>Musca domestica (housefly) adults</td>
<td>53 µg fly⁻¹</td>
<td>89</td>
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</tr>
<tr>
<td>Thymol (thyme oil)**</td>
<td>&gt;10,000 ppm</td>
<td>14</td>
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</table>

Concentrations are from this study (bold) and the sources cited in the table. Values are in EC50 in ppm of pure compound unless otherwise noted. Within each compound, observations are arranged (if applicable) beginning with trypanosomes, then other pathogens, followed by animal cells and insects. Trypanosome EC50 values all refer to in vitro assays of cell cultures. See specific references for methodological details.

*Refers to 3-O-cafeoylquinic acid
*Clove (Syzygium aromaticum) oil: 86.7% eugenol
**Thyme (Thymus) oil: 65.3% thymol
Table 1-2. Phytochemical concentrations in floral tissues, pollen, nectar, and honey (continued onto next few pages).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample type</th>
<th>Plant species</th>
<th>Concentration (ppm)*</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Pyridine alkaloids</td>
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<tr>
<td>Anabasine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>flowers N. noctiflora</td>
<td></td>
<td></td>
<td>2351</td>
<td>40</td>
</tr>
<tr>
<td>flowers N. petunioides</td>
<td></td>
<td></td>
<td>1482</td>
<td>40</td>
</tr>
<tr>
<td>nectar N. glauca</td>
<td></td>
<td></td>
<td>5</td>
<td>63</td>
</tr>
<tr>
<td>nectar 32 Nicotiana spp</td>
<td></td>
<td></td>
<td>0-1.52</td>
<td>40</td>
</tr>
<tr>
<td>nectar N. tabacum</td>
<td></td>
<td></td>
<td>0-1.0</td>
<td>98</td>
</tr>
<tr>
<td>Nicotine</td>
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<td>0-5.38</td>
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<td>nectar N. attenuata</td>
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<td>4</td>
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<td></td>
<td>0.5</td>
<td>63</td>
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<tr>
<td>Cyanogenic glycosides</td>
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<td>Amygdalin</td>
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<td>1889</td>
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<td>Phenolics</td>
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<td>Hydroxycinnamic acids</td>
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<tr>
<td>Caffeic acid</td>
<td></td>
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<tr>
<td>honey Quercus robur</td>
<td></td>
<td></td>
<td>26.8</td>
<td>53</td>
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<tr>
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<td>8.8</td>
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<td>honey Eryngium campestre</td>
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<td>honey Astragalus microcephalus</td>
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<td>5-O-caffeoylquinic acid</td>
<td>pollen</td>
<td>Persea americana</td>
<td>1525 ± 486 (n=30)</td>
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</tr>
<tr>
<td>5-O-caffeoylquinic acid</td>
<td>pollen</td>
<td>Malus domestica</td>
<td>475 ± 862 (n=30)</td>
<td>This study</td>
</tr>
<tr>
<td>5-O-caffeoylquinic acid</td>
<td>pollen</td>
<td>Vaccinium corymbosum (cult.)</td>
<td>430 ± 404 (n=53)</td>
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</tr>
<tr>
<td>5-O-caffeoylquinic acid</td>
<td>pollen</td>
<td>Vaccinium corymbosum (wild)</td>
<td>192 ± 204 (n=30)</td>
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<td>3-O-caffeoylquinic acid</td>
<td>nectar</td>
<td>Prunus dulcis</td>
<td>25.0 ± 14.9 (n=15)</td>
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<tr>
<td>5-O-caffeoylquinic acid</td>
<td>nectar</td>
<td>Malus domestica</td>
<td>15.6 ± 15.2 (n=30)</td>
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<td>14.6 ± 28.2 SD (n=52)</td>
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<td>5-O-caffeoylquinic acid</td>
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<td><em>Vaccinium corymbosum</em> (wild)</td>
<td>7.52 ± 4.23 SD (n=29)</td>
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<td>4-O-caffeoylquinic acid</td>
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<td><em>Vaccinium corymbosum</em> (wild)</td>
<td>6.66 ± 5.11 SD (n=30)</td>
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<tr>
<td>4-O-caffeoylquinic acid</td>
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<td>3.77 ± 7.62 SD (n=55)</td>
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<td>3-O-caffeoylquinic acid</td>
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**Phenylpropenes**

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<td>66.20%</td>
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<tr>
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<td><strong>(methyl eugenol)</strong></td>
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<td><em>Rosa rugosa</em></td>
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<td><em>Rhizophora stylosa</em></td>
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<td><em>Rosa rugosa</em></td>
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<tr>
<td></td>
<td><strong>(eugenol+methyl eugenol)</strong></td>
<td><em>Rosa x hybrida</em></td>
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<td><strong>petals (female)</strong></td>
<td><em>Cucurbita pepo cv. Tosca</em></td>
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<td><strong>Nectar (male and female)</strong></td>
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<td><strong>stigma</strong></td>
<td><em>Cucurbita pepo cv. Tosca</em></td>
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<td><strong>honey</strong></td>
<td><em>Thymus spp</em></td>
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**Trihydroxybenzoic acids**

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<td><strong>Gallic acid</strong></td>
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<td><em>Tilia spp</em></td>
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<td><strong>honey</strong></td>
<td><em>Calluna vulgaris</em></td>
<td>0.61</td>
</tr>
</tbody>
</table>

**Terpenoids**

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>β-caryophyllene</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Compound Type</td>
<td>Species</td>
<td>Concentration</td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>----------------------------------</td>
<td>---------------</td>
<td></td>
</tr>
<tr>
<td>Floral Volatiles</td>
<td>Arabidopsis thaliana</td>
<td>40%</td>
<td></td>
</tr>
<tr>
<td>Floral Volatiles</td>
<td>Nicotiana sylvestris</td>
<td>35%</td>
<td></td>
</tr>
<tr>
<td>Floral Volatiles</td>
<td>Dianthus caryophyllus</td>
<td>23%</td>
<td></td>
</tr>
<tr>
<td>Floral Volatiles</td>
<td>Citrus limon</td>
<td>9.5%</td>
<td></td>
</tr>
<tr>
<td>Pollen Volatiles</td>
<td>Citrus limon</td>
<td>14.5%</td>
<td></td>
</tr>
<tr>
<td>Pollen Volatiles</td>
<td>Papaver rhoaeus</td>
<td>&gt;5%</td>
<td></td>
</tr>
<tr>
<td>Pollen Volatiles</td>
<td>Lupinus polyphyllus</td>
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</tr>
<tr>
<td>Pollen Volatiles</td>
<td>Laurus nobilis</td>
<td>3.4%</td>
<td></td>
</tr>
<tr>
<td>Stamen Volatiles</td>
<td>Laurus nobilis</td>
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<td>Flower Bud Volatiles</td>
<td>Citrus limon</td>
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<tr>
<td>Petal Volatiles</td>
<td>Citrus limon</td>
<td>2.5%</td>
<td></td>
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**Thymol**

<table>
<thead>
<tr>
<th>Type</th>
<th>Species</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nectar</td>
<td>Thymus vulgaris cv. Silver</td>
<td>8.2 (n=1)</td>
</tr>
<tr>
<td>Nectar</td>
<td>Thymus vulgaris cv. German</td>
<td>5.2 ± 2.98 SD (n=11)</td>
</tr>
<tr>
<td>Honey</td>
<td>Apigard™-treated hives</td>
<td>0.5-2.65</td>
</tr>
<tr>
<td>Honey</td>
<td>Calluna vulgaris</td>
<td>0.346</td>
</tr>
<tr>
<td>Honey</td>
<td>Thymus spp.</td>
<td>0.27</td>
</tr>
<tr>
<td>Honey</td>
<td>Tilia spp.</td>
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</tr>
<tr>
<td>Honey</td>
<td>Erica spp.)</td>
<td>0.142</td>
</tr>
<tr>
<td>Honey</td>
<td>Erica spp.</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Concentration measurements for chlorogenic acid and thymol (bold) are from this study’s field sampling of nectar and pollen. Sample sizes are in parentheses. Concentrations of other phytochemicals were compiled through literature searches. Data are arranged in order of decreasing maximum concentration, first for sample types within compounds, and then by observations within a given sample type. SD: Standard Deviation.

*Units are mean concentration by mass in ppm, except for values followed by a “%” sign, which indicates % of total volatiles (for compounds where ppm concentrations were unavailable).
Figure 1-1. Inhibitory effects of (A) anabasine, (B) eugenol, and (C) thymol against 4 strains of *C. bombi*. Points indicate EC50 values in ppm phytochemical. Error bars show 95% credible intervals derived from Bayesian Markov Chain Monte Carlo model fit (see Materials and Methods). For each strain (x axis) and phytochemical (vertically arranged panels), model fit was derived from growth on a 96-well plate at 6 phytochemical concentrations (n=8 (anabasine), 6 (eugenol), or 7 (thymol) replicate samples per concentration). See Supplementary Figures S1-S3 for complete dose-response curves and confidence bands from the fitted models, and Supplementary Figure S4 for representative growth curves of OD over time.
CHAPTER 2

EVOLUTION OF RESISTANCE TO SINGLE AND COMBINED FLORAL PHYTOCHEMICALS BY A BUMBLE BEE PARASITE

Evan C. Palmer-Young1*, Ben M. Sadd2, Lynn S. Adler1

Short running title: Bee parasite evolves resistance to phytochemicals

Data are archived in the Zenodo repository (https://zenodo.org/record/54705) with restricted access for reviewers; at acceptance, data will be made freely available.

Abstract

Repeated exposure to inhibitory compounds can drive the evolution of resistance, which weakens chemical defense against antagonists. Floral phytochemicals in nectar and pollen have antimicrobial properties that can ameliorate infection in pollinators, but evolved resistance among parasites could diminish the medicinal efficacy of phytochemicals. However, multi-compound blends, which occur in nectar and pollen, present simultaneous chemical challenges that may slow resistance evolution.

We assessed evolution of resistance by the common bumble bee gut parasite Crithidia bombi to two floral phytochemicals, singly and combined, over six weeks (~100 generations) of

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chronic exposure. Resistance of C. bombi increased under single and combined phytochemical exposure, without any associated costs of reduced growth under phytochemical-free conditions. After six weeks’ exposure, phytochemical concentrations that initially inhibited growth by >50%, and exceeded concentrations in floral nectar, had minimal effects on evolved parasite lines. Unexpectedly, the phytochemical combination did not impede resistance evolution compared to single compounds. These results demonstrate that repeated phytochemical exposure, which could occur in homogeneous floral landscapes or with therapeutic phytochemical treatment of managed hives, can cause rapid evolution of resistance in pollinator parasites. We discuss possible explanations for submaximal phytochemical resistance in natural populations. Evolved resistance could diminish the antiparasitic value of phytochemical ingestion, weakening an important natural defense against infection.

Key words: experimental evolution, drug resistance, Bombus, Crithidia bombi, thymol, eugenol, EC50, cell culture, dose-response curves, Markov chain Monte Carlo

Introduction

Effective medicinal compounds, whether natural or synthetic, are vulnerable to the evolution of resistance by the parasites that they target. The clinical significance of resistance to antibiotics is considered a major threat to human health (Bonhoeffer et al., 1997). In agriculture, resistance to pesticides has created an ongoing need for new means of genetic and chemical control (Barrett & Antonovics, 1988; Bates et al., 2005). Similarly, in natural systems, the evolution of phytochemical resistance by specialist antagonists necessitates the biosynthetic invention of new plant defenses (Berenbaum & Feeny, 1981) by diminishing the effectiveness of originally toxic compounds. For example, specialist herbivores such as Manduca sexta, which
specialize on Nicotiana plants, have higher resistance to nicotine than do related Lepidopterans (Wink & Theile, 2002); and monarch butterflies are 300-fold more resistant than other Lepidopterans to the cardiac glycosides of their host milkweed plants (Vaughan & Jungreis, 1977). Repeated or chronic exposure to inhibitory phytochemicals exerts strong positive selection for resistance (Elfawal et al., 2015), which can attenuate the effectiveness of the chemical and create the need for additional compounds or higher doses to achieve the same effect (Read et al., 2011).

Plants produce an astounding diversity of phytochemicals that can counteract infection in the plants themselves and also in phytophagous animals that consume phytochemicals (Hartmann, 2007; de Roode et al., 2013). Flowers contain distinct phytochemicals and blends that structure surface microbial communities (Junker et al., 2011) and can protect flowers from infection (Huang et al., 2012). Ingestion of antimicrobial phytochemicals may also ameliorate disease in phytophagous animals. Many animals prefer and seek out particular plants and phytochemicals when infected; ingestion of phytochemical-rich plants and their constituent compounds may reduce levels of infection (de Roode et al., 2013). Among insects, generalist arctiid caterpillars sought out alkaloid-containing host plants when parasitized; consuming these hosts increased the chances of surviving parasitism (Karban & English-Loeb, 1997; Singer et al., 2009). Cardenolide-rich latex from Asclepias improved survival and reduced spore counts of monarch butterfly larvae inoculated with protozoa (Gowler et al., 2015). Like foliage consumed by herbivores, nectar, pollen, and other plant products used by pollinators are rich in antimicrobial phytochemicals (Dobson & Bergstrom, 2000; Heil, 2011). In honey bees, gathering of resins reduced chalkbrood infection (Simone-Finstrom & Spivak, 2012); consumption of plant-derived honeys (Gherman et al., 2014) and the floral phytochemical thymol reduced levels of the microsporidian parasite Nosema ceranae (Costa et al., 2010). Certain nectar phytochemicals
also ameliorated Crithidia bombi infection in bumble bees (Manson et al., 2010; Baracchi et al., 2015; Richardson et al., 2015). The strong effects of phytochemicals on plant and animal parasites may impose selective pressures that could drive the evolution of phytochemical resistance in frequently exposed parasite populations.

The evolution of parasite resistance to natural or artificial compounds could exacerbate the negative impacts of parasites and pathogens on pollinators. Resistance of Varroa mites, which parasitize honey bees, has decreased the effectiveness of conventional miticides in apiculture (Lodesani et al., 1995; Rosenkranz et al., 2010). Phytochemical miticides, such as thymol (Giacomelli et al., 2015) and eugenol (Maggi et al., 2010), have emerged as natural alternatives to acaricides (Rosenkranz et al., 2010). However, the recommended treatment regime, consisting of repeated and prolonged administration of phytochemicals (Imdorf et al., 1996), results in incomplete eradication of the mites (Gregorc & Planinc, 2005), thereby providing conditions under which phytochemical resistance could evolve. In addition, the persistence of prophylactic chemicals at weakly inhibitory concentrations in hive materials (Nozal et al., 2002; Floris et al., 2004) may continue to select for resistant genotypes, even after treatment is complete. Even in the absence of deliberate prophylactic treatment with phytochemicals, chronic exposure to the environmental phytochemicals could create sufficient selective pressure to favor phytochemically resistant parasites. This problem is especially relevant in agricultural landscapes with intentionally low floral diversity, where one or two species may cover the majority of land within a 2 km radius (Long & Krupke, 2016). Low floral diversity is likely to result in a correspondingly low phytochemical diversity in available nectar and pollen. Monotonous exposure of parasites to these chemicals could give rise to chemically resistant parasite populations, thereby reducing the medicinal value of the few compounds available in monocultures. For example, the bumble bee gut parasite, Crithidia bombi, is over
100-fold more resistant to several phytochemicals than are phylogenetically related trypanosomes vectored by blood-feeding insects (Palmer-Young et al., In press). The high resistance of C. bombi, which has more direct exposure to floral phytochemicals than do related trypanosomes, suggests that phytochemical resistance can be increased by exposure to nectar and pollen phytochemicals over evolutionary time.

Whereas monotonous exposure to single chemicals creates strong selection for resistance, chemical combinations are thought to retard the evolution of resistance (Hastings, 2011), and associated costs may curtail the spread of resistance in populations. Pollinator parasites are likely to be frequently exposed to phytochemical combinations when their hosts consume nectar and pollen from multiple plant species or phytochemical blends produced by a single species. For example, nectar of the orchid Epipactis helleborine can contain as many as 100 compounds (Jakubska et al., 2005). In agriculture, models predicted that chemical combinations would be robust to resistance (Roush, 1998); empirically, broccoli plants with two Bacillus thuringiensis toxin genes were less prone than single-toxin plants to the evolution of herbivore resistance (Zhao et al., 2003). Clinically, combination therapy is the recommended treatment for a number of diseases, including protozoan infections such as visceral leishmaniasis (Leishmania donovani) and malaria (Plasmodium spp.) (van Griensven et al., 2010), and has been proposed as an “optimal strategy” to combat resistance (Bonhoeffer et al., 1997). In Plasmodium falciparum, resistance to the antimalarial drug artemisinin developed rapidly, but phytochemically complex Artemisia annua retained its medicinal value (Elfawal et al., 2015). Even if resistance does develop, it may have associated costs in the absence of inhibitory chemicals. These costs may limit the spatial spread and temporal persistence of resistance in populations when chemically mediated selective pressure is sporadic (Vanaerschot et al., 2014), as would be likely in diverse floral landscapes.
To assess whether a pollinator parasite can evolve resistance to single or combined floral phytochemicals under chronic exposure, we tested the ability of the bumble bee parasite, Crithidia bombi, to evolve resistance to the naturally occurring antitrypanosomal floral phytochemicals thymol, eugenol, and a thymol-eugenol blend. We predicted that chronic exposure would (1) increase phytochemical resistance and (2) decrease the growth-inhibiting effects of a given phytochemical concentration, but that (3) resistance would be slower or less likely to develop against the two-phytochemical blend. In addition, we expected that (4) resistance would come at a cost of decreased maximum growth in the absence of phytochemicals.

Materials and methods

Study system

Crithidia bombi is a trypanosome mid- and hindgut parasite of bumble bees (Bombus spp.) (Lipa & Triggiani, 1988; Sadd & Barribeau, 2013). Crithidia bombi is found on multiple continents (Schmid-Hempel & Tognazzo, 2010), including in many species threatened by parasite-related decline (Cameron et al., 2011; Schmid-Hempel et al., 2014). Crithidia bombi lives in the intestinal tract of nectar- and pollen-consuming bees, where it is directly exposed to the phytochemicals ingested by its hosts (Hurst et al., 2014). Although phytochemical concentrations in the gut lumen could be altered by microbial or host metabolism, orally transmitted parasites such as C. bombi are likely to have direct exposure to host-ingested nectar and pollen phytochemicals in the crop, and possibly also in the mid- and hindgut. Parasites can also be exposed to phytochemicals at flowers themselves, which are sites of parasite transmission (Durrer & Schmid-Hempel, 1994; Graystock et al., 2015). Crithidia bombi has several context dependent effects on host fitness (Sadd & Barribeau, 2013), and infection has
been correlated with declining populations of native bees (Schmid-Hempel et al., 2014).

However, ingestion of phytochemicals may ameliorate infection (Manson et al., 2010; Baracchi et al., 2015; Richardson et al., 2015) and directly inhibit parasite growth (Palmer-Young et al., In revision, In press). The phytochemicals encountered by C. bombi are dependent on the spatially and temporally variable floral landscape utilized by bumble bees.

Thymol and eugenol are two widespread phytochemicals to which C. bombi can have prolonged exposure, either alone or in combination. Both of these phytochemicals have recognized antitrypanosomal effects (Santoro et al., 2007a; b), including against C. bombi (Palmer-Young et al., In press, In revision). Thymol occurs in a variety of floral honeys (Nozal et al., 2002; Viñas et al., 2006), but is most well documented in culinary herbs of the Lamiaceae, such as Origanum vulgare (oregano), O. majorana (marjoram), O. dictamus (dictamus), and Thymus vulgaris (common thyme) (Daferera et al., 2000), where thymol was recently quantified (5-8 ppm) in floral nectar (Palmer-Young et al., In press). Eugenol, or its derivative methyl eugenol, has been found in over 450 species from over 80 plant families (Tan & Nishida, 2012), including in the flowers of over 100 species (Tan & Nishida, 2012), making it one of the most common floral phytochemicals. Eugenol has been found in common crop species, such as Cucurbita pepo (Granero et al., 2005) and Ocimum selloi (Martins et al., 1997), ornamentals such as Rosa rugosa (Wu et al., 1985; Dobson et al., 1990), and wild Epipactis (Jakubska et al., 2005) and Gymnadenia (Gupta et al., 2014) orchids. Like thymol, eugenol is most extensively documented in plants of the Lamiaceae (38 species) (Tan & Nishida, 2012). In at least four Lamiaceae species, eugenol is found together with either thymol or thymol's isomer, carvacrol: T. vulgaris (Lee et al., 2005), Ocimum basilicum (Lee et al., 2005; Politeo et al., 2007), Origanum vulgare (Milos et al., 2000), and O. majorana (Deans & Svoboda, 1990).
The flowering periods of thymol- and eugenol-rich plant species may expose pollinators and parasites to these phytochemicals for extended periods of time. The flowering period of thymol- and carvacrol-rich Thymus pulegioides generally lasts for one to two months in late spring and early summer (Senatore, 1996), coinciding with maximal plant monoterpenoid content (Kaloustian et al., 2005); the flowering period of T. vulgaris may last for several months at lower latitudes (McGimpsey et al., 1994; Khazaie et al., 2008). Similarly, the flowering period of Ocimum basilicum lasted three months in Poland, with Bombus spp. comprising 32% of visitors to the nectar- and pollen-rich flowers (Chwil, 2007). In our experiments, we exposed parasites to phytochemicals for six weeks, to reflect both (a) the duration of flowering in thymol- and eugenol-rich plants and (b) the foraging lifetime of a Bombus worker, which typically specializes on a single floral species (Heinrich, 1976b).

**Parasite collection and culturing**

Crithidia bombi cells were isolated from wild bumble bees (B. impatiens) collected near Normal, IL, United States in 2013 (strain “IL13.2”, collected by BMS). The culture was established by flow cytometry-based single cell sorting of bee feces as described previously (Salathé et al., 2012). Cultures were microscopically screened to identify samples with strong Crithidia growth and absence of bacterial or fungal contaminants, then stored at –80°C in a 2:1 ratio of cell culture:50% glycerol until several weeks before the experiments began. Thereafter, cells were incubated in tissue culture flasks at 27°C and propagated twice per week at a density of 100 cells µL-1 in 5 mL fresh culture medium, the composition of which has been previously described (Salathé et al., 2012). The final transfer (to 500 cells µL-1 in 5 mL fresh medium) occurred 48 h before the experiment began.
Phytochemicals

Thymol (Fisher Scientific, Franklin, MA) and eugenol (Acros, Thermo Fisher, Franklin, MA) stock solutions were prepared by pre-dissolving phytochemicals in ethanol (thymol and eugenol: 10 * 10^3 ppm for propagations, 40 * 10^3 ppm for EC50 assays; blend: 10 * 10^3 ppm thymol + 40 * 10^3 ppm eugenol). Stock solutions were sterile-filtered, aliquoted to sterile 2 mL tubes, and stored at –20°C throughout each six-week experiment.

Experimental design

We conducted three six-week exposure experiments, during which *C. bombi* was propagated continuously in either thymol (12 ppm), eugenol (50 ppm), or a 1:4 thymol:eugenol blend (5 ppm thymol + 20 ppm eugenol). Assuming a generation time of ~10 h (Salathé *et al.*, 2012), the six-week exposure period corresponds to approximately 100 generations. Exposure concentrations were chosen to inhibit growth by approximately 50%. Because phytochemical composition of thymol- and eugenol-containing plants varies across species, cultivars, and seasons (Kaloustian *et al.*, 2005; Lee *et al.*, 2005; Wogiatzi *et al.*, 2011), no single phytochemical ratio can encompass the variable proportions at which these compounds occur in plants. The 1:4 thymol:eugenol ratio was chosen to reflect the ratio of EC50 values for these two compounds in previous experiments (Palmer-Young *et al.*, in press), such that each phytochemical would make approximately equal contribution to growth inhibition.

To initiate each of the three experiments, the ancestral *C. bombi* culture was divided into five phytochemical-exposed and five control cell lines at an initial density of 100 cells µL⁻¹ (adjusted using OD (optical density)) in 1 mL of the appropriate phytochemical-containing medium (exposed lines) or phytochemical-free medium (control lines). Sterile ethanol was added to control treatment medium to equalize ethanol concentrations in the two treatments.
(thymol experiment: 0.12% v/v, eugenol experiment: 0.5%, blend experiment: 0.05%). Cells were incubated at 27°C in 12-well plates inside zippered plastic sandwich bags to reduce the chance of contamination. Cells were transferred twice per week (100 cells µL⁻¹ in 1 mL treatment medium) for six weeks after 3 days (odd transfers: 3 d, 10 d, ..., 39 d) or 4 days (even transfers: 7 d, 14 d, ..., 42 d) of growth. An additional two transfers (45 d & 49 d) were made in the blend experiment, for a total exposure time of 49 d. Cell density at time of transfer—a measure of the amount of growth during the preceding incubation period—was estimated by measuring OD (630 nm) of a 200 µL aliquot of each cell line. To obtain an accurate measure of cell density, the 12-well plates containing the cells to be transferred were resuspended (30 s, 600 rpm) on a microplate shaker. The plates were then moved into a laminar flow cabinet, and 200 µL from each well of cultured cells, and also cell-free control media containing the appropriate phytochemical concentration, was transferred to a 96-well plate for spectrophotometric OD (630 nm) measurement. The difference in OD between the cultured cells and the cell-free control media of corresponding phytochemical concentration was calculated for each sample. For analysis, OD readings were standardized relative to the mean OD of the control cell lines of the corresponding experiment and week.

The effects of the exposure treatment on phytochemical tolerance over time were assessed using three different response variables:

1. Cell density at time of transfer, which tested the effects of a fixed phytochemical concentration to which the cells were chronically exposed, and
2. EC50 (i.e., the phytochemical concentration that inhibited growth by 50%) from the weekly assays, which tested growth across a range of concentrations.

In addition, to assess possible costs of resistance, we compared
(3) Growth in phytochemical-free control medium, a measure of the cost of resistance in exposed lines. These values reflect growth in wells with 0 ppm phytochemical during the EC50 assays (described below).

Note that for response variables (2) and (3) above, exposed and control lines were tested under the same respective conditions following 48 h incubation in the absence of phytochemicals.

**EC50 assays**

EC50 assays were conducted weekly on three of the five independently propagated cell lines from each treatment to determine the phytochemical concentration that inhibited growth by 50%. Each assay tested resistance to the same phytochemical or blend used in the exposure treatment, i.e., thymol EC50 for experiment testing effects of thymol exposure; eugenol EC50 for eugenol experiment; and 1:4 thymol:eugenol blend EC50 for the blend experiment. Six concentrations of the appropriate phytochemical (or blend), including a 0 ppm phytochemical control concentration, were prepared by two-fold serial dilution in sterile-filtered growth medium. The maximum concentrations used were 100 ppm w/v thymol (thymol experiment), 400 ppm eugenol (eugenol experiment), and 60 ppm thymol + 240 ppm eugenol (blend experiment). These concentrations resulted in nearly complete growth inhibition, which allowed accurate estimation of dose-response curves and EC50 values. Sterile ethanol was added to control treatment medium to equalize ethanol concentrations in all wells (thymol experiment: 0.25% v/v, eugenol experiment: 1%, blend experiment: 0.6%).

Two days before each week’s EC50 assay began, an aliquot of cells from the lines propagated in 12-well plates was transferred to fresh medium (5 mL) at a density of 500 cells µL-1. These cells were allowed to grow for 48 h in tissue culture flasks in the absence of
phytochemicals. Immediately before the assay, each cell line was adjusted to a cell density of 1,000 cells µL⁻¹ in 8 mL fresh medium. During weeks 0 and 1 of the thymol experiment, cell density was adjusted based on hand counting of C. bombi cells at 400x in a Neubauer hemocytometer. However, C. bombi swim quickly and were difficult to quantify. To more precisely equalize cell densities in subsequent assays, we adjusted cell density based on OD thereafter, using a predetermined linear correlation between cell counts and OD readings (cell density = 1.03 * 10⁵ * OD, r² = 0.93), where cell density is in cells µL⁻¹ and OD is the difference in OD between the sample and an equivalent volume (200 µL) of control medium.

A separate 96-well plate was prepared for each cell line. Each plate contained eight replicate wells at each of six phytochemical concentrations. To each well, 100 µL of 1,000 cells µL⁻¹ cell suspension was added to 100 µL of phytochemical-enriched treatment medium using a multichannel pipette, resulting in a starting cell density of 500 cells µL⁻¹. The outer wells of the plate were used for cell-free controls (100 µL treatment medium + 100 µL control medium) to control for changes in OD unrelated to cell growth. Plates were sealed with laboratory film and incubated inside zippered plastic sandwich bags for 5 d at 27°C. Growth was measured by OD readings (630 nm) at 24 h intervals. OD readings (630 nm) were taken immediately after resuspension of the cells on a microplate shaker (40s, 1000 rpm, 3mm orbit). We calculated net OD (i.e., the amount of OD resulting from parasite growth) by subtracting the average OD reading of cell-free control wells of the corresponding concentration and time point.

For the thymol and blend analyses, we excluded the outermost two replicates (plate columns 3 and 10) of each concentration. Growth in these replicates differed from growth of the interior samples in the same treatments; we attributed this growth variation to volatility of the thymol, which resulted in altered exposure to phytochemicals depending on the contents of the neighboring control wells. In the eugenol experiment, we excluded the final week’s EC50 assays.
(i.e., time = 6 weeks) from analysis due to aberrantly hot lab conditions (40-43°C, due to a
building heating abnormality); cells were exposed to 40°C temperatures for several hours during
the setup of the assay, and to 43°C for an additional hour during the 24 h growth reading.

Statistical analyses

To quantify resistance to phytochemicals, EC50 values (i.e., the phytochemical
concentrations that inhibited growth by 50% relative to phytochemical-free controls) were
interpolated by constructing separate dose-response curves of phytochemical concentration vs.
growth for each cell line (n= 3 lines per treatment) and time point (n= 6-7 weeks per
experiment). All statistical analysis was conducted using the open source software R v3.2.1 (R
Core Team, 2014) following methods used for antimicrobial peptides (Rahnamaeian et al.,
2015). Growth was quantified using the growth integral (i.e., area under the curve of net OD vs.
time) for each well; this integral was calculated by fitting a model-free spline to the observed OD
measurements using grofit (Kahm et al., 2010). The relationship between phytochemical
concentration and growth integral was modeled with a Markov chain Monte Carlo algorithm
using Just Another Gibbs Sampler (Plummer, 2003) in combination with the R-package rjags
(Plummer, 2016). We used the following model to describe the relationship between
phytochemical concentration (c) and growth integral (g):

\[ g = r - \frac{E_{max} c^h}{(C_{50})^h + c^h} \]  \hspace{1cm} (2)

where \( r \) denotes growth in the absence of the phytochemical, \( E_{max} \) represents the maximum
inhibition at high concentrations, and \( C_{50} \) is the phytochemical concentration at which 50% of
the maximum inhibition is reached. The parameter \( h \), the Hill coefficient, indicates how steeply
the inhibition increases around the concentration $C_{50}$. From this model, we derived parameter estimates and 95% highest posterior density credible intervals (CI) of the EC50. For the blend experiment, in which all treatments contained a 1:4 thymol:eugenol ratio, curves were fitted using eugenol concentration as $c$. Growth measurements from the 0 ppm concentration were used to assess costs of resistance in the absence of phytochemicals.

To assess whether cell lines evolved resistance due to chronic phytochemical exposure, the effects of the exposure treatment over time were assessed using linear mixed-effects regression models with the lmer function in R package lme4 (Bates et al., 2015). Each response variable (EC50, cell density at time of transfer, and growth without phytochemicals) was standardized relative to the mean of the control lines at the corresponding time point. Exposure treatment and the treatment by time interaction were used as predictor variables, and cell line was included as a random effect to account for repeated measures. Significance of terms in the model was assessed by chi-squared ($\chi^2$) tests with the Anova function in the R package car (Fox & Weisberg, 2011). Fitted model means and standard errors were obtained using the lsmeans package (Lenth, 2016); graphs were produced with ggplot2 (Wickham, 2009) and cowplot (Wilke, 2016).

**Results**

Chronic phytochemical exposure resulted in increased phytochemical resistance in all three experiments. Changes in cell density at time of transfer indicated remarkably increased resistance to phytochemicals. In each experiment, the highly significant Treatment:Week interaction (Table 1) indicated that the growth-inhibiting effect of the fixed-concentration exposure treatment decreased over the course of the exposure period. Initially, the phytochemical exposure treatments (12 ppm thymol, 50 ppm eugenol, or 5 ppm thymol + 20
ppm eugenol) inhibited growth by over 50% (Fig. 1). However, by the end of the six-week experiment, the same phytochemical concentration had minimal effect on parasite growth in the lines that were chronically exposed to the phytochemical treatment. In other words, after 6 weeks, the exposed lines grew nearly as fast in the presence of phytochemicals as the controls grew in the absence of phytochemicals.

Because the changes in cell density could have reflected both environmental acclimation and genetic changes, we also conducted weekly EC50 assays following a brief relaxation of selection (48 h growth in phytochemical-free media) to minimize contributions of the parental environment to the resistance phenotype. From the two-week assay onward, EC50 values in the exposed lines were consistently higher than those of controls (Fig. 2). Thymol exposure increased resistance to thymol; eugenol exposure increased resistance to eugenol; and exposure to a 1:4 thymol-eugenol blend increased resistance to the same 1:4 blend. For each experiment, the Treatment:Week interaction term was highly significant (Table 1); this indicates that the EC50 ratio between exposed and control lines increased over the exposure period. Increases in EC50 relative to the control were similar across the three experiments (~10%, Fig. 2).

We found little evidence for costs of adaptation in terms of reduced growth in the absence of inhibitory phytochemicals. In the thymol experiment, there was an initial negative effect of the exposure treatment on growth without phytochemicals, but also a significant amelioration of this negative effect over time (Treatment:Week interaction, Table 1; Fig. 3). However, this result was strongly driven by the poor growth in exposed lines at the 1-week time point. When the 1-week time point was removed from the model, the negative effect of treatment was no longer significant ($\chi^2 = 2.23, df = 1, p = 0.14$). However, there remained a significant positive Treatment:Week interaction ($\chi^2 = 8.58, df = 2, p = 0.013$), indicating
progressively better growth relative to controls over time. Across all weeks, growth of thymol-exposed lines in the absence of phytochemicals averaged 98.6% that of controls, or 99.7% after excluding the 1-week time point. In the eugenol experiment, there was again no significant effect of treatment; across all weeks, growth in the absence of phytochemicals differed by only 0.4% between treatments. As in the thymol experiment, there was again a positive Treatment:Week interaction, which was statistically significant, but inconsistent across time (Fig. 3). In the blend experiment, exposed lines tended to have non-significantly higher growth without phytochemicals than the controls ($p = 0.14$, Table 1), and there was a marginally significant tendency of increased relative growth over time ($p = 0.06$, Table 1).

**Discussion**

We tested the effects of chronic phytochemical exposure on the evolution of resistance by the bumble bee parasite *C. bombi* in cell culture. The parasite evolved comparable resistance to both single phytochemicals and a two-compound combination, and resistance had no growth-related costs under phytochemical-free conditions. Thus, chronic exposure to ecologically relevant levels of floral phytochemicals could lead to the evolution of parasite resistance that may weaken the medicinal effects of phytochemicals on pollinators.

**Chronic phytochemical exposure increased resistance**

Initially, phytochemical exposure treatments inhibited *C. bombi* growth by >50%; however, after six weeks of exposure, the same phytochemical concentrations resulted in minimal inhibition (Fig. 1). Our thymol exposure concentration (12 ppm) exceeded levels in *Thymus vulgaris* nectar (5.2-8.2 ppm thymol (Palmer-Young *et al.*, In press)) and honey from thymol-fumigated honey hives (7.5 ppm (Charpentier *et al.*, 2014)). Similarly, our eugenol
exposure concentration (50 ppm) equaled concentrations in *Rosa x hybrida* stamens (Bergougnoux *et al.*, 2007), but far exceeded concentrations in other flowers and honey (Palmer-Young *et al.*, In press). In other words, within a few weeks, parasites became almost completely resistant to the effects of naturally occurring levels of phytochemicals.

Exposure over the six-week time frame used in our experiments is plausible in natural systems. For example, *Ocimum basilicum*, which can contain both thymol and eugenol (Lee *et al.*, 2005; Politeo *et al.*, 2007), flowers for a three-month period, even in northern Europe (Chwil, 2007), and its nectar and pollen are highly attractive to bumble bees. Individual Bombus workers, which live for four to six weeks, tend to specialize on particular plant species (Heinrich, 1976b). Thus, in a worker that specializes on a plant rich in one or several phytochemicals, resident parasites would have ample time to evolve resistance within a single growing season.

We expect that our serial propagation experiments provide a conservative estimate of the ability of natural parasite populations to evolve phytochemical resistance. In contrast to the low initial diversity of our clonal parasite cell lines, *C. bombi* populations are genetically diverse (Tognazzo *et al.*, 2012), and phytochemical resistance can vary several-fold between genotypes (Palmer-Young *et al.*, In press). High levels of preexisting natural variation could result in even more dramatic responses to selection than what we observed using clonal cell lines. Conversely, however, exposure of parasites in nature to nutrient limitation or host immune responses could increase parasite generation times, thereby slowing evolutionary processes and reducing rates of phytochemical adaptation.

**Combined phytochemicals did not curtail the evolution of resistance**

Contrary to our prediction, a two-phytochemical combination of thymol and eugenol did not inhibit the evolution of resistance. This is incongruent with empirical studies (Zhao *et al.*, 2005; Politeo *et al.*, 2007)}
2003; Elfawal et al., 2015), theoretical predictions (Roush, 1998), and clinical recommendations (van Griensven et al., 2010), all of which suggest that resistance should evolve more slowly to blends than to single compounds. Our result may relate to interactions between thymol and eugenol and to their modes of action. First, we have found synergistic effects of thymol and eugenol against C. bombi growth, (Palmer-Young et al., In revision), which may have promoted evolution of resistance by increasing the marginal benefits of resistance to either compound (Yeh et al., 2009). Second, the similar pro-oxidant modes of action of thymol and eugenol may have facilitated simultaneous development of resistance against both compounds. Both the monoterpenoid thymol and the phenylpropanoid eugenol are lipophilic compounds with aromatic rings and free hydroxyl groups. Such compounds penetrate membranes, disrupt ionic gradients and energy production, and increase oxidative stress (Bakkali et al., 2008).

Trypanosomes can counteract oxidative stress by producing thiols (Mehlotra, 1996), heat shock proteins (McCall & Matlashewski, 2012), and glycerol (Husain et al., 2012). In Leishmania donovani, these antioxidant systems can be quickly upregulated by increasing expression of antioxidant enzymes and even duplication of antioxidant-encoding chromosomes (Mannaert et al., 2012), resulting in rapid development of resistance against pro-oxidant drugs (Vanaerschot et al., 2014). Crithidia bombi encounters pro-oxidant floral phytochemicals, osmotic stress, and UV radiation during transmission at flowers (Cisarovsky & Schmid-Hempel, 2014), and had extremely high resistance to phenolics relative to clinically important trypanosomes (Palmer-Young et al., In press). Therefore, C. bombi likely possesses extensive antioxidant mechanisms that could facilitate rapid adaptation to pro-oxidant phytochemicals. If particular genotypes have broad-spectrum resistance against multiple phytochemicals with similar modes of action, resistance to one phytochemical could confer resistance to other phytochemicals as well.
No apparent growth-related cost of resistance in the absence of phytochemicals

The spread and maintenance of chemical resistance in parasite populations is shaped by a balance between the strength of selection favoring resistance and the costs of resistance that favor competing susceptible genotypes (Lenormand, 2002). In our experiments, we found no evidence for resistance-related costs in terms of growth under phytochemical-free conditions. Our previous work, which showed extremely high phytochemical resistance of C. bombi relative to related trypanosomes (Palmer-Young et al., In press), suggests that phytochemical-resistant strains of C. bombi have indeed been quite successful in nature. Although drug resistance appears to be costly in Plasmodium spp. and schistosomes (Vanaerschot et al., 2014), no costs of paromycin resistance were found in Leishmania donovani (Hendrickx et al., 2015); in L. infantum, miltefosine resistance was costly, but paromycin resistance resulted in increased growth and enhanced tolerance to stress (Hendrickx et al., 2016). Resistance to pro-oxidant antimonial drugs can actually improve L. donovani infectivity and establishment in hosts, presumably because the superior antioxidant defenses of resistant lines allow them to tolerate host immune responses and the stress of initial establishment (Vanaerschot et al., 2011). The fitness advantages of chemical resistance in parasites may also be context-dependent. For example, drug-resistant and -susceptible L. donovani competed equally well under optimal conditions, but drug-resistant lines outcompeted susceptible lines under stressful conditions, including heat shock, pH change, starvation, and infection of host cells (García-Hernández et al., 2015). If phytochemical-resistant C. bombi, like resistant L. donovani, gain a competitive advantage under temperature- or food-stressed conditions, then chemically resistant parasites could be favored in communities of stressed or resource-limited pollinators. Food-stressed bees are already immunocompromised (Brunner et al., 2014) and more vulnerable to C. bombi-induced mortality (Brown et al., 2003). Moreover, immunocompromised hosts could promote
the spread of chemically resistant parasites by failing to eradicate residual parasites following chemical treatment (Bloland, 2001), thereby allowing chemically resistant parasites to survive and spread to new hosts. As a result, the spread of phytochemical resistant C. bombi may be most favored under conditions when host bees are most susceptible to infection.

**Ecological determinants of resistance to phytochemicals**

Although Crithidia bombi can evolve resistance to phytochemicals and blends without incurring apparent costs, several factors may constrain parasite adaptation to local phytochemicals in wild populations, thus maintaining submaximal phytochemical resistance that varies among strains (Palmer-Young et al., In press). These factors could include complex and varied phytochemical environments, high rates of migration, periodic population bottlenecks, and possible transmission-related costs of resistance. First, nectar and pollen contain a rich diversity of phytochemicals. For example, more than 60 compounds, including thymol and eugenol, were present in floral essential oils of Helichrysum arenarium (Lemberkovics et al., 2001), and over 100 compounds, including eugenol, were found in nectar of the orchid Epipactis helleborine (Jakubska et al., 2005). As shown in experiments with Artemisia annua and Plasmodium falciparum malaria (Elfawal et al., 2015), it may be difficult for parasites to adapt to these complex blends, particularly when bees consume a mixture of blends from different types of flowers. Second, migration of parasites between different types of landscapes could limit local adaptation. Bumble bees forage over many kilometers (Heinrich, 2004), and founding queens may disperse considerable distances to found new colonies, thereby homogenizing parasite populations from regions with different floral phytochemical characteristics. Furthermore, sexual reproduction in C. bombi could increase the frequency of recombination events (Schmid-Hempel et al., 2011) that break up resistance-conferring gene complexes. Third,
genetic drift may limit the influence of natural selection on C. bombi populations by imposing annual genetic bottlenecks. Unlike honey bee colonies, bumble bee colonies in temperate climates have an annual cycle, and are founded anew each year by queens that mate in autumn, hibernate through the winter, and emerge in spring. Because queens alone survive the winter, and only a small proportion of queens succeed in founding colonies, C. bombi populations can be severely reduced between fall and spring (Erler et al., 2012), with possible random loss of resistance alleles. There may also be subtle costs of resistance that were undetectable in cell cultures. For example, costs related to between-host transmission or within-host growth could reduce the fitness of phytochemically resistant strains in the wild. Any combination of these factors could explain the maintenance of susceptibility to thymol and eugenol in C. bombi populations.

Despite the possibility that migration and genetic drift could weaken the effects of natural selection for phytochemical resistance, C. bombi does appear to have evolved extensive resistance to the nectar phenolic compounds caffeic, chlorogenic, and gallic acids (Palmer-Young et al., In press). We hypothesize that parasites may be more likely to have chronic exposure to these compounds, which are prevalent at considerable concentrations in honey from the nectar of many floral species (Can et al., 2015). Although eugenol in particular is widespread in flowers, both thymol and eugenol are more volatile than the aforementioned phenolics, which may limit the duration of parasite exposure to these compounds. However, repeated prophylactic fumigation of hives with thymol—a common pest-control measure for honey bee hives (Gregorc & Planinc, 2005)—could result in intense and prolonged selection for resistant parasites.

The distribution of phytochemicals in modern landscapes may contribute to evolution of phytochemical resistance. Sequential exposure to single chemicals is known to promote resistance (Bonhoeffer et al., 1997). Bees in agricultural settings may have sequential access to
diets dominated by a single plant species during each period of the growing season (Goulson et al., 2015), which could give parasites ample time to adapt to each plant’s phytochemicals. If phytochemical resistance in C. bombi is minimally costly and stable in the absence of phytochemicals—as observed in L donovani (dos Santos et al., 2008; Hendrickx et al., 2012)—resistance could be maintained between annual periods of exposure to phytochemicals of particular floral species. Progressive augmentation of resistance to each agricultural species’ phytochemicals would decrease the medicinal value of phytochemicals for pollinators.

In contrast to monotony, diversity among plants and hosts could curtail the evolution of phytochemical resistance. Serial infection of related hosts could select for parasites with specialized resistance to the phytochemicals in the host’s preferred food plants. However, transmission of parasites among bumble bee host species with different diets (Goulson & Darvill, 2004) could result in continually varying selective pressures that interrupt the development of phytochemical resistance. Because different pollinators favor different floral species (Heinrich, 1976a; Goulson & Darvill, 2004), pollinator and plant diversity could be mutually stabilizing. Diverse flora may also disrupt the development of resistance by exposing parasites to hundreds of phytochemicals simultaneously (Jakubska et al., 2005), rather than the two phytochemicals used in our thymol/eugenol blend. In addition to possible mitigation of phytochemical resistance among parasites, phytochemically and taxonomically varied landscapes have other known benefits to pollinators. Although thymol and eugenol are relatively benign and even attractive to bees (Goyret & Farina, 2005; Ebert et al., 2007), consumption of other potentially antiparasitic phytochemicals can increase mortality in bumble bees and other insects (Thorburn et al., 2015; Tao et al., 2016). Given that bumble bees are generalist pollinators, we hypothesize that they may be less susceptible to toxicity when allowed to consume mixed diets that do not contain excessive amounts of any particular compound.
Furthermore, varied landscapes are more likely to provide the variety of nutrients needed for colony growth and development, and also to offer a temporally distributed supply of nectar and pollen throughout the growing season (Roulston & Goodell, 2011). Overall, whereas limited floral diversity may decrease pollinator diversity and streamline the evolution of phytochemical resistance, abundant floral diversity could reduce parasite resistance to any particular suite of phytochemicals.

**Conclusion**

Our experiments show that pollinator parasites can evolve resistance to growth-inhibiting floral phytochemicals without associated costs of reduced growth. In contrast to our predictions, resistance was not hindered by a two-phytochemical combination. Given the initially low diversity of our parasite cell lines, these findings represent a conservative estimate of the ability of wild parasite populations to adapt to phytochemicals, a process that could diminish the value of naturally occurring defenses against parasites. Low floral and host diversity can be expected to promote phytochemical resistance. If resistance is not costly, or even confers a fitness advantage, resistance traits could spread quickly, exacerbating vulnerability to infection in already threatened pollinators.

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**Data Availability**

Data are archived in the Zenodo repository (https://zenodo.org/record/54705) with restricted access for reviewers (Palmer-Young et al., 2016); at acceptance, data will be made freely available.
Table 2-1. Effects of exposure treatments on *Crithidia bombi* cell density at time of transfer (estimated using OD (optical density) at 630 nm), EC50, and growth in the absence of phytochemicals. All responses were standardized relative the mean of the control lines of the corresponding experiment and time point. Predictor variables of linear mixed models were tested for statistical significance using $\chi^2$ tests. **Bold:** $p<0.05$.

<table>
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<th>Exposure treatment</th>
<th>Predictor</th>
<th>$\chi^2$</th>
<th>df</th>
<th>$p$</th>
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<tr>
<td><strong>Relative EC50</strong></td>
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Figure 2-1. Chronic exposure of C. bombi to phytochemicals decreased the growth-inhibiting effects of the exposure treatments. The x-axis shows the cumulative duration of exposure to phytochemical treatments. The y-axis shows cell density at time of transfer (estimated using OD (630 nm)) after incubation in thymol (12 ppm), eugenol (50 ppm), or a thymol-eugenol blend (5 ppm thymol + 20 ppm eugenol), standardized relative to the mean of the control lines at the corresponding time point. Points and error bars show mean +/- SE (n = 5 lines per treatment). Lines and shaded bands show predicted means +/- SE from linear mixed model fits. Open circles and solid lines: control treatment; filled circles and dashed lines: phytochemical exposure treatment.
Figure 2-2. Chronic exposure of *C. bombi* to phytochemicals decreased the growth-inhibiting effects of the exposure treatments. The x-axis shows the cumulative duration of exposure to phytochemical treatments. The y-axis shows cell density at time of transfer (estimated using OD (630 nm)) after incubation in thymol (12 ppm), eugenol (50 ppm), or a thymol-eugenol blend (5 ppm thymol + 20 ppm eugenol), standardized relative to the mean of the control lines at the corresponding time point. Points and error bars show mean +/- SE (n = 5 lines per treatment). Lines and shaded bands show predicted means +/- SE from linear mixed model fits. Open circles and solid lines: control treatment; filled circles and dashed lines: phytochemical exposure treatment.
Figure 2-3. Growth without phytochemicals (i.e., at phytochemical concentration of 0 ppm) during each week's EC50 assays. The x-axis shows the cumulative duration of exposure to phytochemical treatments. The y-axis depicts growth in the absence of phytochemicals, standardized relative to the mean of the control lines at the corresponding time point. Points and error bars show mean +/- SE (n = 6 (thymol and blend) or 8 (eugenol) wells each of 3 lines per treatment). Lines and shaded bands show predicted means +/- SE from linear mixed model fits. Open circles and solid lines: control treatment; filled circles and dashed lines: phytochemical exposure treatment.
CHAPTER 3
SYNERGISTIC EFFECTS OF FLORAL PHYTOCHEMICALS AGAINST A BUMBLE BEE PARASITE

Authors

Evan C. Palmer-Young1*, Ben M. Sadd2, Rebecca E. Irwin3, Lynn S. Adler1

Abstract

Floral landscapes comprise diverse phytochemical combinations. Individual phytochemicals in floral nectar and pollen can reduce infection in bees and directly inhibit trypanosome parasites. However, gut parasites of generalist pollinators, which consume nectar and pollen from many plant species, are exposed to phytochemical combinations. Interactions between phytochemicals could augment or decrease effects of single compounds on parasites.

Using a matrix of 36 phytochemical treatment combinations, we assessed the combined effects of two floral phytochemicals, eugenol and thymol, against four strains of the bumble bee gut trypanosome Crithidia bombi. Eugenol and thymol had synergistic effects against C. bombi growth across seven independent experiments, showing that the phytochemical combination can disproportionately inhibit parasites. The strength of synergistic effects varied across strains and experiments. Thus, the antiparasitic effects of individual compounds will depend on both

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the presence of other phytochemicals and parasite strain identity. The presence of synergistic phytochemical combinations could augment the antiparasitic activity of individual compounds for pollinators in diverse floral landscapes.

**Key words:** pollinator-parasite interactions, plant secondary metabolites; antimicrobial synergy; bumble bee; trypanosome; *Crithidia bombi*

**Introduction**

Plant communities comprise species that produce distinct and varied combinations of phytochemicals (Hartmann 1996). Floral phytochemicals, including those found in nectar and pollen, play a variety of ecological roles, including acting as antimicrobials that protect plants and their flowers against pathogens (Huang *et al.* 2012; Junker & Tholl 2013; McArt *et al.* 2014). Phytochemical combinations can have effects that differ from predictions based on activities of isolated components. In the incremental evolution of phytochemical-based defenses in plants, new phytochemicals would be selected for activity in the context of a plant’s pre-existing phytochemical repertoire, rather than for functional value in isolation (Richards *et al.* 2016). Plants can therefore be expected to contain chemical components that, in addition to providing protection from diverse antagonists, act to potentiate each other’s activities, and thereby economize resource allocation to defensive chemicals. However, even in well-established areas of chemical ecology such as plant-herbivore interactions, surprisingly few studies have explicitly examined the interacting effects of chemicals in mixtures (Richards *et al.* 2016), leaving much to be understood regarding the ecological functions of phytochemical mixtures and diversity.

In addition to defending plants against their own pathogens, antimicrobial phytochemicals can also counteract infection in animals, including pollinators (Karban & English-Loeb 1997; Singer, Mace & Bernays 2009; de Roode *et al.* 2013). Medicinal effects of
phytochemicals are especially relevant for bees, given that bees have abundant access to phytochemicals in nectar and pollen, and that some species are threatened by parasite-related population decline (Cameron et al. 2011; Goulson et al. 2015). Several studies have shown that individual floral phytochemicals can reduce parasite infections in bees. High concentrations of thymol (100 ppm) reduced Nosema ceranae infection in honey bees (Costa, Lodesani & Maistrello 2010); realistic nectar concentrations of gelsemine (Manson, Otterstatter & Thomson 2010) and four of eight other floral phytochemicals (Richardson et al. 2015) reduced Crithidia bombi parasitism in Bombus impatiens, and naturally occurring concentrations of nicotine ameliorated C. bombi infection in B. terrestris (Baracchi, Brown & Chittka 2015). In addition, eugenol and thymol had direct inhibitory effects on C. bombi growth, with inhibitory concentrations of thymol (4.5-22 ppm) close to those measured in floral nectar (5.2-8.2 ppm) (Palmer-Young et al. in press).

In nature, pollinators and their parasites encounter phytochemicals in combination rather than individually. Many bees are generalist pollinators that forage from a variety of plants. For example, in grasslands, a single bumble bee species may forage on as many as 13 plant species (Goulson & Darvill 2004). Moreover, phytochemical combinations occur within individual plants. For example, more than 60 compounds were present in floral essential oils of Helichrysum arenarium (Lemberkovics et al. 2001), 37 compounds were identified from Thymus zygus (Pina-Vaz et al. 2004), and over 100 compounds were found in the nectar of Epipactis helleborine (Jakubska et al. 2005). Pollen is similarly rich in phytochemicals (Dobson & Bergstrom 2000; Ketkar et al. 2014). Nectar-derived honey also has abundant floral phytochemicals (Viñas, Soler-Romera & Hernández-Córdoba 2006), with 147 compounds identified from eight types of monofloral honey; these honeys inhibited pro- and eukaryotic
pathogens, including *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* (Isidorov et al. 2015).

Functional interactions among chemicals fall into three general categories: additive, antagonistic, and synergistic effects (Jia et al. 2009). Additive effects indicate that the effects of chemicals are independent of one another. This can occur when the chemicals have similar modes of action, such that adding a second compound has the same effect as adding more of the first compound (Greco, Bravo & Parsons 1995), or when the two compounds target independent processes that have minimal effects on one another (Tallarida 2000). A clinical example of additive effects due to independent actions would be the activities of two phytochemicals, artemisinin and curcumin, against malaria (Nandakumar et al. 2006). Artemisinin interferes with mitochondrial function (Krishna et al. 2006), while curcumin causes DNA damage (Cui, Miao & Cui 2007). Assessments of interactions between compounds often compare results observed to results predicted under a null hypothesis of additivity (Greco et al. 1995).

Antagonistic effects occur when two compounds inhibit one another’s activities, such that mixtures are less effective than predicted based on the activities of each compound in isolation. At the extreme, one compound is an antidote to a compound known to cause toxicity. Antagonistic effects can occur, for example, when one compound alters a structure that is a target of a second compound, or interferes with production of a second compound’s target (Jia et al. 2009). Other mechanisms may include reduced uptake or stimulation of detoxification (Gershenzon & Dudareva 2007). An example of antagonistic effects is the co-precipitation of tomato leaf saponins and phytosterols. Although each can be toxic in isolation, binding between saponins and phytosterols reduces absorption and bioavailability of both compounds (Duffey & Stout 1996).
Synergistic effects occur when two compounds increase one another’s potency, resulting in mixtures that have stronger effects than predicted based on activities of their components in isolation. Synergistic effects are especially useful in clinical situations. By reducing the dose required to achieve a medicinal effect, selectively synergistic drug combinations can both reduce costs and lower the risk of patient toxicity (Greco et al. 1995).

Plants, which have evolved to produce defensive mixtures under conditions of limited resources and diverse antagonists, are an intuitive place to look for synergistic chemical combinations (Richards et al. 2016). Generally speaking, synergy can occur when one compound increases the bioavailability (Smith, Roddick & Jones 2001), inhibits the detoxification (Berenbaum & Neal 1985), or compromises the export of another compound (Stermitz et al. 2000).

Functional interactions between co-occurring phytochemicals could alter how plant chemistry mediates pollinator-parasite relationships, but although several studies have tested the effects of phytochemical mixtures, few have specifically addressed interactions between multiple compounds. For example, phytochemically complex, antimicrobial resins (Simone-Finstrom & Spivak 2012) and certain types of honey (Gherman et al. 2014) may decrease infection in honey bees, and honey derived from multiple plant species had stronger antimicrobial properties than monofloral honey (Erler et al. 2014). However, none of these studies quantified the contributions of individual versus combined phytochemical components to the biological activity of the tested mixtures. The few studies that explicitly tested the effects of mixtures relevant to pollinators have produced results that ranged from potential synergy to antagonism. In one study, neither nicotine nor thymol alone affected C. bombi infection in B. impatiens, but nectar containing both compounds at low concentrations (2 ppm nicotine + 0.2 ppm thymol) tended to reduce infection intensity (Biller et al. 2015), suggesting that the two compounds have synergistic effects. However, resin mixtures gathered by stingless bees had additive and less
than additive effects against several test microbes *in vitro* (Drescher *et al.* 2014), and in *B. impatiens*, a nicotine-anabasine mixture lacked the medicinal value of each compound alone against *C. bombi* (Thorburn *et al.* 2015), suggesting antagonistic effects.

Characterization of parasite-inhibiting interactions between multiple phytochemicals *in vitro* has the potential to link studies of single compounds with studies of complex phytochemical suites that occur in nature. We used cell cultures of the bumble bee parasite *C. bombi* to assess the individual vs. combined effects of two widespread antimicrobial floral phytochemicals, eugenol and thymol, on parasite growth. Parasite cell cultures allow for efficient and high resolution characterization of the direct effects of individual compounds (Palmer-Young *et al.* in press) and their combinations. Such approaches are commonly used for screening clinical drugs; they eliminate variation between individual hosts and allow sufficient replication to test the effects of multiple compounds across a range of doses. Using a statistical approach designed to assess the effects of two-drug combinations (Greco *et al.* 1995), we mathematically defined and graphically illustrated the three classes of interaction between phytochemicals (additive, antagonistic, and synergistic, as introduced above and in Figure 1). When parasite growth isoclines are plotted for concentrations of the two chemicals, each type of interaction produces distinctively shaped isoclines: additive interactions produce straight lines; synergistic interactions produce concave curves; and antagonistic interactions produce convex curves (Figure 1).

**Study system**

The trypanosome gut parasite of bumble bees, *Crithidia bombi*, potentially encounters a diverse suite of phytochemicals throughout its life cycle, making it a relevant system for addressing the effects of individual phytochemicals and combinations. *Crithidia bombi* is
exposed to phytochemicals both directly at flowers, where the parasite is transmitted between hosts (Durrer & Schmid-Hempel 1994; Graystock, Goulson & Hughes 2015), and in the bee intestine, which contains phytochemicals from host-ingested nectar and pollen (Hurst, Stevenson & Wright 2014). Crithidia bombi infects bees in many ecosystems worldwide (Schmid-Hempel et al. 2007; Cameron et al. 2011), where phytochemical exposure will be complex and varied. The parasite’s deleterious effects on infected bees (Brown, Schmid-Hempel & Schmid-Hempel 2003; Sadd & Barribeau 2013), including threatened native species (Schmid-Hempel et al. 2014), indicate its ecological and practical importance (Sadd & Barribeau 2013).

Eugenol and thymol are two widespread floral chemicals to which *C. bombi* is likely to be simultaneously exposed at considerable concentrations (Table 1) when bees forage in diverse floral landscapes. Eugenol or its derivative, methyl eugenol, has been found in over 450 species from 80 plant families (Tan & Nishida 2012), including in the flowers of over 100 species (Tan & Nishida 2012). These numbers refer only to known occurrences; eugenol is recognized as a common volatile (Gupta et al. 2014), and is likely to be present in many additional plant species that have not yet been sampled (Tan & Nishida 2012). Plants known to contain eugenol include common crop species, such as *Cucurbita pepo* and *Ocimum selloi* (Martins et al. 1997), ornamentals such as *Rosa rugosa* (17-40% of anther volatiles (Wu et al. 1985; Dobson, Bergström & Groth 1990)), and wild *Epipactis* (Jakubska et al. 2005) and *Gymnadenia* (Gupta et al. 2014) orchids. Eugenol synthase genes are also found in such common flowering plants as *Arabidopsis* spp., *Glycine max*, *Vitis vinifera*, *Populus* spp., *Betula* spp., *Petunia hybrida*, and *Clarkia breweri* (Gupta et al. 2014). Eugenol’s presence is most extensively documented among plants of the Lamiacae (38 species) (Tan & Nishida 2012), which includes widely cultivated thymol-containing herbs such as *Thymus vulgaris*, *Origanum vulgare*, *O. majorana*, and *O. dictamnus* (Daferera, Ziogas & Polissiou 2000). In at least four Lamiaceae species (Table 1),
eugenol is found together with either thymol or thymol’s isomer, carvacrol: *T. vulgaris* (Lee et al. 2005), *Ocimum basilicum* (Lee et al. 2005; Politeo, Jukic & Milos 2007), *Origanum vulgare* (Milos, Mastelic & Jerkovic 2000), and *O. majorana* (Deans & Svoboda 1990). Thymol, eugenol, and carvacrol all co-occur in inflorescences of the European *Helichrysum arenarium* (Lemberkovics et al. 2001), and eugenol has been found with the thymol isomer carvacrol in honey, although at low concentrations (<1 ppm) (Alissandrakis et al. 2009) that could reflect phytochemical evaporation during storage. In addition to the documented presence of these specific compounds, the biochemical pathways that produce eugenol and thymol give rise to many structurally similar compounds that may have similar individual and interactive effects. Eugenol is produced via the shikimate pathway, and as a phenylpropene, belongs to the second most diverse class of plant volatiles (Pichersky, Noel & Dudareva 2006). Thymol is produced from isoprenoid precursors via the methylerythritol phosphate (MEP) pathway from substrates involved in primary metabolism (Pichersky et al. 2006), meaning that precursors of thymol and related compounds are found in all plant species. As a terpenoid, thymol is a member of the most diverse class of plant volatiles (Pichersky et al. 2006).

Both eugenol and thymol have recognized antitrypanosomal effects, including against *C. bombi* (Palmer-Young et al. 2016b), with 50% growth inhibition of *Trypanosoma cruzi* by 76-246 ppm eugenol and 53-62 ppm thymol (Santoro et al. 2007a; b). Combinations of thymol and eugenol had synergistic effects against *Escherichia coli* (Pei et al. 2009), but antagonistic effects against *Crithidia fasciculata* (Azeredo & Soares 2013). However, compounds with similar or overlapping targets typically have additive effects (Jia et al. 2009). Eugenol and thymol are similar in chemical structure—each is a lipophilic compound with an aromatic ring and free hydroxyl group; eugenol and thymol also had similar effects on cell morphology of *Trypanosoma*
cruzi (Santoro et al. 2007a; b). Therefore, we predicted that eugenol and thymol would have additive effects on C. bombi.

**Materials and methods**

Seven independent experiments were conducted with four C. bombi strains. The first six experiments were conducted on strains tested singly in series, with three rounds of experiments on strain IL13.2 and one experiment each on strains VT1, C1.1, and S08. To account for week-to-week differences between experimental conditions, the final experiment tested all four strains in parallel, i.e., strains were tested concurrently, but with reduced replication of treatments within strains.

**Parasite culturing**

Parasite strains were isolated from wild bumble bees collected near Normal, IL, United States in 2013 (“IL13.2”, from B. impatiens, collected by BMS); Hanover, NH, United States in 2014 (“VT1”, from B. impatiens, by lab of REI); Corsica, France in 2012 (“C1.1”, from B. terrestris, collected by BMS); and Zurich, Switzerland in 2008 (“S08”, from B. terrestris, collected by the group of Paul Schmid-Hempel, which included BMS).

Strains were isolated by flow cytometry-based single cell sorting of bee feces (IL13.2, C1.1, S08) or homogenized intestinal tracts (strain VT1) as described previously (Salathé et al. 2012). All strains were isolated directly from wild bees with the exception of VT1, which was first used to infect laboratory colonies of B. impatiens (provided by Biobest, Leamington, ON, Canada). The cell used to initiate the parasite culture was obtained from an infected worker of one of the commercial colonies. Cultures were microscopically screened to identify samples with strong Crithidia growth and absence of bacterial or fungal contaminants, then stored at -
80°C in a 2:1 ratio of cell culture:50% glycerol until several weeks before the experiments began. Thereafter, strains were incubated in tissue culture flasks at 27°C. Strains were propagated twice per week at a density of 100 cells µL⁻¹ in 5 mL fresh culture medium, the composition of which has been previously described (Salathé et al. 2012). The final transfer (to 500 cells µL⁻¹ in 5 mL fresh medium) occurred 48 h before the experiment began.

**Experimental design**

Eugenol (Acros, Thermo Fisher, Franklin, MA) and thymol (Fisher Scientific, Franklin, MA) treatment media were prepared by pre-dissolving phytochemicals in ethanol to 40 mg mL⁻¹; ethanol solutions were stored at -20°C. Phytochemicals were then dissolved in growth media to create two stock solutions at 4x desired concentrations, one of eugenol (800 ppm in IL13.2, Rounds 1 & 2; 1600 ppm for all other experiments with strains tested in series; 1200 ppm for strains tested in parallel) and another of thymol (200 ppm in IL13.2, Rounds 1 & 2; 400 ppm in other in-series experiments; 300 ppm for strains tested in parallel). Six two-fold dilutions of this stock were made separately for each phytochemical. Ethanol was added to treatments of lesser concentrations to equalize the ethanol concentrations (2-4% v/v for eugenol, 0.5-1% v/v for thymol, depending on the experiment) in all treatments. A fully-crossed phytochemical treatment matrix consisting of all 36 possible combinations at 2x their desired final concentrations was prepared in a 2 mL deep-well 96-well plate, with eugenol treatments in rows and thymol treatments in columns. Using a multichannel pipette, we transferred 100 µL 2x treatment media to the inner 36 wells of six (for experiments in series) or two (for strains tested in parallel) replicate 96-well tissue culture-treated plates. Hence, each plate contained a single well at each of the 36 two-phytochemical treatment combinations, and each experiment included either two (for experiments in series) or six (for strains tested in parallel) biological


replicates at each concentration. The treatment concentrations were chosen with the goal of achieving complete growth inhibition at the highest concentrations, in order to allow construction of dose-response curves without the need for extrapolation of inhibitory effects beyond the tested concentration range (see Statistical analysis). These concentrations (0-400 ppm eugenol, 0-100 ppm thymol) spanned the range of known nectar and pollen phytochemical concentrations, but were less than maximal leaf concentrations of eugenol and thymol (Table 1).

Immediately before the assay, parasite cells from tissue culture flasks were diluted to a density of 1000 cells µL⁻¹ in 6 mL of culture medium. Cells (100 µL) were added to an equal volume of the 2x phytochemical treatment media using a multichannel pipette, thereby diluting the cells to 500 cells µL⁻¹ and phytochemicals to the desired concentrations (1x with 0.625-1.25% v/v ethanol). Two additional plates were seeded with cell-free medium rather than cells; these plates served as negative controls. Sterile distilled water was added to the outer wells of all plates to reduce evaporation and edge effects.

Plates were sealed with laboratory film and incubated inside zippered plastic sandwich bags for 5 d at 27°C. For the experiment with strain S08 tested “in series”, an additional day of growth measurements were included in the model due to slow growth over the first 5 d. Growth was measured by OD (optical density) readings (630 nm) at 24 h intervals. Two techniques were used before each reading to ensure accurate OD measurements: First, cells were resuspended (40s, 1000 rpm, 3mm orbit) using a microplate shaker before each reading. Second, to minimize error due to condensation, the cover of the assay plate was briefly switched with that of an empty, sterile plate under sterile conditions. We calculated net OD (i.e., the amount of OD resulting from parasite growth) by subtracting the average OD reading from cell-free control wells of the corresponding phytochemical treatment and time point.
Statistical analysis

All statistical analyses were carried out using the open source software R v3.2.1 (R Core Team 2014). We used the R package grofit (Kahm et al. 2010) to fit a model-free spline to the observed OD measurements. This spline fit was used to compute each sample’s five-day growth integral (i.e., area under the curve of net OD vs. time). This growth integral was used as the response variable in subsequent analyses.

The effects of the individual phytochemicals and their interaction were assessed with a seven-parameter Universal Response Surface Analysis as described by Greco et al. (Greco et al. 1990; Greco et al. 1995). This method, which provides a statistical estimate of the interactions between compounds, has been deemed both robust and accurate for assessment of drug combinations (Meletiadis et al. 2005; Zhao, Au & Wientjes 2010), and has been used in previous two-compound studies (e.g., Greco et al. 1990; Faessel et al. 1999). The following equations were used:

\[
g(c) = \frac{(g_{\text{max}} - g_{\text{min}})(c/EC_{50})^m}{1 + (c/EC_{50})^m} + g_{\text{min}} \quad (3)
\]

\[
1 = \frac{c_1}{EC_{50(1)}(g_{c_1c_2} - g_{\text{min}})^{m_1} \cdot EC_{50(2)}(g_{c_1c_2} - g_{\text{max}})^{m_2}} + \frac{c_2}{EC_{50(1)} \cdot EC_{50(2)}(g_{c_1c_2} - g_{\text{min}})^{m_1 \cdot m_2}} + \frac{f_{c_1c_2}}{EC_{50(1)} \cdot EC_{50(2)}(g_{c_1c_2} - g_{\text{min}})^{m_1 \cdot m_2}} \quad (4)
\]

Equation (1) describes a sigmoidal dose-response curve in the presence of a single inhibitory compound. On the left side of the equation, “\(g(c)\)” indicates the amount of growth (“\(g\)”) as a function of phytochemical concentration (“\(c\)”). Parameter “\(g_{\text{max}}\)” represents the upper limit of growth in the absence of phytochemicals; “\(g_{\text{min}}\)” represents the lower asymptote of the curve as phytochemical concentration approaches infinity. The “\(EC_{50}\)” (“Effective Concentration”) is the phytochemical concentration at which 50% of maximal growth inhibition
is achieved. Parameter “m” describes the slope of the dose-response curve at the $EC_{50}$ concentration.

Equation (2) extends the single-compound model in Equation (1) to describe the interactive effects of two phytochemicals, which are denoted with subscripts. The parameter “f” classifies the type of interaction between the two phytochemicals as synergy ($f > 0$), additivity ($f = 0$), or antagonism ($f < 0$). This parameter is equivalent to the interaction term of a general linear model, in which a significant interaction indicates that the effect of one factor depends on the level of another factor (Greco et al. 1995). In our case, the factors are the two phytochemicals.

Equation (2) parameters “$c_1$” and “$c_2$” represent the respective concentrations of the two phytochemicals, and “$g_{c_1,c_2}$” predicts the amount of growth at a given combination of “$c_1$” and “$c_2$”. The parameters “$EC_{50}$” and “$m$” are derived by fitting dose-response curves for each individual phytochemical in the absence of the other compound using Equation (1). “$EC_{50(1)}$” and “$EC_{50(2)}$” represent the respective 50% inhibitory concentrations of each phytochemical in the absence of the other compound; and “$m_1$” and “$m_2$” describe how fast growth decreases at the EC50 concentration of each phytochemical in the absence of the other compound. Parameter “$g_{min}$” denotes the lower limit of growth as phytochemical concentrations go to infinity. The units divide out of each term in the equation: within the denominator, the growth parameters divide out and the exponent “$m$” has no units; the units also divide out for the concentration parameters in each term’s numerator and denominator.

A separate model was fit for each strain and experiment round; models were fit by the “ursa” function in package “drc” (Ritz et al. 2015). Results were graphed in R v3.2.1 (R Core Team 2014) packages “plot3D” (Soetaert 2016) and “ggplot2” (Wickham 2009).
Because the scale of the interaction parameter $f$ has a nonlinear relationship to the relative activity of compounds in mixture vs. in isolation, the original interaction parameter $f$ was converted to the linear interaction parameter $s$ (Figure 1), which quantifies the curvature in the growth isoclines (Greco et al. 1990), by solving the equation:

$$f = 4(s^2 - s) \quad (5)$$

Here, $f$ is the parameter derived from Equation (2), and $s$ indicates the ratio of the expected to observed concentrations that result in 50% growth inhibition (Figure 1). For example, an $s$ value of 1 indicates that compounds have additive effects. In contrast, an $s$ value of 2 indicates that the compounds have twice the expected inhibitory activity when in mixture, such that only half of the expected concentrations are sufficient for 50% growth inhibition.

**Results**

Eugenol and thymol had synergistic effects on the growth inhibition of *C. bombi* in each of the ten analyses, as evidenced by the shape of the growth contour lines (Figures 2-3) and values of the interaction parameter “$s$” (Figure 4; $s > 1$ indicates synergy). The highly concave contour lines in strain IL13.2 (Figure 2A-C, Table 1) indicate that synergistic effects were most pronounced against this strain. The increase in potency due to co-occurrence of the compounds in IL13.2 varied from 23% in Round 3 to 84% in Round 2, with statistically significant synergy in all strains and experimental rounds (Figure 4, Supplementary Table S1). Synergistic interactions were weaker but still statistically significant in strains VT1 (15% and 38% potentiation in series and in parallel, respectively), C1.1 (8% and 27%), and S08 (11% and 50%, Figure 4; see Supplementary Table S1 for full model parameters). In general, the in-series experiments with VT1, C1.1, and S08 were characterized by poor growth, with low levels of synergy, phytochemical tolerance, and maximum growth in the absence of phytochemicals. When strains
were tested in parallel, all strains grew strongly, with higher EC50 values, but also more apparent synergistic effects of the combined phytochemicals (Figure 4). The relative strength of synergy in the four strains was reasonably consistent across the in-series and in-parallel experiments. In both the in-series and in-parallel experiments, synergistic effects were strongest against strain IL13.2, weakest against C1.1, and intermediate against VT1 and S08.

**Discussion**

The existence and nature of combinatorial interactions will determine how phytochemical blends can mediate plants’ interactions with mutualists, antagonists, and their diseases—including pollinator infections—in nature, where exposure to compound combinations at variable doses is inevitable. Synergistic interactions, in which chemical combinations are more effective than single components, are of particular clinical and ecological interest. Synergistic combinations can have greater efficacy against infection, or achieve medicinal effects at lower total dosage, which may reduce the risk of host toxicity (Jia et al. 2009). Our results quantitatively demonstrate how a naturally occurring phytochemical combination influences the growth of an important pollinator parasite, and provide a model for future work on the role of phytochemical combinations in plant-pollinator-parasite interactions.

Eugenol and thymol exhibited synergistic inhibitory effects that varied in strength across strains and experiments. Previous work has indicated that interactions between eugenol and thymol are dependent on the focal taxon. Eugenol and thymol synergistically inhibited *E. coli* (Pei 2009) and porcine gut microbiota (Michiels *et al.* 2007), and a eugenol-thymol-citral combination had synergistic toxicity to *Trypanosoma cruzi* (Azeredo & Soares 2013). However, eugenol and thymol had antagonistic effects against *Crithidia fasciculata* (Azeredo & Soares 2013). *C. bombi* is known to be genetically diverse (Salathé & Schmid-Hempel 2011), with
genotype-specific infection ability (Barribeau et al. 2014) and growth rate (Ulrich & Schmid-Hempel 2012). Our results show that C. bombi strains also varied in resistance to both inter-
phytochemical synergy and isolated phytochemicals (Palmer-Young et al. in press). This finding
has ecological importance, because, in contrast to the organisms above, C. bombi is naturally
exposed to these phytochemicals from flowers.

The mode of action of phytochemicals can influence their interactions when in
combination. Eugenol and thymol have generally similar effects against trypanosomes and other
eukaryotes, although these effects can vary across taxa. Eugenol and thymol are both
hydrophobic volatiles with free hydroxyl groups; they can penetrate membranes, disrupt ionic
gradients needed for energy production, and precipitate oxidative stress that damages vital
lipids and proteins (Bakkali et al. 2008). In T. cruzi, both eugenol (Santoro et al. 2007a) and
thymol (Santoro et al. 2007b) caused cytoplasmic swelling, rounding of the cell body, and
altered nuclear morphology. In Leishmania major, both eugenol (Ueda-Nakamura et al. 2006)
and thymol (de Medeiros et al. 2011) affected mitochondria. In the yeast Candida albicans, both
eugenol and thymol altered membrane morphology (Braga et al. 2007). Although neither
compound affected the plasma membrane of T. cruzi (Santoro et al. 2007a; b), eugenol altered
the mitochondrial membrane in L. donovani (Ueda-Nakamura et al. 2006), and thymol caused
membrane wrinkling and sub-membrane accumulation of lipid droplets in L. amazonensis (de
Medeiros et al. 2011). Given the similar chemical structures and modes of action of eugenol and
thymol, we predicted that these compounds would behave additively. To our surprise, eugenol
and thymol had synergistic effects against all four C. bombi strains. Generally, compounds with
synergistic effects have related but distinct cellular targets (Jia et al. 2009), rather than identical
targets. Although eugenol and thymol had similar effects on trypanosome cell morphology
(Azeredo & Soares 2013), our results suggest that these compounds may have distinct complementary effects at a finer scale.

From an ecological perspective, the synergistic effects found in our study suggest that combinations of eugenol and thymol could ameliorate parasite infection in pollinators. Both eugenol and thymol are tolerated by bees at considerable concentrations. In *Apis mellifera* adults, the eugenol LD50 over 8 d was 7800 ppm (Ebert *et al*. 2007), well above the 44-185 ppm EC50 of our *C. bombi*. Similarly, the thymol LD50 of *A. mellifera* exceeded 1,000 ppm (Ebert *et al*. 2007), far higher than the 8.5-49.8 ppm EC50 of *C. bombi*. However, a mere 50 ppm thymol delayed *A. mellifera* larval development (Charpentier *et al*. 2014), and could have similar sub-lethal but deleterious effects on *Bombus* spp. Synergy between the antitrypanosomal effects of co-occurring phytochemicals could reduce the total phytochemical dose needed to ameliorate infection, thereby reducing the risk of side effects in hosts and their offspring.

Additional sampling is needed to determine the phytochemical concentrations in nectar and pollen relative to the inhibitory concentrations reported here. Although the concentrations that inhibited growth in this study were higher than those documented to date in nectar and pollen, they were well below the levels found in leaves (Table 1). Few studies have measured pollen and nectar phytochemical concentrations. Those that have reported generally lower phytochemical concentrations in nectar and pollen than in leaves (Detzel & Wink 1993; Kessler & Halitschke 2009), but in some cases pollen concentrations were actually higher than in leaf tissue (Frölich, Hartmann & Ober 2006), and were orders of magnitude higher than those in nectar (Detzel & Wink 1993; London-Shafir, Shafir & Eisikowitch 2003; Palmer-Young *et al*. 2016b). Even if pollen phytochemical concentrations are less than 10% of those in leaves, such concentrations of thymol (100-820 ppm) would still be highly inhibitory (EC50 <50 ppm).

Moreover, we tested for inhibition under conditions optimized for *C. bombi* growth. In the wild,
C. bombi is exposed to complex phytochemical blends, host immune responses (Barribeau & Schmid-Hempel 2013), and abiotic stresses including temperature fluctuation, osmotic stress, and dessication (Cisarovsky & Schmid-Hempel 2014). Under such stressful conditions, lower concentrations might be sufficient to impede growth.

To understand the ecological importance of phytochemical combinations, future research must address not only direct effects on parasites, but also how interactions between phytochemicals are altered by host-mediated effects. First, phytochemicals that stimulate the host immune system (Mao, Schuler & Berenbaum 2013), or affect intestinal muscle contraction (Tomizawa & Casida 2003), could synergize with directly antimicrobial phytochemicals to kill or expel gut parasites. Second, if different phytochemicals are detoxified by different enzymes (Mao, Schuler & Berenbaum 2011), then host detoxification of a phytochemical combination might be more efficient than detoxification of a single phytochemical. As a result, gut-dwelling parasites might experience a relatively small proportion of the ingested phytochemical combination, and parasite inhibition would require greater total ingestion of the phytochemical combination versus the single phytochemical. This result would be interpreted as antagonism between compounds. Third, although phytochemical combinations may have synergistic effects against parasites, compound combinations can also have synergistic toxic and immunosuppressive effects against insects (Berenbaum & Neal 1985; Duffey & Stout 1996; Richards et al. 2012), which could exacerbate the deleterious effects of floral phytochemicals on bees (Nibret & Wink 2010; Hurst et al. 2014). Finally, insects in the wild make behavioral choices involving nonrandom collection and use of phytochemicals, and may alter foraging behavior and preferences when diseased (Karban & English-Loeb 1997; Simone-Finstrom & Spivak 2012; de Roode et al. 2013; Baracchi et al. 2015; Erler & Moritz 2015). Hence, cell culture experiments,
which detect direct effects of phytochemicals, should be complemented by studies in live 
insects, which account for host-mediated indirect effects.

Our quantification of the interactive effects of a phytochemical combination is a start 
towards integration of the effects of single chemicals with those of chemically complex 
ecosystems. In our experiments, interactions between two phytochemicals had synergistic 
inhibitory effects of varying magnitude on a pollinator parasite. Given the actual diversity of 
floral blends, and the possibility of additional interactions between phytochemicals and host- 
mediated effects, our study alone cannot quantify the ecological significance of interactions 
between co-occurring phytochemicals. Phytochemical composition of the floral community may 
interface with the genotypic interactions of hosts and parasites (Sadd & Barribeau 2013) to 
structure patterns of infection. Further research on single and multi-plant blends is needed to 
determine the ecological relevance of phytochemical combinations consumed by generalist and 
specialist pollinators, including the effects of phytochemical combinations on disease of 
threatened species. Because the generalist foraging habits of many pollinators results in novel 
phytochemical combinations, interactions between phytochemicals of similar and distinct 
species are equally plausible, and offer immense opportunities for future investigation, from the 
scale of molecules to ecosystems.

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Data accessibility

All supporting data are available in the Zenodo repository with restricted access at https://zenodo.org/record/167602. Data will be made freely available upon manuscript acceptance (Palmer-Young et al. 2016a).

Competing interests

The authors declare that there are no competing interests.
Authors’ contributions

BMS and ECPY conceived the study. EPY conducted the experiments, analyzed the data, and wrote the first draft of the manuscript. All authors revised the manuscript and have agreed to its submission.
Table 3-1. **Published concentrations of eugenol and thymol in selected plants.** Concentrations are given in ppm fresh mass when possible. Where references quantified concentrations in percent of essential oil per unit dry mass, concentrations were converted based on other studies that quantified leaf moisture content and/or essential oil yield, as explained in the “Notes” column (continued onto next few pages).

<table>
<thead>
<tr>
<th>A. Plant species high in Eugenol</th>
<th>Species</th>
<th>Sample type</th>
<th>Concentration</th>
<th>Reference</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ocimum selloi</strong></td>
<td>leaves</td>
<td>~1200 ppm</td>
<td>(Martins <em>et al.</em> 1997)</td>
<td>0.2% essential oil by fresh mass, 63% eugenol in oil</td>
<td></td>
</tr>
<tr>
<td></td>
<td>flowers</td>
<td>~2400 ppm</td>
<td>(Martins <em>et al.</em> 1997)</td>
<td>0.4% essential oil by fresh mass, 63% eugenol in oil</td>
<td></td>
</tr>
<tr>
<td><strong>Ocimum basilicum</strong></td>
<td>leaves (broad-leaf variety)</td>
<td>~70 ppm</td>
<td>(Wogiatzi <em>et al.</em> 2011)</td>
<td>500 ppm in dried leaves; 86% leaf moisture (Rocha, Lebert &amp; Marty-Audouin 1993). <em>O. basilicum</em> may also contain thymol (Lee <em>et al.</em> 2005)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>leaves (narrow-leaf variety)</td>
<td>~100 ppm</td>
<td>(Wogiatzi <em>et al.</em> 2011)</td>
<td>700 ppm in dried leaves; 86% moisture (Rocha <em>et al.</em> 1993).</td>
<td></td>
</tr>
<tr>
<td><strong>Rosa x hybrida</strong></td>
<td>stamens</td>
<td>50 ppm</td>
<td>(Bergougnoux <em>et al.</em> 2007)</td>
<td>13.1% of 380.6 ppm total analytes</td>
<td></td>
</tr>
<tr>
<td><strong>Cucurbita pepo cv. Tosca</strong></td>
<td>petals</td>
<td>0.99-1.2 ppm</td>
<td>(Granero <em>et al.</em> 2005)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nectar</td>
<td>0.02-0.57 ppm</td>
<td>(Granero <em>et al.</em> 2005)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dianthus caryophyllus</strong></td>
<td>floral volatiles</td>
<td>trace-84.1% of emissions</td>
<td>(Clery <em>et al.</em> 1999)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Gymnadenia densiflora</strong></td>
<td>flower headspace</td>
<td>0.839 ppm</td>
<td>(Gupta <em>et al.</em> 2014)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rosmarinus spp.</strong></td>
<td>monofloral honey</td>
<td>0.02-0.03 ppm</td>
<td>(Castro-Vázquez, Pérez-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## B. Plant species high in Thymol

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample type</th>
<th>Concentration</th>
<th>Reference</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lippida sidoides</em></td>
<td>leaves</td>
<td>~8200</td>
<td>(de Medeiros <em>et al.</em> 2011)</td>
<td>1.06% oil in leaves (Veras <em>et al.</em> 2012), 78% thymol in oil</td>
</tr>
<tr>
<td><em>Origanum dictaminus</em></td>
<td>leaves</td>
<td>~1300</td>
<td>(Daferera <em>et al.</em> 2000)</td>
<td>1.05% essential oil by mass (Argyropoulou <em>et al.</em> 2014), 78% thymol in oil, 84% moisture in leaves (Loghmanieh, Bakhoda &amp; Issa 2014).</td>
</tr>
<tr>
<td><em>Origanum vulgare</em></td>
<td>leaves &amp; flowers</td>
<td>~990 ppm</td>
<td>(De Martino <em>et al.</em> 2009)</td>
<td>2.3% essential oil by dry mass. 63% thymol in oil. 84% moisture in leaves (Loghmanieh <em>et al.</em> 2014). O. vulgare may also contain eugenol (Milos <em>et al.</em> 2000; De Martino <em>et al.</em> 2009)</td>
</tr>
<tr>
<td><em>Thymus vulgaris</em></td>
<td>leaves</td>
<td>~3200 ppm</td>
<td>(Daferera <em>et al.</em> 2000)</td>
<td>~0.5% essential oil by fresh mass (Hudaib <em>et al.</em> 2002), 64% thymol in oil</td>
</tr>
<tr>
<td><em>Thymus vulgaris</em></td>
<td>leaves</td>
<td>~1370 ppm</td>
<td>(Lee <em>et al.</em> 2005)</td>
<td>8550 ppm in dried leaves; assume 84% moisture in leaves (Loghmanieh <em>et al.</em> 2014). T. vulgaris may</td>
</tr>
<tr>
<td>Species</td>
<td>Part</td>
<td>Concentration (ppm)</td>
<td>Reference</td>
<td>Notes</td>
</tr>
<tr>
<td>-------------------------------</td>
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<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td><em>Thymus pulegioides</em> L.</td>
<td>leaves &amp; flowers</td>
<td>~1500 ppm</td>
<td>(Senatore 1996)</td>
<td>0.5% essential oil by fresh mass; 30% thymol in oil</td>
</tr>
<tr>
<td><em>Satureja montana</em></td>
<td>leaves</td>
<td>~1000 ppm</td>
<td>(Nikolić <em>et al.</em> 2014)</td>
<td>1.5% essential oil by dry mass (Sefidkon, Jamzad &amp; Mirza 2004), 44% thymol in oil, 84% moisture in leaves (Loghmanieh <em>et al.</em> 2014).</td>
</tr>
<tr>
<td><em>Origanum majorana</em></td>
<td>leaves</td>
<td>~1100 ppm</td>
<td>Daferera <em>et al.</em> 2000</td>
<td>Assumes 0.5% essential oil by fresh mass (Hudaib <em>et al.</em> 2002), 14% thymol in oil. <em>O. majorana</em> may also contain eugenol (Deans &amp; Svoboda 1990).</td>
</tr>
<tr>
<td><em>Thymus vulgaris</em></td>
<td>nectar</td>
<td>5.2-8.2 ppm</td>
<td>(Palmer-Young <em>et al.</em> 2016b)</td>
<td></td>
</tr>
<tr>
<td><em>Thymus</em> spp.</td>
<td>honey</td>
<td>0.27 ppm</td>
<td>(Nozal <em>et al.</em> 2002)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3-1. Schematic depiction of the shapes of growth isoclines for different patterns of interaction. Interactions between the two compounds are quantified by the parameter $s$, which reflects the ratio of the Expected to Observed concentrations that result in 50% inhibition. The solid black line represents the shape of the growth isocline under the null hypothesis of additivity, corresponding to $s = 1$. The red parabola depicts the concave shape of the isocline when there is synergy between the two compounds ($\text{Expected} > \text{Observed}, s > 1$), whereas the gray parabola depicts a convex isocline, which occurs when the compounds have antagonistic effects ($s < 1$). For clarity, the distance $\text{Observed}$ is only shown for the case of synergy.
Figure 3-2. Combinatorial effects of eugenol and thymol against *C. bombi* strains tested in series over six experiments. Panels show the results of six separate experiments in separate weeks: three with *C. bombi* strain IL13.2—referred to as “Rounds 1-3”, and one each with strains VT1, C1.1, and S08. The solid line shows the isocline of 50% growth inhibition. The dashed line that connects thymol EC50 (y-intercept) and eugenol EC50 (x-intercept) represents the expected growth isocline if the compounds have additive effects. Concave isoclines indicate synergistic effects (see Figure 1). The plot area is color-coded according to the predicted growth at any given vector of concentrations, with red indicating highest growth, and blue indicating least growth. Growth was measured as the 5-day growth integral, i.e., area under the curve of net OD vs. time. Within each panel, growth is scaled relative to growth in the absence of phytochemicals, such that maximal growth is always equal to 1. For absolute growth measurements, refer to Figure 4D: Maximum growth. Each experiment included *n* = 216 samples (6 replicate wells at each of 36 combinations of eugenol and thymol). Rd.: round. ppm: parts per million.
Figure 3-3. Combinatorial effects of eugenol and thymol against four *C. bombi* strains, assayed in parallel. As in Figure 2, the solid line shows the isocline of 50% growth inhibition. The dashed line that connects thymol EC50 (y-intercept) and eugenol EC50 (x-intercept) represents the expected growth isocline if the compounds have additive effects. Concave isoclines indicate synergistic effects (see Figure 1). Tests of each strain included n = 72 samples (2 replicate wells at each of 36 combinations of eugenol and thymol). ppm: parts per million.
Figure 3-4. Universal Response Surface Analysis model parameters across all experiments. The y-axis shows the round of the experiment. The first six experiments were conducted on strains tested singly in series, with three experiments on strain IL13.2 (“Rounds 1-3”) and one experiment each on strains VT1, C1.1, and S08. The final four experiments were conducted on all four strains tested in parallel, i.e., strains were tested concurrently. The vertical line divides the experiments conducted in series from the experiments conducted in parallel. The x-axis shows model estimates and 95% CI’s for four parameters: (A) $s$ is the interaction parameter from Equation (3), which indicates the relative potency of each compound in mixture versus in isolation. Values $s>1$ indicate synergy. The null hypothesis of additivity is indicated by the dashed green line. (B) Eugenol and (C) Thymol EC50’s are the individual phytochemical concentrations necessary for 50% growth inhibition. (D) Max. growth shows growth in the absence of phytochemicals, i.e., at a concentration of 0 ppm. The legend shows color-coding of points and confidence intervals by strain. Where no error bars are shown for maximum growth, this parameter was fixed as the average of growth in control samples exposed to 0 ppm phytochemicals.
BIBLIOGRAPHY


158. Fox, J. & Weisberg, S. An R companion to applied regression. (Sage, 2011).


