Nectar and Pollen Phytochemicals Stimulate Honey Bee (Hymenoptera: Apidae) Immunity to Viral Infection

Evan C. Palmer-Young,1,2 Cansu O. Tozkar,3,4 Ryan S. Schwarz,3,5 Yanping Chen,3 Rebecca E. Irwin,6 Lynn S. Adler,1 and Jay D. Evans3

1Department of Biology, University of Massachusetts, Amherst, MA (ecp52@cornell.edu; lsadler@ent.umass.edu), 2Corresponding author, e-mail: ecp52@cornell.edu, 3Bee Research Lab, Agricultural Research Service, US Department of Agriculture, Beltsville, MD (tozkar@gmail.com; rsschwarz@fortlewis.edu; judy.chen@ars.usda.gov; jay.evans@ars.usda.gov), 4Current address: Department of Biology, University of Yuzuncu Yil, Van, Turkey, 5Current address: Department of Biology, Fort Lewis College, Durango, CO, and 6Department of Applied Ecology, North Carolina State University, Raleigh, NC 27695 (reirwin@ncsu.edu)

Subject Editor: David Tarpy

Received 19 March 2017; Editorial decision 6 June 2017

Abstract

Parasites and pathogens are implicated in honey bee colony losses, and honey bees may also spread infection to wild pollinators. Bees consume nectar and pollen, which contain phytochemicals that can positively or negatively affect pollinator health. Certain phytochemicals can reduce parasite loads in humans and other animals. Understanding how phytochemicals affect honey bee infection and survival could help identify optimal forage sources and phytochemical treatments to ameliorate disease. We fed honey bees seven dietary phytochemicals to evaluate whether phytochemical consumption would treat preexisting infection in mature bees, or mitigate infection in young bees either inside or outside of their colonies. Phytochemicals were generally well-tolerated at levels documented in nectar, honey, and pollen, although clove oil and thymol increased mortality at high doses. Six of seven tested phytochemicals significantly increased antimicrobial peptide expression by 12.9 to 61-fold in older bees after 7 d consumption. Short-term (<24 h) phytochemical consumption reduced levels of Deformed wing virus (DWV) up to 500-fold in young bees released into field colonies. However, with the exception of high-dose clove oil, our phytochemical treatments did not alter infection with Lotmaria passim or Nosema ceranae. Phytochemicals also lacked antiviral effects for pollen-deprived bees reared outside the colony. Our results suggest that phytochemicals have potential therapeutic value for honey bees infected with DWV. Short-term phytochemical consumption may be sufficient to confer benefits against infection. Phytochemical concentrations that reduced disease were comparable with naturally occurring floral concentrations, suggesting that flowers could serve as seasonally varied, serially consumed pollinator medicines.

Key words: immune priming, plant secondary metabolite, medicinal plant, tritrophic interaction, colony collapse disorder

Honey bees (Apis mellifera L.) are key contributors to pollination of crops and wild plants worldwide. Honey bees provide an estimated >US$150B per annum, or nearly 10% of agricultural production (Gallai et al. 2009). However, honey bees as well as wild pollinators are challenged by abiotic and biotic stress factors that cause queen and worker mortality and impact colony health (Goulson et al. 2015). In the United States, 30% of honey bee hives are lost each winter and nearly 50% of hives are replaced annually, both historically high averages that put intense economic pressures on beekeepers and growers (Steinhauer et al. 2014). These losses have been attributed to a number of factors that include pesticides, pathogens, and land use changes that reduce the quality of forage (vanEngelsdorp et al. 2009). To preserve the services that honey bees provide, it is essential to determine how bees survive in the face of stress and to develop strategies that protect honey bee health.

Honey bees are threatened by a variety of infectious diseases that have been correlated with colony collapse (vanEngelsdorp and Meixner 2010, Cormann et al. 2012). The transmission of many diseases has been aided by the immunosuppressant ectoparasite and disease vector Varroa destructor Anderson and Trueman (Arachnida: Acari: Varroidae) (Guzmán-Novoa et al. 2010). In addition to bacterial and fungal pathogens, key threats to honey bees include viruses, such as Deformed wing virus (DWV; de Miranda and Genersch 2010), the microsporidia Nosema apis and Nosema ceranae (Higes et al. 2008), and trypanosomatids (Cormann et al. 2012, Schwarz et al. 2015). Besides infection of honey bees, honey bee
pathogens can also be transmitted to and elevate mortality of wild bee species such as bumble bees (Graystock et al. 2013, Furst et al. 2014, Graystock et al. 2014), many species of which are endangered (Williams et al. 2009).

Nutritional factors can strongly influence immunity and infection outcome, not only in humans, but also in bees (Brunner et al. 2014, Conroy et al. 2016). Bee diets are comprised almost exclusively of plant products (pollen and nectar) that supply sugars, lipids, and amino acids as well as vitamins and minerals (Yang et al. 2013). In addition to these macro- and micronutrients, nectar and pollen are rich in diverse phytochemicals that include alkaloids, phenolics, and terpenoids (Dobson 1988, Adler 2001, Heil 2011, Negri et al. 2011). In plants, many of these chemicals function in defense against both pathogens (Bennett and Wallsgrove 1994, Huang et al. 2012) and herbivorous insects (Agrawal 2011). Infected insects may employ antimicrobial phytochemicals against their own diseases (Singer et al. 2009, de Roode et al. 2013, Erler and Moritz 2015), although medicinal effects may be offset by mortality at high phytochemical concentrations (Detzel and Wink 1993, Singaravelan et al. 2006, Ebert et al. 2007, Arnold et al. 2014).

To gauge the potential for phytochemicals in bee diets to ameliorate trypanosomatid (Lotmaria passim), microsporidian (Nosema ceranae), and viral (DWV) infection, and to assess potential risks of increased mortality, we exposed honey bees to seven different plant nectar compounds (Table 1) under incubator and field conditions. Many of the tested phytochemicals have been shown to affect human and insect pathogens, but also to have dose-dependent toxicity to bees (Table 2). We used naturally occurring concentrations to gain a better understanding of how floral phytochemicals affect infection, but also tested higher concentrations of several

### Table 1. List of phytochemicals, concentrations found in nectar, honey, and pollen, and concentrations used in experiments

<table>
<thead>
<tr>
<th>Compound (class)</th>
<th>Natural concn</th>
<th>Sample</th>
<th>Reference</th>
<th>Experimental concn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdalin (cyanogenic glycoside)</td>
<td>2.9–3.17 ppm</td>
<td><em>Prunus dulcis</em> Miller</td>
<td>(London-Shafir et al. 2003)</td>
<td>50 ppm</td>
</tr>
<tr>
<td></td>
<td>4.9–6.7 ppm</td>
<td><em>P. dulcis</em> nectar</td>
<td>(London-Shafir et al. 2003)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,889 ppm</td>
<td><em>P. dulcis</em> pollen</td>
<td>(London-Shafir et al. 2003)</td>
<td></td>
</tr>
<tr>
<td>Anabasine (alkaloid)</td>
<td>5 ppm</td>
<td><em>Nicotiana glauca</em> Graham</td>
<td>(Tadmor-Melamed et al. 2004)</td>
<td>5 ppm (Cup: Mature Bees; Cup: Young Bees; Colony: Young Bees, with inoculation)</td>
</tr>
<tr>
<td></td>
<td>0–1.5 ppm</td>
<td><em>Nicotiana spp.</em> nectar</td>
<td>(Adler et al. 2012)</td>
<td>50 ppm (Colony: Young Bees, without inoculation)</td>
</tr>
<tr>
<td>Aucubin (iridoid glycoside)</td>
<td>1,100 ppm</td>
<td><em>Chelone glabra</em> L.</td>
<td>(Richardson et al. 2016)</td>
<td>1,600 ppm</td>
</tr>
<tr>
<td></td>
<td>100 ppm</td>
<td><em>C. glabra</em> pollen</td>
<td>(Richardson et al. 2016)</td>
<td></td>
</tr>
<tr>
<td>Catalpol (iridoid glycoside)</td>
<td>230 ppm</td>
<td><em>C. glabra</em> nectar</td>
<td>(Richardson et al. 2016)</td>
<td>1,400 ppm</td>
</tr>
<tr>
<td></td>
<td>3,600 ppm</td>
<td><em>Chelone glabra</em> pollen</td>
<td>(Richardson et al. 2016)</td>
<td></td>
</tr>
<tr>
<td>Nicotine (alkaloid)</td>
<td>0–5.4 ppm</td>
<td><em>Nicotiana spp.</em> nectar</td>
<td>(Adler et al. 2012)</td>
<td>2 ppm (Cup: Mature Bees; Cup: Young Bees; Colony: Young Bees, with inoculation)</td>
</tr>
<tr>
<td></td>
<td>1.6–16 ppm</td>
<td><em>N. attenuata</em> Torrey ex S. Watson (unresolved)</td>
<td>(Kessler et al. 2012)</td>
<td>20 ppm (Colony: Young Bees)</td>
</tr>
<tr>
<td>Thymol (monoterpenic alcohol)</td>
<td>0.2 ppm</td>
<td><em>Tilia spp.</em> honey</td>
<td>(Guyot et al. 1998)</td>
<td>0.2 ppm (Cup: Mature Bees); 0.16 ppm (Cup: Young Bees; Colony: Young Bees, with inoculation)</td>
</tr>
<tr>
<td></td>
<td>0.27 ppm</td>
<td><em>Thymus spp.</em> honey</td>
<td>(Nozal et al. 2002)</td>
<td>16 ppm (Colony: Young Bees, without inoculation)</td>
</tr>
<tr>
<td></td>
<td>0–10 ppm</td>
<td><em>Thymus vulgaris</em> L.</td>
<td>(Palmer-Young et al. 2016)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5–2.65 ppm</td>
<td>Honey from thymol-fumigated hives</td>
<td>(Nozal et al. 2002)</td>
<td></td>
</tr>
</tbody>
</table>

Clove oil (5,000 ppm for Cup: Mature Bees; 20,000 ppm for Colony: Young Bees (Without Inoculation)) and the Nosema treatment fumagillin (25 ppm for Colony: Young Bees (Without Inoculation)) were also tested, but are not shown here because concentrations were not based on naturally occurring levels in flowers. See Palmer-Young et al. (2016, 2017b) for summaries of floral and foliar concentrations of eugenol, the dominant compound in clove oil. The recommended fumagillin concentration in sugar water fed to managed colonies is 25 ppm (Huang et al. 2013).
Table 2. Effective concentrations of phytochemicals for bee pathogens and their viral or trypanosomatid phylogenetic relatives, and for bees

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose</th>
<th>Target</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdalin</td>
<td>50 ppm</td>
<td>Bombus impatiens Cresson 1863 (Hymenoptera: Apidae)</td>
<td>Nonsignificant reduction in infection intensity</td>
<td>(Richardson et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>1,600 ppm</td>
<td>Apis mellifera</td>
<td>8 d LD50</td>
<td>(Ebert et al. 2007)</td>
</tr>
<tr>
<td>Anabasine</td>
<td>5–20 ppm</td>
<td>Crithidia in Bombus impatiens</td>
<td>Significant reduction in infection vs. phytochemical-free nectar (4 of 5 published trials)</td>
<td>(Anthony et al. 2015, Richardson et al. 2015, Thorburn et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>628–2160 ppm</td>
<td>Crithidia bombi (in vitro)</td>
<td>5 d IC50</td>
<td>(Palmer-Young et al. 2016)</td>
</tr>
<tr>
<td>Aucubin</td>
<td>1,600 ppm</td>
<td>Crithidia in Bombus impatiens</td>
<td>Nonsignificant reduction in infection intensity</td>
<td>(Richardson et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>148 ppm</td>
<td>Trypanosoma brucei Hepatitis B virus in vitro</td>
<td>IC50</td>
<td>(Tasdemir et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>1,417 ppm</td>
<td>Trypanosoma brucei</td>
<td>Significant reduction in infection vs. phytochemical-free nectar</td>
<td>(Richardson et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>151 ppm</td>
<td></td>
<td></td>
<td>(Tasdemir et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>19.7–23.5 ppm eugenol&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Crithidia bombi (in vitro)</td>
<td>5 d IC50</td>
<td>(Palmer-Young et al. 2016)</td>
</tr>
<tr>
<td>Clove oil</td>
<td>57.5–99.5 ppm</td>
<td>Trypanosoma cruzi Hepatitis simplex virus (8 of 12 strains inhibited)</td>
<td>IC50</td>
<td>(Santoro et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>50–74 ppm (dried ethanol extract)</td>
<td>Herpes simplex virus</td>
<td>IC50</td>
<td>(Tragoolpua and Jatisatienr 2007)</td>
</tr>
<tr>
<td></td>
<td>16.2–25.6 ppm eugenol&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Herpes simplex virus</td>
<td>IC50</td>
<td>(Benencia and Courrèges 2000)</td>
</tr>
<tr>
<td></td>
<td>12,300 ppm</td>
<td>Varroa destructor A. mellifera workers</td>
<td>24 h LD50</td>
<td>(Maggi et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>7,800 ppm</td>
<td>A. mellifera workers</td>
<td>8 d LD50</td>
<td>(Ebert et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>25 ppm</td>
<td>Nosema apis and N. ceranae infection in A. mellifera</td>
<td>Eliminated infection</td>
<td>(Huang et al. 2013)</td>
</tr>
<tr>
<td>Nicotine</td>
<td>2–2.5 ppm</td>
<td>Crithidia in Bombus impatiens</td>
<td>Significant reduction in infection vs. phytochemical-free nectar (3 of 5 published trials)</td>
<td>(Baracchi et al. 2015, Biller et al. 2015, Richardson et al. 2015, Thorburn et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>&gt;1,000 ppm</td>
<td>Crithidia bombi (in vitro)</td>
<td>5 d IC50</td>
<td>(Palmer-Young et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>2,000 ppm</td>
<td>A. mellifera workers</td>
<td>2 d LD50</td>
<td>(Detzel and Wink 1993)</td>
</tr>
<tr>
<td></td>
<td>300 ppm</td>
<td>A. mellifera workers</td>
<td>50% reduced consumption relative to phytochemical-free solution</td>
<td>(Detzel and Wink 1993)</td>
</tr>
<tr>
<td></td>
<td>50 ppm</td>
<td>A. mellifera larvae</td>
<td>&gt;50% reduction in survival</td>
<td>(Singaravelan et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>~30 ppm</td>
<td>A. mellifera workers</td>
<td>50% reduction in nectar consumption</td>
<td>(Köhler et al. 2012a)</td>
</tr>
<tr>
<td>Thymol</td>
<td>0.2 ppm</td>
<td>Crithidia in Bombus impatiens</td>
<td>Significant reduction in infection vs. phytochemical-free nectar (1 of 3 published trials)</td>
<td>(Biller et al. 2015, Richardson et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>4.5–22 ppm</td>
<td>Crithidia bombi (in vitro)</td>
<td>5 d IC50</td>
<td>(Palmer-Young et al. 2016)</td>
</tr>
<tr>
<td></td>
<td>100 ppm</td>
<td>Nosema ceranae in A. mellifera</td>
<td>56% reduction in spore counts 2.5 d p.i.</td>
<td>(Costa et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>30 ppm</td>
<td>Herpes simplex virus Varroa destructor</td>
<td>IC50</td>
<td>(Astani et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>250,000 ppm (as fumigant)</td>
<td>Varroa destructor</td>
<td>76% mite mortality</td>
<td>(Giacomelli et al. 2015)</td>
</tr>
<tr>
<td></td>
<td>&gt;1,000 ppm</td>
<td>A. mellifera workers</td>
<td>8 d LD50 (43% mortality at 1,000 ppm)</td>
<td>(Ebert et al. 2007)</td>
</tr>
<tr>
<td></td>
<td>700 ppm</td>
<td>A. mellifera larvae</td>
<td>2 d LD50</td>
<td>(Charpentier et al. 2014)</td>
</tr>
<tr>
<td></td>
<td>500 ppm</td>
<td>A. mellifera larvae</td>
<td>Reduced growth and survival</td>
<td>(Charpentier et al. 2014)</td>
</tr>
<tr>
<td></td>
<td>100 ppm (thyme oil&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>A. mellifera workers</td>
<td>50% reduced consumption relative to phytochemical-free solution</td>
<td>(Detzel and Wink 1993)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Eugenol is dominant component of clove oil (86.7%; Maggi et al. 2010).

<sup>b</sup> Thyme oil contained 65.3% thymol (Damiani et al. 2009).
frames of their colonies and reared them in cups (Evans et al. 2009). In "Cup: Mature bees" experiments, we captured worker bees from the outer preexisting infection or affected immune gene expression ("Cups: "Backyard" apiary at the USDA-ARS Beltsville Area Research insects) or fed phytochemicals for 24 h before release to the colony ("Without Inoculation"). Treated bees were marked with distinctive colors (using paint pens) and allowed to live in their natal colonies for 5–7 d, at which time the colonies were opened, and marked bees were collected by aspiration and then frozen for analysis of infection. Each experiment used bees from a single colony, except for the experiment with mature bees, which included bees from four colonies. Experiments without inoculations tested 6–7 phytochemicals in parallel, but for feasibility, only two phytochemicals (amygdalin and thymol) were tested in experiments that involved inoculations.

Phytochemical Treatments

Phytochemicals (listed in Table 1) were chosen from among those documented in nectar, with inclusion of those tested previously for medicinal activity in honey bees or other bee species (Costa et al. 2010, Baracchi et al. 2015, Richardson et al. 2015). We were particularly interested in chemicals to which bees are often exposed. These include amygdalin, which is found in almond nectar and pollen (London-Shafir et al. 2003), and thymol, which is used to treat mite infestation (Gregorc and Planinc 2005). Clove oil was tested owing to evidence that eugenol, a primary constituent (Maggi et al. 2010), is antiparasitic against both viruses (Benencia and Courrèges 2000) and trypanosomatids (Palmer-Young et al. 2016, 2017b), as well as its historic use as an antiparasitic treatment for bees (Imdorf et al. 1999, Ebert et al. 2007, Maggi et al. 2010).

Parasites

We tested the effects of phytochemicals on infection with three parasites that have been found in honey bees and, in some studies, correlated with colony loss. *Lotmaria passim* is a hindgut trypanosomatid parasite recently differentiated from *Crithidia mellificae* (Schwarz et al. 2015). Infection with *L. passim* was significantly correlated with colony loss in Belgium (Ravoet et al. 2013) and the United States (Cormann et al. 2012), and can reach >75% prevalence during the growing season in managed bee populations (Runcell et al. 2011). *Lotmaria passim* is a relative of the bumble bee pathogen *Crithidia bombi*, which has been the subject of several studies that tested for medicinal effects of phytochemicals in bumble bees (Manson et al. 2010, Baracchi et al. 2015, Biller et al. 2015, Richardson et al. 2015, Thorburn et al. 2015).

*Nosema ceranae* is a microsporidian intracellular parasite that infects midgut epithelial cells, causing cell lysis that appears to compromise nutrient absorption (Mayack and Naug 2009). Over the past three decades, *N. ceranae* has rapidly spread from Asia throughout the world (Klee et al. 2007) and replaced *N. apis* in all but high-latitude regions (Natsopoulou et al. 2015). Infection prevalence is now extremely high, with up to 95% infection of bees in Hawaii (Martin et al. 2013) and >90% summertime infection of commercial bees in the continental United States (Runcell et al. 2011). *Nosema ceranae* can suppress bee immunity (Antúnez et al. 2009), and has been implicated in colony collapse in Spain (Higes et al. 2008), although not in the United States (Cormann et al. 2012) or other countries (Stevanovic et al. 2010, 2013). *Nosema ceranae* can also cross-infect and increase mortality in bumble bees (Graystock et al. 2013).

Deformed wing virus is one of the many viruses that infect honey bees (de Miranda and Genersch 2010). Although many infections are asymptomatic, virulence is increased by *Varroa* mite-mediated transmission of the virus to the developing pupa (Ryabov et al. 2014), which results in characteristic wing deformities and death.
soon after emergence (de Miranda and Genersch 2010). Deformed wing virus infection was strongly correlated with U.S. colony collapse (Corman et al. 2012); DWV from honey bees can also increase mortality in bumble bees (Fürst et al. 2014). Unlike *N. ceranae*, which can be controlled to some extent by fumagillin (Huang et al. 2013), no direct treatments of DWV are currently available.

**Inoculations**

Trypanosomatids

Lotmaria passim cells representing the type collection strain “BRL” (ATCC PRA-422) were cultured in axenic media from an isolate purified in Beltsville, MD (Schwarz et al. 2013). Cryogenically preserved cultures were thawed 3 d prior to inoculation and grown in DS2 medium supplemented with 5% (v/v) fetal bovine serum (Cellgro, Manassas, VA), 100 IU ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin. On the day of inoculation, cells were pelleted by centrifugation (3,500 rpm, 5 min, 4°C), growth medium aspirated, and the cell pellet resuspended in 1× PBS (1 ml). Cell density was determined with a hemocytometer by counting cells at 400× magnification. Cells were initially diluted to 10⁻⁰ final concentration in 1:10 w/v sugar water (Schwarz and Evans 2013), because *Lotmaria* spores can lose their viability at high sugar concentrations (Cisarovsky and Schmid-Hempel 2014). Immediately before the inoculation, cells were diluted at 1,200 cells ml⁻¹ in 1:2.5 w/v sugar:water with or without phytochemicals. For inoculation, 6,000 *Lotmaria* spores were given in a 5-μl droplet of sugar water from a micropipette tip. Bees that did not consume the droplet were removed from the experiment.

**How Does Continuous Phytochemical Exposure Affect Gene Expression and Preexisting Infection (Cup: Mature Bees)?**

Mature bees were removed from their colonies and placed in groups of ~30 bees in 340-ml polystyrene cups with phytochemical treatments in sterile 1:1 w/v sugar water. The treatments were administered via a disposable polystyrene Pasteur pipette, inserted into a hole in the cup’s lid. Cups were incubated at 34°C and 55% relative humidity (RH). Bees were not fed pollen; phytochemical-treated sugar water was the only food available during the 7-d trial. Each phytochemical treatment was represented by eight cups. Cups were checked daily for new deaths. New deaths were recorded, but dead bees were not removed from the cups. After 7 d, bees were frozen for assessment of infection intensity and gene expression (see “Methods: Molecular analyses”). Groups of 8–10 bees from the same cup were pooled for RNA extraction.

**How Does a Single Phytochemical Feeding Affect Infection of Newly Emerged Bees Under Incubator Conditions (Cup: Young Bees)?**

Newly emerged workers were collected by removal of brood frames from the colony, followed by overnight incubation in an incubator (34°C, 55% RH). Workers that had emerged overnight were collected the following morning for inoculation with parasites. Three replicate cups of ~30 bees each were used for each infection and phytochemical treatment.

**How Does Short-Term Phytochemical Feeding Affect Infection of Newly Emerged Bees in the Colony (Colony: Young Bees)?**

With Inoculation (Single Phytochemical Feeding)

Newly emerged workers were inoculated on the first day post-emergence with *L. passim*, *N. ceranae*, or DWV, either with or without phytochemicals, as described in Cup: Young bees: Inoculations. After inoculation, bees were marked with distinctive colors that corresponded to infection and phytochemical treatments, and then placed in their original colony. Thirty bees per treatment were marked. Bees were collected after 7 d in the colony. Hives were opened and each frame was systematically removed from the hive. Frames were scanned methodically and all bees were collected with
Inferior intensity owing to low sample size, a consequence of high oil (7 (Anabasine), 8 (Catalpol), 6 (Fumagillin), and 9 (Nicotine). Clove RPS5), sample sizes for analyses of infection were successful (defined as successful amplification of the marker gene "Molecular analyses"). Because not all RNA extractions were successful (defined as successful amplification of the marker gene "Molecular analyses"). Between 10 and 12 bees per treatment, except for thymol (n = 4 survivors), were tested individually for infection (see “Methods: Molecular analyses”).

Without Inoculation (24-h Phytochemical Feeding)

Newly emerged bees were placed in groups of ~30 bees in 340-ml polystyrene cups and fed phytochemical treatments in sterile 1:2.5 w/v sugar water for 24 h (one cup per phytochemical treatment). After 24-h phytochemical consumption, bees were painted and released directly to their colonies. Bees were collected after 5 d in the colony, i.e., 6-d postemergence, as described in the preceding paragraph. Between 10 and 12 bees per treatment, except for thymol (n = 4 surviving bees) and clove oil (n = 0 survivors), were tested individually for infection and gene expression (see “Methods: Molecular analyses”). Because not all RNA extractions were successful (defined as successful amplification of the marker gene RPS5), sample sizes for analyses of infection were n = 10 (Control), 7 (Anabasine), 8 (Catalpol), 6 (Fumagillin), and 9 (Nicotine). Clove oil (n = 0) and thymol (n = 2) were excluded from the analysis of infection intensity owing to low sample size, a consequence of high mortality in these treatments.

Molecular Analyses of Infection Intensity and Gene Expression

Bees were stored at ~80 °C prior to RNA extraction. RNA was extracted from frozen groups of bees (Cup: Mature Bees) or individual bees (all other experiments) using Trizol reagent (Life Technologies, Carlsbad, CA) as described in the BeeBook protocols (Evans et al. 2013). Frozen bee abdomens were homogenized with a sterile plastic pestle in a 1.5-ml microcentrifuge tube with 500 μl Trizol acid-phenol reagent. An additional 500 μl Trizol was added; the tube was inverted several times to mix the contents, and then incubated for 3–5 min at room temperature to dissociate nucleotides from proteins. After addition of 200 μl chloroform, samples were shaken by hand (1 min), incubated at room temperature (3 min), and then centrifuged (1,430 rcf, 10 min, 4 °C). The aqueous phase of the tube was transferred to a clean tube for RNA precipitation, which was accomplished by addition of 500 μl isopropanol followed by incubation (10 min, room temperature) and centrifugation (1,710 rcf, 15 min, 4 °C). The supernatant was decanted and the pellet washed with 1 ml 75% ethanol. RNA was dissolved in 50 μl RNAse-free water and stored at ~80 °C. RNA quantity and purity was measured on a Nanodrop8000 (Thermo, Wilmington, DE) following manufacturer’s protocol.

cDNA Synthesis

Synthesis of cDNA was performed as previously described (vanEngelsdorp et al. 2009). The RNA was first treated with DNase in an 11-μl reaction that consisted of 8 μl total RNA, 2U DNase1 with appropriate 1× buffer (Ambion, Foster City, CA), 20 U RNaseout (Invitrogen, Carlsbad, CA), 50 ng poly-dT nucleotide mix (12–18 nt), 100 ng random heptamers, and 2 mM dNTPs. The reaction was incubated for 37 °C for 1 h, followed by 75 °C for 10 min, then cooled on ice and briefly centrifuged. Reverse transcriptase was used to synthesize cDNA from each of the RNA samples by heating the reactions to 42 °C for 2 min and adding Superscript II with appropriate buffer and DTT (Invitrogen). The plate was incubated at 42 °C for 10 min, then 70 °C for 15 min. The cDNA was diluted 1:5 with ddH2O and used as the template for quantitative real-time PCR.

Quantitative Real-Time PCR of cDNA

Quantitative real-time PCR analyses were performed in 96-well Microseal PCR plates as described previously (Schwarz et al. 2016). Individual samples were amplified in a 20-μl reaction mix that consisted of 1 μl cDNA template, 0.4 μM each primer pair, and 10 μl SsoAdvanced Universal SYBR Green Supermix (Biorad, Hercules, CA). The following PCR conditions were run on an iCyclerCFX96 Real-Time System (Biorad): 95 °C for 2 min, then 50 cycles of 95 °C for 5 s, 60 °C for 30 s. Melt curve analysis between 69 °C and 95 °C confirmed PCR product identity and integrity. Samples that did not reach the threshold fluorescence level after 50 cycles were recorded as having a CT of 50 cycles.

Primers targeted three pathogens (Lotmaria passim, Nosema ceranae, and DWV) and genes encoding the honey bee protein vitellogenin and the antimicrobial peptide hymenoptaecin. Honey bee Ribosomal protein S5 (RPS5) was used as a normalizer gene. This gene has been shown to be stable across development and infection status in honey bees (Jefferson et al. 2013, Schwarz and Evans 2013). Primer sequences are given in Table 3.

Statistical Analysis

Expression levels were normalized relative to honey bee Ribosomal protein S5 (RPS5) by subtraction of the CT score (i.e., number of cycles to reach threshold fluorescence) of the target gene from the CT score of the reference gene:

$$\Delta CT = CT \text{ reference gene} - CT \text{ target gene}$$

(1)

The ΔCT score is on a log base 2 scale. In other words, a ΔCT score of 0 corresponds to equal amplification of the target and reference genes. A ΔCT score of −1 indicates half as many transcripts of the target gene as of the reference gene, and a ΔCT score of 1 indicates twice as many target as reference transcripts.

Table 3. Primers used for qPCR analysis: Target name, primer sequences, and references

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lotmaria passim*</td>
<td>GTGCAGTTCGCGGGAAGTCCTT</td>
<td>CTGAGCTGCTGCCTAGGAGCAC</td>
<td>(Teixeira et al. 2008)</td>
</tr>
<tr>
<td>Nosema ceranae</td>
<td>CTTAAAGTTGGATAGAAGATGTT</td>
<td>GACTTTAGTGCGGGTCCTT</td>
<td>(Schwarz and Evans 2013)</td>
</tr>
<tr>
<td>DWV</td>
<td>GAGATTGAAGGCGCATGAAA</td>
<td>TGAATTCAGTGTCGCCCCATA</td>
<td>This study</td>
</tr>
<tr>
<td>Hymenoptaecin</td>
<td>CTCTTTGTGCGTGGTGCTA</td>
<td>GCGTCTCCTGTCATTCCTAA</td>
<td>(Evans 2006)</td>
</tr>
<tr>
<td>Vitellogenin</td>
<td>AGTTCGCGCGGCGGACGAC</td>
<td>TCCCCCTCCACCGAGGTCC</td>
<td>(Boncristiani et al. 2012)</td>
</tr>
<tr>
<td>Ribosomal protein S5</td>
<td>AATTATTTGTCGCTGGAAATTG</td>
<td>TAACGTCCAGCAGAATGTTG</td>
<td>(Evans 2006)</td>
</tr>
</tbody>
</table>

* Generic primer for Crithidia and Lotmaria trypanosomatids.
In the Results, we express effects of phytochemical treatments in ΔACT units, where

\[ ΔACT = ΔCT_{\text{control group}} - ΔCT_{\text{treated group}} \]  

(2)

Like the ΔCT score, the ΔACT score is on a log base 2 scale, such that a ΔACT score of −1 indicates half as many transcripts of the target in the treated group as compared with the control group.

Analysis was performed in R v3.2.2 (R Core Team 2014). ACT scores for qPCR analyses were used as the response variables in analyses of infection intensity and host gene expression. Phytochemical treatment was used as the predictor variable. Bee colony of origin and replicate cup were included as random effects in the “Cap: Mature Bees” experiment, which was the only experiment that used bees from multiple colonies. Because only two measurements were collected per colony and phytochemical treatment combination, we did not test for variations in the effects of phytochemicals across colonies. Replica cup was included as a random effect in “Cap: Young Bees.” A separate linear model was fit for each experiment (Bates et al. 2015). For experiments that involved inoculations, we first pooled bees across all phytochemical treatments to test whether inoculation significantly increased levels of infection with the target parasite. Only inoculated bees were used for analyses of L. passim and N. ceranae infection in “Cap: Young Bees” and “ Colony: Young Bees (With inoculation)” experiments. In these experiments, oral inoculation treatments significantly increased levels of these parasites (ΔACT > 10, \( P < 0.001 \)), which were rare in uninoculated bees. In contrast, inoculation with DWV did not significantly affect DWV infection in either “Cap: Young Bees” or “ Colony: Young Bees (With inoculation)” (\( P > 0.10 \) for each). This result is consistent with previous experiments, where injection of pupae resulted in symptomatic infection but feeding of DWV-contaminated sucrose or food did not (de Miranda and Genersch 2010, Mocqué et al. 2011, Ryabov et al. 2014, Doublet et al. 2015). Therefore, within each phytochemical treatment, bees from all inoculation treatments (sham-inoculated control, L. passim, N. ceranae, and DWV) were pooled for DWV analyses. Posthoc tests were used to determine differences in infection intensity and gene expression between individual phytochemical treatments and the phytochemical-free controls (Lenth 2016). Within each model, \( P \)-values were corrected for multiple tests by Duncan’s method (Lenth 2016). For experiments that showed significant effects of phytochemicals, we also checked whether phytochemical treatments altered expression of the normalizer gene RPS5, which could lead to systematic errors in ΔCT scores. No effects of phytochemicals on the normalizer gene were found (\( P > 0.14 \) for all).

Effects of phytochemical treatments on mortality were assessed with a Cox proportional hazards mixed-effects model (“Cap: Mature Bees”; mortality checked daily) or binomial model (“Colony: Young Bees (Without inoculation)”; endpoint mortality checked after 6 d). In the Cox proportional hazards model (Therneau 2015), death hazard rate was used as the response variable, phytochemical treatment was a fixed predictor, and replicate cup and colony of origin were included as random effects. In the binomial model (Kosmidis 2013), proportion dead was used as the response variable, with phytochemical treatment as a fixed predictor. Raw data can be found in Supp. Data 1 (online only).

Results

Cup: Mature Bees

In bees removed from their colonies and fed phytochemicals for 7 d, consumption of each phytochemical significantly upregulated transcription of the antimicrobial peptide gene hymenoptaecin by 3.69–5.93 ΔACT units relative to a control diet of sucrose solution without phytochemicals (Fig. 2A). This change corresponds to a 12.9 to 61.0-fold increase in expression on a linear scale. This increase was statistically significant for all phytochemicals except amygdalin (\( t_{27} = 2.68, P_{\text{adjusted}} = 0.055 \)).

Clove oil was the only treatment that affected L. passim infection (\( \Delta CT = -10.94 \pm 3.31 \text{ SE}, t_{50} = -3.31, P = 0.01; \) Fig. 2A), which corresponds to a 1600-fold change. However, this reduction may partly reflect declining bee health, which could have rendered hosts unfit to support parasites (see following paragraph). Phytochemical treatments did not alter N. ceranae (\( \chi^2 = 5.27, df = 7, P = 0.63 \)) or DWV infection (\( \chi^2 = 6.26, df = 7, P = 0.51 \)). However, naturally occurring levels of both these parasites were low (phytochemical-free control group mean \( \Delta CT = -11.63 \pm 2.35 \text{ SE for } N. \ ceranae, \Delta CT = -10.12 \pm 3.01 \text{ SE for DWV} \)). Phytochemical treatments also did not affect expression of the storage protein vitellogenin (\( \chi^2 = 5.02, df = 7, P = 0.66 \)). However, we note that older bees generally have quite low levels of vitellogenin relative to younger bees (Amdam and Omholt 2002).

Most of the phytochemicals did not affect mortality, with one exception. The 5,000-ppm dose of clove oil substantially increased mortality (hazard ratio = 7.17 ± 1.21 SE, \( z = 10.1, P < 0.001 \); Fig. 2B). In three of the eight replicate cups of clove oil-fed bees, all 30 bees died within 7 d.

Cup: Young Bees

In young bees fed phytochemicals continuously for 10 d, the thymol (0.16 ppm) and amygdalin (50 ppm) diet treatments did not affect infection with any of the tested parasites (Fig. 3), including L. passim (\( \chi^2 = 1.9071, df = 2, P = 0.39 \)), N. ceranae (\( \chi^2 = 0.6638, df = 2, P = 0.72 \)), or DWV (\( \chi^2 = 4.37, df = 2, P = 0.11 \)).

Colony: Young Bees (With Inoculation)

In young bees fed a single dose of parasites with phytochemicals before being released to the colony for 7 d, thymol (0.16 ppm) and amygdalin (50 ppm) did not affect infection with L. passim (\( F_{2,33} = 0.04, P = 0.96 \)) or N. ceranae (\( F_{2,30} = 0.13, P = 0.88 \)). In contrast, the thymol treatment significantly reduced DWV infection (\( \Delta CT = -4.72 \pm 1.11 \text{ SE}, t_{12} = -4.27, P < 0.001 \); Fig. 4) This corresponds to a 26-fold decrease in infection level.

Colony: Young Bees (Without Inoculation)

A 24-h prefeeding with relatively high concentrations of phytochemicals tended to reduce levels of DWV relative to the phytochemical-free sucrose solution (Fig. 5A). This reduction was significant for bees fed anabasine (\( \Delta CT = -8.05 \pm 2.40 \text{ SE}, t_{56} = -3.36, P = 0.0084 \)), catalpol (\( \Delta CT = -7.81 \pm 2.31 \text{ SE}, t_{56} = -3.38, P = 0.0078 \)), and nicotine (\( \Delta CT = -6.01 \pm 2.23 \text{ SE}, t_{56} = -2.69, P = 0.045 \)), but not fumagillin (\( t_{56} = 0.64, P = 0.91 \)) or thymol (\( t_{56} = -0.26, P = 0.99 \)). Note that a ΔACT score of −8 corresponds to a 256-fold reduction in infection intensity. Infection with L. passim and N. ceranae was not analyzed owing to low prevalence (<10% of bees had detectable infection).

Both clove oil (log-odds ratio = 5.25 ± 1.51 SE, \( z = 3.48, P = 0.003 \)) and thymol (log-odds ratio = 2.91 ± 0.67 SE, \( z = 4.34, P < 0.001 \)) increased mortality relative to the control (Fig. 5B). All 30 clove oil-fed bees, and 26 of the 30 thymol-fed bees, died during their time in the colony. Because of this high mortality (RNA extraction was successful for only 2 thymol-fed bees), we could not test effects of clove oil and thymol on DWV infection.
Discussion

To better understand the ecological roles and possible applications of putatively medicinal nectar and pollen chemicals and the plants that contain them, we tested the effects of phytochemical consumption on honey bee immunity and mortality under lab and field conditions. Our experiments provide the first evidence that phytochemicals can have substantial antiviral effects in bees, and indicate that consumption of many different phytochemicals can augment immunity, even when relatively low concentrations are consumed for short periods of time.

We found that consumption of nearly all tested phytochemicals upregulated expression of the antimicrobial peptide gene hymenoptaecin (Fig. 2A). Antimicrobial peptides, induced by the IMD and Toll pathways (Casteels et al. 1993), are key components of invertebrate humoral immunity, with broad-spectrum inhibitory effects against bacteria, fungi, viruses, and protozoa (Klotman and Chang 2006, McMenamin et al. 2016). Our finding that phytochemicals modulate bee gene expression has precedence in prior experiments that showed upregulation of detoxification genes after consumption of p-coumaric acid, a common pollen phytochemical (Mao et al. 2017).

Fig. 2. Effects of phytochemical consumption on gene expression, infection, and mortality of mature bees reared in cups (“Cup: Mature bees”). (A) All phytochemicals except amygdalin significantly increased expression of the antimicrobial peptide gene hymenoptaecin. (B) Only clove oil reduced infection with the trypanosomatid *L. passim*. (C) Only clove oil altered mortality relative to the control. Points and error bars show means and 95% confidence intervals; triangles show raw data. Asterisks indicate significance of posthoc pairwise comparison between each treatment and the control: *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. Sample sizes: $n = 8$ cups of 30 bees each per phytochemical treatment. Abdomens of 8–10 bees per cup were pooled for genetic analyses, whereas survival data WERE recorded for individual bees.
2013); the same study also found upregulation of the antimicrobial peptides defensin and abaecin (Mao et al. 2013). Increased expression of antimicrobial peptides could prime bees against infection, and help to prevent the spread of infection through colonies and possibly even populations and communities. The importance of phytochemical-rich nectar and pollen for bee health was further supported by the higher survival and lower Nosema and black queen cell virus infection in bees that foraged on Brassica rapa.
plants, rather than being fed protein supplements (DeGrandi-Hoffman et al. 2015).

Given that six of the seven tested phytochemicals significantly increased hymenoptaecin expression in our study—including five tested at naturally occurring concentrations—the beneficial effects of phytochemicals on immunity appear to be general rather than unique to a few compounds. Moreover, these beneficial effects were from compounds from a range of chemical classes, including alkaloids, terpenoids, and iridoid glycosides. Future studies that test a greater diversity of phytochemicals would be able to clarify how general these effects may be, and whether there are any structural motifs or chemical properties that predict which compounds stimulate immunity. Similarly, field manipulations that assess gene expression on bees that forage in areas with various types of wildflowers or crop species could assess whether and how plant communities modulate immunity.

To test whether differences in immune gene expression would translate into reduced infection intensity, we first experimentally inoculated young bees with pathogens and reared them with constant access to phytochemicals (“Cup: Young Bees”). However, neither amygdalin nor thymol improved resistance to L. passim, N. ceranae, or DWV, which may reflect both the concentrations used and the experimental conditions. Although one study showed beneficial effects of 0.2 ppm thymol on infection with the Lotmaria relative Crithidia in bumble bees (Richardson et al. 2015), a subsequent study found no effect of this concentration on infection (Biller et al. 2015). Similarly, although Nosema infection was reduced by feeding on thymol-supplemented syrup (Costa et al. 2010), the 100 ppm concentration used in that study was 500 times greater than our 0.2 ppm dose. Our 0.2 ppm concentration is also lower than the dose required for direct inhibition of trypanosomatids and viruses (Table 2).

Besides our use of low phytochemical concentrations that precluded direct antimicrobial effects of phytochemicals, the lack of pollen fed to young bees in the Cup experiments may have blunted phytochemical-mediated increases in immune gene expression, which might otherwise have augmented resistance to infection. In bumble bees, dietary pollen is necessary for infection-induced gene expression (Brunner et al. 2014). In honey bees, young bees generally consume more pollen than old bees (Crailsheim 1990), and as in bumble bees, pollen had a positive effect on transcription of antimicrobial peptides (Alaux et al. 2011). Hence, pollen deprivation could constrain the plasticity of honey bee immune gene expression in response to stimulation by infection or phytochemical consumption.

Fig. 5. Effects of 24-h pre-exposure to phytochemicals on infection and mortality in bees that were subsequently released to their colonies and collected after 6 d (“Colony: Young Bees (Without Inoculation)”)(A) All phytochemicals tended to reduce infection with DWV; this reduction was significant for anabasine, catalpol, and nicotine. Sample sizes: n = 10 (Control), 7 (Anabasine), 8 (Catalpol), 6 (Fumagillin), 9 (Nicotine). (B) Thymol and clove oil significantly increased mortality relative to the control group. Sample sizes for survival: n = 30 bees per treatment. No infection data are shown for clove oil and thymol in A owing to low sample sizes (n = 0 for clove oil and n = 2 for thymol), a consequence of high mortality during the experiment. Points and error bars show means and 95% confidence intervals triangles show raw data. Asterisks indicate significance of posthoc pairwise comparison between each treatment and the control: *: P < 0.05, **: P < 0.01, ***: P < 0.001.

In young bees released to the colony, a single feeding of thymol at the time of inoculation (0.2 ppm; Fig. 4) or a 24-h feeding with anabasine (50 ppm), catalpol (1417 ppm), or nicotine (20 ppm) resulted in lower levels of infection with DWV 7 d posttreatment (Fig. 5). Because we did not sample bees prior to treatment, we cannot say whether the treatment reduced preexisting infection, or slowed the buildup of infection in adults. In the “Young Bees: With inoculation” experiment, antiviral effects are not likely to reflect direct effects of thymol on viruses for two reasons. First, the same inoculation procedure was used in the “Cup: Young Bees” experiments, in which there were no effects of thymol on infection with DWV. Second, the oral DWV inoculation itself did not significantly alter viral infection (P > 0.05), which suggests that most DWV infection was either preexisting or acquired while in the colony.

An alternative explanation for the observed antiviral effects would be that phytochemicals stimulated or primed expression of immune genes that augmented bee resistance to preexisting DWV or DWV to which they were subsequently exposed. This would be consistent with the findings of the “Cup: Mature bees” experiments. Thymol, catalpol, and nicotine are known immunomodulators used in traditional medicines. For example, thymol induced proliferation of lymphocytes, augmented the oxidative burst, and increased the lysosomal activity of human macrophages in vitro (Chauhan et al. 2014). Like mammals, insects have dedicated immune cells (hemocytes) that neutralize or engulf pathogens (Lemaître and Hoffmann 2007) and could be potentiated by thymol consumption. Catalpol has been used as an anti-inflammatory in Chinese medicine (Liu et al. 2009); it can protect kidney tissues from excess inflammation (Zhu et al. 2015) and neutralized hepatitis B virus in vitro (Mehrotra et al. 1990). The iridoid glycosides catalpol and aucubin also improved the melanization response (a proxy for immune function) in Junonia coenia larvae (Richards et al. 2012). Nicotine, too, can augment immunity through induction of human antimicrobial peptides (Nakamura et al. 2010), which can directly inactivate viral particles and block transcription of viral RNA (Klotman and Chang 2006). We can only speculate on exactly how this induction occurs.
It is possible that phytochemicals act as immune adjuvants that alter presentation of pathogen-associated molecular patterns to immune cells, a principal that has been exploited in medicine to augment immune responses to vaccination (Rajput et al. 2007, Garçon et al. 2011). Another possibility is that phytochemicals themselves comprise immunogenic stimuli (Kimata 2004). More research is needed to evaluate these potential mechanisms and their practical, ecological, and evolutionary significance in plant–insect–pathogen interactions.

Nicotine, and perhaps other phytochemicals, can strengthen the function of the intestine as a barrier to pathogens (McGilligan et al. 2007, Costantini et al. 2012) and augment mucous production in several taxa and insect life stages, conferring immunomodulatory functions in humans (Borchers et al. 1997). In several taxa and insect life stages, consumption of plant foods or pesticides upregulate production of the peritrophic matrix (Abedi and Brown 1961, Barbehenn 2001, Pymale et al. 2008), which increased resistance to viruses in mosquitoes and Lepidoptera (Houk et al. 1979, Cory and Hoover 2006, Pymale et al. 2008). This matrix is selectively produced in response to protein-rich bloodmeals in mosquitoes (Houk et al. 1979). If honey bees also require dietary protein to upregulate matrix production, this could explain why phytochemicals were not medicinal in the experiments with pollen-starved young bees in cups. The matrix could have particular importance for resistance to DWV, which replicates in the midgut epithelium (de Miranda and Genersch 2010). Future experiments should examine how the peritrophic matrix of both larval and adult bees responds to ingestion of different phytochemicals and nectar- and pollen-based versus synthetic diets.

Future studies should explore the immunoregulatory activities of additional plants and phytochemicals, including context dependency of these effects, and how they differentially affect resistance to different types of infection. Plants and compounds could be selected for testing based on importance and availability in the wild, or immunomodulatory functions in humans (Borchers et al. 1997). Host variation in responsiveness to phytochemicals should also be explored. Our experiment used bees from a single Italian breeding stock. If phytochemicals act mainly through induction of host immunity, inducibility of immune genes by phytochemicals might vary among bee genotypes, just as bumble bee genotypes vary in immune responsiveness to Crithidia bombi infection (Barrieau and Schmid-Hempel 2013). Finally, our results showed that phytochemical treatments were effective against DWV but generally not against N. ceranae or L. passum. We hypothesize that L. passum and N. ceranae, both obligate gut pathogens, may be less affected by systemic immune responses than is DWV, which is not limited to the gut (de Miranda and Genersch 2010); experiments with other pairs of systemic versus enteric pathogens are needed to test this hypothesis. Although more work is needed to clarify the mechanisms by which phytochemicals modulate resistance to viruses, knowledge of the exact mechanisms is not essential for the application of phytochemicals to improve bee health.

The effectiveness of short-term phytochemical consumption on antiviral immunity of bees in the colony suggests the potential for flowering plants in the landscape to protect bees from disease without causing toxicity. In the “Young Bees: Without inoculation” experiment, the magnitude of phytochemical-mediated reduction in DWV was large (ΔΔCT = −8.05 (anabasine), −7.81 (capto), and 6.01 (nicotine)). This is close to the difference in virus titers for bees infected with avirulent versus virulent DWV strains (ΔΔCT = −11.0; Ryabov et al. 2014). Moreover, antiviral effects were achieved with a single feeding of thymol at a concentration well within the range found in plant nectar and honey. Similarly, benefits of anabasine, capto, and nicotine accrued from just one day of phytochemical consumption.

In contrast, the lethal and sublethal effects of phytochemicals on bees are well established. In our study, clove oil (5,000 and 20,000 ppm) and thymol (16 ppm, higher than concentrations in thyme nectar; Table 1) increased mortality. Previous work has shown that sufficient concentrations of many compounds can kill honey bees outright and deter consumption (Detzel and Wink 1993, Ebert et al. 2007). Sublethal effects can occur at far lower doses. For example, 50 ppm nicotine had little (Köhler et al. 2012a) or no effect on worker survival (Singaravelan et al. 2006), but reduced nectar consumption, sugar storage, and larval survival by >50% (Köhler et al. 2012a). Similarly, thymol had an LD50 of >1,000 ppm in adult bees (Ebert et al. 2007) and 700 ppm in larvae (Charpentier et al. 2014), but just 50 ppm altered larval development (Charpentier et al. 2014). Negative effects of phytochemicals may also be amplified by infection. Nicotine increased mortality at lower doses when bees were simultaneously immune-challenged (Köhler et al. 2012b). Similarly, expression of a cytochrome p450 detoxification gene was negatively correlated with expression of antimicrobial peptides in bumble bees (Barrieau and Schmid-Hempel 2013), suggesting tradeoffs between detoxification and immunity. Chronic phytochemical exposure can also be costly in the field, where long-term (30-d) thymol exposure decreased immune gene expression in thymol-treated colonies (Boncristiani et al. 2012).

Short-term phytochemical consumption, which yielded the greatest benefits in our study, is reminiscent of natural patterns of phytochemical exposure in varied floral landscapes. Previous studies have indicated benefits of mixed-pollen diets for immunity (Alaux et al. 2010, Pascale et al. 2013). Flowering plant diversity has the additional benefit of improving the temporal consistency of food availability, if different species flower at different times of the year (Roulston and Goodell 2011, Goulson et al. 2013). Our findings suggest that floral diversity across time, which provides a dynamically varied assortment of phytochemicals, may allow colonies to sustain resistance to infection while avoiding phytochemical toxicity. Other possible benefits of diet diversity include consumption of different chemicals that have complementary effects against different parasite taxa (Drescher et al. 2014), synergistic effects against a single parasite taxon (Palmer-Young et al. 2017b), and avoidance of phytochemical resistance in parasites over time (Palmer-Young et al. 2017a).

In conclusion, this is the first study to show that phytochemical consumption confers immunity to viruses in honey bees. Our results highlight benefits of brief (<24 h) phytochemical exposure, which may modulate immune gene expression to achieve some of the same antiparasitic benefits of long-term exposure, but avoid potentially costly side effects on worker survival. The majority of tested phytochemicals were immune adjuvants or had antiviral effects at levels found in floral nectar or pollen, which suggests that plant community composition could influence patterns of disease in honey bees. Landscape management to encourage season-long availability of a diversity of phytochemical-containing flowers could promote biodiversity and adequate pollinator nutrition, and protect pollinators from infection.

Acknowledgments

This research was funded by the National Science Foundation (NSF: nsf.gov) under NSF-DEB-1258096 (to L.S.A., R.E.I., and J.D.E.), and NSF DDIG (NSFDEB-1501907 to E.C.P.Y. and L.S.A.); by the National Research Initiative (NRI) Arthropod and Nematode Biology and Management Program of the United States Department of Agriculture (USDA: usda.gov)
Cooperative State Research, Education, and Extension Service (CSREES) Grant USDA-AFRI 2013-02536 (to L.S.A., R.E.L., and J.D.E.); by the North American Pollinator Protection Campaign (J.D.E., L.S.A., and R.E.L.) and by a National Institutes of Food and Agriculture Predoctoral Fellowship (USDA- AFRI-2016-67011-24698 to E.C.P.Y.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. We are grateful to two anonymous reviewers whose comments improved the manuscript.

References Cited


