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Effects of short-term exposure to naturally occurring thymol concentrations on transmission of a bumble bee parasite

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Abstract. 1. Plants produce antimicrobial phytochemicals that can reduce growth and infectivity of parasites in animals. Pollinator parasites are transmitted between hosts that forage on shared flowers. Floral transmission directly exposes parasites to phytochemicals on floral surfaces and in nectar, both at flowers and, post-ingestion, in the crop. This exposure could directly affect parasite transmission to new hosts.

2. Nectar chemical analyses were combined with field and cell culture experiments to test the effects of the floral phytochemical thymol on the transmission potential of the trypanosomatid gut parasite *Crithidia* in *Bombus impatiens*. First, thymol concentrations in *Thymus vulgaris* nectar were measured. Second, the effect of adding thymol to floral nectaries on parasite transmission to foraging bees was tested. Third, cell cultures were used to determine direct, dose-dependent effects of short-term thymol exposure on subsequent *in vitro* parasite growth.

3. A total of 26.1 ppm thymol was found in *T. vulgaris* nectar, five-fold higher than previously documented in this species. However, addition of thymol to flowers of parasite-inoculated inflorescences of four plant species did not affect acquisition of *Crithidia* infection during a foraging bout. Cell culture experiments showed that the thymol concentrations needed to reduce subsequent *Crithidia* growth by 50% (120 ppm) were 4.6-fold higher than the highest detected nectar concentration.

4. Although thymol exposure can influence *Crithidia* viability, *Crithidia* are robust to the duration and magnitude of exposure encountered during floral foraging under natural conditions. These experiments suggest that any effects of thymol alone on *Crithidia*–host infection dynamics probably reflect indirect, possibly host-mediated, effects of chronic thymol ingestion.

Key words. Floral trait manipulation, horizontal transmission, plant secondary metabolites, terpenoids, tritrophic interactions, trypanosomatids.

Introduction

Antimicrobial phytochemicals have a long history of use in human medicine (Wink, 2012), and can alter the outcome of infection in a variety of animals, including pollinators (Gowler *et al.*, 2015). Given recent concerns regarding infectious disease-related decline of pollinators (Cameron *et al.*, 2011; Goulson *et al.*, 2015), interest has grown in the potential for phytochemicals to ameliorate the severity and consequences of infection in bees (Simone-Finstrom & Spivak, 2012; Gherman *et al.*, 2014; Baracchi *et al.*, 2015; Erler & Moritz, 2015). Most recent studies have tested the antiparasitic effects of chronic phytochemical ingestion after inoculation with a fixed quantity

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of parasites (Costa *et al.*, 2010; Gherman *et al.*, 2014; Richardson *et al.*, 2015). However, the environment experienced by parasites during transmission between unrelated hosts can shape both the genetic composition and subsequent intensity of infection (Schmid-Hempel *et al.*, 1999). Florally transmitted parasites must survive direct exposure to floral phytochemicals, which have been shown to act as strong filters of microbial communities (Junker & Tholl, 2013; Junker & Keller, 2015). Hence, the direct effects of floral phytochemical exposure during parasite transmission could reduce the survival or infectivity of parasites before they enter the host. However, only two studies have tested how direct exposure to floral phytochemicals influences subsequent infectivity of parasites to pollinators (Manson *et al.*, 2010; Baracchi *et al.*, 2015).

Flowers are potential hotspots for pathogen transmission in bumble bees and other pollinators because they are frequently visited by a wide variety of organisms (Graystock *et al.*, 2015; McFrederick *et al.*, 2017). Disease transmission on floral substrates can occur through infected pollen (Singh *et al.*, 2010) or via faecal contamination (Durrer & Schmid-Hempel, 1994). While floral traits, including flower longevity (Thrall & Jarosz, 1994; Shykoff *et al.*, 1996), morphology (Elmqvist *et al.*, 1993; Shykoff *et al.*, 1997; Biere & Honders, 2006), and phytochemistry (Dötterl *et al.*, 2009; Sasu *et al.*, 2010; Huang *et al.*, 2012), are known to affect the transmission of plant pathogens, little is known about how floral characteristics affect the transmission of animal pathogens (McArt *et al.*, 2014). Although three prior studies demonstrated the potential for parasite transmission among bee individuals and species that visit the same flowers (Durrer & Schmid-Hempel, 1994; Singh *et al.*, 2010; Graystock *et al.*, 2015), the effects of floral chemicals on transmission of pollinator parasites remain unexplored, and no study has experimentally manipulated floral traits to test how chemistry influences transmission of pollinator infection.

We focused on transmission of the intestinal parasite *Crithidia* in the common Eastern bumble bee, *Bombus impatiens* Cresson (Apidae). Two related *Crithidia* species, *C. expoeki* and *C. bombi* Lippa and Triggiani (Trypanosomatidae), have been found in bumble bees (Schmid-Hempel & Tognazzo, 2010) and are distinguishable only by molecular methods. Because we did not conduct molecular analyses of the parasites used here, we will refer to *Crithidia* by its generic epithet. *Crithidia* are trypanosomatid protozoans that reduce bumble bee fitness, shortening both individual and colony life span and reducing colony production of new queens (Schmid-Hempel, 1998). Infection is transmitted via faecal-oral contact, which has been shown to occur at flowers (Durrer & Schmid-Hempel, 1994; Graystock *et al.*, 2015). While *B. impatiens* populations are stable, spread of *Crithidia* is correlated with the dramatic decline of a South American bumble bee species, *Bombus dahlbomii* (Schmid-Hempel *et al.*, 2014). *Bombus impatiens*, due to its high abundance and ease of rearing (Velthuis & van Doorn, 2006), serves as a model organism for the investigation of disease transmission.

We investigated the effects of thymol, a monoterpene phenol, on *Crithidia* transmission. Thymol is a naturally occurring floral volatile, found in *Thymus vulgaris* L. (common thyme) as well as in a variety of relatives in the Lamiaceae and other families (Zamureenko *et al.*, 1989; Rota *et al.*, 2008; Figiel *et al.*, 2010; Ozkan *et al.*, 2010; Novy *et al.*, 2015). Thymol inhibited growth of the trypanosomatids *Trypanosoma cruzi* and *Crithidia fasciculata* (Azeredo & Soares, 2013), and of *Leishmania amazonensis* (de Medeiros *et al.*, 2011), which are closely related to bumble bee-infective *Crithidia* (Schwarz *et al.*, 2015). More recently, naturally occurring concentrations of thymol have been shown to inhibit growth of *Crithidia* isolated from bumble bees (Palmer-Young *et al.*, 2016) and to reduce *Crithidia* infection intensity in live *B. impatiens* (Richardson *et al.*, 2015), suggesting the ability of thymol to influence infection in nature. However, other studies have shown no antiparasitic effects of dietary thymol across a range of concentrations (Biller *et al.*, 2015). Once ingested, dietary phytochemical concentrations are rapidly reduced by metabolic enzymes (du Rand *et al.*, 2015) and absorption into the haemolymph (Hurst *et al.*, 2014), which may reduce the concentrations to which hindgut parasites like *Crithidia* are exposed in the intestine. If thymol directly affects *Crithidia* growth at ecologically relevant concentrations, then it could have its strongest effects on parasite transmission at flowers, where parasites are directly exposed to the full chemical concentration produced by the plant.

We combined nectar sampling with field transmission and cell culture experiments to assess the effects of short-term thymol exposure on *Crithidia* transmission in *B. impatiens*. We first used chemical analyses of *T. vulgaris* nectar to determine naturally occurring thymol concentrations. We then used these measurements to design experiments that tested the effects of naturally occurring concentrations on acquisition of *Crithidia* infection. In these experiments, we allowed bees to forage on *Crithidia*-treated inflorescences, to which we experimentally added sucrose solutions with or without thymol (Fig. 1a). We complemented these *in vivo* transmission trials with *in vitro* experiments that tested direct effects of short-term phytochemical exposure on subsequent parasite growth in culture medium (Fig. 1b).

Methods

Nectar sampling

We analysed freshly collected *T. vulgaris* nectar. Note that this contrasts with methods used in a prior study, where nectar was mixed with ethanol and evaporated at room temperature (Palmer-Young *et al.*, 2016) prior to analysis. The evaporation process probably resulted in loss of thymol, which is volatile. Nectar was pooled from approximately 30 flowers of four *T. vulgaris* plants at Royal Botanic Gardens, Kew, (Richmond, Surrey, England) in June 2015. Samples were collected from flowers using microcapillary tubes inserted in the corolla. Briefly, sample volume was estimated by measurement of the length of the tube filled by the sample. The pooled nectar (~16 µl) was diluted to a volume of 80 µl by addition of methanol [high-performance liquid chromatography (HPLC) grade]. The diluted nectar was analysed directly by HPLC using a Waters Alliance system (Elstree, U.K.) hyphenated to a photodiode array detector and ZQ LC–MS detector. Compounds were separated on a Phenomenex (Macclesfield,

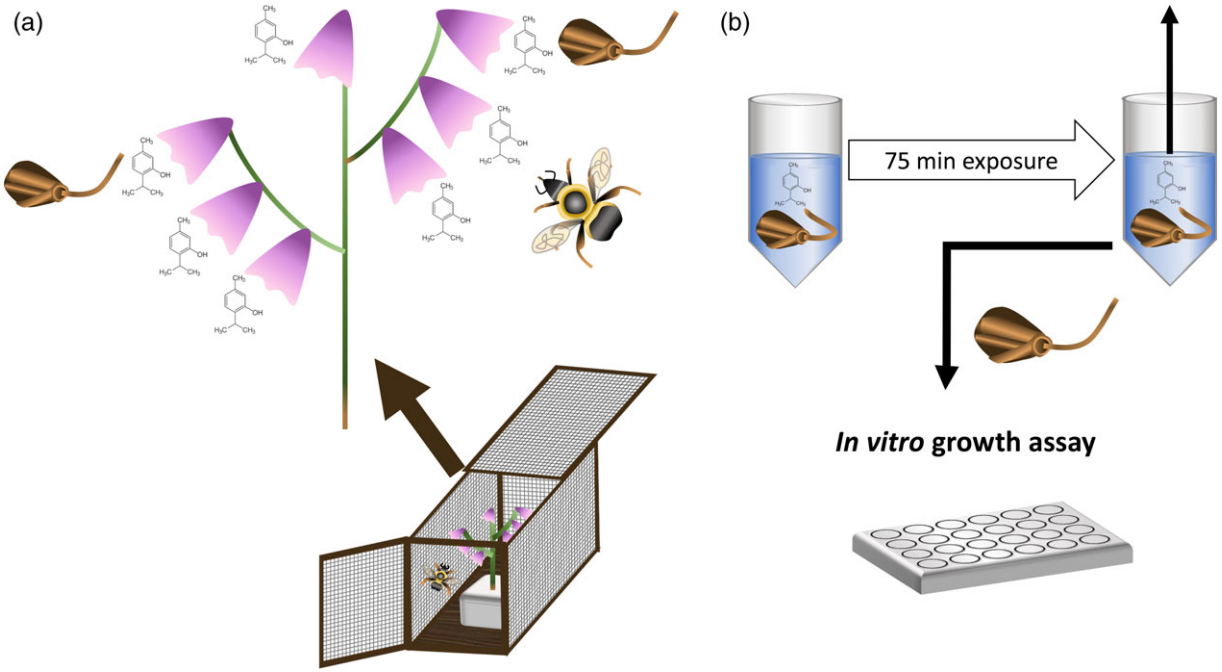

Fig. 1. Schematic of experimental design. (a) Floral transmission. Individual bumble bees (*Bombus impatiens*) were allowed to forage on cut inflorescences inside a screened cage. A subset (two to four flowers, depending on floral species) were inoculated with *Crithidia* (6000 parasite cells in 10 µl per flower). Nectaries of every flower on the inflorescence received either 18 ppm thymol-containing sucrose solution (2 µl per flower) or a 0 ppm thymol control solution. Bees were allowed to forage until at least five flowers, including one inoculum droplet, had been probed, then reared in the lab for 7 days prior to assessment of infection intensity. (b) *In vitro* thymol exposure of parasite cell cultures. *Crithidia* cell cultures were incubated with 0–500 thymol-containing Ringer's solution for 75 min. Cultures were then centrifuged and washed with Ringer's solution to remove thymol, and resuspended in growth medium at a final concentration of 250 cells μl^{-1}. Post-treatment growth was measured by spectrophotometry over the subsequent 70 h. [Colour figure can be viewed at wileyonlinelibrary.com].

U.K.) Luna C18(2) column (150 × 4.0 mm inner diameter, 5 µm particle size) with a gradient elution of solvents MeOH (A), H_2O (B), and 1% HCO_2H (C) 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. Solvent C was held at 10% throughout the run. Column temperature was 30 °C with a flow rate of 0.5 ml min^{-1}. Thymol eluted at 18.0 min and was quantified at 275 nm in the diode array detector against a standard curve of thymol purchased from Sigma–Aldrich (Gillingham, U.K.).

Floral transmission experiment

Having established a reference concentration of thymol in floral nectar, we tested whether addition of thymol-treated sucrose solutions to floral nectar of *Crithidia*-inoculated inflorescences would influence acquisition of *Crithidia* infection during foraging by *B. impatiens*.

Parasite collection and propagation

Crithidia used for the floral transmission trials originated from three wild *B. impatiens* workers collected at Stone Soup Farm (Hadley, Massachusetts: 42.363911, −72.567747) in summer 2015. Gut extracts from these wild bees were fed to bees in commercial *Crithidia* 'source' colonies (Biobest, Leamington, Ontario, Canada), which served as reservoirs of infection. The infection was transferred to new source colonies every 4–6 weeks by feeding of diluted gut extracts to workers of the young source colony. Colonies were stored in darkness at room temperature, and fed multi-floral pollen (Koppert Biological Systems, Howell, Mississippi) and sucrose solution (30% w/v in deionized water) *ad libitum*. Uninfected colonies, referred to as 'experimental colonies', provided *B. impatiens* for experimental trials. Source and experimental colonies were stored in separate cabinets, and five workers per experimental colony were screened weekly via microscopy of gut samples to verify the absence of *C. bombi*. In total, 10 experimental colonies and six source colonies were used.

Crithidia *inoculum*

Crithidia inoculum was prepared daily from bees in source colonies on each day of transmission trials. Intestinal tracts from *B. impatiens* were homogenized in quarter-strength Ringer's solution (300 µl) composed of sodium chloride (2.25 g l^{-1}), potassium chloride (0.105 g l^{-1}), calcium chloride (0.12 g l^{-1}), and sodium bicarbonate (0.05 g l^{-1}), obtained from Sigma Aldrich (St Louis, Missouri). After a 4-h settling period, a 10-µl

aliquot of supernatant was transferred to a Neubauer haemocytometer and all moving parasite cells in a 0.02-μl subsample were counted under 400× magnification. Extracts from two to three bees were mixed and diluted with Ringer's solution to 1200 cells μl^{-1}, then mixed with an equal volume of sucrose solution (50% w/v in deionized water) to obtain an inoculum with 6000 cells per 10 μl inoculum in a final concentration of 25% sucrose.

Thymol treatments

Thymol solutions (18 ppm) were prepared weekly by dissolution of 36 mg thymol in 2 litres of 30% sucrose in deionized water. This concentration was chosen to lie between the concentration of the fresh nectar sample measured here (see Results) and the maximum concentration of thymol documented previously in dried nectar from *T. vulgaris* (Palmer-Young *et al.*, 2016). Control sucrose solutions were prepared identically, but without the addition of thymol.

Plant species

We used flowers of four plant species to investigate the effects of thymol on *Crithidia* transmission at flowers: *Echium vulgare* L. (Boraginaceae), *Lobelia siphilitica* L. (Campanulaceae), *Lythrum salicaria* L. (Lythraceae), and *Penstemon digitalis* Nuttall ex Sims (Plantaginaceae; all species hereafter referred to by genus). Seed sources and rearing conditions are given in the Supporting Information (Supplementary methods in File S1). Use of a variety of floral species allowed us to test the generality of thymol's effects in a variety of phytochemical and morphological backgrounds. *Echium*, *Lythrum*, and *Penstemon* are not known to emit thymol, although they produce other volatiles that may include monoterpenoids (Filella *et al.*, 2011; Parachnowitsch *et al.*, 2013; Manayi *et al.*, 2014). We were unable to find information on volatiles of *L. siphilitica*, but distillates of the aerial parts of the related *Lobelia pyramidalis* Wall did not contain thymol, although other monoterpenes were found (Joshi *et al.*, 2011). By using species that are not known to produce thymol, we could manipulate thymol without unknown variation due to thymol produced by the test plant itself.

Floral transmission assay

Trials were conducted over a 7-week period from mid-June to early August. Each trial consisted of a foraging bout by a single bee caged for ∼20 min with a single cut inflorescence (Fig. 1a, Figure S1). We used inflorescences of each plant species as they came into flower (*Penstemon* in June, *Echium* in July, *Lythrum* in early August, and *Lobelia* in late August). All flowers were covered with breathable white, organza mesh bags from Uline (New York, New York) for at least 48 h before use in trials to prevent contamination from wild bee visits. To perform a trial, an inflorescence was cut from the perennial garden and placed in a floral tube with deionized water. The requirements for the number of flowers per inflorescence varied for each species, and when necessary two inflorescences were used to meet the requirements. We used five to 10 flowers per inflorescence for *Penstemon*, five to seven flowers per inflorescence for *Echium*, and at least 10 flowers for *Lobelia* and *Lythrum*. For the first two species, we chose flowers to reflect typical numbers per inflorescence, but ultimately realized there was no reason to limit the number of flowers as even the maximum number per inflorescence represents only a very small fraction of the number of flowers a bee would encounter on a typical foraging trip. We therefore had no maximum flower number for *Lobelia* and *Lythrum*. Wilted flowers were manually removed from the inflorescence.

Either thymol-treated or control sucrose solution (2 μl) was added inside the corolla tube of each flower on the inflorescence. To minimize floral handling, we did not remove existing nectar from the nectaries. Although this pre-existing nectar would have diluted the thymol treatment, preliminary measurements indicated that standing nectar volume was, on average, < 2 μl for all species [*Echium*, < 0.15 μl $flower^{-1}$ (Corbet, 1978); *Lobelia* $\bar{x} = 1.42 \pm 1.79$ μl (mean ± SD), $n = 11$; *Lythrum*, $\bar{x} = 0.24 \pm 0.15$, $n = 10$; *Penstemon*, $\bar{x} = 0.92 \pm 0.96$, $n = 26$]. Therefore, the added solutions probably comprised the majority of the nectar available during the trial, especially for *Echium* and *Lythrum*. *Crithidia* inoculum (10 μl per flower) was then added to a subset of flowers (two per inflorescence for *Echium* and *Penstemon*, and four per inflorescence for *Lobelia* and *Lythrum*). These inoculated flowers were labelled with a paint pen (Craftsmart, Irving, Texas) at the base of the petals or on the receptacle.

On the morning of each trial, experimental bees were removed from colonies, placed in individual clean, vented 20 ml vials, starved at room temperature for 2–3 h, and transported to the field site in an ice-filled cooler. Cut, thymol-treated, inoculated inflorescences were placed in a flight cage constructed from wood and insect screening (45.7 × 71.0 × 55.6 cm). To initiate each trial, a single bee was removed from the cooler, placed in the cage, and allowed to forage. The trial was considered complete when the bee had probed at least three flowers for *Penstemon* or *Echium* and at least five flowers for *Lythrum* and *Lobelia*, including at least one inoculated flower in all cases. Bees that did not meet these criteria within 20 min of the trial start were removed from the experiment. We recorded the length of the trial, the total number of flowers probed, the number of inoculated flowers probed, the total amount of time spent foraging, the number of flowers on the inflorescence, and the amount of time from inoculum preparation to trial. After each trial, the cage was left in the sun to dry for 30 min to minimise cross-contamination between trials; *Crithidia* survive poorly when subjected to desiccation outside of bees (Schmid-Hempel *et al.*, 1999).

Assessment of infection intensity

Upon completion of the trial, experimental bees were again chilled in individual vials until all trials for the day were completed (up to 2–3 h), then brought back to the laboratory and provided with 500 μl of sucrose solution (30% w/v in deionized water) and 6 mg of multi-floral pollen (Koppert Biological

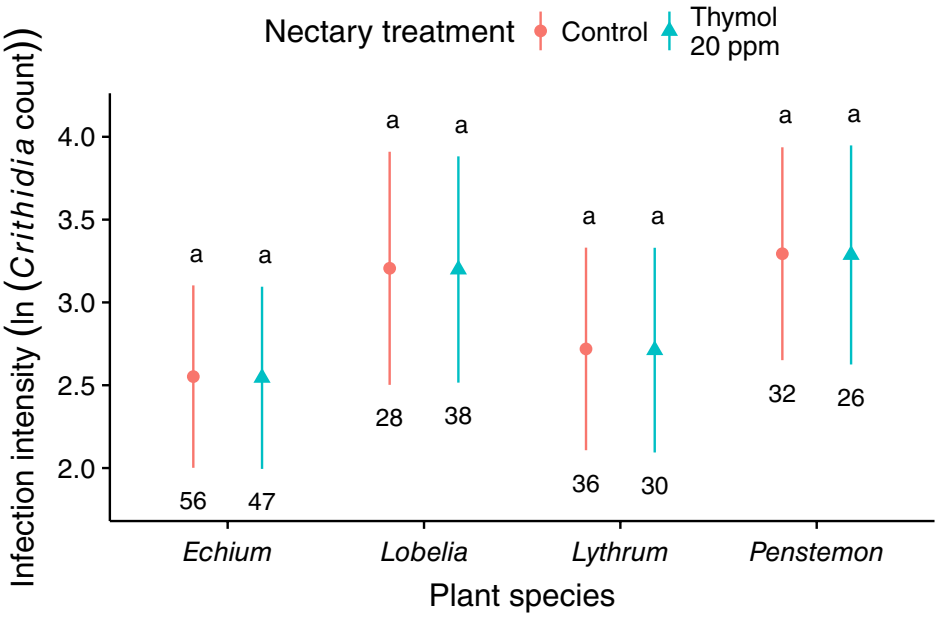

Fig. 2. Addition of thymol-containing sucrose solutions to nectaries did not affect acquisition of *Crithidia* infection by *Bombus impatiens* during a foraging bout on *Crithidia*-inoculated flowers. The *x*-axis indicates flowering plant species, and the *y*-axis shows infection intensity as the natural log of number of parasite cells counted in 0.02 µl of gut extract from bees dissected at 7 days post-trial. Points and error bars show model means and 95% confidence intervals. Different lower-case letters indicate significant differences in *post hoc* pairwise comparisons for effects of thymol within each plant species. Plant species had a non-significant effect on infection (Table 1). Numbers within the plot area indicate sample size (number of bees) for each treatment group. [Colour figure can be viewed at wileyonlinelibrary.com].

Systems). Bees were kept in individual 20 ml vials in a dark incubator at 28 °C and moved to a clean vial daily, provisioned with fresh sucrose (from a dental cotton wick) and pollen. They were dissected 7 days after the transmission trial, by which time infection generally plateaus (Otterstatter & Thomson, 2006). To assess parasite load, individual intestinal tracts were removed and treated as described earlier for the preparation of inoculum. The length of the radial cell of each bee's right forewing was measured as an estimate of body size (Schiestl & Barrows, 1999), which was used as a covariate in the analysis (Wilfert *et al.*, 2007; Manson *et al.*, 2010). Sample sizes are shown in Fig. 2.

Statistical analysis of floral transmission experiments

Statistical analysis was conducted using the open source software R v3.2.1 (R Core Team, 2014). Effects of thymol treatment and plant species on infection intensity at 7 days post-trial were analysed with **a** generalised linear mixed model in R package glmmTMB (Magnusson *et al.*, 2017). The number of *Crithidia* cells counted in a 0.02-µl gut extract was used as the response variable. Thymol treatment, plant species, and their interaction were included as fixed effects. The number of inoculum drops probed (an estimate of parasite exposure during the trial) and forewing marginal cell length (an index of bee size) were included as covariates. We also included an interaction between thymol treatment and number of flowers probed, to test whether thymol had a stronger effect on infection in bees that were exposed to greater amounts of the phytochemical. Because *Crithidia* can fare poorly under high-sugar conditions (Cisarovsky & Schmid-Hempel, 2014), the amount of time elapsed between inoculum preparation and foraging trial was initially included as a covariate, but removed from the final model because it did not explain significant variation in infection intensity ($\chi^2 = 0.19$, d.f. = 1, $P = 0.66$). Date of inoculation was used as a random effect to account for the independent preparation of inoculum on each trial date, and experimental bee colony was included as an additional random effect to account for non-independence of bees within a colony. The model used a negative binomial error distribution with zero inflation. The negative binomial is commonly used for non-negative count data that are overdispersed relative to the Poisson distribution (Bliss & Fisher, 1953); *Crithidia* infection intensities are often characterized by skewed distributions with long tails (Wilfert *et al.*, 2007). The zero-inflation parameter allows for the existence of two processes that can generate zero counts (Martin *et al.*, 2005), e.g. whether the infection was acquired during the foraging bout, and the intensity of parasitism in bees that did become infected. The significance of individual terms was tested with likelihood ratio χ^2 tests, conducted with the drop1 function, which compares relative goodness of fit between models with and without the term under consideration. The main effects of thymol and plant species were tested after removal of higher-order interaction terms. Estimated group means, confidence intervals, and pairwise comparisons for effects of thymol within each species were derived using the lsmeans package (Lenth, 2016). Figures were produced with

the R packages cowplot (Wilke, 2016) and ggplot2 (Wickham, 2009).

Cell culture experiments

We complemented our floral transmission assays with a cell culture assay that used *Crithidia* cell cultures to determine dose-dependent effects of a 75 min thymol exposure on subsequent *in vitro* growth. We chose this exposure period because it is within the range of durations for normal foraging trips made by *Bombus vosnesenskii* (Allen *et al.*, 1978). Hence, this time period approximates the total duration of thymol exposure for parasites that are deposited at thymol-rich flowers, then incubated in the crop of foragers that consume thymol-rich nectar.

Parasite collection and culture conditions

Crithidia cells were isolated from wild bumble bees (*B. impatiens*) collected near Normal, Illinois, in 2013 (strain 'IL13.2', collected by BMS) by flow cytometry-based single cell sorting of bee faeces (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 of fresh culture medium (Salathé *et al.*, 2012). The final transfer (to 500 cells μl^{-1} in 5 ml of fresh medium) occurred 48 h before the experiment began.

Experimental design

Thymol treatments (six concentrations, 0–500 ppm by volume at intervals of 100 ppm) were prepared at 1.2× final concentration in sterile Ringer's solution from a sterile-filtered stock solution of 40×10^3 ppm thymol dissolved in ethanol. A preliminary experiment indicated minimal effects of concentrations below 100 ppm (Figure S2); therefore, aside from the 0 ppm control, we tested only concentrations between 100 and 500 ppm. Ethanol was added to treatments of lower thymol concentrations to equalise ethanol concentrations (1.25% by volume) in all treatments. Cell cultures were diluted to a density of 1500 cells μl^{-1} in growth medium. An aliquot of the cell suspension (200 µl) was then added to 1 ml of each thymol treatment in a 2 ml tube; two replicate tubes were used for each of the six concentrations. Cells were incubated with thymol treatments for 75 min at 26 °C. Immediately thereafter, tubes were centrifuged (12 min, 3200 ***g***) and 1 ml of supernatant was removed. The cell pellet was then washed twice by the addition of 1 ml of sterile Ringer's solution, centrifugation (12 min, 3200 ***g***), and removal of 1 ml of supernatant. Because each removal of supernatant removed 83.3% of the liquid in the tube, we estimate that the three centrifugation and aspiration steps removed all but 0.5% (0.167^3) of the thymol used in the exposure. Hence, effects of the treatment are probably due to thymol's effects during the 75 min exposure, rather than due to the inhibitory effects of residual thymol during subsequent growth, which generally requires 20–25 ppm thymol for this strain (Palmer-Young *et al.*, 2017b; Palmer-Young *et al.*, 2017c). The resulting cell suspensions (250 cells μl^{-1}) were aliquoted to a 96-well plate (five wells per tube, 200 µl per well, $n = 10$ total wells per treatment concentration). The plate was sealed with laboratory film and incubated at 26 °C inside a zippered plastic sandwich bag. Optical density (OD, $\lambda = 630$ nm) was measured three times per day through 70 h by spectrophotometry; cells were resuspended (30 s, 1000 rpm, 2 mm orbit) on a microplate shaker before each measurement. Net OD was computed by subtracting the mean OD of 12 cell-free blanks that contained growth medium without cells.

Statistical analysis of cell culture experiments

The effects of thymol on *Crithidia* growth were determined by maximum likelihood estimation of dose–response curves in the R package drc (Ritz *et al.*, 2015). First, growth was quantified using the growth integral (i.e. area under the curve of net OD versus time) for each well; this integral was calculated by fitting a model-free spline to the observed OD measurements, as implemented in R package grofit (Kahm *et al.*, 2010). Measurements from the final time point (70 h) were removed prior to calculation of integrals, because by this time OD of the controls had begun to fall. The relationship between phytochemical concentration and growth integral was modelled with a three-parameter log-logistic model with the lower limit fixed at zero, corresponding to no growth as exposure concentration approaches infinity.

$$g = f\left(x, b, g_{\max}, \mathrm{e}_{50}\right) = \frac{g_{\max}}{\left\{1 + \exp\left[b\left(\log x - \log \mathrm{e}_{50}\right)\right]\right\}} \quad (1)$$

where g denotes growth integral, x refers to thymol concentration, $g_{\max}$ denotes growth in the absence thymol, and e_{50} is the phytochemical concentration at which inhibition is reached (effective concentration, or (EC_{50})). The parameter b indicates the slope of the curve. From this model, we derived parameter estimates and 95% confidence intervals for the EC_{50}, and predictions for growth at each thymol concentration.

Results

Nectar thymol concentration in *T. vulgaris* was 26.1 ppm volume (i.e. 0.17 mM) for our single pooled sample, more than double the maximum of 10 ppm found among samples of dried nectar (Palmer-Young *et al.*, 2016).

In the floral transmission experiment, there was no effect of thymol nectar treatment on intensity of infection 7 days after the foraging bout (Table 1, Fig. 2). There were also no significant effects of the plant species used for the trial (Table 1), nor was there evidence for differential effects of thymol across plant species (thymol × plant species interaction, $\chi^2 = 1.791$, d.f. = 3, $P = 0.62$, removed from the final model). There was also no trend for an increase in the effect of thymol with an increase in number of thymol-containing flowers

Table 1. Predictors of infection intensity in *Bombus impatiens* 7 days after a foraging bout on *Crithidia*-inoculated inflorescences. Wing size refers to length of the right forewing marginal cell.

Predictor	χ^2	d.f.	*P*
Thymol	0.000 94	1	0.98
Plant species	5.13	3	0.16
Inoculum drops probed	2.12	1	0.15
Wing size	6.89	1	**0.0086**

Bold print indicates $P < 0.05$.

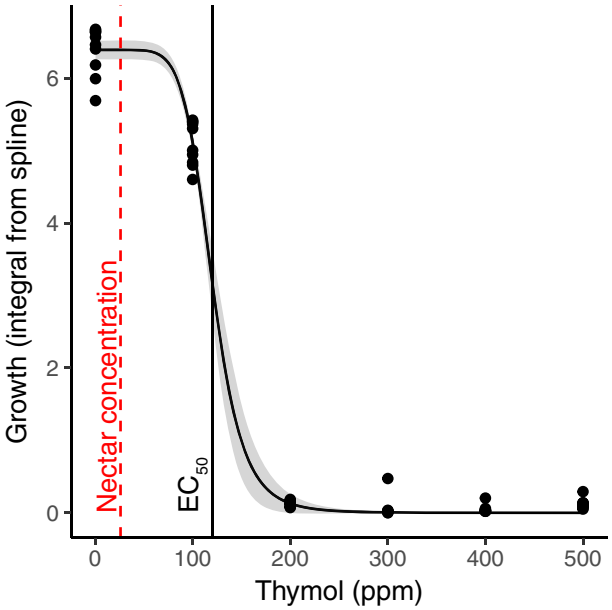

Fig. 3. Effects of 75 min exposure of *Crithidia* cell cultures to thymol on subsequent *in vitro* growth. The dose response curve relates thymol concentration (*x*-axis) to the area under the growth curve (*y*-axis). Shaded bands show 95% confidence intervals from log-logistic model. Solid black line, 50% effective concentration (EC_{50}); dashed red line, thymol concentration in *Thymus vulgaris* nectar sample; OD, optical density (630 nm wavelength). See Figure S3 for full growth curves. [Colour figure can be viewed at wileyonlinelibrary.com].

probed (non-significant thymol × flowers probed interaction, $\chi^2 = 0.14$, d.f. = 1, $P = 0.71$, removed from final model). The number of inoculum drops probed during the trial also did not explain the significant variation in infection intensity [$\beta = 0.10 \pm 0.07$ (SE); Table 1]. Wing size was negatively correlated with infection intensity ($\beta = -1.56 \pm 0.59$; Table 1), indicative of lower infection intensity in larger bees, which is consistent with previous results (Manson *et al.*, 2010; Palmer-Young *et al.*, 2017a).

In *Crithidia* cell cultures, although a 75 min exposure to at least 200 ppm thymol completely inhibited subsequent growth (Fig. 3), the EC_{50} for growth integral was 120 ± 2.3 ppm, or 4.6-fold higher than the fresh nectar concentration (Fig. 3). However, growth was only slightly affected by concentrations of 100 ppm (five-fold higher than mean nectar concentrations), and a preliminary trial showed negligible effects of concentrations similar to those found in nectar (26 ppm; Figure S2). The estimated concentration needed to reduce the growth integral by only 10% (89.8 ± 2.35 ppm) was still over 3.4-fold higher than the concentration in the nectar.

Changes in cell morphology observed at the end of the exposure period were good indicators of subsequent viability, with striking changes in both appearance and behaviour at concentrations > 100 ppm (Figure S4, Videos S1–S7).

Discussion

By analysing fresh nectar samples, we found higher concentrations of nectar thymol than reported in previous analyses that used dried nectars (Palmer-Young *et al.*, 2016). However, even these higher thymol concentrations were insufficient to affect acquisition of infection by foraging bees. A 75 min direct exposure to nectar thymol concentrations was also insufficient to inhibit growth of *Crithidia* cell cultures. These results suggest that any effects of nectar thymol on bee–parasite infection dynamics are not likely to reflect direct effects of thymol alone on parasites during horizontal transmission events.

The nectar concentration in our freshly collected, pooled sample (26.1 ppm) was nearly five-fold the mean concentration found in evaporated *T. vulgaris* nectar (Palmer-Young *et al.*, 2016). We expect that these higher concentrations reflect the fact that we analysed fresh nectar. Thymol is a volatile substance, and some nectar thymol will evaporate during the drying of samples. Due to the importance of volatile compounds in pollinator foraging and behaviour (Junker & Parachnowitsch, 2015), we suggest that future studies analyse fresh nectar when feasible.

Given that our measured floral nectar concentrations were above those necessary to inhibit growth of *Crithidia* cell cultures (Palmer-Young *et al.*, 2016; Palmer-Young *et al.*, 2017b), and 100-fold higher than the concentrations that reduced infection intensity when fed to bees (Richardson *et al.*, 2015), we hypothesized that exposure of parasites to thymol during bee foraging would mitigate acquisition of infection. However, addition of 18 ppm thymol-treated sucrose solutions to *Crithidia*-inoculated inflorescences did not alter acquisition of infection during a foraging bout. This absence of effect was consistent across plant species (Fig. 2).

Variability in consumption of both thymol and parasite inoculum by bees during the foraging trial may have reduced our power to detect an effect of the thymol treatment, but it is also possible that the duration and magnitude of thymol exposure in the trials were insufficient to alter parasite viability. To distinguish between low power to detect effects (due to experimental variability) and true robustness of parasites to short-term thymol exposure, we performed a controlled *in vitro* trial to determine the thymol concentrations necessary to inhibit parasite growth after a 75 min chemical exposure. In reality, bumble bee flowers may be visited – and presumably drained of nectar – multiple times per hour (Ruiz-González *et al.*, 2012). Although longer periods between visits are possible, *Crithidia* infectivity was reduced by 75% during just 40 min outside

the host (Schmid-Hempel *et al.*, 1999). Therefore, the effects of thymol over longer time periods may be irrelevant due to reduction in *Crithidia* infectivity for other reasons. Hence, our experiments probably tested upper estimates of the durations and concentrations of thymol exposure during transmission, yet still showed no direct effects on parasite viability. These results indicate that: (i) higher phytochemical concentrations are necessary for inhibition of parasite growth when exposure is acute (75 min) rather than chronic (5 days; Palmer-Young *et al.*, 2016, Palmer-Young *et al.*, 2017b, Palmer-Young *et al.*, 2017c); and (ii) nectar thymol concentrations appear too low to have direct effects on parasite viability over durations typical of horizontal transmission at flowers.

Given the absence of an effect of thymol on parasite transmission, does this floral phytochemical likely play a role in pollinator–parasite infection dynamics? One possibility is that chronic thymol exposure in hosts has direct effects on parasite replication. However, this appears to be theoretically and empirically questionable. First, although nectar concentrations may be high enough to inhibit growth when parasites are directly exposed to those concentrations over many days (Palmer-Young *et al.*, 2016), *Crithidia* inhabit the distal gut, and are exposed to concentrations that are probably much lower than those in ingested nectar. We expect that thymol is passively absorbed across cell membranes (Bakkali *et al.*, 2008), such as those in the midgut, and also actively diluted by digestive secretions and detoxified by cytochrome p450 and other enzymes. These enzymes have been shown to mediate detoxification of nicotine (du Rand *et al.*, 2017) and quercetin (Mao *et al.*, 2017), and are up-regulated by thymol exposure in honey bees (Boncristiani *et al.*, 2012). Second, empirical tests of chronic oral thymol consumption by bumble bees may cause no reduction in live bee infection intensity, even when phytochemicals are consumed at concentrations sufficient to inhibit growth *in vitro*. Whereas just 12 ppm thymol and 50 ppm eugenol resulted in strong inhibition of parasite growth in 12-well plates *in vitro* (Palmer-Young *et al.*, 2017b), one study found no effects of up to 7 days' consumption of 20 ppm dietary thymol on *Crithidia* infection in *B. impatiens* (Biller *et al.*, 2015), and another found no effects of 7 days' consumption of 50 ppm dietary eugenol on *Crithidia* infection (Palmer-Young *et al.*, 2018).

Despite these negative results, consumption of only 0.2 ppm thymol reduced *B. impatiens* infection of *Crithidia* (Richardson *et al.*, 2015). Moreover, studies in honey bees (Palmer-Young *et al.*, 2017d) suggested a possible host-mediated mechanism for thymol's effects on infections. Consumption of six of seven phytochemicals, including thymol, up-regulated transcription of the antimicrobial peptide hymenoptaecin (Palmer-Young *et al.*, 2017d), and consumption of the hydroxycinnamic acid *p*-coumaric acid up-regulated transcription of the antimicrobial peptide abaecin and defensin1 (Mao *et al.*, 2013). Even a single exposure of newly emerged bees to 0.2 ppm thymol resulted in reduced titres of Deformed Wing Virus after 5 days in the colony (Palmer-Young *et al.*, 2017d), and 20 ppm thymol reduced *Nosema* infection (Costa *et al.*, 2010). These reductions in infection could reflect thymol-mediated immune stimulation. In contrast, application of concentrated thymol to honey bee hives – a treatment used to reduce *Varroa* mite infestation (Imdorf *et al.*, 1999) – reduced transcription of several immune genes (Boncristiani *et al.*, 2012). Similarly, dietary thymol did not affect infection with the trypanosomatid *Lotmaria passim*, a relative of *Crithidia* (Palmer-Young *et al.*, 2017d), nor did thymol reduce *Crithidia* infection in *B. impatiens* in all cases (Biller *et al.*, 2015). Future experiments that measure both infection intensity and immune function are necessary to clarify the mechanism by which thymol affects *Crithidia* and other pathogens, and the reasons for variability in the effects of thymol and other phytochemicals on infection (Thorburn *et al.*, 2015; Palmer-Young *et al.*, 2017a).

Our integration of nectar chemistry, field transmission studies, and *in vitro* experiments indicates that thymol concentrations in floral nectar can be higher than previously documented, and that brief exposure to high thymol concentrations can dramatically affect parasite morphology and viability. However, the robustness of *Crithidia* floral transmission and *in vitro* growth to short-term thymol exposure at natural nectar concentrations suggests that this compound has limited direct effects on *Crithidia* transmission at flowers. Thymol and other phytochemicals may still play immunoregulatory roles that have context-dependent effects on *Bombus* infection with *Crithidia* and other parasites. Experiments that explore the mechanisms by which secondary compounds do and do not affect parasitism *in vivo*, and the specific doses and time periods of application of the compounds, are needed to clarify the role of phytochemicals in pollinator health and disease.

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LSA, REI and PCS conceived the collection of data on naturally occurring phytochemical levels and experimental manipulation of floral chemistry, and ECPY and BMS conceived the cell culture study. PCS measured natural thymol concentrations, KWR conducted the field manipulative study with guidance

from LSA, and ECPY conducted the cell culture experiment. ECPY, LSA, and BMS analysed the data. KWR and ECPY wrote the manuscript. All authors revised the manuscript and agreed to its submission.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/een.12631

File S1. Supplementary methods. Plant seed sources and rearing conditions.

Figure S1. *Bombus impatiens* forages on *Penstemon digitalis* inflorescence inside experimental cage. One-litre water bottle in background (for scale).

Figure S2. Results of preliminary experiment that tested effects of short-term (75 min) exposure to thymol at lower concentrations. (a) Growth curves showing growth (measured by optical density) over time following exposure to different thymol concentrations, indicated by different coloured lines. Points show means and 95% confidence intervals for $n = 10$ replicates per treatment concentration. (B) Dose–response curve that relates thymol concentration (x-axis) to area under the growth curve (shown in a). Note the poor precision of fit when compared with Fig. 3b, due to lack of observations between 125 and 500 ppm. Solid black line, EC_{50} concentration; dashed red line, thymol concentration in *Thymus vulgaris* nectar sample.

Figure S3. Growth curves of *Crithidia* cell cultures after 75-min exposure to thymol. Growth was measured by optical density following exposure to different thymol concentrations, indicated by different coloured lines. Points show means and 95% confidence intervals for $n = 10$ replicates per treatment concentration. Integrals used for dose–response curves were calculated after exclusion of the final time point, due to fall in OD of control samples (0 ppm).

Figure S4. Micrographs showing morphological effects of thymol exposure on *Crithidia* cell cultures across a range of concentrations from 0 to 500 ppm. The photographed field was chosen haphazardly from a 1-ml sample volume; cell densities in the images are not meant to be representative of the entire sample.

Videos S1–S6. Video recordings showing morphological effects of thymol exposure on *Crithidia* cell cultures across a range of concentrations from 0 to 500 ppm. Control cells were oblong and characterised by relatively sharp anterior and posterior ends and rapid swimming. Cells exposed to 100 ppm appeared somewhat compressed along the major axis, with squared-off appearance, but were still motile, although they swam less rapidly than the control cells. Cells exposed to 200 ppm assumed a curled, spheroid appearance and did not swim at all. Cells exposed to 300 ppm had a spheroid appearance as in the 200 ppm treatment, but internal morphology appeared more granular. Cells exposed to 400 ppm were a mix of bulging spheroids and shrivelled, possibly lysed cells with a frayed and ragged appearance. After exposure to 500 ppm, almost all cells appeared shrivelled and deformed.

Video S7. Video recording of *Crithidia* cell cultures following exposure to 0 ppm (first half) or 500 ppm thymol (second half). Note the lack of movement and wrinkling of cell membrane and organelles in thymol-exposed parasite cells.

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