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

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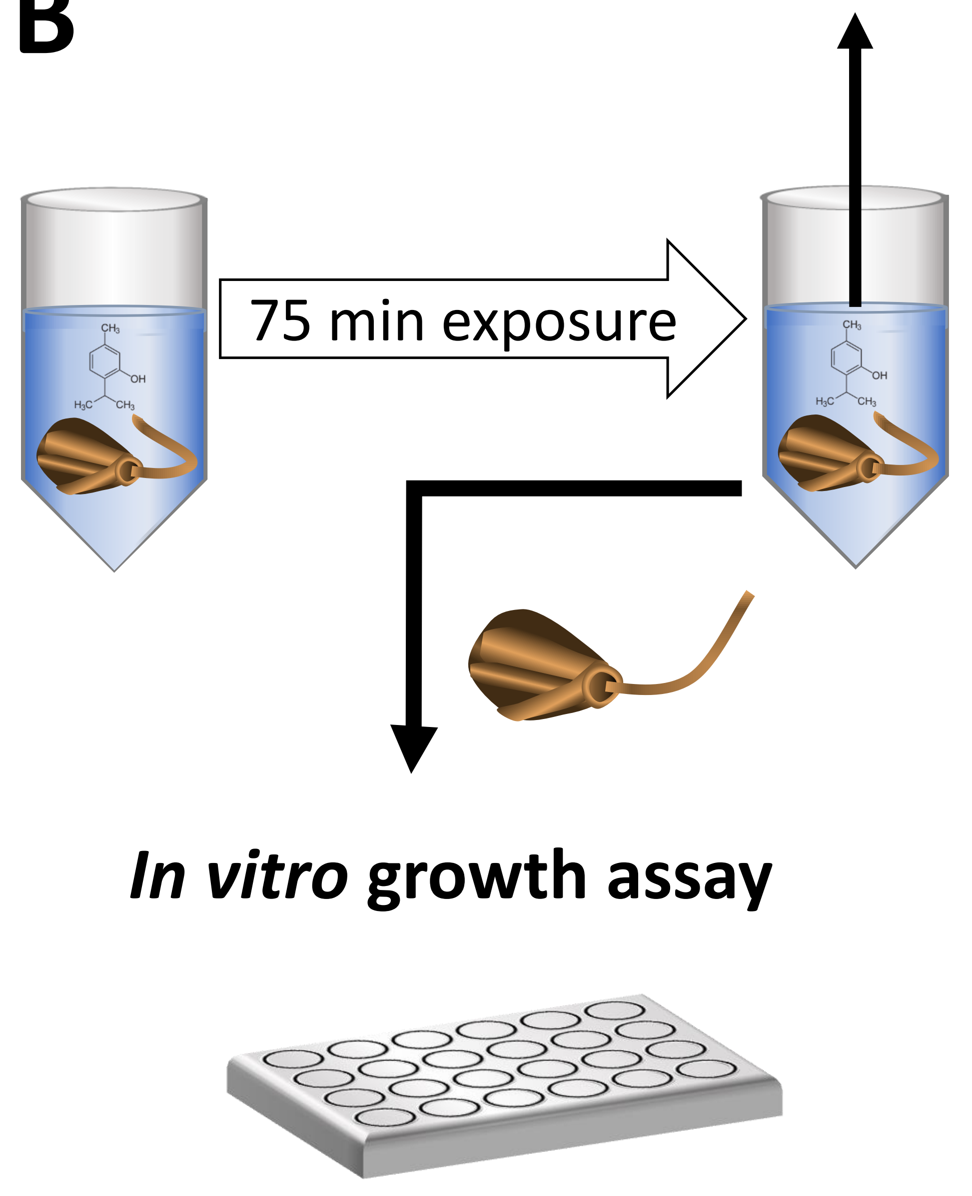

1 # Title

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

4 Running head: Effects of thymol on parasite transmission

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16 # Abstract

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

22 **Approach:** We combined nectar chemical analyses with field and cell culture experiments to test
23 effects of the floral phytochemical thymol on transmission potential of the trypanosomatid gut
24 parasite *Crithidia* in *Bombus impatiens*. First, we measured thymol concentrations in *Thymus vulgaris*
25 nectar. Second, we tested how addition of thymol to floral nectaries affected parasite transmission
26 to foraging bees. Third, we used cell cultures to determine direct, dose-dependent effects of short-
27 term thymol exposure on subsequent *in vitro* parasite growth.

28 **Results:** We found 26.1 ppm thymol in *Thymus vulgaris* nectar, 5-fold higher than previously
29 documented in this species. However, addition of thymol to flowers of parasite-inoculated
30 inflorescences of four plant species did not affect acquisition of *Crithidia* infection during a foraging
31 bout. Cell culture experiments showed that thymol concentrations needed to reduce subsequent
32 *Crithidia* growth by 50% (120 ppm) were 4.6-fold higher than the highest detected nectar
33 concentration.

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

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38 **Key words:** tritrophic interactions, plant secondary metabolites, terpenoids, trypanosomatids,

39 horizontal transmission, floral trait manipulation

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40 # Introduction

41 Antimicrobial phytochemicals have a long history of use in human medicine (Wink 2012), and can
42 alter the outcome of infection in a variety of animals, including pollinators (Gowler et al. 2015).
43 Given recent concerns regarding infectious disease-related decline of pollinators (Cameron et al.
44 2011, Goulson et al. 2015), interest has grown in the potential for phytochemicals to ameliorate the
45 severity and consequences of infection in bees (Simone-Finstrom and Spivak 2012, Gherman et al.
46 2014, Baracchi et al. 2015, Erler and Moritz 2015). Most recent studies have tested the antiparasitic
47 effects of chronic phytochemical ingestion after inoculation with a fixed quantity of parasites (Costa
48 et al. 2010, Gherman et al. 2014, Richardson et al. 2015). However, the environment experienced by
49 parasites during transmission between unrelated hosts can shape both the genetic composition and
50 subsequent intensity of infection (Schmid-Hempel et al. 1999). Florally transmitted parasites must
51 survive direct exposure to floral phytochemicals, which have been shown to act as strong filters of
52 microbial communities (Junker and Tholl 2013, Junker and Keller 2015). Hence, the direct effects of
53 floral phytochemical exposure during parasite transmission could reduce the survival or infectivity of
54 parasites before they enter the host. However, only two studies have tested how direct exposure to
55 floral phytochemicals influences subsequent infectivity of parasites to pollinators (Manson et al.
56 2010, Baracchi et al. 2015).

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57 Flowers are potential hotspots for pathogen transmission in bumble bees and other pollinators
58 because they are frequently visited by a wide variety of organisms (Graystock et al. 2015,
59 McFrederick et al. 2017). Disease transmission on floral substrates can occur through infected pollen
60 (Singh et al. 2010) or via fecal contamination (Durrer and Schmid-Hempel 1994). While floral traits,
61 including flower longevity (Thrall and Jarosz 1994, Shykoff et al. 1996), morphology (Elmqvist et al.
62 1993, Shykoff et al. 1997, Biere and Honders 2006), and phytochemistry (Dötterl et al. 2009, Sasu et
63 al. 2010, Huang et al. 2012) are known to affect the transmission of plant pathogens, little is known
64 about how floral characteristics affect the transmission of animal pathogens (McArt et al.

65 2014). Although three prior studies demonstrated the potential for parasite transmission among
66 bee individuals and species that visit the same flowers (Durrer and Schmid-Hempel 1994, Singh et al.
67 2010, Graystock et al. 2015), the effects of floral chemical traits on transmission of pollinator
68 parasites remain unexplored, and no study has experimentally manipulated floral traits to test how
69 chemistry influences transmission of pollinator infection.

70 We focused on transmission of the intestinal parasite *Crithidia* in the common Eastern bumble bee,
71 *Bombus impatiens* Cresson (Apidae). Two related *Crithidia* species, *C. expoeki* and *C. bombi* Lippa
72 and Triggiani (Trypanosomatidae) have been found in bumble bees (Schmid-Hempel and Tognazzo
73 2010), and are distinguishable only by molecular methods. Because we did not conduct molecular
74 analyses of the parasites used here, we will refer to *Crithidia* by its generic epithet. *Crithidia* are
75 trypanosomatid protozoans that reduce bumble bee fitness, shortening both individual and colony
76 lifespan and reducing colony production of new queens (Schmid-Hempel 1998). Infection is
77 transmitted via fecal-oral contact, which has been shown to occur at flowers (Durrer and Schmid-
78 Hempel 1994, Graystock et al. 2015). While *B. impatiens* populations are stable, spread of *Crithidia* is
79 correlated with the dramatic decline of a South American bumble bee species, *B. dahlbomii* (Schmid-
80 Hempel et al. 2014). *B. impatiens,* due to its high abundance and ease of rearing (Velthuis and van
81 Doorn 2006), serves as a model organism for the investigation of disease transmission.

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82 We investigated the effects of thymol, a monoterpene phenol, on *Crithidia* transmission. Thymol is a
83 naturally occurring floral volatile, found in *Thymus vulgaris* L. (common thyme) as well as in a variety
84 of relatives in the Lamiaceae and other families (Zamureenko et al. 1989, Rota et al. 2008, Figiel et al.
85 2010, Ozkan et al. 2010, Novy et al. 2015). Thymol inhibited growth of the trypanosomatids
86 *Trypanosoma cruzi, Crithidia fasiculata* (Azeredo and Soares 2013), and of *Leishmania amazonensis*
87 (de Medeiros et al. 2011), which are closely related to bumble bee-infective *Crithidia* (Schwarz et al.
88 2015). More recently, naturally occurring concentrations of thymol have been shown to inhibit
89 growth of *Crithidia* isolated from bumble bees (Palmer-Young et al. 2016), and to reduce *Crithidia*

90 infection intensity in live *B. impatiens* (Richardson et al. 2015), suggesting the ability of thymol to
91 influence infection in nature. However, other studies have shown no antiparasitic effects of dietary
92 thymol across a range of concentrations (Biller et al. 2015). Once ingested, dietary phytochemical
93 concentrations are rapidly reduced by metabolic enzymes (du Rand et al. 2015) and absorption into
94 the hemolymph (Hurst et al. 2014), which may reduce the concentrations to which hindgut parasites
95 like *Crithidia* are exposed in the intestine. If thymol directly affects *Crithidia* growth at ecologically
96 relevant concentrations, then it could have its strongest effects on parasite transmission at flowers,
97 where parasites are directly exposed to the full chemical concentration produced by the plant.

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

107

108 Methods

109 Nectar sampling

110 We analyzed freshly collected *Thymus vulgaris* nectar. Note that this contrasts with methods used in
111 a prior study, where nectar was mixed with ethanol and evaporated at room temperature (Palmer-
112 Young et al. 2016) prior to analysis. The evaporation process likely resulted in loss of thymol, which

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113 is volatile. Nectar was pooled from approximately 30 flowers of 4 *Thymus vulgaris* plants at Royal
114 Botanic Gardens, Kew, (Richmond, Surrey, England) in June 2015. Samples were collected from
115 flowers using microcapillary tubes inserted in the corolla. Briefly, sample volume was estimated by
116 measurement of the length of the tube filled by the sample. The pooled nectar (~16 µL) was diluted
117 to a volume of 80 µL by addition of methanol (HPLC grade). The diluted nectar was analysed directly
118 by HPLC using a Waters Alliance system (Elstree, Herts, United Kingdom) hyphenated to a photo-
119 diode array detector and ZQ LC–MS detector. Compounds were separated on a Phenomenex
120 (Macclesfield, Cheshire, United Kingdom) Luna C18(2) column (150 X 4.0 mm inner diameter, 5 µm
121 particle size) with a gradient elution of solvents A = MeOH, B = H2O, and C = 1% HCO_2H in MeCN: A =
122 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%
123 at t = 31 min. Solvent C was held at 10% throughout the run. Column temperature was 30°C with a
124 flow rate of 0.5 ml min^{-1}. Thymol eluted at 18.0 minutes and was quantified at 275 nm in the diode
125 array detector against a standard curve of thymol purchased from Sigma–Aldrich (Gillingham, Dorset,
126 United Kingdom).

127 Floral transmission experiment

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

131 Parasite collection and propagation

132 *Crithidia* used for the floral transmission trials originated from three wild *B. impatiens* workers
133 collected at Stone Soup Farm (Hadley, MA, USA: 42.363911, -72.567747) in summer 2015. Gut
134 extracts from these wild bees were fed to bees in commercial *Crithidia* "source" colonies (Biobest,
135 Leamington, Ontario, Canada), which served as reservoirs of infection. The infection was transferred
136 to new source colonies every 4-6 weeks by feeding of diluted gut extracts to workers of the young

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137 source colony. Colonies were stored in darkness at room temperature, and fed multi-floral pollen
138 (Koppert Biological Systems, Howell, MI, USA) and sucrose solution (30% w/v in deionized water) *ad*
139 *libitum*. Uninfected colonies, referred to as “experimental colonies” provided *B. impatiens* for
140 experimental trials. Source and experimental colonies were stored in separate cabinets, and five
141 workers per experimental colony were screened weekly via microscopy of gut samples to verify the
142 absence of *C. bombi*. In total, 10 experimental colonies and 6 source colonies were used.

143 ## *Crithidia* inoculum

144 *Crithidia* inoculum was prepared daily from bees in source colonies on each day of transmission trials.
145 Intestinal tracts from *B. impatiens* were homogenized in ¼ strength Ringers solution (300 μL)
146 composed of sodium chloride (2.25 g/L), potassium chloride (0.105 g/L), calcium chloride (0.12 g/L),
147 and sodium bicarbonate (0.05 g/L), obtained from Sigma Aldrich (St. Louis, MO, USA). After a 4 h
148 settling period, a 10 μL aliquot of supernatant was transferred to a Neubauer hemocytometer and
149 all moving parasite cells in a 0.02 μL subsample were counted under 400x magnification. Extracts
150 from 2-3 bees were mixed and diluted with Ringer’s solution to 1200 cells μL^{-1}, then mixed with an
151 equal volume of sucrose solution (50% w/v in deionized water) to obtain an inoculum with 6000 cells
152 per 10 μL inoculum in a final concentration of 25% sucrose.

153 ## Thymol treatments

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

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159 ## Plant species

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

172 ## Floral transmission assay

173 Trials were conducted over a seven-week period from mid-June to early August. Each trial consisted
174 of a foraging bout by a single bee caged for ~20 min with a single cut inflorescence (Figure 1A,
175 Supplementary figure 1). We used inflorescences of each plant species as they came into flower
176 (*Penstemon* in June, *Echium* in July, *Lythrum* in early August, and *Lobelia* in late August). All flowers
177 were covered with breathable white, organza mesh bags from Uline (New York, NY, USA) for at least
178 48 h before use in trials to prevent contamination from wild bee visits. To perform a trial, an
179 inflorescence was cut from the perennial garden and placed in a floral tube with deionized water.
180 The requirements for the number of flowers per inflorescence varied for each species, and when
181 necessary two inflorescences were used to meet the requirements. We used 5-10 flowers per
182 inflorescence for *Penstemon,* 5-7 flowers per inflorescence for *Echium,* and at least 10 flowers for

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183 *Lobelia* and *Lythrum.* For the first two species, we chose flowers to reflect typical numbers per
184 inflorescence, but ultimately realized there was no reason to limit the number of flowers since even
185 the maximum per inflorescence represents only a very small fraction of the number of flowers a bee
186 would encounter on a typical foraging trip. We therefore had no maximum flower number for
187 *Lobelia* and *Lythrum*. Wilted flowers were manually removed from the inflorescence.

188 Either thymol-treated or control sucrose solution (2 μL) was added inside the corolla tube of each
189 flower on the inflorescence. To minimize floral handling, we did not remove existing nectar from the
190 nectaries. Although this pre-existing nectar would have diluted the thymol treatment, preliminary
191 measurements indicated that standing nectar volume was on average <2 μL for all species (*Echium*
192 <0.15 μL flower^{-1} (Corbet 1978); *Lobelia* $\bar{x}$ = 1.42 μL ± 1.79 SD, n = 11; *Lythrum* $\bar{x}$ = 0.24 ± 0.15 SD, n =
193 10; *Penstemon* $\bar{x}$ = 0.92 ± 0.96 SD, n = 26). Therefore, the added solutions likely comprised the
194 majority of the nectar available during the trial, especially for *Echium* and *Lythrum*. *Crithidia*
195 inoculum (10 μL per flower) was then added to a subset of flowers (2 per inflorescence for *Echium*
196 and *Penstemon,* and 4 per inflorescence for *Lobelia* and *Lythrum*). These inoculated flowers were
197 labeled with a paint pen (Craftsmart, Irving, TX, USA) at the base of the petals or on the receptacle.

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198 On the morning of each trial, experimental bees were removed from colonies, placed in individual
199 clean, vented 20 mL vials, starved at room temperature for 2-3 hours, and transported to the field
200 site in an ice-filled cooler. Cut, thymol-treated, inoculated inflorescences were placed in a flight cage
201 constructed from wood and insect screening (45.7 cm x 71.0 cm x 55.6 cm). To initiate each trial, a
202 single bee was removed from the cooler, placed in the cage, and allowed to forage. The trial was
203 considered complete when the bee had probed at least 3 flowers for *Penstemon* or *Echium* and at
204 least 5 flowers for *Lythrum* and *Lobelia,* including at least 1 inoculated flower in all cases. Bees that
205 did not meet these criteria within 20 min of the trial start were removed from the experiment. We
206 recorded the length of the trial, the total number of flowers probed, the number of inoculated
207 flowers probed, the total amount of time spent foraging, the number of flowers on the inflorescence,

208 and the amount of time from inoculum preparation to trial. After each trial, the cage was left in the
209 sun to dry for 30 min to minimize cross-contamination between trials; *Crithidia* survive poorly when
210 subject to desiccation outside of bees (Schmid-Hempel et al. 1999).

211 Assessment of infection intensity

212 Upon completion of the trial, experimental bees were again chilled in individual vials until all trials
213 for the day were completed (up to 2-3 h), then brought back to the laboratory and provided with
214 500 μL of sucrose solution (30% w/v in deionized water) and 6 mg of multi-floral pollen (Koppert
215 Biological Systems, Howell, MI, USA). Bees were kept in individual 20 mL vials in a dark incubator at
216 28°C and moved to a clean vial daily, provisioned with fresh sucrose (from a dental cotton wick) and
217 pollen. They were dissected 7 d after the transmission trial, by which time infection generally
218 plateaus (Otterstatter and Thomson 2006). To assess parasite load, individual intestinal tracts were
219 removed and treated as described above for the preparation of inoculum. The length of the radial
220 cell of each bee's right forewing was measured as an estimate for body size (Schiestl and Barrows
221 1999), which was used as a covariate in the analysis (Wilfert et al. 2007, Manson et al. 2010). Sample
222 sizes are shown in Figure 2.

223 Statistical analysis of floral transmission experiments

224 Statistical analysis was conducted using the open source software R v3.2.1 (R Core Team 2014).
225 Effects of thymol treatment and plant species on infection intensity at 7 d post-trial were analyzed
226 with a generalized linear mixed model in R package glmmTMB (Magnusson et al. 2017). Number of
227 *Crithidia* cells counted in 0.02 μL gut extract was used as the response variable. Thymol treatment,
228 plant species, and their interaction were included as fixed effects. Number of inoculum drops probed
229 (an estimate of parasite exposure during the trial) and forewing marginal cell length (an index of bee
230 size) were included as covariates. We also included an interaction between thymol treatment and
231 number of flowers probed, to test whether thymol had a stronger effect on infection in bees that

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232 were exposed to greater amounts of the phytochemical. Because *Crithidia* can fare poorly under
233 high-sugar conditions (Cisarovsky and Schmid-Hempel 2014), the amount of time elapsed between
234 inoculum preparation and foraging trial was initially included as a covariate, but removed from the
235 final model because it did not explain significant variation in infection intensity (χ^2 = 0.19, Df = 1, P =
236 0.66). Date of inoculation was used as a random effect to account for the independent preparation
237 of inoculum on each trial date, and experimental bee colony included as an additional random effect
238 to account for non-independence of bees within a colony. The model used a negative binomial error
239 distribution with zero inflation. The negative binomial is commonly used for non-negative count data
240 that are over-dispersed relative to the Poisson distribution (Bliss and Fisher 1953); *Crithidia* infection
241 intensities are often characterized by skewed distributions with long tails (Wilfert et al. 2007). The
242 zero-inflation parameter allows for the existence of two processes that can generate zero counts
243 (Martin et al. 2005), e.g., whether the infection was acquired during the foraging bout, and the
244 intensity of parasitism in bees that did become infected. Significance of individual terms was tested
245 with likelihood ratio chi-squared tests, conducted with the drop1 function, which compares relative
246 goodness of fit between models with and without the term under consideration. Main effects of
247 thymol and plant species were tested after removal of higher-order interaction terms. Estimated
248 group means, confidence intervals, and pairwise comparisons for effects of thymol within each
249 species were derived with the lsmeans package (Lenth 2016). Figures were produced with R
250 packages cowplot (Wilke 2016) and ggplot2 (Wickham 2009).

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251 Cell culture experiments

252 We complemented our floral transmission assays with a cell culture assay that used *Crithidia* cell
253 cultures to determine dose-dependent effects of a 75 min thymol exposure on subsequent *in vitro*
254 growth. We chose this exposure period because it is within the range of durations for normal
255 foraging trips made by *Bombus vosnesenskii* (Allen et al. 1978). Hence, this time period

256 approximates the total duration of thymol exposure for parasites that are deposited at thymol-rich
257 flowers, then incubated in the crop of foragers that consume thymol-rich nectar.

258 Parasite collection and culture conditions

259 *Crithidia bombi* cells were isolated from wild bumble bees (*B. impatiens*) collected near Normal, IL,
260 United States in 2013 (strain "IL13.2", collected by BMS) by flow cytometry-based single cell sorting
261 of bee feces (Salathé et al. 2012). Cultures were microscopically screened to identify samples with
262 strong *Crithidia* growth and absence of bacterial or fungal contaminants, then stored at –80°C in a
263 2:1 ratio of cell culture:50% glycerol until several weeks before the experiments began. Thereafter,
264 cells were incubated in tissue culture flasks at 27°C and propagated twice per week at a density of
265 100 cells μL^{-1} in 5 mL fresh culture medium (Salathé et al. 2012). The final transfer (to 500 cells μL^{-1} in
266 5 mL fresh medium) occurred 48 h before the experiment began.

267 Experimental design

268 Thymol treatments (6 concentrations, 0-500 ppm by volume at intervals of 100 ppm) were prepared
269 at 1.2x final concentration in sterile Ringer's solution from a sterile-filtered stock solution of $40 * 10^3$
270 ppm thymol dissolved in ethanol. A preliminary experiment indicated minimal effects of
271 concentrations below 100 ppm (Supplementary figure 2); therefore, aside from the 0 ppm control,
272 we tested only concentrations between 100 and 500 ppm. Ethanol was added to treatments of
273 lesser thymol concentrations to equalize ethanol concentrations (1.25% by volume) in all treatments.
274 Cell cultures were diluted to a density of 1,500 cells μL^{-1} in growth medium. An aliquot of the cell
275 suspension (200 µL) was then added to 1 mL of each thymol treatment in a 2 mL tube; 2 replicate
276 tubes were used for each of the 6 concentrations. Cells were incubated with thymol treatments for
277 75 min at 26°C. Immediately thereafter, tubes were centrifuged (12 min, 3200 g) and 1 mL
278 supernatant removed. The cell pellet was then washed twice by addition of 1 mL sterile Ringer's
279 solution, centrifugation (12 min, 3200 g), and removal of 1 mL supernatant. Because each removal of

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280 supernatant removed 83.3% of the liquid in the tube, we estimate that the three centrifugation and
281 aspiration steps removed all but 0.5% (0.167^3) of the thymol used in the exposure. Hence, effects of
282 the treatment are likely due to thymol's effects during the 75-minute exposure, rather than due to
283 inhibitory effects of residual thymol during subsequent growth, which generally requires 20-25 ppm
284 thymol for this strain (Palmer-Young, Sadd, and Adler 2017, Palmer-Young, Sadd, Irwin, et al. 2017).
285 The resulting cell suspensions (250 cells μL^{-1}) were aliquoted to a 96-well plate (5 wells per tube, 200
286 µL per well, n =10 total wells per treatment concentration). The plate was sealed with laboratory
287 film and incubated at 26°C inside a zippered plastic sandwich bag. Optical density (OD, λ = 630 nm)
288 was measured three times per day through 70 h by spectrophotometry; cells were resuspended (30
289 s, 1000 rpm, 2 mm orbit) on a microplate shaker before each measurement. Net OD was computed
290 by subtracting the mean OD of 12 cell-free blanks that contained growth medium without cells.

291 Statistical analysis of cell culture experiments

292 Effects of thymol on *Crithidia* growth were determined by maximum likelihood estimation of dose-
293 response curves in R package drc (Ritz et al. 2015). First, growth was quantified using the growth
294 integral (i.e., area under the curve of net OD vs. time) for each well; this integral was calculated by
295 fitting a model-free spline to the observed OD measurements, as implemented in R package grofit
296 (Kahm et al. 2010). Measurements from the final time point (70 h) were removed prior to calculation
297 of integrals, because by this time OD of the controls had begun to fall. The relationship between
298 phytochemical concentration and growth integral was modeled with a 3-parameter log-logistic
299 model with the lower limit fixed at zero, corresponding to no growth as exposure concentration
300 approaches infinity.

301 $$g = f(x, (b, g_{max}, e) = \frac{g_{max}}{(1+exp(b(\log(x)-(\log(e))))} \quad (1)$$

302 where g denotes growth integral, x refers to thymol concentration, g_{max} denotes growth in the
303 absence thymol, and e is the phytochemical concentration at which 50% of the maximum inhibition

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304 is reached. The parameter *b* indicates the slope of the curve. From this model, we derived parameter
305 estimates and 95% confidence for the EC50 concentration *e,* and predictions for growth at each
306 thymol concentration.

307

308 Results

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

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312 In the floral transmission experiment, there was no effect of thymol nectar treatment on intensity of
313 infection 7 d after the foraging bout (Table 1, Figure 2). There were also no significant effects of the
314 plant species used for the trial (Table 1), nor was there evidence for differential effects of thymol
315 across plant species (thymol x plant species interaction, $\chi^2 = 1.791$, Df = 3, $P = 0.62$, removed from
316 final model). There was also no trend for an increase in effect of thymol with an increase in number
317 of thymol-containing flowers probed (non-significant thymol x flowers probed interaction, $\chi^2 = 0.14$,
318 Df = 1, $P = 0.71$, removed from final model). Number of inoculum drops probed during the trial also
319 did not explain significant variation in infection intensity ($\beta = 0.10 \pm 0.07$ SE, Table 1). Wing size was
320 negatively correlated with infection intensity ($\beta = -1.56 \pm 0.59$ SE, Table 1), indicative of lower
321 infection intensity in larger bees, which is consistent with previous results (Manson et al. 2010,
322 Palmer-Young, Hogeboom, Kaye, et al. 2017).

323 In *Crithidia* cell cultures, although a 75 min exposure to at least 200 ppm thymol completely
324 inhibited subsequent growth (Figure 3), the EC50 for growth integral was 120 ppm ± 2.3 ppm SE, or
325 4.6 fold higher than the fresh nectar concentration (Figure 3). However, growth was only slightly
326 affected by concentrations of 100 ppm (5-fold higher than mean nectar concentrations), and a

327 preliminary trial showed negligible effects of concentrations similar to those found in nectar (26 ppm;
328 Supplementary Figure 2). The estimated concentration needed to reduce the growth integral by only
329 10% (89.8 ppm ± 2.35 SE) was still over 3.4-fold higher than the concentration in the nectar.

330 Changes in cell morphology observed at the end of the exposure period were good indicators of
331 subsequent viability, with striking changes in both appearance and behavior at concentrations above
332 100 ppm (Supplementary Figure 4, Supplementary movies 1-7).

333

334 Discussion

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

342 The nectar concentration in our freshly collected, pooled sample (26.1 ppm) was nearly 5-fold the
343 mean concentration found in evaporated *Thymus vulgaris* nectar (Palmer-Young et al. 2016). We
344 expect that these higher concentrations reflect the fact that we analyzed fresh nectar. Thymol is a
345 volatile substance, and some nectar thymol will evaporate during the drying of samples. Due to the
346 importance of volatile compounds in pollinator foraging and behavior (Junker and Parachnowitsch
347 2015), we suggest that future studies analyze fresh nectar when feasible.

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

355 Variability in consumption of both thymol and parasite inoculum by bees during the foraging trial
356 may have reduced our power to detect an effect of the thymol treatment, but it is also possible that
357 the duration and magnitude of thymol exposure in the trials was insufficient to alter parasite
358 viability. To distinguish between low power to detect effects (due to experimental variability) and
359 true robustness of parasites to short-term thymol exposure, we performed a controlled *in vitro* trial
360 to determine the thymol concentrations necessary to inhibit parasite growth after a 75 minute
361 chemical exposure. In reality, bumble bee flowers may be visited– and presumably drained of
362 nectar—multiple times per hour (Ruiz-González et al. 2012). Although longer periods between visits
363 are possible, *Crithidia* infectivity was reduced by 75% during just 40 min outside the host (Schmid-
364 Hempel et al. 1999). Therefore, the effects of thymol over longer time periods may be irrelevant due
365 to reduction in *Crithidia* infectivity for other reasons. Hence, our experiments likely tested upper
366 estimates of the durations and concentrations of thymol exposure during transmission, yet still
367 showed no direct effects on parasite viability. These results indicate that (1) higher phytochemical
368 concentrations are necessary for inhibition of parasite growth when exposure is acute (75 min)
369 rather than chronic (5 d, (Palmer-Young et al. 2016, Palmer-Young, Sadd, and Adler 2017, Palmer-
370 Young, Sadd, Irwin, et al. 2017), and (2) nectar thymol concentrations appear too low to have direct
371 effects on parasite viability over durations typical of horizontal transmission at flowers.

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372 Given the absence of an effect of thymol on parasite transmission, does this floral phytochemical
373 likely play a role in pollinator-parasite infection dynamics? One possibility is that chronic thymol
374 exposure in hosts has direct effects on parasite replication. However, this appears to be theoretically
375 and empirically questionable. First, although nectar concentrations may be high enough to inhibit
376 growth when parasites are directly exposed to those concentrations over many days (Palmer-Young
377 et al. 2016), *Crithidia* inhabit the distal gut, and are exposed to concentrations that are likely much
378 lower than those in ingested nectar. We expect that thymol is passively absorbed across cell
379 membranes (Bakkali et al. 2008), such as those in the midgut, and also actively diluted by digestive
380 secretions and detoxified by cytochrome p450 and other enzymes. These enzymes have been shown
381 to mediate detoxification of nicotine (du Rand et al. 2017) and quercetin (Mao et al. 2017), and are
382 upregulated by thymol exposure in honey bees (Boncristiani et al. 2012). Second, empirical tests of
383 chronic oral thymol consumption by bumble bees may cause no reduction in live bee infection
384 intensity, even when phytochemicals are consumed at concentrations sufficient to inhibit growth *in*
385 *vitro.* Whereas just 12 ppm thymol and 50 ppm eugenol resulted in strong inhibition of parasite
386 growth in 12-well plates *in vitro* (Palmer-Young, Sadd, and Adler 2017), one study found no effects of
387 up to 7 d consumption of 20 ppm dietary thymol on *Crithidia* infection in *B. impatiens* (Biller et al.
388 2015), and another found no effects of 7 d consumption of 50 ppm dietary eugenol on *Crithidia*
389 infection (Palmer-Young et al. (In review)).

390 Despite these negative results, consumption of only 0.2 ppm thymol reduced *B. impatiens* infection
391 of *Crithidia* (Richardson et al. 2015). Moreover, studies in honey bees (Palmer-Young, Tozkar,
392 Schwarz, et al. 2017) suggested a possible host-mediated mechanism for thymol's effects on
393 infections. Consumption of six of seven phytochemicals, including thymol, upregulated transcription
394 of the antimicrobial peptide hymenoptaecin (Palmer-Young, Tozkar, Schwarz, et al. 2017), and
395 consumption of the hydroxycinnamic acid *p*-coumaric acid upregulated transcription of the
396 antimicrobial peptide abaecin and defensin1 (Mao et al. 2013). Even a single exposure of newly

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397 emerged bees to 0.2 ppm thymol resulted in reduced titers of Deformed Wing Virus after 5 d in the
398 colony (Palmer-Young, Tozkar, Schwarz, et al. 2017), and 20 ppm thymol reduced *Nosema* infection
399 (Costa et al. 2010). These reductions in infection could reflect thymol-mediated immune stimulation.
400 In contrast, application of concentrated thymol to honey bee hives—a treatment used to reduce
401 *Varroa* mite infestation (Imdorf et al. 1999)—reduced transcription of several immune genes
402 (Boncristiani et al. 2012). Similarly, dietary thymol did not affect infection with the trypanosomatid
403 *Lotmaria passim,* a relative of *Crithidia* (Palmer-Young, Tozkar, Schwarz, et al. 2017), nor did thymol
404 reduce *Crithidia* infection in *B. impatiens* in all cases (Biller et al. 2015). Future experiments that
405 measure both infection intensity and immune function are necessary to clarify the mechanism by
406 which thymol affects *Crithidia* and other pathogens, and the reasons for variability in the effects of
407 thymol and other phytochemicals on infection (Thorburn et al. 2015, Palmer-Young, Hogeboom,
408 Kaye, et al. 2017).

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

419

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435

436 ## Contribution of authors

437 LSA, REI and PCS conceived the collection of data on naturally-occurring phytochemical levels and
438 experimental manipulation of floral chemistry, and ECPY and BMS conceived the cell culture study.
439 PCS measured natural thymol concentrations, KWR conducted the field manipulative study with
440 guidance from LSA, and ECPY conducted the cell culture experiment. ECPY, LSA, and BMS analyzed
441 the data. KWR and ECPY wrote the manuscript. All authors revised the manuscript and agreed to its
442 submission.

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Tables

645 **Table 1. Predictors of infection intensity in *B. impatiens* 7 d after a foraging bout on *Crithidia*-**

646 **inoculated inflorescences.** Wing size refers to length of the right forewing marginal cell.

Predictor	χ^2	Df	P
Thymol	0.00094	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**

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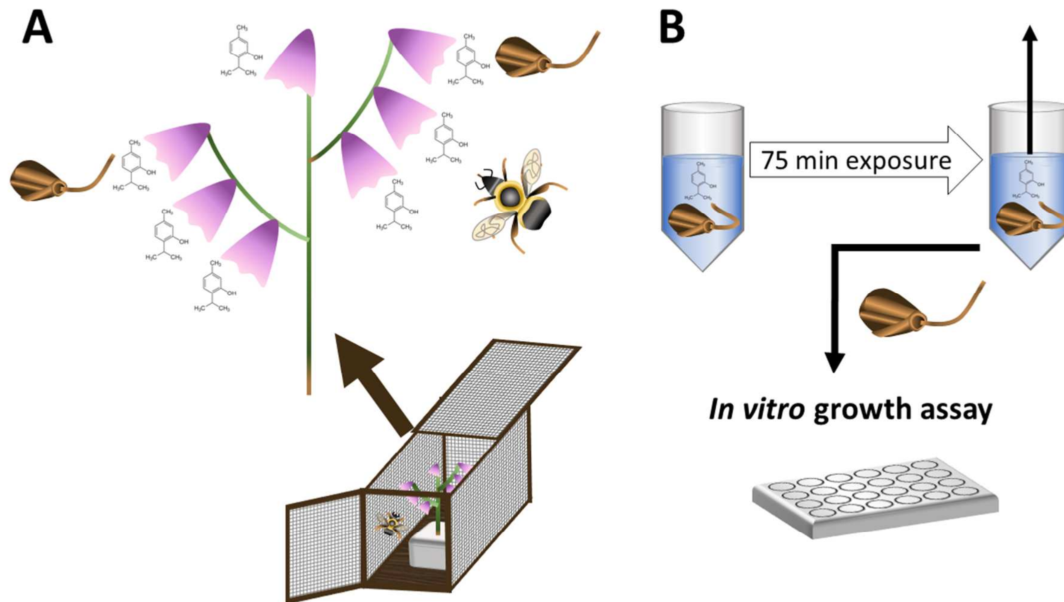
649 # Figure legends



650

651 **Figure 1. Schematic of experimental design. (A) Floral transmission**. Individual bumble bees (*B.*
652 *impatiens)* were allowed to forage on cut inflorescences inside a screened cage. A subset (2-4
653 flowers, depending on floral species) were inoculated with *Crithidia* (6000 parasite cells in 10 µL
654 flower^{-1}). Nectaries of every flower on the inflorescence received either 18 ppm thymol-containing
655 sucrose solution (2 µL flower^{-1}) or a 0 ppm thymol control solution. Bees were allowed to forage
656 until at least 5 flowers, including one inoculum droplet, had been probed, then reared in the lab for
657 7 d prior to assessment of infection intensity. **(B) *In vitro* thymol exposure of parasite cell cultures**.
658 *Crithidia* cell cultures were incubated with 0-500 thymol-containing Ringer's solution for 75 min.
659 Cultures were then centrifuged and washed with Ringer's solution to remove thymol, and
660 resuspended in growth medium at a final concentration of 500 cells µL^{-1}. Post-treatment growth was
661 measured by spectrophotometry over the subsequent 70 h.

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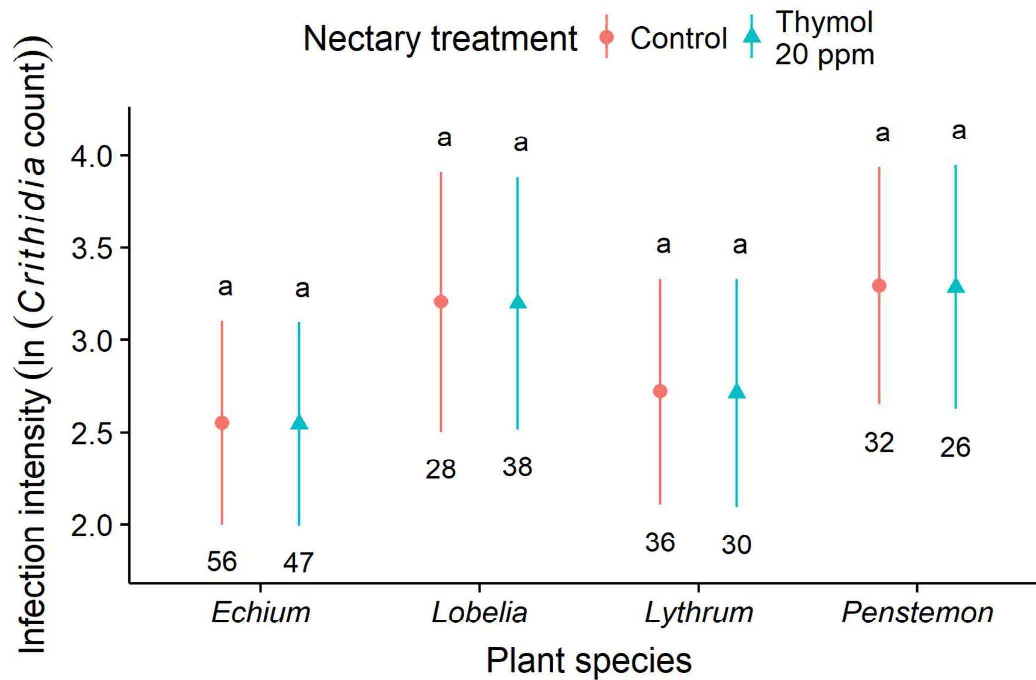

663

664 **Figure 2. Addition of thymol-containing sucrose solutions to nectaries did not affect acquisition of**
665 ***Crithidia* infection by *Bombus impatiens*** during a foraging bout on *Crithidia*-inoculated flowers. X-
666 axis indicates flowering plant species. Y-axis shows infection intensity as the natural log of number of
667 parasite cells counted in 0.02 µL gut extract from bees dissected 7 d post-trial. Points and error bars
668 show model means and 95% confidence intervals. Different lower-case letters indicate significant
669 differences in *post hoc* pairwise comparisons for effects of thymol within each plant species. Plant
670 species had a non-significant effect on infection (Table 1).

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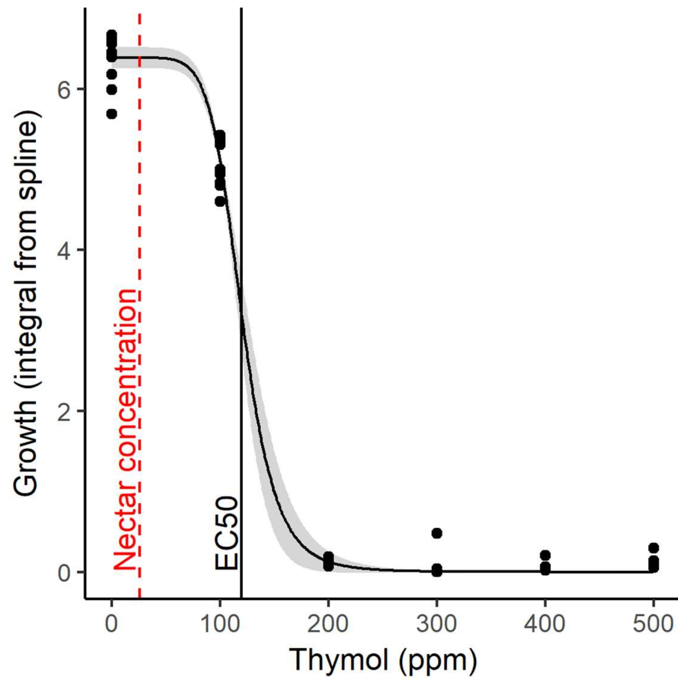

673

674 **Figure 3. Effects of 75 minute exposure of *Crithidia* cell cultures to thymol on subsequent *in vitro***
675 **growth.** Dose-response curve relates thymol concentration (x-axis) to area under the growth curve
676 (y-axis). Shaded bands show 95% confidence intervals from log-logistic model. Solid black line: EC50
677 concentration. Dashed red line: thymol concentration in *Thymus vulgaris* nectar sample. EC50:
678 Effective concentration 50%. OD: Optical density (630 nm wavelength). See Supplementary figure 3
679 for full growth curves.

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681 # Supporting Information

682 **Supplementary methods.** Plant seed sources and rearing conditions.

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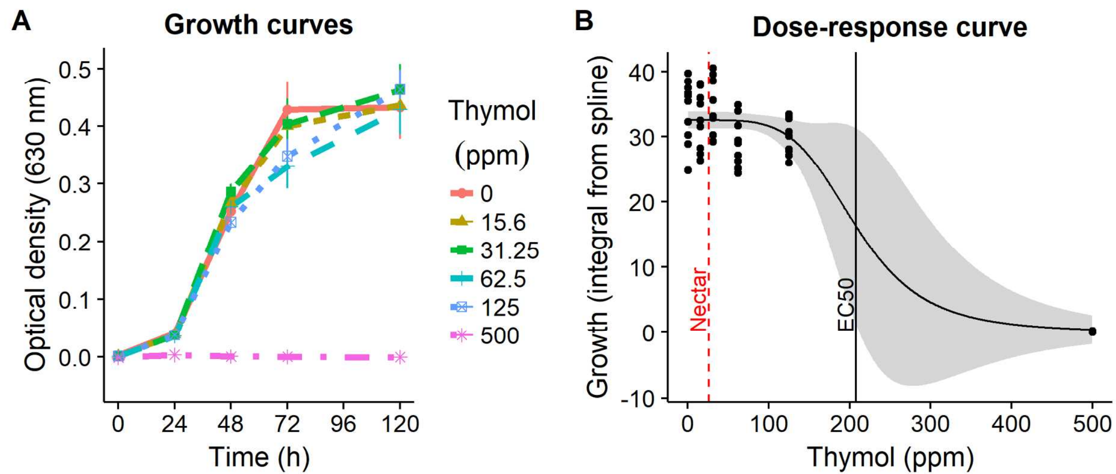
685 **Supplementary figure 1.** *Bombus impatiens* forages on *Penstemon digitalis* inflorescence inside of

686 experimental cage. One-liter water bottle in background (for scale).



687

688 **Supplementary figure 2.** Results of preliminary experiment that tested effects of short-term (75 min)
689 exposure to thymol at lower concentrations. (A) Growth curves showing growth (measured by
690 optical density) over time following exposure to different thymol concentrations, indicated by
691 different colored lines. Point show means and 95% confidence intervals for n = 10 replicates per
692 treatment concentration. (B) Dose-response curve that relates thymol concentration (x-axis) to area
693 under the growth curve (shown in (A)). Note poor precision of fit when compared to Figure 3B, due
694 to lack of observations between 125 and 500 ppm. Solid black line: EC50 concentration. Dashed red
695 line: thymol concentration in *Thymus vulgaris* nectar sample.

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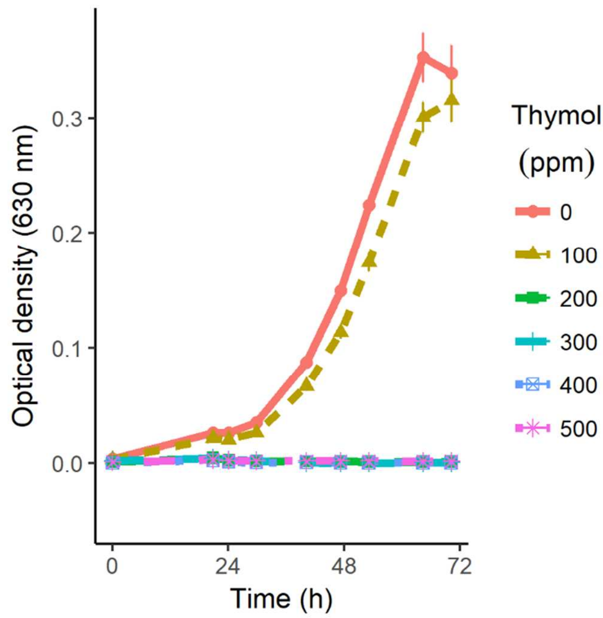

697

698 **Supplementary figure 3.** Growth curves of *Crithidia* cell cultures after 75 minute exposure to thymol.
699 Growth was measured by optical density following exposure to different thymol concentrations,
700 indicated by different colored lines. Points show means and 95% confidence intervals for n = 10
701 replicates per treatment concentration. Integrals used for dose-response curves were calculated
702 after exclusion of the final time point, due to fall in OD of control samples (0 ppm).

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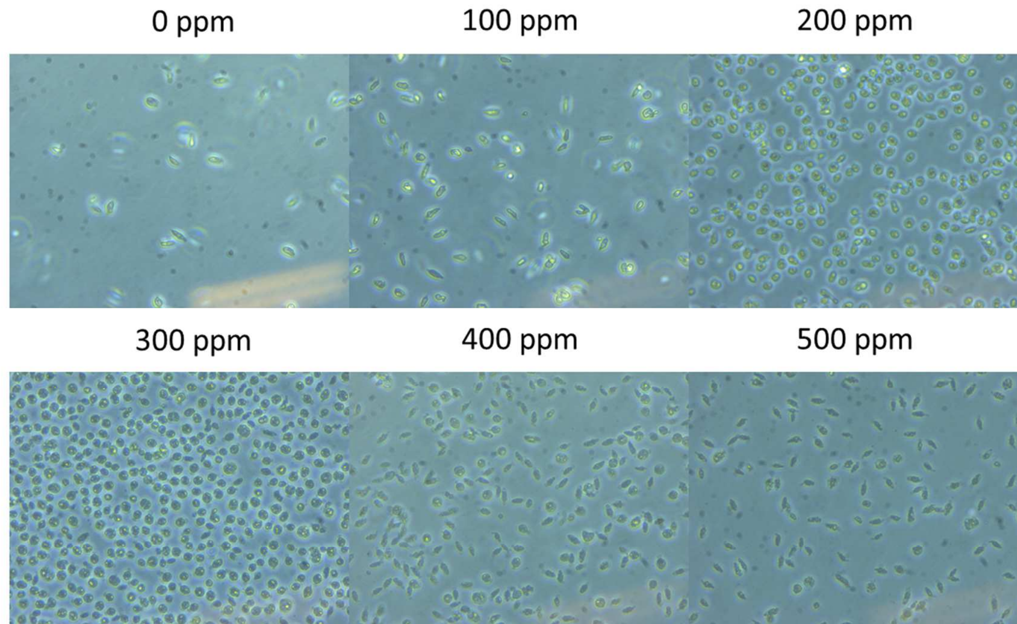

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705 **Supplementary figure 4.** Micrographs showing morphological effects of thymol exposure on
706 *Crithidia* cell cultures across a range of concentrations from 0 to 500 ppm. The photographed field
707 was chosen haphazardly from a 1 mL sample volume; cell densities in the images are not meant to
708 be representative of the entire sample.

709 **Supplementary movies 1-6.** Video recordings showing morphological effects of thymol exposure on
710 *Crithidia* cell cultures across a range of concentrations from 0 to 500 ppm. Control cells were oblong
711 and characterized by relatively sharp anterior and posterior ends and rapid swimming. Cells exposed
712 to 100 ppm appeared somewhat compressed along the major axis, with squared-off appearance, but
713 were still motile, although they swam less rapidly than the control cells. Cells exposed to 200 ppm
714 assumed a curled, spheroid appearance and did not swim at all. Cells exposed to 300 ppm had a
715 spheroid appearance as in the 200 ppm treatment, but internal morphology appeared more granular.
716 Cells exposed to 400 ppm were a mix of bulging spheroids and shriveled, possibly lysed cells with a
717 frayed and ragged appearance. After exposure to 500 ppm, almost all cells appeared shriveled and
718 deformed.

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719 **Supplementary movie 7.** Video recording of *Crithidia* cell cultures following exposure to 0 ppm (first
720 half) or 500 ppm thymol (second half). Note lack of movement and wrinkling of cell membrane and
721 organelles in thymol-exposed parasite cells.

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