Pollen extracts and constituent sugars increase growth of a trypanosomatid parasite of bumble bees

Evan C Palmer-Young Corresp., [1] , **Lucy Thursfield** [2]

[1] Organismic and Evolutionary Biology, University of Massachusetts at Amherst, Amherst, MA, United States

[2] Royal Botanic Gardens, Kew, Richmond, Surrey, United Kingdom

Corresponding Author: Evan C Palmer-Young
Email address: ecp52@cornell.edu

Phytochemicals produced by plants, including at flowers, function in protection against plant diseases, and have a long history of use against trypanosomatid infection. Floral nectar and pollen, the sole food sources for many species of insect pollinators, contain phytochemicals that have been shown to reduce trypanosomatid infection in bumble and honey bees when fed as isolated compounds. Nectar and pollen, however, consist of phytochemical mixtures, which can have greater antimicrobial activity than do single compounds. This study tested the hypothesis that pollen extracts would inhibit parasite growth.Extracts of six different pollens were tested for direct inhibitory activity against cell cultures of the bumble bee trypanosomatid gut parasite *Crithidia bombi*.

Surprisingly, pollen extracts increased parasite growth rather than inhibiting it. Pollen extracts contained high concentrations of sugars, mainly the monosaccharides glucose and fructose. Experimental manipulations of growth media showed that supplemental monosaccharides (glucose and fructose) increased maximum cell density, while a common floral phytochemical (caffeic acid) with inhibitory activity against other trypanosomatids had only weak inhibitory effects on*Crithidia bombi*. These results indicate that, although pollen is essential for bees and other pollinators, pollen may promote growth of intestinal parasites that are uninhibited by pollen phytochemicals and, as a result, can benefit from the nutrients that pollen provides.

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Authors:

Evan C Palmer-Young[1] and Lucy Thursfield[2]

Affiliations:

[1]Organismic & Evolutionary Biology, University of Massachusetts Amherst, Amherst MA 01003, United States

[2] Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AE, United Kingdom

Corresponding author: Evan C Palmer-Young[1]

Email: ecp52@cornell.edu[1]

Abstract

Phytochemicals produced by plants, including at flowers, function in protection against plant diseases, and have a long history of use against trypanosomatid infection. Floral nectar and pollen, the sole food sources for many species of insect pollinators, contain phytochemicals that have been shown to reduce trypanosomatid infection in bumble and honey bees when fed as isolated compounds. Nectar and pollen, however, consist of phytochemical mixtures, which can have greater antimicrobial activity than do single compounds. This study tested the hypothesis that pollen extracts would inhibit parasite growth. Extracts of six different pollens were tested for direct inhibitory activity against cell cultures of the bumble bee trypanosomatid gut parasite *Crithidia bombi*.

Surprisingly, pollen extracts increased parasite growth rather than inhibiting it. Pollen extracts contained high concentrations of sugars, mainly the monosaccharides glucose and fructose. Experimental manipulations of growth media showed that supplemental monosaccharides (glucose and fructose) increased maximum cell density, while a common floral phytochemical (caffeic acid) with inhibitory activity against other trypanosomatids had only weak inhibitory effects on *Crithidia bombi*. These results indicate that, although pollen is essential for bees and other pollinators, pollen may promote growth of intestinal parasites that are uninhibited by pollen phytochemicals and, as a result, can benefit from the nutrients that pollen provides.

Introduction

Plants provide nutrients that sustain the growth and reproduction of many animal species, but also contain antimicrobial phytochemicals that may counteract infection in plant-eating animals (de Roode et al., 2013). Insect pollinators such as bumble bees, whose diets consist exclusively of phytochemical-rich nectar and pollen, are important for agricultural production. However, like honey bees, bumble bees are threatened by parasite-related decline (Goulson et al., 2015). Because bumble bees have abundant natural access to phytochemicals, antimicrobials from flowers could provide a natural source of medicinal compounds that could counteract infection in pollinator populations.

Trypanosomatids are parasites that, in addition to afflicting over 12 million humans, infect and decrease fitness of many species of insects (Maslov et al., 2013). For example, the newly described *Jaenimonas drosophilae* increases mortality of pupae and decreases fecundity of adult *Drosophila melanogaster* (Hamilton et al., 2015). Trypanosomatid infection can also be pervasive in populations of wild and managed bees (Shykoff & Schmid-Hempel, 1991; Schwarz et al., 2015). With spread of parasites facilitated by use of shared flowers (Durrer & Schmid-Hempel, 1994; Graystock, Goulson & Hughes, 2015), infection in some areas may exceed 80% in bumble bees (Shykoff & Schmid-Hempel, 1991; Gillespie, 2010; Popp, Erler & Lattorff, 2012) and 90% in honey bees (Runckel et al., 2011). Correlative evidence implicates trypanosomatid infection as a factor in honey bee colony loss. In Belgian honey bees, infection with *Lotmaria passim* (formerly named and reported as *Crithidia mellificae* (Schwarz et al., 2015)) was correlated with colony death (Ravoet et al., 2013). In the United States, *Lotmaria passim* infection intensity was over six-fold higher in hives that suffered from Colony Collapse

Disorder than in hives that did not collapse (Cornman et al., 2012). In bumble bees, *Crithidia bombi* infection is similarly detrimental for individuals and colonies. Infection increased the rate of death in starved workers (Brown, Schmid-Hempel & Schmid-Hempel, 2003), provoked potentially costly immune responses (Sadd & Barribeau, 2013), altered foraging behavior and learning (Gegear, Otterstatter & Thomson, 2005), and decreased colony fitness (Shykoff & Schmid-Hempel, 1991).

Phytochemicals have well-known antimicrobial properties that inhibit infection not only in plants (Bennett & Wallsgrove, 1994; Huang et al., 2012), but also in animals that consume phytochemical-rich plant materials (Karban & English-Loeb, 1997; Singer, Mace & Bernays, 2009; de Roode et al., 2013). Plant-based therapeutics have a long history of traditional use against trypanosomatid infection, and recent studies have confirmed the inhibitory activity of both plant extracts and isolated phytochemicals against trypanosomatid cell cultures (Merschjohann & Steverding, 2006; Santoro et al., 2007a; Wink, 2012). Similarly, phytochemicals may have medicinal effects in bees infected with the trypanosomatid *C. bombi* (Manson, Otterstatter & Thomson, 2010; Baracchi, Brown & Chittka, 2015; Richardson et al., 2015; Biller et al., 2015), such that nectar and pollen of phytochemical-rich wildflowers and crops could provide medicinal resources for pollinators.

To date, all studies on the medicinal effects of phytochemicals on bees have tested single compounds. However, plants contain mixtures of phytochemicals that can have synergistic effects against both insects (Berenbaum & Neal, 1985; Berenbaum, Nitao & Zangerl, 1991) and microbes (Fewell & Roddick, 1993), including *C. bombi* (Palmer-Young et al., 2017). The defensive compounds in phytochemical-rich plants, such as milkweed (*Danaus* spp.) (Gowler et

al., 2015), can also counteract pathogens of plant-eating insects and other animals (de Roode et al., 2013). In a mouse model of *Plasmodium falciparum* malaria, crude *Artemisia annua* plant extract had a stronger medicinal effect than did equivalent amounts of purified artemisinin (Elfawal et al., 2012). *Artemisia* spp. extracts can also have inhibitory effects against trypanosomatids, such as the *C. bombi* relative *Leishmania major*, where the effects of crude plant extracts and phytochemically complex essential oils can have greater inhibitory activity than do individual compounds (Efferth et al., 2011). Similarly, essential oil from *Thymus vulgaris* plants was a more powerful inhibitor of *Trypanosoma cruzi* growth than was the purified main constituent thymol (Santoro et al., 2007b). Together, these studies suggest that the phytochemical mixtures found in natural plant materials may be more effective inhibitors of parasites than are isolated chemicals.

Pollen and nectar consumed by bees contain diverse phytochemicals (Dobson & Bergstrom, 2000; Adler, 2001; Jakubska et al., 2005). For example, over 100 compounds were found in the nectar of a single orchid species (Jakubska et al., 2005), and bumble bees forage from a variety of floral species throughout the growing season (Goulson & Darvill, 2004; Heinrich, 2004; Vaudo et al., 2015). Pollen contains particularly high phytochemical concentrations that can exceed those in nectar by several orders of magnitude (Detzel & Wink, 1993; London-Shafir, Shafir & Eisikowitch, 2003, p.; Palmer-Young et al., 2016). Hence, pollen could be expected to have particularly strong effects on parasites that are susceptible to inhibition by phytochemicals. However, studies in bees have found that, even though pollen consumption increased expression of immune genes (Brunner, Schmid-Hempel & Barribeau, 2014), dietary pollen increased *C. bombi* infection intensity (Logan, Ruiz-González & Brown, 2005; Conroy et al., 2016) in *Bombus terrestris* and *B. impatiens*. One hypothesis to explain the positive effects of

pollen was that nutrients in pollen promote parasite growth. Pollen is rich in carbohydrates, proteins, and other nutrients that are essential for bee reproduction (Roulston & Cane, 2000), but these nutrients could also benefit parasites that can tolerate high phytochemical concentrations (Palmer-Young et al., 2016).

To test the alternative hypotheses that (a) pollen phytochemicals inhibit parasites and (b) pollen nutrients benefit parasites, we tested the direct effects of six pollens, a nectar phytochemical with demonstrated effects against trypanosomatids, and monosaccharides on *C. bombi* growth in cell culture.

Materials and Methods

Overview

Three experiments were conducted to elucidate the effects of pollen extracts on *in vitro* growth of *C. bombi* cell cultures. Experiments evaluated the effects of (1) extracts of single pollens, (2) extracts of mixed pollens, and (3) specific chemicals (sugar and the floral phytochemical caffeic acid) in order to better understand the mechanisms by which pollen extracts affected growth. In addition, the pollen extracts were chemically analyzed by HPLC to determine sugar content.

Parasite culturing

Crithidia bombi is a flagellated trypanosomatid parasite that infects bumble bees (Lipa & Triggiani, 1988; Schmid-Hempel & Tognazzo, 2010). Phylogenetic analyses (Schwarz et al., 2015) showed that *C. bombi* belongs to one of four clades in the subfamily Leishmaniinae, whih is one of roughly 12 trypanosomatid subfamilies (Maslov et al., 2013). The clade of *C. bombi*

121 also includes the honey bee parasites *C mellificae* and *L. passim* as well as *Leptomonas* spp..
122 Other clades within the Leishmaniinae include the *Leishmania* spp. human parasites and the
123 mosquito-infecting model organism *Crithidia fasciculata* (Schwarz et al., 2015).

124 *Crithidia bombi* was isolated in 2013 by Ben Sadd from feces of wild *Bombus impatiens*
125 near Normal, IL by flow cytometry (Salathé et al., 2012, p.) and kept frozen at -80 °C until
126 several weeks before the experiments. Thereafter, cells were grown at 27 °C in 50 cm^2 tissue
127 culture flasks and transferred to fresh media every 3-4 days. No special permits were required
128 for the collection. Cultures are available upon request, provided appropriate documentation and
129 permissions are supplied. The growth medium was composed of a tryptose-liver broth
130 (containing 2.2 mM glucose) supplemented with B vitamins, haemin, 10% heat-inactivated fetal
131 bovine serum. In addition, the medium contained 10 mM fructose and 2.5 mM free proline
132 (Salathé et al., 2012, p.).

133 Pollen and phytochemical treatments

134 Six types of bee-collected pollen—buckwheat *(Fagopyrum esculentum)*, lotus (*Nelumbo*
135 *nucifera)*, poppy *(Papaver somniferum)*, rapeseed *(Brassica napus)*, sunflower *(Helianthus*
136 *annuus)*, and tea *(Camellia sinensis)*— were obtained from Changge Huading Wax Industry
137 (Henan, China) in 2015. The pollens were stored at -20 °C and sorted to remove heterogeneous
138 granules. For extraction, 6 g of pollen was incubated for 24 h at room temperature in constant
139 darkness with 20 mL of 50% v/v aqueous methanol in a 50 mL conical tube. The 50% methanol
140 was used as a solvent due to its widespread application in phytochemical extraction of pollen
141 (Serra Bonvehi, Soliva Torrentó & Centelles Lorente, 2001) and other plant tissues (Keinänen,
142 Oldham & Baldwin, 2001). Samples were shaken at 180 rpm on a shaker table for the first 20

143 min of the extraction. After 24 h, tubes were centrifuged (30 min, 2700 g) and the supernatant
144 removed, sterile-filtered, aliquoted, and stored at -80°C until use. The mixed-pollen extract
145 consisted of equal volumes of buckwheat, rapeseed, and sunflower extracts, which were
146 combined immediately before the experiment.

147 Caffeic acid was used as a representative phytochemical to evaluate possible negative and
148 positive effects of pollen constituents on *C. bombi.* This hydroxycinnamic acid is likely to be
149 widespread in bee diets, as it was the most widespread phytochemical in honey extracts, with
150 occurrence in all 14 tested types of Turkish honey (Can et al., 2015); cinnamic acids and other
151 phenolics are also abundant in pollen (Campos et al., 1997; Serra Bonvehi, Soliva Torrentó &
152 Centelles Lorente, 2001; Almaraz-Abarca et al., 2004). Caffeic acid inhibited the
153 trypanosomatids *Leishmania donovani, Trypanosoma cruzi,* and *T. brucei* (Tasdemir et al., 2006;
154 Grecco et al., 2014, p. 20), which suggested that caffeic acid could inhibit *C. bombi* as well.
155 However, caffeic acid is also a powerful antioxidant, with ability to scavenge reactive oxygen
156 species that exceeds that of ascorbic acid and is comparable to that of tocopherols (Chen & Ho,
157 1997; Almaraz-Abarca et al., 2004). Antioxidant activity of caffeic acid and other pollen
158 components (Almaraz-Abarca et al., 2004) might protect *Crithidia bombi* from stress incurred
159 during the experiment, such as shaking and handling, and in the wild, where parasites encounter
160 temperature changes, osmotic shock, UV light, and pro-oxidant enzymes of the bee immune
161 system that may contribute to oxidative stress (Sadd & Barribeau, 2013; Vanaerschot et al.,
162 2014). For experiments, caffeic acid was dissolved to a concentration of 22.2 mM (4 mg mL^{-1})
163 in 50% methanol and tested at final concentrations of up to 1.85 mM. This concentration is more
164 than 30-fold the levels that occur in most types of honey (Can et al., 2015) and 10-fold the mean
165 cinnamic acid concentration in pollen (Serra Bonvehi, Soliva Torrentó & Centelles Lorente,

166 2001). Therefore, the tested concentration range was likely to detect any direct effects against
167 trypanosomatids that could be attributed to phytochemical consumption by bees.

168 The addition of sugar to the medium was also tested for positive or negative effects on
169 growth. Bee-collected pollen, such as that used to create the extracts in this study, is rich in
170 sugars from nectar, which are added to the pollen by bees during collection (Roulston & Cane,
171 2000). Previous experiments with *C. bombi* cell cultures showed that growth was strongly
172 inhibited when 20% of growth medium was replaced by sugar syrup (Cisarovsky & Schmid-
173 Hempel, 2014). However, many trypanosomatids prefer sugars to proline as a carbon source
174 (Lamour et al., 2005; Bringaud, Rivière & Coustou, 2006), which suggests that addition of sugar
175 to the tryptose- and liver-based growth medium (Salathé et al., 2012) could enhance growth. To
176 evaluate the effects of pure sugars relative to pollen extracts, a 1.1 M sugar solution, consisting
177 of equimolar amounts of glucose and fructose in 50% methanol, was added to the growth
178 medium at final concentrations of up to 93 mM. This sugar concentration was chosen to slightly
179 exceed the likely sugar concentration in the pollen extracts, which was estimated *a priori* as
180 ~500-660 mM. This estimate was based on a sugar content of ~1.7-2.2 mol kg^{-1} (~30-40%
181 monosaccharides by weight in the pollen (Todd & Bretherick, 1942; Herbert & Shimanuki,
182 1978; Roulston & Cane, 2000; Campos et al., 2008), with ~30% pollen in the extract. The sugar
183 composition was chosen to reflect the roughly equal amounts of glucose and fructose that have
184 been found in nectar and honey (London-Shafir, Shafir & Eisikowitch, 2003, p.; Ohmenhaeuser
185 et al., 2013). Although pollen can contain sugars other than monosaccharides (Herbert &
186 Shimanuki, 1978), monosaccharides were used because the bee intestine rapidly hydrolyzes
187 disaccharides to glucose and fructose (Nicolson, 1998), which likely leaves only
188 monosaccharides in the distal intestine where trypanosomatids become established (Lipa &

189 Triggiani, 1988; Schwarz et al., 2015). The 1.1 M sugar solution was tested at up to 8.3%
190 concentration by volume (93 mM monosaccharides in growth medium).

191 Experimental design

192 Each experiment tested the effects of treatments on growth of parasite cell cultures in 96-well
193 microplates. The first experiment tested extracts of six different species of pollen. The second
194 experiment tested the effects of buckwheat, rapeseed, and sunflower pollen extracts, individually
195 and in a mixture that consisted of equal proportions of each of the three extracts (i.e., one-third
196 buckwheat, one-third rapeseed, and one-third sunflower extract by volume). The third
197 experiment tested the effects of added chemicals, which included the common floral
198 phytochemical caffeic acid and a sugar solution. This third experiment included buckwheat
199 pollen extract as a positive control to verify the effects of pollen extracts observed in the
200 previous two experiments.

201 To test the effects of pollen extracts, extract of each of the six pollens was dissolved at
202 six concentrations by two-fold serial dilution. Concentrations ranged from 0-5% (for pollen
203 extracts) or 0-8.3% (for chemical additions) final concentration by volume in growth medium.
204 Additional 50% methanol was added to samples of lesser concentrations to equalize methanol
205 concentrations across samples. The inner 48 wells of a 96-well plate were filled with 100 µL of
206 treatment solution (at 2x final concentration) and 100 µL of a suspension of *C. bombi* cells (10^3
207 cells μL^{-1}), to achieve an initial cell density of 500 cells μL^{-1} (250 cells μL^{-1} for chemical
208 addition experiment). Outer wells were filled with 200 µL sterile water to mitigate edge effects.
209 Plates were sealed with laboratory film and placed inside zippered sandwich bags to minimize
210 evaporation. Samples were incubated without shaking at 27 °C in a dark incubator. Growth was

measured as optical density (630 nm) at 24 h intervals for 5 d by a spectrophotometer. Before each growth measurement, plates were shaken on a microplate shaker (30 s, 1000 rpm, 4 mm orbit) to homogenize and resuspend the cells. In addition, immediately before each spectrophotometer reading, the plate cover used during incubation was exchanged for a dry plate cover under sterile conditions, to prevent condensation from interfering with optical density measurements. Wells that contained treatment media without cells and were used to control for changes in optical density independent of parasite growth. Experiments included n = 8 (for pollen extracts) or n = 5 (for chemical additions) replicate samples per treatment concentration, plus the n = 2 negative control wells of cell-free treatment medium. Final concentrations of methanol were 2.5% (for pollen extracts) or 4.17% (for chemical additions) by volume.

Analysis

Because no extracts or chemicals fully inhibited growth, EC50 values could not be calculated. Instead, we used linear regression to test for concentration-dependent changes in parasite growth. Treatment concentration (in percent extract for pollen extracts and mM for chemical additions) was used as the predictor variable. For the Caffeic acid + Sugar treatment in the chemical additions experiment, mM sugar was used as the predictor, to better compare the effects of sugar with versus without caffeic acid. Maximum optical density at 630 nm, estimated using a model-free spline fit, (Kahm et al., 2010), was used as the response variable. Separate models were fitted for each pollen extract or chemical. P-values were adjusted with a Bonferroni correction to account for multiple tests within each experiment. Graphs were produced with the R package ggplot2 (Wickham, 2009, p. 2).

232 Chemical analyses

233 Sugar contents of each type of pollen extract were determined by HPLC (Alliance e2695 HPLC
234 system, Waters Co., USA) coupled with an evaporative light scattering detector (ELSD, Waters
235 2424, USA). Extracts were separated on a COSMOSIL Sugar-D column (4.6 mm I.D. × 250 mm
236 length, Nacalai Tesque, Kyoto, Japan) at a column temperature of 30 °C. The mobile phase was
237 80% acetonitrile and 20% methanol throughout the run, with a flow rate of 0.9 mL min^{-1}.
238 Quantities of sugar were determined based on external standard curves from analysis of pure
239 solutions of aqueous fructose, glucose, and sucrose. Each sample had a total run time of 30
240 minutes (including column regeneration). Sugar contents are expressed as means of two (fructose
241 and sucrose) or three (glucose) technical replicates.

242 # Results

243 Extracts of each of the six pollens increased *C. bombi* growth, as measured by the maximum cell
244 density achieved during the 5 d incubation period (Figure 1); the increase in growth was
245 significant in analysis by linear regression (Table 1). Relative to the pollen-free control, addition
246 of 5% extract of each pollen resulted in approximately 50% higher maximum density. A mixture
247 of buckwheat, rape, and sunflower extracts had effects that were similar to those of the
248 individual pollens in isolation (Figure 2).

249 In HPLC analyses, pollen extracts were found to contain considerable amounts of sugar,
250 mainly the monosaccharides fructose and glucose (Figure 3), with sucrose found in tea and
251 sunflower extracts, but in relatively small amounts. Total sugar content ranged from 192 mM in
252 tea pollen extract to 789 mM in rape pollen extract.

253 In the test of specific additional chemicals, both the buckwheat pollen extract (positive
254 control) and addition of sugar solution resulted in increased growth (Figure 4, Table 1), whereas
255 the common floral phytochemical caffeic acid had only weakly inhibitory effects at up to 1.85
256 mM (Figure 4, Table 1), which is an order of magnitude higher than any concentration
257 documented among different types of honey (Can et al., 2015). Additional sugar had similar
258 effects whether it included caffeic acid (coefficient = 0.0028 ± 0.00060 SE) or not (coefficient =
259 0.0034 ± 0.00024 SE, Table 1). Although a transient decrease in growth was found at
260 intermediate caffeic acid concentrations (Supplementary Figure 3), the greatest growth inhibition
261 occurred at intermediate concentrations, suggesting that inhibition reflected the position of the
262 sample on the plate rather than the effects of the phytochemical. As can be seen from the growth
263 curves of the buckwheat, sugar, and caffeic acid + sugar treatments, early growth was often poor
264 in the samples of intermediate concentration that were incubated in the center of the plate. We
265 attribute this effect to toxicity of the methanol, which would have dissipated relatively slowly
266 from the samples in the center of the plate as compared to the samples at the periphery. In
267 previous tests of three different *C. bombi* strains, including the IL13.2 strain used here (Palmer-
268 Young et al., 2016), only weak effects of caffeic acid occurred at concentrations up to 1.39 mM
269 (Supplementary Figure 4). None of the tested concentrations resulted in >50% growth inhibition,
270 which precluded estimation of an EC50 concentration.

271

272 Discussion

273 These experiments indicate that pollen extracts can increase growth of an intestinal parasite, and
274 that the growth-promoting effects of pollen extracts can be reproduced by addition of similar

amounts of a sugar solution. Pollen phytochemicals appear to be insufficient to stop growth of *C. bombi*, and moreover, pollen appears to contain substances that improve trypanosomatid growth. This result is consistent with previous experiments that showed a decrease in infection intensity in bees deprived of pollen; our results suggest a mechanism by which pollen may directly promote trypanosomatid infection. The positive effects of pollen nutrients on *C. bombi*, a hindgut trypanosomatid, suggests the potential for facilitation of nutrient-limited hindgut parasites by midgut parasites that interfere with nutrient absorption. In addition, the phytochemical tolerance of *C. bombi* relative to that of bloodstream trypanosomatids invites further study on adaptation to phytochemicals in different trypanosomatid species, and variation in tolerance across life stages.

Phytochemical insensitivity

Crithidia bombi growth was not inhibited by any of the pollen extracts. This was unexpected in the context of current literature on bumble bee-*Crithidia* interactions, which has suggested that phytochemical ingestion can reduce *C. bombi* infection (Manson, Otterstatter & Thomson, 2010; Baracchi, Brown & Chittka, 2015; Richardson et al., 2015). On the contrary, growth was increased by addition of pollen extracts. Similarly, growth of *C. bombi* in the present study was only weakly inhibited (<50% decrease in maximum OD, Figure 3) by caffeic acid at concentrations of over 1.8 mM. From an ecological perspective, the 1.8 mM concentration is 6-fold greater than the concentration found in pollen (0.3 mM (Šarić et al., 2009)) and 12-fold greater than the 0.15 mM (26.8 ppm) maximum concentration found in any type of honey, including the *Quercus* spp. honey from bees that foraged on the oak tree sap (Can et al., 2015). No other type of honey exceeded 0.05 mM (8.8 ppm (Can et al., 2015)). Hence, it appears that *C. bombi* is little inhibited by naturally occurring concentrations of caffeic acid or other compounds

297 that may have been present in the six different pollen extracts. The insensitivity of *C. bombi* to
298 naturally occurring levels of hydroxycinnamic acids is consistent with previous results (Palmer-
299 Young et al., 2016).

300 The phytochemical tolerance observed here is greater than that found in some
301 bloodstream-form trypanosomatids, but not unprecedented for trypanosomatid life stages found
302 in insects. Concentrations needed for 50% growth inhibition (EC50) ranged from 0.006 to 0.31
303 mM (1.1-56 ppm) caffeic acid in bloodstream forms of *L. donovani*, *T. brucei,* and *T. cruzi*,
304 (Tasdemir et al., 2006; Grecco et al., 2014, p. 20). However, tolerance can be much higher in
305 other trypanosomatid species and life stages. *Leishmania* promastigotes, the stage found in the
306 insect gut, retained viability at concentrations comparable to those tested in our study, with
307 inhibitory concentrations of 1.05 mM for *Leishmania amazonensis* and >2.78 mM for *L.*
308 *braziliensis* (Passero et al., 2011). Similarly, caffeic acid EC50 was only 3.9-6.1 nM for
309 intracellular bloodstream amastigotes of four tested *Leishmania* spp., but >2800 nM (2.8 μM) for
310 promastigotes, the stage found in the insect gut; the same trend was observed for seven other
311 compounds (Radtke et al., 2003). The higher phytochemical tolerance of extracellular,
312 promastigote *Leishmania* relative to bloodstream-form, intracellular amastigotes was confirmed
313 in other studies (Kolodziej & Kiderlen, 2005). These differences in sensitivity may reflect
314 different levels of exposure to phytochemicals during different life stages, or costs of resistance
315 in bloodstream forms. More study is needed to understand the basis of differential resistance
316 across life stages and species, which could have relevance to development of drug resistance.

317 Commensurate with its evolutionary history of chronic phytochemical exposure in the
318 bee gut, *C. bombi* appears to be well adapted to phytochemicals, including those that are toxic to

other trypanosomatids and even those initially shown to reduce infection intensity. For example, *C. bombi* exhibited EC50 values for several phenolics that were orders of magnitude higher than those reported for other trypanosomatid species (Palmer-Young et al., 2016), and infection was robust to thymol, anabasine, and nicotine under controlled conditions (Biller et al., 2015; Thorburn et al., 2015). Although the present study did not address possible host-mediated effects of phytochemicals on infection, such as phytochemical-induced stimulation of immune responses (Borchers et al., 1997; Mao, Schuler & Berenbaum, 2013) or changes in gut kinetics (Tadmor-Melamed et al., 2004) that have been observed in other species and could alter trypanosomatid attachment to the gut wall (Schwarz et al., 2015), the fact that none of the six pollens inhibited growth demonstrates that *C. bombi* is robust to many of the phytochemicals in the diet of its hosts.

Many trypanosomatids complete their life cycle in two hosts, which may include insects, mammals, and plants (Maslov et al., 2013). It would be intriguing to use the comparative method to test whether evolutionary history is predictive of phytochemical tolerance. To accomplish this, future studies could compare phytochemical tolerance of species that occupy niches with different levels of phytochemical exposure. In order of decreasing intensity of phytochemical exposure, these could include (a) species that utilize plants as hosts, (b) those that are gut parasites of herbivores, and (c) species and life stages that live in the blood and are transmitted by blood-feeding insects. Trypanosomatids with an evolutionary history of phytochemical exposure would be expected to have higher phytochemical tolerance than those that encounter phytochemicals only occasionally or indirectly.

Increase in growth was reproduced by addition of sugar

The growth-promoting properties of the pollen extracts may be attributable to its constituent nutrients, in particular monosaccharides, and possibly amino acids. Pollen collected by corbiculate bees, such as bumble bees, is moistened with nectar, which renders it sufficiently sticky to be carried in the bee's corbicula (pollen basket) (Roulston & Cane, 2000). Thus, bee-collected pollen contains considerable amounts of carbohydrate, including up to 40% sugars by weight (Todd & Bretherick, 1942; Roulston & Cane, 2000). Although the osmolarity of very high (~20% w/v, unspecified composition) sugar concentrations can kill *C. bombi* as well as other microbes (Cisarovsky & Schmid-Hempel, 2014), the effects found in this study indicate that addition of sugar at low concentrations is beneficial for trypanosomatid growth.

The monosaccharides added to the growth medium would have increased the sugar content of growth medium several-fold, providing up to 93 mM monosaccharides in addition to the 12.2 mM in the base medium (10 mM from fructose + 2.2 mM from glucose in liver broth (Salathé et al., 2012)). This additional sugar may have increased the quality of the media for *C. bombi* energy production. Insect and bloodstream-form trypanosomatids can use glucose as a carbon source (Mazet et al., 2013). Although proline is the normal carbon source for trypanosomatids in insect guts where glucose is scarce (Bringaud, Rivière & Coustou, 2006), proline metabolism is dramatically reduced in the presence of glucose, which suggests that glucose is a preferred energy source (Lamour et al., 2005, p. 200). The trypanosomatids that infect bees, which consume carbohydrate-rich diets, may be particularly adapted to use of carbohydrates. In a genomic comparison between *Leishmania major* and the honey bee gut parasite *Lotmaria passim* (n.b. Originally reported as *C. mellificae*), genes related to carbohydrate metabolism were enriched in the bee parasite compared to its bloodborne relative (Runckel, DeRisi & Flenniken, 2014). Genomic studies may reveal whether carbohydrate

364 metabolism is also well developed in *C. bombi*, which is a close relative of *L. passim* (Schwarz et
365 al., 2015).

366 The stimulatory effect of sugar on *C. bombi* growth raises the question of possible
367 facilitation of hindgut trypanosomatid infections by co-occurring infections that impair nutrient
368 absorption, such as *Nosema ceranae* and *Nosema apis. Nosema* spp. have been implicated in
369 collapse of honey bee colonies (Higes et al., 2009; Cornman et al., 2012) and may infect bumble
370 bees as well (Graystock et al., 2013). Field studies have found positive correlations between
371 *Nosema apis* and trypanosomatid infections in honey bees (Cornman et al., 2012), which
372 provides suggestive evidence for positive effects of *Nosema* spp. on gut trypanosomatids. The
373 present study suggests a mechanism by which *Nosema* infection could contribute to
374 trypanosomatid infection via negative effects on sugar absorption in bees. Healthy bees and other
375 nectivorous insects have an excellent ability to digest and absorb sugars from nectar (Nicolson,
376 1998), which may explain why there was no effect of dietary sugar concentration on infection
377 intensity in *B. impatiens* (Conroy et al., 2016) . However, gut infection by microsporidians can
378 disrupt the midgut epithelium (Higes et al., 2008). Injury to midgut tissue may decrease
379 absorption of sugar in hosts, as suggested by the decreased hemolymph sugar concentrations and
380 increased hunger observed in *Nosema*-infected bees (Mayack & Naug, 2009). As a result of
381 *Nosema*-induced malabsorption, more sugar may reach the hindgut, and thereby increase the
382 supply growth-limiting carbohydrate to trypanosomatids. To test whether *Nosema*-related
383 malabsorption facilitates infection by trypanosomatids, future experiments could test the effects
384 of microsporidian infection on fecal carbohydrate content and trypanosomatid infection intensity.

385 Although pollens differed considerably in sugar content, each extract had similar effects
386 on growth. This suggests that, in addition to sugars, pollen may contain additional substances
387 that promote *C. bombi* growth. One such substance may be proline, which is the normal carbon
388 source for trypanosomatids in insect guts where glucose is scarce (Bringaud, Rivière & Coustou,
389 2006). Proline has many functions in plants, including regulation of osmolarity and resistance to
390 abiotic stress (Verbruggen & Hermans, 2008), and is the most abundant amino acid in pollen.
391 For example, pollen of nine Asteraceae species contained 85-420 mmol kg^{-1} free proline
392 (Mondal, Parui & Mandal, 1998); another study found 173 mM kg-1 free proline in Spanish bee
393 pollens (Paramás et al., 2006). A proline content of 200 mmol kg^{-1} in pollen would correspond to
394 approximately 60 mM proline in the pollen extract and 3 mM additional proline in samples
395 treated with 5% pollen extract. This would roughly double the 2.5 mM proline in the base growth
396 medium (Salathé et al., 2012). Although we were not able to conduct an amino acid analysis,
397 which requires a special type of HPLC column, proline is generally agreed to be found in all
398 pollens (De Simone et al., 1980) as the dominant amino acid, composing 15-69% of total amino
399 acids (Yang et al., 2013). More study is needed to determine which, if any, additional pollen
400 compounds alter trypanosomatid growth *in vitro* and *in vivo*.

401

402 **Pollen in pollinator communities**

403 Pollen, like nectar, is an indispensable source of nutrients for bees and other pollinators that
404 supports insect immunity (Brunner, Schmid-Hempel & Barribeau, 2014), survival (Conroy et al.,
405 2016), and reproduction (Vaudo et al., 2015). However, pollen may nourish parasites as well as
406 hosts. Although phytochemicals may reduce growth of some microbes, parasites of

phytophagous animals are likely adapted to the phytochemicals and concentrations found in the diets of their hosts. Moreover, in coevolved obligate parasites such as *Crithidia* and other trypanosomatids (Maslov et al., 2013), there may be substantial overlap in the nutrient requirements of parasites and hosts. Shared nutritional requirements, and the increased fitness of parasites in well-nourished hosts, may result in tradeoffs between starvation of parasites and starvation of hosts, and may explain the utility of anorexia as a defense against parasites in some taxa (Parker et al., 2011). The results found here exemplify the trade-offs between host health and defense, underline the difficulty of eradicating well-adapted parasites without compromising host fitness, and suggest that natural selection may act across all levels of tritrophic interactions.

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Table 1(on next page)

Effects of pollen extracts and supplemental chemicals on growth.

Estimates and p-values are for linear regression after Bonferroni correction for multiple testing within each experiment. Coefficients are expressed as change in maximum optical density per percent pollen extract (A, B, and C: Buckwheat) or per mM chemical (C: Caffeic acid, Sugar, and Caffeic acid + Sugar).

*Mix treatment consisted of equal proportions of buckwheat, rape, and sunflower extracts.

#Coefficient expressed as change in OD per mM sugar

Table 1. **Effects of pollen extracts and supplemental chemicals on growth.** Estimates and p-values are for linear regression after Bonferroni correction for multiple testing within each experiment. Coefficients are expressed as change in maximum optical density per percent pollen extract (A, B, and C: Buckwheat) or per mM chemical (C: Caffeic acid, Sugar, and Caffeic acid + Sugar).

A. Single pollens				
Treatment	Coefficient (β)	Std. Error	T	*p* (T)
Buckwheat	0.037	0.0028	13.02	<0.001
Lotus	0.024	0.0021	11.21	<0.001
Poppy	0.034	0.0042	8.18	<0.001
Rape	0.032	0.0028	11.25	<0.001
Sunflower	0.021	0.0024	8.80	<0.001
Tea	0.032	0.0031	10.28	<0.001
B. Mixed Pollens				
Buckwheat	0.025	0.0021	11.99	<0.001
Rape	0.036	0.0028	13.16	<0.001
Sunflower	0.033	0.0025	13.36	<0.001
Mix*	0.025	0.0016	15.08	<0.001
C. Chemical additions				
Buckwheat	0.029	0.0030	9.95	<0.001
Caffeic acid	-0.013	0.0068	-1.99	0.23
Sugar	0.0034	0.00024	13.84	<0.001
Caffeic acid + Sugar[#]	0.0028	0.00060	4.73	<0.001

*Mix treatment consisted of equal proportions of buckwheat, rape, and sunflower extracts.

[#]Coefficient expressed as change in OD per mM sugar

Figure 1(on next page)

Figure 1. Individual pollen extracts increased parasite growth.

Extracts of six types of pollen were tested at up to 5% concentration. Each panel (A-F) shows the maximum optical density (OD 630 nm) for parasites exposed to 50% methanol extracts of one of the six types of pollen. Additional 50% methanol was added to samples of lesser concentrations to equalize methanol concentrations (2.5% by volume) across samples. Points and error bars show means and standard errors for each concentration (n = 8). OD: optical density (630 nm). See Supplementary Figures 1-3 for complete growth curves.

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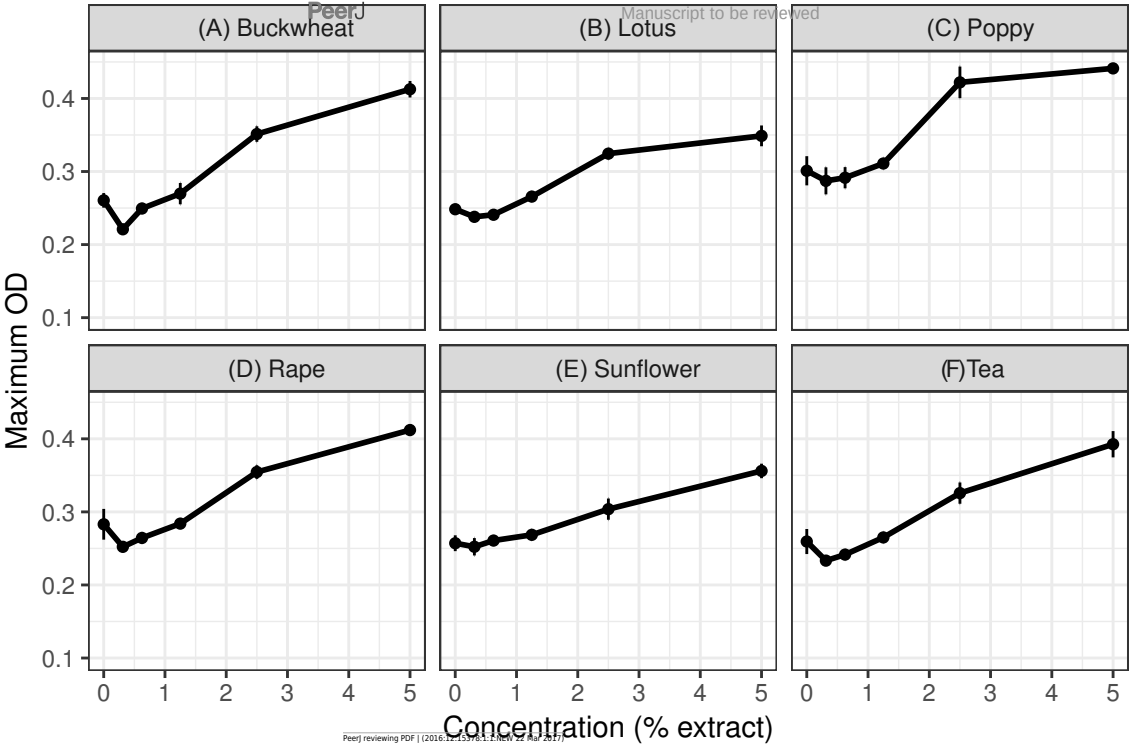

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Figure 2(on next page)

Figure 2. Mixed and individual pollen extracts each increased growth.

The treatments consisted of extracts of (A-C) individual pollens and (D) a mixture (“mix”) of equal proportions of buckwheat, rapeseed, and sunflower pollen. Each panel represents a different pollen extract. Additional 50% methanol was added to samples of lesser concentrations to equalize methanol concentrations (2.5% by volume) across samples. Points and error bars show means and standard errors for each concentration (n = 8).

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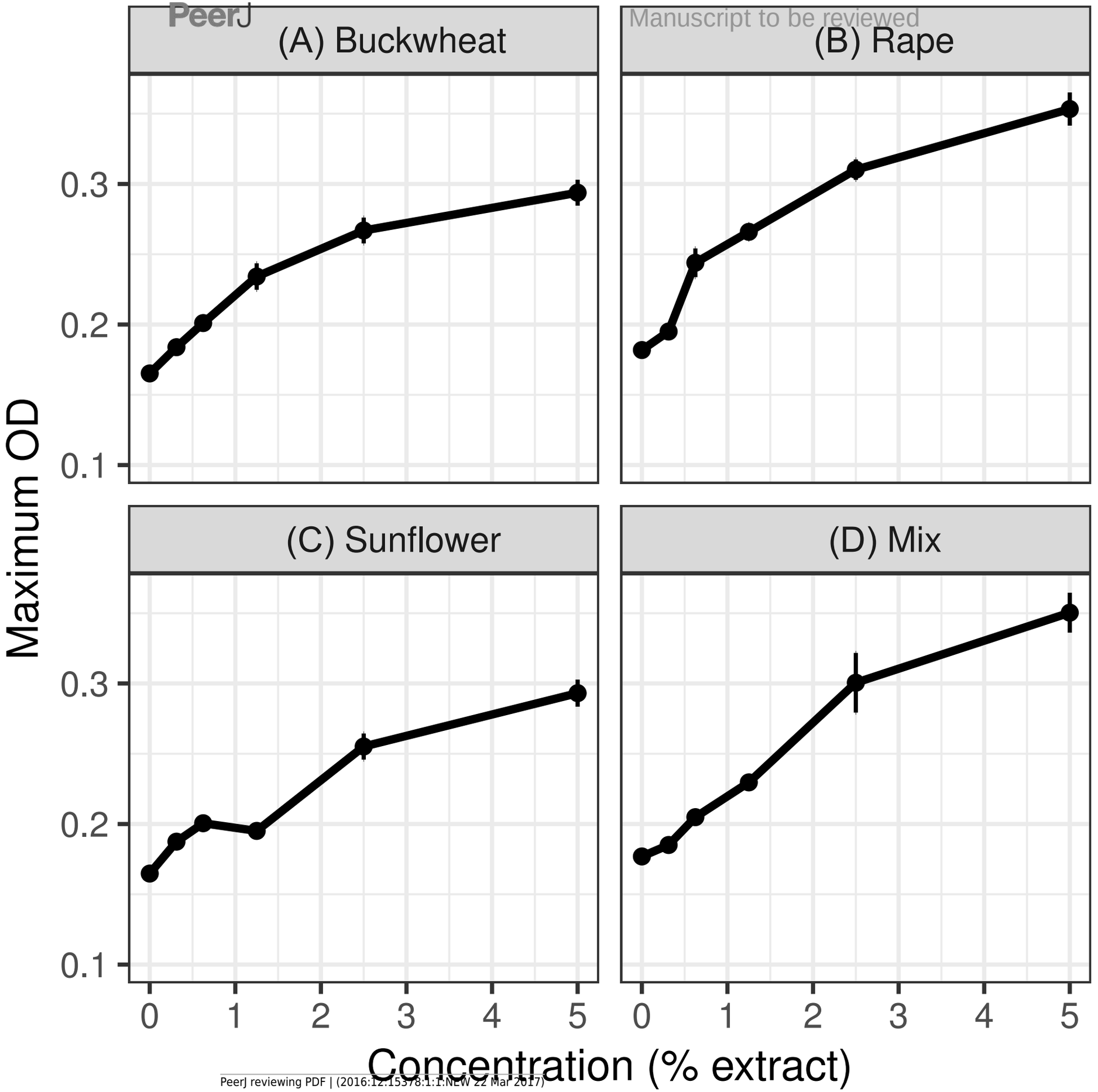

Figure 3(on next page)

Figure 3. Sugar composition of pollen extracts.

Gray bars represent fructose; orange bars represent glucose; blue bars represent sucrose. Concentrations were determined by HPLC with refractive index detector. Bars show mean of technical replicates (2 for fructose and sucrose, 3 for glucose).

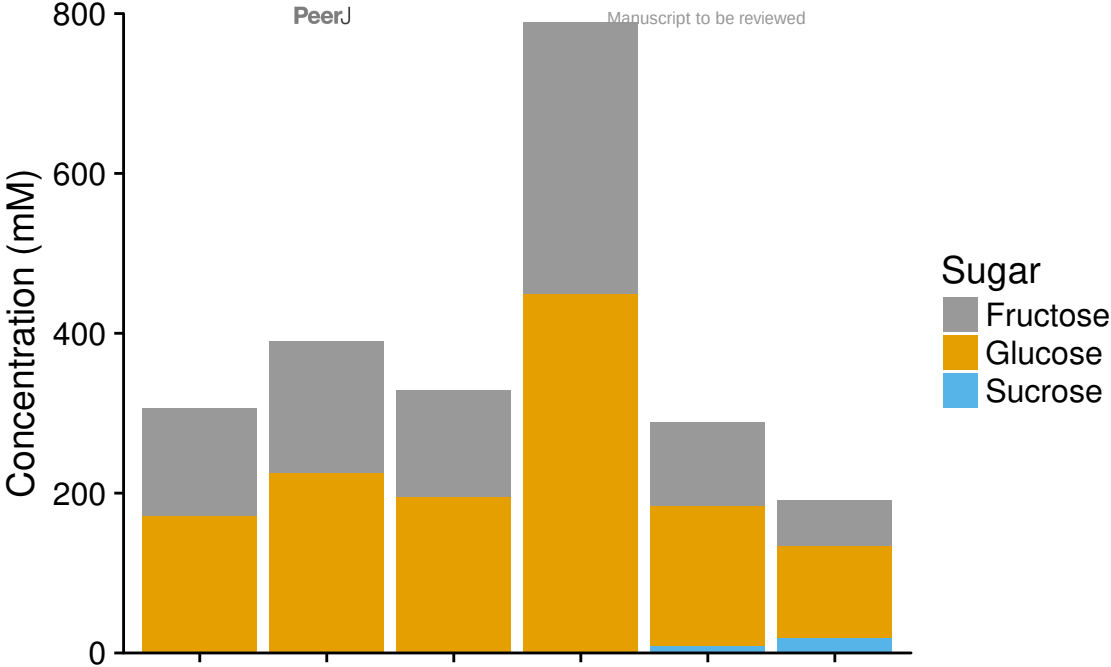
800
600
400
200
0
Concentration (mM)
Buckwheat
Lotus
Poppy
Rape
Sunflower
Tea
Pollen
Sugar
Fructose
Glucose
Sucrose

Figure 4(on next page)

Figure 4. A floral phytochemical had weak effects on growth, whereas supplemental sugar increased growth.

Each panel shows the growth curve for parasites exposed to one of the chemical treatments. (A) Buckwheat pollen extract was used as a positive control to confirm increased growth in the presence of pollen extract. (B) The sugar treatment consisted of equimolar amounts of glucose and fructose; both (C) caffeic acids and (B) sugars were dissolved in 50% methanol. (D) In the caffeic acid + sugar treatment, concentrations are shown for caffeic acid (top line) and sugars (bottom line). Additional 50% methanol was added to samples of lesser concentrations to equalize methanol concentrations (4.17% by volume) across samples. Points and error bars show means and standard errors for each concentration (n = 5).

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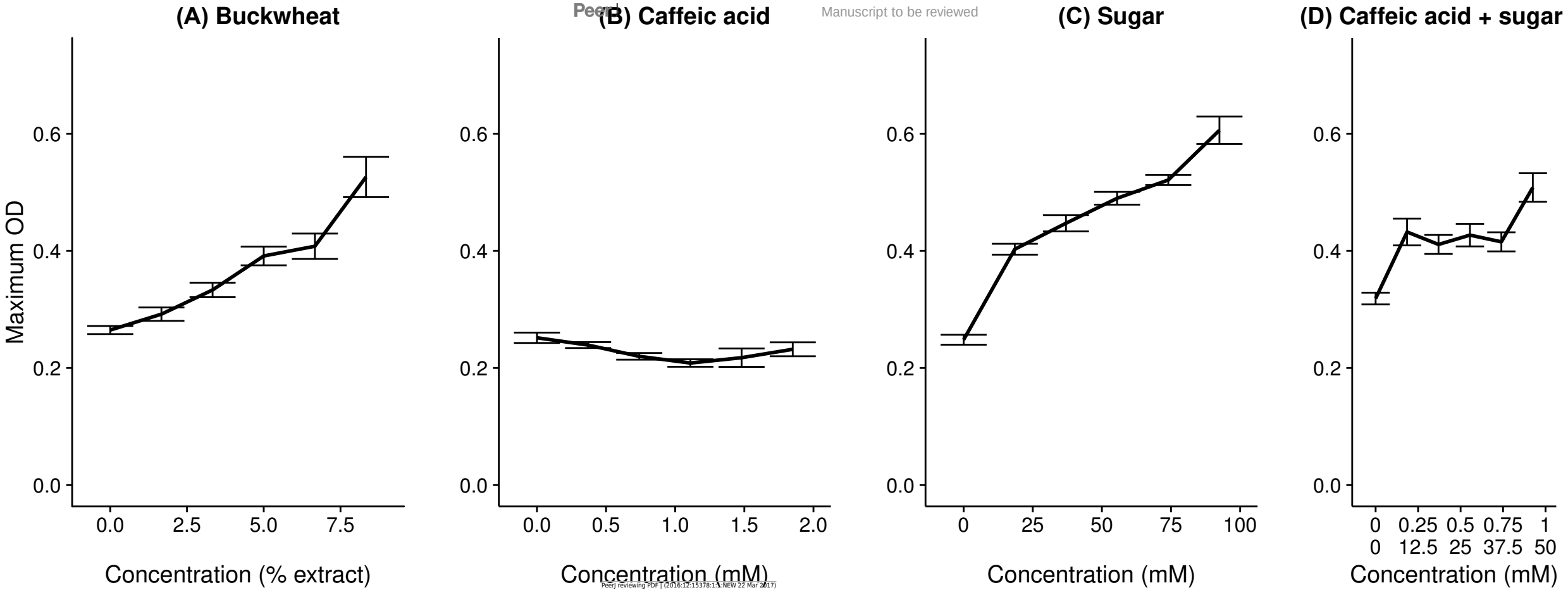

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