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Assessing Chemical Mechanisms Underlying the Effects of Sunflower Pollen on a Gut Pathogen in Bumble Bees

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Abstract

Many pollinator species are declining due to a variety of interacting stressors including pathogens, sparking interest in understanding factors that could mitigate these outcomes. Diet can affect host-pathogen interactions by changing nutritional reserves or providing bioactive secondary chemicals. Recent work found that sunflower pollen (*Helianthus annuus*) dramatically reduced cell counts of the gut pathogen *Crithidia bombi* in bumble bee workers (*Bombus impatiens*), but the mechanism underlying this effect is unknown. Here we analyzed methanolic extracts of sunflower pollen by LC-MS and identified triscoumaroyl spermidines as the major secondary metabolite components, along with a flavonoid quercetin-3-*O*-hexoside and a quercetin-3-*O*-(6-*O*-malonyl)-hexoside. We then tested the effect of triscoumaroyl spermidine and rutin (as a proxy for quercetin glycosides) on *Crithidia* infection in *B. impatiens*, compared to buckwheat pollen (*Fagopyrum esculentum*) as a negative control and sunflower pollen as a positive control. In addition, we tested the effect of nine fatty acids from sunflower pollen individually and in combination using similar methods. Although sunflower pollen consistently reduced *Crithidia* relative to control pollen, none of the compounds we tested had significant effects. In addition, diet treatments did not affect mortality, or sucrose or pollen consumption. Thus, the mechanisms underlying the medicinal effect of sunflower are still unknown; future work could use bioactivity-guided fractionation to more efficiently target compounds of interest, and explore non-chemical mechanisms. Ultimately, identifying the mechanism underlying the effect of sunflower pollen on pathogens will open up new avenues for managing bee health.

Keywords Bee pathogens · *Bombus impatiens* · *Crithidia bombi* · *Helianthus annuus* · Pollen chemistry · Pollinator health

Introduction

Parasites and pathogens have been implicated in the declines and even disappearance of bee species, including bumble bees (Cameron et al. 2016; Cameron et al. 2011; Schmid-Hempel et al. 2014). Pollinators provide billions of dollars in crop pollination annually, and are critical for preserving plant biodiversity (reviewed in Potts et al. 2016). Species declines have been attributed to a wide range of stressors, including loss of habitat, pesticide exposure, nutritional deficits, and parasites that may all interact synergistically (Goulson et al. 2015; Vanbergen et al. 2013). Diet may play a central role in mediating how pollinators interact with their pathogens. For example, poor nutrition can reduce bee immunity and increase the detrimental effects of parasites (Alaux et al. 2010; Brown et al. 2003; Roger et al. 2017). Furthermore, quality as well as quantity of floral resources may affect bee health. Consuming a diverse pollen diet increased longevity of honey bees infected with *Nosema* (Di Pasquale et al. 2013), a pathogen that has been implicated in widespread colony losses.

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The consumption of *p*-coumaric acid, a component of pollen and honey, upregulated honey bee genes involved in pesticide detoxification and antimicrobial function (Mao et al. 2013). Fatty acids are necessary for bee reproduction and development (Manning 2001), including fat bodies that play a role in energy storage and release as well as immune function in insects (Arrese and Soulages 2010). Plant secondary compounds can also influence pollinator-pathogen interactions. For example, multiple nectar secondary compounds reduced intensity of infection with a gut pathogen in bumble bees at natural concentrations (e. g., Baracchi et al. 2015; Koch et al. 2019; Manson et al. 2010; Richardson et al. 2015) although this is not always the case (Biller et al. 2015; Thorburn et al. 2015). Thus, both nutrition and secondary chemicals may play important roles in mediating pollinator health and interactions with pathogens.

Although previous research asking how floral reward chemistry affects bee pathogens has focused on nectar, pollen can contain greater diversity and orders of magnitude higher concentrations of secondary compounds than nectar (Cook et al. 2013; Palmer-Young et al. 2019b), consistent with optimal defense theory predicting greater investment in tissues more tightly linked with fitness (McKey 1979). Pollen chemistry could therefore have greater efficacy on pollinator pathogens than the less consistent effects of nectar secondary chemicals (Biller et al. 2015; Thorburn et al. 2015). Pollen nutrients including protein, lipids, amino acids and starches vary widely among plant taxa (Roulston and Cane 2000; Roulston et al. 2000; Yang et al. 2013), and could additionally influence bee susceptibility to pathogens.

We recently discovered that sunflower pollen (*Helianthus annuus*) consistently and dramatically reduced infection by *Crithidia bombi* in one species of bumble bee, *Bombus impatiens* (Giacomini et al. 2018). Pathogen cell counts were typically 30- to 50-fold lower in bees fed sunflower pollen than in bees fed buckwheat pollen (*Fagopyrum esculentum*), and at least 4-fold lower than in bees fed mixed wildflower pollen. Furthermore, this effect was consistent across 13 *H. annuus* cultivars and wild accessions, two *Helianthus* congeners, and two *Solidago* species, suggesting a potentially widespread effect in Asteraceae pollen (LoCascio et al. 2019a). These results are consistent with previous work in which the pathogen *Nosema ceranae* was most strongly reduced by sunflower honey compared to three other honeys (Gherman et al. 2014). Furthermore, mason bee (*Osmia*) species that specialize on Asteraceae pollen experienced almost no brood parasitism, whereas *Osmia* generalists had up to 50% parasitism (Spear et al. 2016). All these lines of evidence suggest some mechanism by which sunflower and related pollen reduces pathogen and parasite infection.

The mechanism by which these pollens reduce pathogens could be pollen chemistry, including both secondary chemicals and nutritional components. Sunflower pollen is antimicrobial and inhibited the causal agent of American foulbrood, a honey bee brood pathogen, *in vitro* (Fatrcova-Sramkova et al. 2016). Sunflower pollen methanolic extracts also have anti-inflammatory and antitumor-promoting effects in mice (reviewed in Ukiya et al. 2003). Nutritionally, sunflower pollen is low in protein (Human et al. 2007; Yang et al. 2013) and lacked two essential amino acids necessary for honey bee development in one study (Nicolson and Human 2013), although another study did not find the same deficiencies (McAulay and Forrest 2019). However, sunflower pollen is high in saturated fatty acids such as palmitic, lauric, stearic and caprylic acids (Kostic et al. 2017) and another study also found high concentrations of myristic acid (Farag et al. 1978). Palmitic and stearic acids together comprise 20–35% of the total lipid composition of adult honey bees, suggesting important roles in nutrition (Manning 2001; Robinson and Nation 1970). Lauric acid had antimicrobial properties against the honey bee pathogen *Paenibacillus larvae larvae* (Feldlaufer et al. 1993), and several analogues of myristic acid, a common saturated fatty acid of plant oils, have potent antitrypanosomal activity (Doering et al. 1994). Thus, fatty acids from sunflower pollen could also play important roles in bee health.

The goal of this study was to assess major chemical components of sunflower pollen to determine whether they represent the mechanism underlying the dramatic reduction of *Crithidia* gut pathogen cells. First, we isolated major secondary compounds of sunflower pollen. We then synthesized one compound and purchased another and tested their effects on *Crithidia* infection in the bumble bee *B. impatiens* in separate experiments. In addition, we assessed the effects of an array of fatty acids, singly and in combination, to determine their effects on *Crithidia*. If we can discover a chemical mechanism underlying the effect of sunflower pollen on pathogen infection, this could lead to breeding programs selecting for these traits in agricultural crops or choosing wild plants for pollinator habitat partially based on pollen composition.

Methods and Materials

Study System *Bombus impatiens* (Apidae), or the common eastern bumble bee, is one of the most abundant bumble bee species in the eastern United States (Cordes et al. 2012; Gillespie 2010). *Crithidia bombi* (Trypanosomatidae; *Crithidia* hereafter) is a gut pathogen of bumble bees that can infect up to 80% of *Bombus* at sites in western Massachusetts (Gillespie 2010) and up to 80% of *B. terrestris* workers near Basel, Switzerland (Shykoff and Schmid-Hempel 1991). *Crithidia* can be relatively benign when bees have sufficient resources (Brown et al. 2003), but under food-limited conditions can reduce queen fitness and colony performance (Brown et al. 2003) and worker survival

(Brown et al. 2000). In the field, infection with *Crithidia* was associated with reduced reproduction in wild colonies (Goulson et al. 2018).

Helianthus annuus, or sunflower, is native to North America (Reagon and Snow 2006) and is a major oilseed crop worldwide whose yield is improved by bee visitation (reviewed in Nicolson and Human 2013). Buckwheat pollen (*Fagopyrum esculentum)* has protein content comparable to sunflower (Yang et al. 2013) but bees that consume it have much higher *Crithidia* cell counts (Giacomini et al. 2018), and so it can be used as a control pollen treatment to compare diets with comparable protein but different effects on *Crithidia*.

Chemical Analysis We analyzed *H. annuus* pollen sourced from China (Changge Hauding Wax Industry, China Co. LTD). Pollen samples (50 mg) were sonicated in 100% methanol for 10 min and left to extract at room temperature for 24 h. Each sample was analyzed by LC–MS on a Waters Alliance LC solvent delivery system with a ZQ MS detector. Compounds were separated over a Phenomenex Luna C18(2) column (150 × 4.0 mm i.d., 5 μm particle size) operating under gradient elution conditions, with A = MeOH, B = H_2O, C = 1% HCO_2H in MeCN; A = 0%, B = 90% at t = 0 min; A = 90%, B = 0% at t = 20 min; A = 90%, B = 0% at t = 30 min; A = 0%, B = 90% at t = 31 min; column temperature 30 °C and flow rate of 0.5 ml min^{-1}. Analysis of our samples by this method identified two major peaks eluting after 10.4 and 11.0 min as a quercetin-3-*O*-hexoside and a quercetin-3-*O*-(6-*O*-malonyl)-hexoside respectively. These compounds were characterised by a UV spectrum that was similar to that of rutin (quercetin-3-*O*-rutinoside) with band maxima at 357 and 260 nm and pseudo molecular ions at *m/z* = 463 and 549 $[M\text{-}H]^-$ in negative mode and 465 and 551 $[M + H]^+$ in positive mode respectively. Fragmentation in the mass spectrometer in positive mode revealed ions at *m/z* = 303 in both compounds corresponding to the aglycone $[quercetin+H]^+$. These identifications correspond with those of Kostic et al. (2019).

Two additional major peaks were recorded in the LC-MS chromatogram of the sunflower pollen methanol extract that eluted between 13 and 15 min and corresponded to two triscoumaroyl spermidines. These compounds were characterised by pseudomolecular ions with *m/z* = 584 $[M + H]^+$ and a UV spectrum similar to that recorded in our laboratory (Kew) for coumaric acid derivatives (with a band max = 313 nm). The most abundant triscoumaroyl spermidine co-eluted and showed similar fragmentation, including three losses of a *m/z* = 146.03 fragment in the mass spectrometer, to N^1, N^5, N^{10}- tri(*E*)-*p*-coumaroyl spermidine which was synthesised in our laboratory (Online Resource 1). Minor components were also recorded in the chromatogram with similar pseudo-molecular ions and fragmentation in the mass spectrometer and were likely to be structural analogues of the main compound, with various *E* and *Z* isomerization at the propenyl of the three coumaroyl groups since high levels of variation in isomerization of the cinnamoyls in these compounds are reported (Kite et al. 2013).

Polynomial calibration curves for each compound via quantification of the $[M + H]^+$ molecular ion of a commercial standard of rutin (Sigma-Aldrich, Dorset, UK) and a sample of triscoumaroyl spermidine synthesized in our laboratory were used to quantify compounds. The two quercetin glycosides occurred at 0.56 and 1.37 mg/g pollen while the mean concentration of the triscoumaroyl spermidine was 12.16 mg/g pollen.

Bee Bioassay General Approach The general approach was similar for all assays and followed previously published protocols (Giacomini et al. 2018; LoCascio et al. 2019a; Richardson et al. 2015). Commercial experimental colonies (BioBest, Leamington, Canada) were confirmed to be *Crithidia*-free by biweekly subsampling, and the *Crithidia* strain used in these assays was originally collected in 2015 from infected wild *B. impatiens* at a farm in Hadley, Massachusetts USA (42°21′51.93”N, 72°33′55.88”W) and maintained in commercial colonies thereafter. All colonies were fed mixed wildflower pollen (Koppert Biological Systems, Howell, Michigan, USA or CC Pollen Inc., Phoenix, Arizona, USA). Worker bees were inoculated after a 1–2 h starving period with either 10 μl (secondary compounds) or 15 μl (fatty acids) of a 25% sucrose solution containing 600 cells/μl, well within the range of natural fecal *Crithidia* concentrations (Otterstatter and Thomson 2006). Upon inoculation, bees were placed in individual 18.5 ml vials (secondary compounds) or Placon 473 ml cups (fatty acids) and assigned to diet treatments that they received for 7 days along with 30% sucrose solution that was replaced daily (secondary compounds) or every other day (fatty acids). Bees were maintained in darkness at 27 °C in an incubator during assays. After 7 days, bees were dissected to remove the gut, which was ground in 300 μl of ¼ strength Ringer's solution (Sigma-Aldrich – Fluka 96,724). After 4 h, a 10 μl subsample was removed and moving *Crithidia* cells were counted on a 0.02 μl field of vision on the grid of a hemacytometer.

For the secondary compound assays we used callows, which are bees that had emerged from pupae within the last 24 h. We isolated pupal clumps from colonies, collected callows as they emerged and fed them wildflower pollen for two days before inoculating them to enter the experiment. Because callows emerged without nestmates, this was an extra precaution to ensure they were free of *Crithidia* (which infects adults), but these bees also did not receive gut microbiota from their nestmates that could influence interactions with *Crithidia* (Koch and Schmid-Hempel 2011). For the fatty acid assays, we pulled adult workers directly from colonies and inoculated them to enter the experiment. This method does not control for worker age, but allows bees to acquire their colony's gut

microbiome. Within each assay, bees with pollen treatments were compared to controls under the same conditions, so while our methods varied, we can still assess diet effects within each experiment. We also included a measure of bee size for all assays since smaller bees often have higher infections (e.g., Richardson et al. 2015). For the secondary compound assays we used callow mass, and for fatty acids we used marginal cell length, which is highly correlated with other measures of body size (Nooten and Rehan 2020). In the triscoumaryl spermidine experiment, we measured both callow mass and marginal cell length and they were highly correlated (Pearson's r = 0.85, $n = 259$).

For all assays we recorded daily mortality, and measured daily pollen and sucrose consumption once per bee, except for omitting sucrose consumption in the triscoumaroyl spermidine assay. Pollen and sucrose rations were typically weighed upon providing them to bees and then approximately 24 h later (48 h in the first fatty acid assay).

Secondary Chemical Bioassays We tested the effects of two major secondary compounds found in sunflower pollen, a triscoumaroyl spermidine and rutin, at four concentrations each. We used rutin as a proxy for the quercetin glycosides identified in sunflower pollen because rutin is also a quercetin glycoside and available commercially. Quercetin glycosides are reported to show equivalent activity across a range of analogues against microorganisms including trypanosomes (da Silva et al. 2019; Marin et al. 2017), suggesting that rutin provides a suitable proxy for other quercetin glycosides.

Tri-*p*-coumaroyl spermidine ('triscoumaryl spermidine' hereafter) was synthesized in the laboratory as described in Online Resource 1, while rutin was purchased commercially (Sigma-Aldrich, R5143-50G). Both were added to buckwheat pollen at 1% (very high), 0.1% (high), 0.01% (medium), or 0.001% (low) concentrations wt/wt. For context, we found 12 mg/g of triscoumaroyl spermidine in pollen (1.2%; comparable with the 'very high' treatment) and 0.2% quercetin glycosides in pollen, similar to the 'high' level. Thus, our concentrations are within natural ranges. Compounds were mixed with buckwheat pollen and compared to pure buckwheat pollen as a negative control and sunflower pollen as a positive control (sunflower and buckwheat pollen came from Changge Huading Wax Industry Co., Ltd., Henan, China; except that buckwheat came from Fuyang Import and Export Ltd. for the final fatty acid assay). Compounds were added to dry pollen, which was then mixed with distilled water for all treatments to create a paste that was frozen at -20 °C until use. Details of mixing secondary compounds with pollen and preparing pollen balls are in Online Resource 2.

The triscoumaryl spermidine assay was conducted from July 17 through August 19, 2015, using 108 bees from three experimental colonies, ultimately including 15–20 bees/treatment. The rutin experiment was conducted from November 3, 2015 through April 7, 2016 and used 292 bees from 12 experimental colonies, ultimately including 45–54 bees/treatment.

Fatty Acid Bioassays We tested the effects of several fatty acids singly and in combination. We chose the most common ones detected in sunflower pollen that were commercially available, and tested each at typical concentrations based on published literature (Nicolson and Human 2013; Yang et al. 2013). We conducted three assays that each tested individual or combinations of fatty acids added directly to control pollen as powders or liquids. Compounds tested in the first assay were linoleic (1.67 mg/g; CAS 60–33-3), lauric (12.39 mg/g; CAS 143–07-7), caprylic (1.46 mg/g; CAS 124–07-2), palmitic (8.46 mg/g; CAS 57–10-3), and decanoic (0.14 mg/g; CA: 334–48-5) acids. The second assay included linolenic (7.61 mg/g; CAS 463–40-1), myristic (1.62 mg/g; CAS 544–63-8), stearic (0.65 mg/g; CAS 57–11-4) and oleic (5.39 mg/g; CAS 112–80-1) acids. The third assay tested all nine fatty acids combined in a single treatment (using the same concentrations as in individual assays) and sunflower oil, which contains a mix of fatty acids (37.07 mg/g; Organic 365 Everyday Value Products, Whole Foods Market, Austin, Texas, USA), to assess whether fatty acids had interactive effects that would not be detected in assays of individual compounds. Details of suppliers and concentration calculations are provided in Online Resource 2. Fatty acids were added to dry pollen with a coffee grinder, and then distilled water was added in a 6:1 or 7:1 (third assay only) ratio to make a paste. As with the secondary chemical assays, compounds were added to buckwheat pollen and compared to buckwheat and sunflower pollen as negative and positive controls, respectively. However, in the second assay we did not have sufficient buckwheat pollen and instead added fatty acids to mixed wildflower pollen (CC Pollen Inc. Phoenix, Arizona, USA) and used wildflower pollen as the negative control.

We conducted the first assay from September 28, 2018 to February 6, 2019 using 246 bees from 14 experimental colonies, including 32–37 bees per fatty acid treatments and 47 in the buckwheat control. The second was conducted from February 20 to April 12, 2019, using 167 bees from 5 experimental colonies, including 25–31 bees/treatment. The third assay was conducted from July 18 to 26, 2019 using 106 bees from 4 experimental colonies, including 25–27 bees/treatment.

Statistical Analysis We used the open source software R v3.3.3 (R Development Core Team 2014). All data CSV files and R scripts are available from the Dryad Digital Repository (http://www.datadryad.org); (https://doi.org/10.5061/dryad.dv41ns1v9). For all analyses, the significance of model terms was tested with likelihood ratio χ^2 tests, conducted with the Anova function, which compares relative goodness of fit between models with and without each term (car

package, Fox and Weisberg 2019). To analyze the number of *Crithidia* cells counted in a 0.02-μl gut extract as the response, we used generalized linear mixed models (Bates et al. 2015). Cell counts were over-dispersed, and thus we used a negative binomial error distribution and the model function glmer.nb (lme4 package, Bates et al. 2015). Each model included diet treatment and callow mass or wing marginal cell size as fixed effects. Colony of origin was included as a random effect, along with inoculation date to account for the independent preparation of inoculation on each trial date. When diet treatment was significant, means were compared with post-hoc Tukey's tests using the glht function (multcomp package, Hothorn et al. 2008). To analyze the amount of pollen and sucrose consumed, we used general linear models with diet and callow mass or wing marginal cell size as fixed effects and colony of origin and inoculation date as random effects. To analyze death hazard rate by pollen treatment, we used a survival analysis with a Cox proportional hazards mixed model using the coxme function (coxme package, Therneau 2019), survfit, and Surv functions (survival package, Therneau 2015; Therneau and Grambsch 2000)) including diet treatment, callow mass or wing marginal cell length, date of inoculation and colony of origin as predictors. In the rutin experiment, inoculation date was not recorded for dead bees and so was not included in the survival analysis. In the two individual fatty acid assays, we did not measure wing cell length on dead bees and so did not include this predictor in the survival analysis. All figures were made using lsmeans function (lsmeans package, Lenth 2016)) and ggplot function (ggplot2 package, Wickham 2016).

Results

Crithidia cells replicated in hosts; final raw counts in the control treatments across assays ranged from 29.7–59.9 cells per 0.02 μl. On average, this is approximately 100 times more *Crithidia* cells than the initial inoculation.

In every assay, diet significantly affected *Crithidia* cell counts (combined fatty acid assay: $\chi^2 = 16.02$, $P = 0.007$; all other assays: $\chi^2 > 21.3$, $P < 0.001$). However, in every case this effect was driven by the difference between the sunflower pollen and all other treatments (Figs. 1 and 2); sunflower pollen reduced *Crithidia* counts by 64–94% per assay compared to the control treatment. None of the secondary compounds or fatty acids, singly or in combination, significantly reduced *Crithidia* cell counts compared to the control treatments, and all were significantly higher than *Crithidia* counts with sunflower pollen.

Diet did not affect mortality in any assay ($\chi^2 < 4.3$, $P > 0.5$ for all), suggesting that neither sunflower pollen nor any of the compounds tested had notable costs or benefits for bee survival. In the rutin and combined fatty acid assays, smaller bees had higher mortality (rutin: $\chi^2 = 14.39$, $P < 0.001$; combined fatty acid: $\chi^2 = 8.47$, $P = 0.004$) but there was no relationship between bee size and mortality in the triscoumaroyl spermidine assay ($\chi^2 = 1.63$, $P = 0.214$); relationships between bee size and mortality could not be assessed for the other two assays because wings were not collected from dead bees.

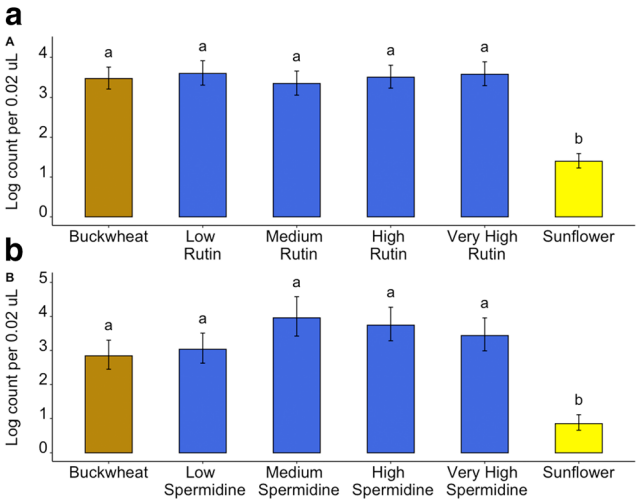

Fig. 1 Effect of (a) rutin and (b) triscoumaroyl spermidine in pollen on $\log_{10}$-transformed *Crithidia* cell counts in *B. impatiens* workers. In both experiments, compounds were added to buckwheat pollen (*Fagopyrum esculentum*), which was used as a negative control, and compared to sunflower (*Helianthus annuus*) pollen as a positive control. "Low," "Medium," "High" and "Very High" refer to 0.001%, 0.01%, 0.1%, or 1% of compound added to pollen (wt/wt) prior to adding water. Different letters above means represent significantly different treatments ($P < 0.05$). Error bars indicate standard error

Diet treatments also did not affect consumption of pollen ($\chi^2 < 5.3$, $P > 0.38$ for all) or sucrose (rutin assay: $\chi^2 = 9.99$, $P = 0.075$; all others: $\chi^2 < 5.9$, $P > 0.43$). Larger bees consumed more pollen than smaller bees in all experiments ($\chi^2 > 5.8$, $P < 0.017$ for all) and also consumed more sucrose in all assays in which this was measured ($\chi^2 > 4.8$, $P < 0.03$ for all).

Discussion

We isolated major secondary compounds of sunflower and tested their efficacy against a common bumble bee gut pathogen; we also assessed the effects of numerous fatty acids from sunflower pollen, singly and in combination. Although we found that sunflower pollen consistently reduced *Crithidia* compared to control pollens, matching previous work (Giacomini et al. 2018; LoCascio et al. 2019a, b), none of the compounds we tested appear to be the sole mechanism driving this result, as none of them significantly reduced pathogen counts relative to control pollens. Although we did not test protein content in this study, it seems unlikely that protein content is the underlying mechanism since protein

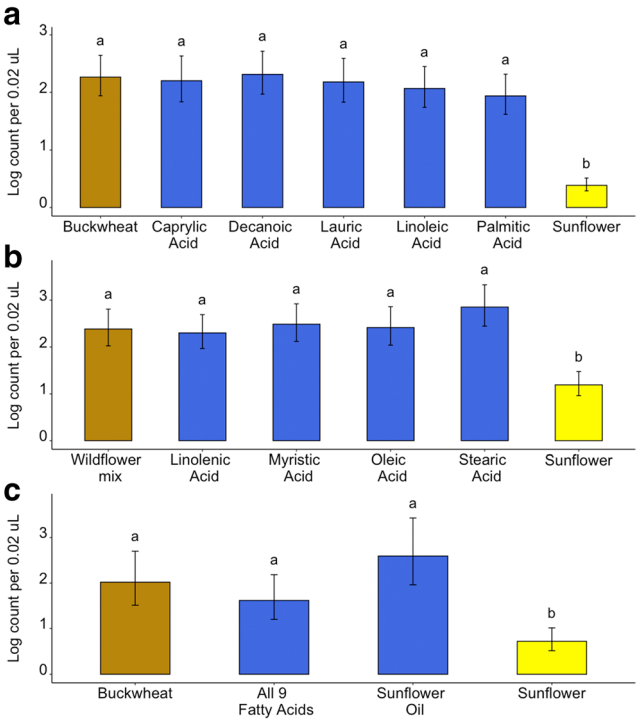

Fig. 2 Effect of (a, b) individual fatty acids and (c) combined fatty acids and sunflower oil in pollen on $\log_{10}$-transformed *Crithidia* cell counts in *B. impatiens* workers. In (a) and (c), compounds were added to buckwheat pollen (*Fagopyrum esculentum*), which was used as a negative control, and compared to sunflower (*Helianthus annuus*) pollen as a positive control. In (b), compounds were instead added to mixed wildflower pollen, which was used as the control. In (c), "All 9 fatty acids" refers to a treatment in which the nine fatty acids tested individually in (a) and (b) were added in combination to a single pollen treatment. Different letters above means represent significantly different treatments ($P < 0.05$). Error bars indicate standard error

concentrations are similarly low in buckwheat and sunflower pollen (Yang et al. 2013) but outcomes of these diets for *Crithidia* infection are very different. Rather than focusing on specific compounds, it may be more efficient in the future to assess potential chemical mechanisms of sunflower pollen by testing sequential extractions to more rapidly facilitate identification of the key biologically active components. Progressing from apolar to more polar fractions (to selectively extract compounds first that can be most easily isolated via evaporation) and adding each to control pollen to assess effects against *Crithidia* may provide a more efficient method to identify the specific bioactive compounds or combinations that make sunflower so consistently effective against infection by this pathogen. Alternatively, the slow digestibility of pollen could result in compounds being naturally released in the hind gut where infection occurs. Mixing commercial compounds with pollen might cause earlier release, which could be less effective. However, some secondary compounds are metabolized in the mid-or hind-gut and early exposure in the crop can have prophylactic effects on infection (Koch et al. 2019). Further work is needed to elucidate whether sunflower pollen digestion and metabolism processes play a role in its effect on infection.

The mechanism underlying the interaction between sunflower pollen and *Crithidia* could be mechanical rather than chemical. For example, the roughness of some plant leaves can rid chimpanzees of intestinal parasites via scouring intestinal walls (Huffman 1997), a mechanical mechanism. Sunflower and other Asteraceae pollen is notable for spines on the outer coat (Blackmore et al. 2009). *Crithidia* is a gut parasite that establishes infection by attaching to the ileum wall, a portion of the hindgut (Gorbunov 1996; Koch et al. 2019). An exciting recent study showed that a compound from heather nectar (*Calluna vulgaris*) dramatically reduced *Crithidia* infection in *B. terrestris* by removing the flagellum, preventing attachment (Koch et al. 2019). Although we do not know how spines could interfere in attachment, it is possible that spines make it difficult for cells to attach to the gut wall, which we can examine in future work. We note that in Koch et al. (2019), nectar compounds were not effective at reducing infections that were already established, likely because compounds were metabolized before reaching the hindgut where infection occurs. By contrast, sunflower is effective even after infection has established (Giacomini et al. 2018), suggesting a mechanism acting in the hindgut itself. Interestingly, species in the family Malvaceae also have spiny pollen that is hypothesized to be a defense preventing consumption by bees (Lunau et al. 2015). Pollen from hollyhocks, *Alcea rosea*, were not collected by *B. terrestris* unless spines and sticky pollenkitt were removed; extracted chemicals did not affect pollen collection, suggesting that deterrence was due to mechanical rather than chemical mechanisms (Lunau et al. 2015). Future work can use a similar approach or take advantage of sunflower pollen 'shells' used for nanodrug delivery (Mundargi et al. 2016) to dissect the role of mechanical and chemical mechanisms underlying the effect on *Crithidia*.

We identified flavonoid glycosides and triscoumaroyl spermidines in high quantities as the major secondary metabolites in the sunflower pollen. The occurrence of flavonoids in pollen and honey is well reported (Ciappini 2019; Palmer-Young et al. 2019a, b) so their occurrence in sunflower pollen was not unexpected. Indeed, Kostic et al. (2019) recently analyzed bee-collected pollen from sunflower and also identified flavonoid glycosides in methanol extracts of sunflower pollen including a quercetin 3-*O*-(6-*O*-acetyl)-hexoside as the major flavonoid component, mirroring the occurrence of the quercetin-3-*O*-(6-*O*-malonyl)-hexoside identified here. It is likely that the acetylated quercetin hexoside recorded by Kostic et al. (2019) was in fact a decarboxylated derivative and occurred naturally as a malonylated analogue, as identified in our material and reported here. Facile decarboxylation in solution of malonyl to acetyl has been reported, and naturally occurring acetyl glycosides are rare in nature compared to malonyl derivatives (Agrawal 1992; Stevenson and Veitch 1996).

Triscoumaroyl spermidines have not previously been reported in sunflower pollen extracts but are otherwise known to occur widely although not ubiquitously in the pollen of angiosperms (Kite et al. 2013; Martin-Tanguy et al. 1978; Palmer-Young et al. 2019a, b). Their role in flower growth and reproduction has been determined using metabolomic and genomic studies (Fellenberg et al. 2009; Hanhineva et al. 2008), while deficiency of spermidine conjugates caused pollen grains to become deformed, indicating a developmental role for these compounds (Grienenberger et al. 2009). Their potential as plant defense compounds against fungi has been demonstrated (Bassard et al. 2010; Martin-Tanguy et al. 1978; Walters et al. 2001), and although some compounds show biological activity against insect neuroreceptors, they have low toxicity in bioassays when ingested by insects (Fixon-Owoo et al. 2003). Mori et al. (2019) suggest their occurrence in apricot pollen (*Prunus mume* L.) was associated with non-fluorescing pollen so they may absorb UV light and protect the male gamete against harmful UV radiation (Gill and Tuteja 2010). Despite the variety of biological activities attributable to acyl spermidines and quercetin glycosides, they do not explain the biological activity of sunflower pollen against *Crithidia*.

Fatty acids are important components of the bee diet (Vaudo et al. 2016). They typically occur in the pollen kit (Chichiricco et al. 2019) and can make up to 10% of the dry weight of pollen (Arien et al. 2015), providing a significant dietary component for larvae and adult bumble bees. Overall, the most common free fatty acids in pollen are α-linolenic acid, α-linoleic acid, palmitic acid, stearic acid, and oleic acid (Manning 2001). However, they differ in sunflower, with myristic acid dominant and including palmitic, lauric, stearic and caprylic acids (Kostic et al. 2019). The role of lipids in colony growth and individual bee behaviors has been studied in honey bees and commercial bumble bees (Arien et al. 2015; Muth et al. 2018), but how they influence the development or establishment of bee pathogens such as *Crithidia* is unknown. Fatty acids have antimicrobial properties (Manning 2001) and several analogues of myristic acid, a common saturated fatty acid of plant oils, have potent anti-trypanosomal activity (Doering et al. 1994). Thus, we hypothesized that pollen fatty acids could contribute to the bioactivity of sunflower pollen against *Crithidia*. However, we found no activity among the fatty acids tested here. The most active myristate derivatives reported by Doering et al. (1994) were oxidized and sulfonated analogues of myristic acid rather than myristic acid itself, suggesting that the common fatty acids occurring widely in floral pollen as saturated or unsaturated fats may not have the structural characters to challenge *Crithidia*.

If we can discover the mechanism by which sunflower pollen reduces bee pathogen infection, this can open new avenues for management of bee health. Because sunflower pollen has low protein, if the mechanism was known we could search for this trait in other pollens to identify potential species that are more nutritionally beneficial. If we discover chemical components of pollen responsible for reducing pathogen infection, this could lead to breeding programs selecting for these traits in agricultural crops or choosing wild plants for pollinator habitat partially based on these traits. Other recent work in our labs showed that blueberry domestication reduced levels of a nectar caffeic acid below the threshold that is effective in reducing a bee pathogen (Egan et al. 2018), suggesting that there is an opportunity to incorporate selective breeding of crop floral traits to improve bee health. If the mechanism is chemical, another possibility would be isolating or synthesizing those compounds for incorporation into commercial bee diet. We note that sunflower pollen often leads to poor performance of generalist bees such as honey bees (Human et al. 2007; Nicolson et al. 2018; Nicolson and Human 2013). and bumble bees (McAulay and Forrest 2019; Tasei and Aupinel 2008), although this is not always the case (Giacomini et al. 2018; Treanore et al. 2019). However, mixing sunflower pollen with other pollens can ameliorate negative effects on worker survival (McAulay and Forrest 2019). Thus, sunflower pollen could be an effective treatment for *Crithidia* infection without sacrificing bee performance if it were mixed with other pollens (Giacomini et al., in preparation), but isolating the underlying mechanism may open up new avenues for development of supplements that could be added to more nutritious pollen diets.

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Author Contributions LSA, REI and PCS conceived of and designed the study. PCS and IWF conducted chemical analysis of pollen and synthesized spermidines. AEF, RLM, PRA, LMC, PMD, and SL carried out bioassay experiments. AEF analyzed data and prepared figures. LSA wrote the manuscript with substantial contributions from PCS. All co-authors read and provided feedback on the manuscript.

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