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Pollen extracts increase growth of a trypanosome parasite of bumble bees

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Phytochemicals produced by plants, including at flowers, function in protection against plant diseases, and have a long history of use against trypanosome infection. Floral nectar and pollen, the sole food sources for many species of insect pollinators, contain phytochemicals that have been shown to reduce trypanosome 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 trypanosome gut parasite Crithidia bombi.

Surprisingly, pollen extracts increased parasite growth rather than inhibiting it. Experimental manipulations of growth media showed that supplemental monosaccharides (glucose and fructose) were sufficient to promote growth, while a common floral phytochemical (caffeic acid) with inhibitory activity against other trypanosomes 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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Abstract

Phytochemicals produced by plants, including at flowers, function in protection against plant diseases, and have a long history of use against trypanosome infection. Floral nectar and pollen, the sole food sources for many species of insect pollinators, contain phytochemicals that have been shown to reduce trypanosome 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 trypanosome gut parasite *Crithidia bombi*.

Surprisingly, pollen extracts increased parasite growth rather than inhibiting it. Experimental manipulations of growth media showed that supplemental monosaccharides (glucose and fructose) were sufficient to promote growth, while a common floral phytochemical (caffeic acid) with inhibitory activity against other trypanosomes 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.

Trypanosomes are parasites that, in addition to afflicting over 12 million humans (Maslov et al., 2013), can also be pervasive in populations of wild and managed bees (Schwarz et al., 2015; Shykoff and Schmid-Hempel, 1991). With spread of parasites facilitated by use of shared flowers (Durrer and Schmid-Hempel, 1994; Graystock et al., 2015), infection in some areas may exceed 80% in bumble bees (Gillespie, 2010; Popp et al., 2012; Shykoff and Schmid-Hempel, 1991) and 90% in honey bees (Runckel et al., 2011). Correlative evidence implicates trypanosome 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 et al., 2003), provoked potentially costly immune responses.
(Sadd and Barribeau, 2013), altered foraging behavior and learning (Gegear et al., 2005), and decreased colony fitness (Shykoff and Schmid-Hempel, 1991).

Phytochemicals have well-known antimicrobial properties that inhibit infection not only in plants (Bennett and Wallsgrove, 1994; Huang et al., 2012), but also in animals that consume phytochemical-rich plant materials (Karban and English-Loeb, 1997; de Roode et al., 2013; Singer et al., 2009). Plant-based therapeutics have a long history of traditional use against trypanosome infection, and recent studies have confirmed the antitrypanosomal activity of both plant extracts and isolated phytochemicals (Merschjohann and Steverding, 2006; Santoro et al., 2007a; Wink, 2012). Similarly, phytochemicals may have medicinal effects in bees infected with the trypanosome *C. bombi* (Baracchi et al., 2015; Biller et al., 2015; Manson et al., 2010; Richardson 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 and Neal, 1985; Berenbaum et al., 1991) and microbes (Fewell and Roddick, 1993), including *C. bombi* (Palmer-Young et al., In revision). 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 trypanosomes, such as the *C. bombi* relative *Leishmania major*, where the effects of crude plant
extracts and phytochemically complex essential oils can have greater antitrypanosomal 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 (Adler, 2001; Dobson and Bergstrom, 2000; 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 and 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 and Wink, 1993; London-Shafir et al., 2003; 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 et al., 2014), dietary pollen increased *C. bombi* infection intensity (Conroy et al., 2016; Logan et al., 2005) 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 and 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, I tested the direct effects of six pollens, a known antitrypanosomal nectar phytochemical, 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. These 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.

**Parasite culturing**

*Crithidia bombi* was isolated in 2013 by Ben Sadd from feces of wild *Bombus impatiens* near Normal, IL by flow cytometry (Salathé et al., 2012) and kept frozen at -80 °C until several weeks before the experiments. Thereafter, cells were grown at 27 °C in 50 cm² tissue culture flasks and transferred to fresh media every 3-4 days. No special permits were required for the collection. Cultures are available upon request, provided appropriate documentation and permissions are supplied.
Pollen and phytochemical treatments

Six types of bee-collected pollen—buckwheat (*Fagopyrum esculentum*), lotus (*Nelumbo nucifera*), poppy (*Papaver somniferum*), rapeseed (*Brassica napus*), sunflower (*Helianthus annuus*), and tea (*Camellia sinensis*)—were obtained from Changge Huading Wax Industry (Henan, China) in 2015. The pollens were stored at -20 °C and sorted to remove heterogeneous granules. For extraction, 6 g of pollen was incubated for 24 h at room temperature in constant darkness with 20 mL of 50% aqueous methanol in a 50 mL conical tube. The 50% methanol was used as a solvent due to its widespread application in phytochemical extraction of pollen (Serra Bonvehi et al., 2001) and other plant tissues (Keinänen et al., 2001). Samples were shaken at 180 rpm on a shaker table for the first 20 min of the extraction. After 24 h, tubes were centrifuged (30 min, 2700 g) and the supernatant removed, sterile-filtered, aliquoted, and stored at -80°C until use. The mixed-pollen extract consisted of equal volumes of buckwheat, rapeseed, and sunflower extracts, which were combined immediately before the experiment.

Caffeic acid was used as a representative phytochemical to evaluate possible negative and positive effects of pollen constituents on *C. bombi*. This hydroxycinnamic acid is likely to be widespread in bee diets, as it was the most widespread phytochemical in honey extracts, with occurrence at detectable levels in all 14 tested types of Turkish honey (Can et al., 2015); cinnamic acids and other phenolics are also abundant in pollen (Almaraz-Abarca et al., 2004; Campos et al., 1997; Serra Bonvehi et al., 2001). Caffeic acid has strong antitrypanosomal effects on *Leishmania donovani*, *Trypanosoma cruzi*, and *T. brucei* (Grecco et al., 2014; Tasdemir et al., 2006), which suggested that caffeic acid could inhibit *C. bombi* as well. However, caffeic acid is also a powerful antioxidant, with ability to scavenge reactive oxygen.
species that exceeds that of ascorbic acid and is comparable to that of tocopherols (Almaraz-Abarca et al., 2004; Chen and Ho, 1997). Antioxidant activity of caffeic acid and other pollen components (Almaraz-Abarca et al., 2004) might protect Crithidia bombi from stress incurred during the experiment, such as shaking and handling, and in the wild, where parasites encounter temperature changes, osmotic shock, UV light, and pro-oxidant enzymes of the bee immune system that may contribute to oxidative stress (Sadd and Barribeau, 2013; Vanaerschot et al., 2014). For experiments, caffeic acid was dissolved to a concentration of 4 mg mL$^{-1}$ in 50% methanol and tested at a final concentration of up to 333 ppm. This concentration is more than 30-fold the levels that occur in most types of honey (Can et al., 2015) and 10-fold the mean cinnamic acid concentration in pollen (Serra Bonvehi et al., 2001). Therefore, the tested concentration range was likely to detect any antitrypanosomal effects that could be attributed to phytochemical consumption by bees.

The addition of sugar to the medium was also tested for positive or negative effects on growth. Bee-collected pollen, such as that used to create the extracts in this study, is rich in sugars from nectar, which are added to the pollen by bees during collection (Roulston and Cane, 2000). Previous experiments with C. bombi cell cultures showed that growth was strongly inhibited by addition of 20% sugar to growth medium (Cisarovsky and Schmid-Hempel, 2014). However, other trypanosomes such as T. brucei prefer sugars to proteins as a carbon source (Lamour et al., 2005), which suggests that addition of sugar to the peptone- and liver-based growth medium (Salathé et al., 2012) could enhance growth. The sugar solution consisted of 20% w/v (10% dextrose (d-glucose) + 10% fructose) in 50% methanol. This sugar concentration was chosen to slightly exceed the likely sugar concentration in the pollen extracts, which was estimated as ~10%. This estimate was based on a sugar content of ~30-40% by weight in the...
pollen (Campos et al., 2008; Herbert and Shimanuki, 1978; Roulston and Cane, 2000; Todd and Bretherick, 1942), with ~30% pollen in the extract. The sugar composition was chosen to reflect the roughly equal amounts of dextrose and fructose that have been found in nectar and honey (London-Shafir et al., 2003; Ohmenhaeuser et al., 2013). Although pollen contains sugars other than monosaccharides (Herbert and Shimanuki, 1978), monosaccharides were used because the bee intestine rapidly hydrolyzes disaccharides to glucose and fructose (Nicolson, 1998), which likely leaves only monosaccharides in the distal intestine where trypanosomes become established (Lipa and Triggiani, 1988; Schwarz et al., 2015). The sugar solution was tested at up to 8.3% concentration by volume (16.7 g L\(^{-1}\) monosaccharides in growth medium).

**Experimental design**

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

To test the effects of pollen extracts, extract of each of the six pollens was dissolved at six concentrations by two-fold serial dilution. Concentrations ranged from 0-5% (for pollen extracts) or 0-8.3% (for chemical additions) final concentration by volume in growth medium.
Additional 50% methanol was added to samples of lesser concentrations to equalize methanol concentrations across samples. The inner 48 wells of a 96-well plate were filled with 100 µL of treatment solution (at 2x final concentration) and 100 µL of a suspension of *C. bombi* cells ($10^3$ cells µL$^{-1}$), to achieve an initial cell density of 500 cells µL$^{-1}$ (250 cells µL$^{-1}$ for chemical addition experiment). Outer wells were filled with 200 µL sterile water to mitigate edge effects. Plates were sealed with laboratory film and placed inside zippered sandwich bags to minimize 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.

**Analysis**

Linear regression was used to test for concentration-dependent changes in parasite growth. Treatment concentration was used as the predictor variable. Growth integral (i.e., area under the curve of growth vs. time, estimated using a model-free spline (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).

Results

Extracts of each of the six pollens resulted in increased C. bombi growth, as measured by the 5-day growth integral (Figure 1); the increase in growth was significant in analysis by linear regression (Table 1). Relative to the pollen-free control, addition of 5% extract of each pollen resulted in approximately 50% more growth over 5 d. Effects were similar for a mixture of buckwheat, rape, and sunflower extracts (Figure 2).

In the test of specific additional chemicals, both the buckwheat pollen extract (positive control) and 20% sugar solution resulted in increased growth (Figure 3, Table 1), whereas the common floral phytochemical caffeic acid (4 * 10³ ppm stock solution) had only weakly inhibitory effects at up to 333 ppm, which is an order of magnitude higher than any concentration documented among different types of honey (Can et al., 2015). Although inhibition was statistically significant (Table 1), the highest concentration resulted in <50% growth inhibition, which precluded estimation of a standard dose-response curve to determine the EC₅₀ concentration.
These experiments indicate that pollen extracts can increase growth of an intestinal parasite, and 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 trypanosome growth. This result is consistent with previous experiments that showed a decrease in infection intensity in bees deprived of pollen, and the results presented here suggest a mechanism by which pollen may promote trypanosome infection. The positive effects of pollen nutrients on *C. bombi*, a hindgut trypanosome, 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 trypanosomes invites further study on adaptation to phytochemicals in different trypanosome species.

**Phytochemical insensitivity**

*C. bombi* growth was not inhibited by any of the pollen extracts. This was unexpected in the context of the current literature on bumble bee-*Crithidia* interactions, which has suggested that phytochemical ingestion can counteract proliferation of *C. bombi* (Baracchi et al., 2015; Manson et al., 2010; Richardson et al., 2015). On the contrary, growth was actually increased in the presence of the extracts. Similarly, growth of *C. bombi* in the present study was only weakly inhibited (<50% decrease in growth integral, Figure 3) by caffeic acid at concentrations of over 300 ppm. This concentration is far higher than the levels that occur naturally in honey and pollen (Serra Bonvehi et al., 2001); for example, among 14 types of honey, none contained >27 ppm (Can et al., 2015). Insensitivity of *C. bombi* to hydroxycinnamic acids is consistent with previous
results (Palmer-Young et al., 2016), and striking in light of work on other trypanosomes. For comparison, concentrations needed for 50% growth inhibition ("EC$_{50}$") of $L$. donovani, $T$. brucei, and $T$. cruzi ranged from 1.1-56 ppm caffeic acid (Grecco et al., 2014; Tasdemir et al., 2006), and

$C$. bombi appears to be well adapted to phytochemicals, including those that are toxic to other trypanosomes and even those initially shown to reduce infection intensity. For example, $C$. bombi exhibited EC$_{50}$ values for several phenolics that were orders of magnitude higher than those reported for other trypanosome species (Palmer-Young et al., 2016), and 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 et al., 2013) or change in gut kinetics (Tadmor-Melamed et al., 2004) that have been observed in other species and could alter trypanosome attachment to the gut wall (Schwarz et al., 2015), the fact that none of the six pollens showed inhibitory activity demonstrates that $C$. bombi is robust to many of the phytochemicals in the diet of its hosts.

Many trypanosomes 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 the 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. Trypanosomes 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. Pollen collected by corbiculate bees, such as bumble bees, is moistened with nectar, which renders it sufficiently sticky to be carried back to the hive in the bee’s corbicula (pollen basket) (Roulston and Cane, 2000). As a result, bee-collected pollen contains considerable amounts of carbohydrate, including up to 40% sugars by weight (Roulston and Cane, 2000; Todd and Bretherick, 1942). Although the osmolarity of very high (~20%) sugar concentrations can kill *C. bombi* as well as other microbes (Cisarovsky and Schmid-Hempel, 2014), the effects found in this study indicate that addition of sugar at low concentrations is beneficial for trypanosome growth.

The monosaccharides added to the growth medium would have increased the sugar content several-fold, providing up to 16.7 additional g L\(^{-1}\) monosaccharides in addition to the 2.2 g L\(^{-1}\) in the original growth medium (1.8 g L\(^{-1}\) from fructose + 0.4 g L\(^{-1}\) from dextrose in liver broth (Salathé et al., 2012)). This additional sugar may have increased the quality of the media for *C. bombi* energy production. Trypanosomes rely on glycolysis for energy production (Mazet et al., 2013). Although they are capable of using peptides for energy (Bringaud et al., 2006), peptide metabolism is dramatically reduced in the presence of glucose, which suggests that glucose is a preferred energy source (Lamour et al., 2005). The trypanosomes that infect bees, which consume carbohydrate-rich diets, may be particularly adapted to utilization 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 et al., 2014*). Genomic studies may reveal whether carbohydrate metabolism is also well developed in *C. bombi*, which is a close relative of *L. passim* (*Schwarz et al., 2015*).

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

**Pollen in pollinator communities**

Pollen, like nectar, is an indispensable source of nutrients for bees and other pollinators that supports insect immunity (Brunner et al., 2014), survival (Conroy et al., 2016), and reproduction (Vaudo et al., 2015). However, pollen may nourish parasites as well as 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 trypanosomes (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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the separate funding statement.
References


**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.

*Mix treatment consisted of equal proportions of buckwheat, rape, and sunflower extracts.*
<table>
<thead>
<tr>
<th>A. Single pollens</th>
<th>Treatment</th>
<th>Slope ($\beta$)</th>
<th>Std. Error</th>
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<th>p (T)</th>
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<td>Buckwheat</td>
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<td>B. Mixed Pollens</td>
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<td>11.11</td>
<td>&lt;0.001</td>
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</table>

*Mix treatment consisted of equal proportions of buckwheat, rape, and sunflower extracts.
Individual pollen extracts increased parasite growth.

Extracts of six types of pollen were tested at up to 5% concentration. Each panel shows the 5 d growth integral (i.e., area under the curve of OD vs. time) 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 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.
Mixed and individual pollen extracts each increased growth.

The treatments consisted of extracts of individual pollens and 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 across samples. Points and error bars show means and standard errors for each concentration (n = 8).

Figure 2 (on next page)
Figure 3 (on next page)

An antitrypanosomal 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. Buckwheat pollen extract was used as a positive control to confirm increased growth in the presence of pollen extract. The caffeic acid solution contained $4 \times 10^3$ ppm caffeic acid in 50% methanol. The sugar solution consisted of 20% w/v monosaccharides (10% dextrose + 10% fructose in 50% methanol). Chemical solutions and pollen extract were added at the same final concentrations (up to 8.3% in prepared samples), which corresponds to up to 333 ppm caffeic acid and up to 16.7 mg L$^{-1}$ additional sugar. Additional 50% methanol was added to samples of lesser concentrations to equalize methanol concentrations across samples. Points and error bars show means and standard errors for each concentration (n = 5).
Buckwheat Extract

Caffeic acid (4000 ppm)

Sugar (20% w/v)

Concentration (percent by volume)