Secondary metabolites in a neotropical shrub: spatiotemporal allocation and role in fruit defense and dispersal

Lauren D. Maynard, Heather L. Slinn, Andrea E. Glassmire, Bernal Matarrita-Carranza, Craig D.

Dodson, Trang T. Nguyen, Megan J. Burroughs, Lee A. Dyer, Christopher S. Jeffrey, Susan R.

Whitehead

This is a post-peer-review, pre-copy edit version of an article published in Ecology. The final authenticated version is available online at: <https://doi.org/10.1002/ecy.3192>.

Copyright by the Ecological Society of America

Suggested Citation: Maynard, L. D., Slinn, H. L., Glassmire, A. E., Matarrita-Carranza, B., Dodson, C. D., Nguyen, T. T., Burroughs, M. J., Dyer, L. A., Jeffrey, C. S., and Whitehead, S. R.. 2020. Secondary metabolites in a neotropical shrub: spatiotemporal allocation and role in fruit defense and dispersal. *Ecology* 101(12):e03192. 10.1002/ecy.3192.

ABSTRACT (max 350 words)

Deciphering the ecological roles of plant secondary metabolites requires integrative studies that assess both the occurrence patterns of compounds and their bioactivity in ecological interactions. Secondary metabolites have been primarily studied in leaves, but many are unique to fruits and can have numerous potential roles in interactions with both mutualists (seed dispersers) and antagonists (pathogens and predators). We described 10 secondary metabolites of the class alkenylphenols from the plant species *Piper sancti-felicis*, quantified their patterns of intraplant allocation across tissues and fruit development, and examined their ecological role in fruit interactions. We found that fruit pulp had the highest concentrations and diversity of alkenylphenols, followed by flowers; leaves and seeds had only a few compounds at detectable concentrations. We observed a nonlinear pattern of alkenylphenol allocation across fruit development—increasing as the flowers developed into unripe pulp then decreasing as the pulp ripened. This pattern is consistent with the hypothesis that alkenylphenols function to defend fruits from pre-dispersal antagonists and are allocated based on the contribution of the tissue to the plant's fitness. To assess the impacts of alkenylphenols in interactions with antagonists and mutualists, we performed fungal bioassays, field observations, and feeding experiments with vertebrate dispersers. In fungal bioassays, we found that increasing concentrations of alkenylphenols had a negative effect on the growth of most fungal taxa. In field observations, nocturnal dispersers (bats) removed the majority of infructescences, and diurnal dispersers (birds) removed a larger proportion of unripe infructescences. In feeding experiments, bats had an aversion to alkenylphenols, but birds did not. This aversion observed in bats, combined with our results showing a decrease in alkenylphenols during ripening, suggests that alkenylphenols in fruits represent a trade-off (defending against pathogens but reducing disperser preference). Together,

this research provides a broad overview of the ecological significance of a little studied class of secondary metabolites in seed dispersal and fruit defense. It can also serve as a roadmap for integrating the study of intraplant spatiotemporal allocation patterns with ecological experiments to further our understanding of the evolutionary ecology of plant chemical traits.

Keywords: *Antagonism; Alkenylphenols; Defense trade-off hypothesis; La Selva Biological Station; Mutualism; Optimal defense theory; Piper sancti-felicis; Specialized metabolites*

INTRODUCTION

One of the most extraordinary features of plants is their biosynthetic capacity to generate diverse secondary metabolites. Secondary metabolites (also referred to as specialized metabolites), are thought to function primarily in plant interactions with the abiotic and biotic environment. They can have broad consequences for the ecology and evolution of plants, consumers, and entire communities (Kessler and Kalske 2018). However, only a small fraction of secondary metabolites have been structurally elucidated, and an even smaller fraction have any ascribed function. A key step toward understanding the ecological roles of secondary metabolites is to describe the intraplant spatiotemporal patterns of secondary metabolite occurrence (i.e. where and when are they occurring in the plant). This variation may be a crucial, yet overlooked, function for plant fitness (reviewed in Moore et al. 2014). While most ecological studies of secondary metabolites have focused on leaves, many compounds are produced primarily in other organs, such as fleshy fruits.

Fleshy fruits function primarily to attract animal mutualists who will effectively transport seeds away from the parent plant. However, the same rewards that attract dispersers are a resource

for many antagonists, including pathogens and seed predators. The high risk of attack of fruits, combined with their high fitness value, leads to predictions based on plant defense theory that fruits should be heavily defended (McKey 1974, Rhoades and Cates 1976, Zangerl and Rutledge 1996). Indeed, fruits often have higher diversity and concentrations of secondary metabolites compared to leaves (Herrera 1982, Cipollini and Levey 1997, Çirak and Radušienė 2007, Whitehead et al. 2013, Whitehead and Bowers 2014). Many secondary metabolites in fruits may also serve as frugivore attractants or function to mediate frugivore behavior and physiology (Thies et al. 1998, Cipollini 2000, Rodríguez et al. 2013, Baldwin and Whitehead 2015). Understanding the functional significance of fruit secondary metabolites could provide valuable insight into ecological processes, including seed dispersal—a critical ecological process that determines plant distribution and abundance (Cipollini and Levey 1997, Tewksbury 2002).

Fruits are complex organs, and different tissues and developmental stages likely experience an array of selective pressures and constraints that may shape their chemical traits. Studies that have compared the within-fruit spatial distribution of secondary metabolites (e.g. pulp vs seeds) have shown that the composition of secondary metabolites in these tissues can be highly variable and tissue-specific (Cappelletti et al. 1992, Barnea et al. 1993, Whitehead and Bowers 2013, Whitehead et al. 2013, Beckman 2013, Kolniak-Ostek 2016, D’Abrosca et al. 2017). For example, capsaicin in chilies occurs only in fruits and is highly concentrated in the placental tissue surrounding seeds (Iwai et al. 1979, Fujiwake et al. 1982). Secondary metabolite composition, at least in domesticated fruits, can also change dramatically during development (Hall et al. 1987, Kulkarni 2005, Zhang et al. 2010, Tohge et al. 2014), but these patterns, and the potential to inform our understanding of ecological function, are less explored in wild fruits.

There are different hypothesized adaptive functions of fruit secondary metabolites that

generate different predictions for changes in their allocation during fruit development. Here, we offer three hypotheses that explain the allocation of secondary metabolites in fruit development based on function: manipulation of mutualists, defense allocated by risk, and defense allocated by fitness (Table 1). First, a number of adaptive hypotheses explaining the occurrence of fruit secondary metabolites (e.g. gut retention time hypothesis, directed toxicity hypothesis, and attraction/repulsion hypothesis; Cipollini and Levey 1997) are united by the idea that certain secondary metabolites may function primarily to mediate interactions with vertebrate consumers of ripe fruits. For example, the gut retention time hypothesis (Cipollini and Levey 1997, Baldwin and Whitehead 2015) posits that certain secondary metabolites in fruits could function to mediate the passage rate of seeds in frugivore guts, thereby impacting dispersal distance and the exposure of seeds to gut conditions. If manipulation of disperser behavior or physiology is the primary adaptive function driving the patterns of occurrence of a particular secondary metabolite, we would predict maximum allocation to that metabolite in ripe fruits: the stage of fruit development with the greatest amount of interaction with frugivores.

Alternatively, fruit secondary metabolites may function primarily to defend fruits against insect pests and microbial pathogens, as posited by the defense trade-off hypothesis (Cipollini and Levey 1997, Dyer et al. 2001, Cazetta et al. 2008, Whitehead and Bowers 2014, Whitehead et al. 2016). Our second and third hypotheses both explain allocation patterns in fruits based on this idea. In both cases, the same secondary metabolites that defend fruits may also deter beneficial dispersers, leading to costly trade-offs when they are produced in ripe fruit pulp. Thus, if a secondary metabolite functions primarily in defense, we would expect the concentrations to decline with final ripening. However, the overall patterns during development may depend on the costs and benefits of defense.

Optimal defense theory predicts that plants allocate chemical defenses across different tissues based on the cost of defending that tissue, the relative risk of attack, and the fitness consequences of tissue loss (McKey 1974, Rhoades and Cates 1976). Depending on the relative importance of these factors, this could lead to various predictions for defense allocation during fruit development. If risk of attack is the main driver of allocation, we would predict that defenses are highest in immature fruits, which are composed of rapidly expanding and highly nutritious tissues that are not yet protected by physical defenses (e.g. a tough exocarp). The same general pattern is seen in leaves, where young leaves experience much higher rates of damage compared to mature leaves and are often more highly-defended (Kursar and Coley 1991, McCall and Fordyce 2010, Barton et al. 2019). If instead the fitness consequences of tissue loss are the main driver of allocation, we would predict a non-linear change in fruit secondary metabolites during development. Early in development, the metabolic investment in a fruit is still minimal, but as fruits mature, their fitness value increases. The negative fitness costs of consumption would peak immediately before fruit maturity: the plant has invested heavily in producing fruit, but the seeds are not yet viable. Once seeds mature, the fitness consequences of consumption can shift from a net fitness loss to a net gain (depending on the consumer), and maintaining high levels of defenses could limit dispersal. Thus, in this scenario, we would predict that the concentration of secondary metabolites will increase over development, peak before maturation, and then decrease as fruits enter the final ripening stage and are ready for dispersal (Table 1).

The hypotheses described above rest on the assumption that secondary metabolites in fruits provide adaptive benefits in biotic interactions and are specifically regulated in plants according to their fitness costs and benefits. However, it is also critical to consider that the occurrence patterns of many secondary metabolites may be the result of neutral or non-adaptive processes. At

least two non-adaptive processes may contribute to spatial and developmental patterns. First, certain secondary metabolites in fruits may be present due to strong selection for defense of leaves and other plant parts, combined with physiological constraints on their exclusion from fruit tissues (Swain 1977, Eriksson and Ehrlén 1998). In this case, we might expect that: 1) secondary metabolites should be more diverse and abundant in leaves than in fruits, and 2) concentrations in fruits and leaves should be correlated. These predictions have not been supported in other systems (Whitehead and Bowers 2013), but could be true for other plant species or classes of compounds. Second, temporal variation in secondary metabolite abundance during fruit development may occur as a passive consequence of other physiological processes, rather than the specific adaptive regulation of particular compounds. For example, a reduction in the concentration of a compound during ripening could be simply due to enzymatic degradation that occurs during fruit softening (Brady 1987). In this case, as with any non-adaptive scenario, there may be limited or neutral consequences of fruit secondary metabolites in fruit defense or seed dispersal. Thus, furthering our understanding of the evolutionary ecology of secondary metabolites in fruits requires a combination of descriptive documentation of spatiotemporal occurrence patterns and ecological experiments to examine the bioactivity of fruit secondary metabolites in interactions with fruit consumers—including both mutualists and antagonists.

In this study, we combine structure elucidation of secondary metabolites, quantitative descriptions of spatiotemporal chemical variation, field observations, fungal bioassays, and behavioral experiments with birds and bats to provide a broad overview of the evolutionary ecology of fruit secondary metabolites in *Piper sancti-felicitis* Trel. (Piperaceae). *Piper sancti-felicitis* is a widespread and abundant neotropical shrub and was chosen for a case study because it fruits abundantly throughout the year and represents a dietary staple for bats and birds (Fleming

2004, Thies and Kalko 2004). Little was known about the secondary chemistry of this species, and our initial analyses suggested infructescences were dominated by compounds structurally related to alkenylphenols described from other species of *Piper* (Orjala et al. 1998, Valdivia et al. 2008, de Oliveira et al. 2012, Yang et al. 2013, Varela et al. 2017, Yoshida et al. 2018). This study had four specific objectives: 1) to elucidate the structures of the major alkenylphenol compounds present in *P. sancti-felicitis*; 2) to assess the extent to which spatial patterns of alkenylphenol occurrence across tissues (leaves, flowers, unripe fruit pulp, ripe fruit pulp, and seeds) and temporal patterns during fruit development are consistent with different hypothesized functions of fruit secondary metabolites (Table 1); 3) to test the effects of alkenylphenols in interactions with fruit-associated fungi (antagonists); and 4) to test the effects of fruit alkenylphenols in interactions with vertebrate seed dispersers (mutualists). Together, these investigations provide an overview of the ecological significance of a group of secondary metabolites, demonstrate the value of using intraplant spatiotemporal variation to understand ecological roles, and, more broadly, contribute a holistic understanding of the functions of secondary metabolites in biotic interactions, including fruit defense and seed dispersal.

METHODS

Study site and system

All plant collection and experiments were conducted at La Selva Biological Station (LSBS), Heredia Province, Costa Rica. The station is managed by the Organization for Tropical Studies (OTS) and comprises approximately 1600 ha of tropical wet forest. The site has high diversity of the genus *Piper*, and hosts over 60 species (OTS 2020). *Piper* is one of the largest genera of flowering plants, containing well over 1,000 species. The greatest diversity of *Piper* is

found in the neotropics and lowland tropical forest sites, such as LSBS (Gentry 1990). The genus has distinctive inflorescences (spikes containing hundreds of small, reduced flowers along a rachis), and each flower matures into a single-seeded drupelet, creating an infructescence (Greig 2004; Fig. 1). For most species of *Piper*, all fruits on an infructescence mature simultaneously and are dispersed as a single unit. Previous investigations into the genus have described the presence of a broad range of secondary metabolites in leaves, including amides, alkaloids, lignans, terpenes, and steroids (Dyer et al. 2004). Structurally-related compounds to the alkenylphenols in *P. sancti-felicis* that have been described from several other species of *Piper* and have known antifungal, antimicrobial, and cytotoxic properties (Valdivia et al. 2008, Yang et al. 2013). Yet, the occurrence of alkenylphenols in *P. sancti-felicis* and their ecological significance was, to our knowledge, previously undescribed.

The primary dispersers of the infructescences of *Piper* in neotropical forests are a group of bats in the genus *Carollia* (Phyllostomidae), which depend on the infructescences as a year-round staple in their diet (Fleming 2004, Maynard et al. 2019). Although species of *Carollia* are the dominant dispersers, several species of birds also consume the infructescences of *Piper*, including tanagers (Thraupidae), sparrows (Emberizidae), manakins (Pipridae), toucans (Ramphastidae), cuckoos (Cuculidae), pigeons and doves (Columbidae; Palmeirim et al. 1989, Thies and Kalko 2004). After consumption, the seeds passed by both bats and birds are viable (Palmeirim et al. 1989). However, the two groups of dispersers handle the infructescences differently. Birds typically consume infructescences at the plant, stripping the pulp and seeds and leaving the rachis (Appendix S1: Fig. S1). Bats use a combination of echolocation and olfaction to locate ripe infructescences, which readily abscise from the plant (Thies et al. 1998). They collect the entire infructescence and carry it to a roost for consumption (Fleming 2004; Appendix S1: Fig. S2).

Furthermore, birds tend to defecate while perched, whereas Phyllostomid bats defecate more often during flight (Charles-Dominique 1986). Thus, seeds consumed by bats may be moved further where they may not be shaded or outcompeted by the parent plant (Levey 1987, Thies and Kalko 2004). These differences in handling behaviors, as well as infructescence removal rate and dispersal distance, likely all play a role in the relative seed dispersal effectiveness of birds versus bats (Schupp et al. 2010).

Structure elucidation of alkenylphenols in Piper sancti-felicis

For structure elucidation of alkenylphenols, unripe and ripe infructescences were collected from approximately 20 individuals of *P. sancti-felicis* occurring in and around the lab clearing at LSBS during June–August 2011. Approximately 200 infructescences were oven-dried at 50°C for 48 h, ground to a fine powder in a coffee grinder, and transported to the University of Nevada, Reno. An analysis of the crude ¹H-NMR extracts of the infructescences was performed first. The major components were further fractionated using flash column chromatography and preparatory TLC on silica gel using mixtures of hexanes and ethyl acetate, followed by detailed 1D and 2D ¹H-, ¹³C-NMR, and EI-MS analysis.

Quantification of alkenylphenols across tissues and developmental stages

To examine variation in alkenylphenols across tissues and developmental stages (Objective 2), one branch with fruits spanning a range of developmental stages (one ripe infructescence, two unripe infructescences, two inflorescences, and one developing inflorescence; Fig. 1) was collected from each of 21 *P. sancti-felicis* individuals (*n*=21), during June–July 2017 and June–July 2018. Similar to other species of *Piper*, infructescences of *P. sancti-felicis* ripen in the afternoon and are typically removed by bats the first night they are ripe (Fleming 2004). Thus,

each branch was collected in the afternoon, usually between 1300 h and 1600 h, so that it included a ripe infructescence that had matured on that day. Mature, fully expanded leaves from the branches were also collected. Each sample was dried in a separate envelope in the field with silica, transported to Virginia Tech, and further lyophilized prior to alkenylphenol extractions.

To separate reproductive tissues (i.e. pulp, seeds, and rachis), the dried samples were processed through stainless steel mesh sieves (0.01 mm or 0.0075 mm, depending on sample stage and seed size). Seeds were separated from the pulp for both ripe and unripe infructescences. However, only seeds from ripe infructescences were able to be fully cleaned of pulp; thus, only ripe seeds were analyzed. Dried leaves were ground whole. All samples were extracted and analyzed by GC-MS using a process similar to that in Whitehead et al. (2013) and Aziz et al. (2017). Additional methodological details are provided in Appendix S2.

Effects of alkenylphenols on fruit-associated fungi (antagonists)

To assess whether alkenylphenols that occur in *P. sancti-felicis* have a potential defensive role against fruit-associated fungi (Objective 3), we conducted a microdilution assay in September 2018 using methods modified from Zgoda and Porter (2001). To extract large quantities of alkenylphenols, ripe infructescences of *P. sancti-felicis* were locally collected at LSBS, oven dried at 60°C, and ground. A scaled-up version of the extraction procedure described above was used, beginning with a 10 g aliquot of dried plant material.

Three of the most common fungal taxa were selected from *P. sancti-felicis* seed fungi cultures (Slinn, unpublished data): *Microdochium lycopodium*, *Fusarium A*, and *Fusarium B* (Appendix S4: Table S4). Fungi were initially cultured on 2% malt extract agar (Thermo Fisher Scientific Oxoid Malt Extract) and plated at 26°C for 6–7 d. Mycelia were harvested from the plate by adding 1 mL of sterile water and probing the culture with the end of a tip to dislodge fungi.

Fungi were stored in a sterile microcentrifuge tube at 4°C until needed. For details on DNA extraction, PCR and sequencing see Appendix S2.

BLAST was used to align sequences to taxa in the UNITE v8.2 database which features additional quality control checks for fungi deposited in GenBank (Altschul et al. 1990, Kõljalg et al. 2005, accessed 28 February 2020). Taxa were assigned to ecological guilds using the FUNGuild database (Nguyen et al. 2016, accessed 14 May 2019). Two of the three taxa were classified to the genus *Fusarium* (*Fusarium* A and B; guilds: endophyte, plant and animal pathogen, wood saprotroph), and the third taxa was classified as *Microdochium lycopodium* (guilds: endophyte, plant pathogen; White et al. 1990, Nguyen et al. 2016; Appendix S4: Table S4). Sequences were deposited at the National Center for Biotechnology Information (NCBI) on GenBank under accession numbers MT093652 - MT093654 (Appendix S4: Table S4).

To determine if the compounds had an effect on fungal growth, we performed a microdilution assay with eight-serial dilutions. Each well received half of the alkenylphenol extract concentration compared to the previous well, fungal inoculum, 2% malt extract to provide nutrients for fungal growth, and sterile DI water. The final volume of each well was 200 µl. The first well received the highest concentration of extract with 5 µl at 73.15 mg/ml in ethanol and an additional 195 µl of water. Next, the remaining 7 wells received 100ul of sterile DI water. The water and extract in well 1 was mixed by pipetting before 100 µl of the 200 µl solution was aliquoted into the second well. This process of mixing newly aliquoted extract into 100 µl of water in subsequent wells was continued across the wells until the eighth well where the 100 µl taken was discarded. Once the wells had the appropriate concentration gradient of alkenylphenols, each well received 80 µl of 2% malt extract and 20 µl of fungal inoculum at a spore concentration of 10⁶ cells/ml. Thus, the final concentration of extract in the growth media at the highest concentration was 0.91

mg/mL, approximately equivalent to 0.0003 proportion fresh weight of a ripe infructescence and 6.2% of average alkenylphenol concentration found in one ripe infructescence. A sterile control with no fungi and no alkenylphenol extract was mixed, including 95 µl of water, 80 µl of 2% malt extract, and 25 µl of 100% ethanol. A negative control was also included with 20 µl of fungal inoculum and no alkenylphenols. These wells then received 95 µl of water, 80 µl of 2% malt extract, 5 µl of 100% ethanol in addition to the inoculum. To measure absorbance of fungi, a spectrophotometer was used at 450 nm with 96 well plates. Hyphal growth was measured as the difference in optical density (or absorbance) at 72h minus 0h. Additional methodological details are provided in Appendix S2.

Effects of alkenylphenols on seed dispersers (mutualists)

To determine if alkenylphenols that occur in *P. sancti-felicis* impact interactions with mutualistic vertebrate seed dispersers (Objective 4), we paired field observations with flight cage feeding experiments. For the field studies, we collected observational data on the removal of infructescences of *P. sancti-felicis* from natural plant populations. We chose 10 individual plants for monitoring. All plants were at least 30 m apart in clearings and along trails within 1 km of the field station. On each plant, we marked and mapped all unripe and ripe infructescences on up to 11 branches. Each plant was then visited twice daily (at dawn and dusk) during 26 May–31 May 2009, and we recorded all nocturnal and diurnal removal events for unripe and ripe infructescences on marked branches. In addition, to further describe the bird species that use plants of *P. sancti-felicis*, we conducted focal observations of six individual plants during 5 July–10 July 2018. Visiting bird species and their behavior (i.e. frugivory, gleaning, calling, etc.) were recorded (Appendix S4: Fig. S2).

To better understand vertebrate responses to the compounds, we conducted feeding trials in a controlled flight cage setting with the dominant consumers of the infructescences of *Piper*. One representative species was chosen from each group of dispersers: Seba's Short-tailed Bat (*Carollia perspicillata* Linnaeus, 1758) and Passerini's Tanager (*Ramphocelus passerinii* Desmarest, 1805; Appendix S1: Fig. S1 & S2). Feeding trials were conducted during January–March 2018. An experimental diet of mashed bananas and agar was used, which allowed us to test the effects of alkenylphenols on animal preference without the confounding effects of natural variation in alkenylphenols found in infructescences of *Piper*. The amount of food provided was equivalent to the fresh weight of one ripe infructescence of *P. sancti-felicis* (approximately 3 g). For the treatment diet, 1 mL of alkenylphenol extract was added at an estimated concentration of 14.6 mg/mL (0.0049 proportion fresh weight). For the control diet, 1 mL of ethanol was applied. All ethanol was evaporated before trials began by allowing diet to air-dry at room temperature (~26°C). For each choice test, one dish each of control and treatment diet were presented simultaneously to each animal (bats $N = 16$, birds $N = 10$) for between one and four trials that occurred over consecutive nights (bat trials $N = 58$) or days (bird trials $N = 27$). Each dish was pre-weighed and then placed in the flight cage on separate trays to account for any food displaced from the dish but not consumed. Bat trials began at 1800 h and bird trials began at 0600 h. Animals were checked every 30–60 min. After the animal had participated by consuming some portion of either diet, the dishes were removed and weighed to determine the amount eaten from each dish. Any food found in the trays was added to the respective dish. Additional methodological details are provided in Appendix S2.

Statistical analyses

All analyses were performed in R v3.6.1 (R Core Team 2020). Linear models, chi-squared analyses, and paired t-tests were performed using the base package, beta-regressions were performed using the package betareg v3.1-2 (Zeileis et al. 2019), estimated marginal means were performed using package emmeans v1.3.5.1 (Lenth et al. 2019), AIC model comparisons were performed using package AICcmodavg v2.2-2 (Linden 2019), analysis of variance (ANOVA) was performed using package car v3.0-3 (Fox et al. 2019), and plots were created with package ggplot2 (Wickham et al. 2019). All annotated code, data, and metadata are publicly archived at GitHub (https://github.com/ldmaynard/Alkenylphenols_Psf) and will be permanently archived at Dryad Digital Repository upon publication (DOI TBD).

Variation in alkenylphenols across tissue types and stages of development

To determine how alkenylphenol concentration varied across tissue types, we fit the data to a beta-regression. The response variable was the proportion dry weight of total alkenylphenols (summed across all detected compounds). Tissue type was a categorical predictor with five levels: leaves, late flowers (stage 4), late unripe pulp (stage 2), ripe pulp (stage 1), and ripe seeds. Estimated marginal means were computed for tissue type.

To determine how alkenylphenol concentration changed during fruit development and to assess support for linear and non-linear patterns (Table 1), we fit the data to two beta-regression models: one linear and one nonlinear (i.e. including a quadratic term). The proportion dry weight of total alkenylphenols was the response and developmental stage was a continuous predictor variable (as described in Fig. 1). AIC model comparison was used to select the model of best fit.

Effects of alkenylphenols on fruit-associated fungi (antagonists)

To determine the effect of alkenylphenol concentration and species of fungi on fungal growth, we fit the data to a linear model (LM). The response variable was the difference in

absorbance values of wells containing fungi, measured in average optical density (OD), at 72 h minus 0 h, averaged across triplicate readings taken from each well. The predictor variables were fungal taxa, alkenylphenol concentration in the growth media, and the interaction between the two. AIC model comparison was used to compare the full model to all possible component models. A two-way ANOVA was performed on the model of best fit (full model), which indicated a significant interaction between the species of fungi and concentration of alkenylphenols. Based on this result, the data were analysed separately for each fungi, using only concentration of alkenylphenols as a predictor variable.

Effects of alkenylphenols on seed dispersers (mutualists)

To examine the temporal differences in removal of unripe and ripe infructescences in our field observational study, a chi-squared analysis was used to test for independence between ripening stage and removal period in predicting the number of infructescences removed. To determine the effect of alkenylphenol presence on disperser response in our feeding trials, we performed paired t-tests, conducted separately for birds and bats, comparing the total amount of control eaten versus total amount of treatment eaten by each animal in the behavioral trials.

RESULTS

Structure elucidation of alkenylphenols in *Piper sancti-felicis*

Analysis of the crude ¹H-NMR extracts of the infructescences revealed the presence of *para*-alkenylphenols due to characteristic AB sets of coupled doublets in aromatic region (6-7ppm, $J = 8.5-9.0$ Hz), multiplets in the alkene region ($-\underline{CH}=\underline{CH}-$ 5.4-4.5 ppm), and characteristic aliphatic resonances of a long-chain hydrocarbon [$(\underline{CH}_2)_n$ 1.2 ppm and CH_3 0.88 ppm] (Fig. 2). GC-MS analysis revealed the presence of up to 10 distinct chromatographic peaks corresponding

to compounds A-J (Appendix S3: Fig. S1). Compound F ($R_t=13.6$) was the dominant peak in the extract with compounds B and D ($R_t=12.4$ and 12.9) as the other major components. Analysis of the mass spectra of all components suggested *para*-alkenylphenol in all cases based upon dominant fragmentation to the hydroxytropolium ion ($m/z = 107$) and compared favorably to literature data for other alkenylphenols that have been isolated from other species of *Piper* (Vieira et al. 1980, Galinis and Wiemer 1993, Jinno and Okita 1998, Li et al. 2008, Valdivia et al. 2008, Yang et al. 2013, Rajeev and Jain 2014, Dung et al. 2015, Yoshida et al. 2018). Further analysis of the molecular ion of these peaks suggested that the compounds A, B, E, F, I and J were phenols with C_{12} (A, B), C_{14} (E, F), C_{16} (I, J) *para*-alkenyl substituents containing one unit of unsaturation (C_nH_{2n-2}). Analysis of the molecular ion of compounds C, D, G and H demonstrated that the *para*-substituent was a doubly unsaturated dienyl chain of the C_{12} (C and D) C_{14} (G and H) series. Isomeric relationships between compound A and B, E and F, and I and J suggested that these compounds only differed by the double bond position in the mono-unsaturated *para*-substituent. The major components were further fractionated using flash column chromatography and preparatory TLC on silica gel using mixtures of hexanes and ethyl acetate. Detailed 1D and 2D ^1H -, ^{13}C -NMR, and EI-MS analysis of the major component were strongly consistent with the structure of a *p*-alkenylphenol. Double bond position was tentatively assigned in the mono-unsaturated series (A, B, E, F, I and J) based upon EI-MS fragmentation and comparisons to the literature. EI-MS analysis was consistent with the $\Delta^{3',4'}$ position of the double bond for compounds A, E, and I and the $\Delta^{4',5'}$ position for compounds B, F, and J. Comparison of all spectral data to known alkenylphenols that have been previously isolated from other *Piper* species strongly supported the structures of E and I, and supported the proposed structure of the major component F by comparison (Vieira et al. 1980, Yoshida et al. 2018). Double bond position and

stereochemistry of the doubly unsaturated components (C, D, G, and H) in the series could not be unequivocally established based upon NMR and EI-MS analysis. Full structural characterization data for F and tentative assignments of A-E and G-J are reported in the supplementary information (Appendix S3).

Variation of alkenylphenols across tissue types and stages of development

Alkenylphenols were abundant in unripe and ripe fruit pulp, present in flowers, and almost undetectable in leaves and seeds (Fig. 3). All 10 compounds were found in flowers and fruit pulp, nine were found in developing flowers, and only two were detected in seeds and leaves (compounds B and F; Appendix S4: Fig. S1 & Table S1). Our statistical analyses showed that pulp had higher concentrations of alkenylphenols compared to other tissues, including leaves, seeds, and late flowers. The average total concentration of alkenylphenols in unripe pulp was 1.5x higher than ripe pulp, 2.6x higher than flowers, 36.2x higher than seeds, and 534.9x higher than leaves (Fig. 3, Appendix S4: Fig. S2).

When examining alkenylphenol concentration across reproductive structure developmental stages, AIC model comparison indicated that the nonlinear model (with the quadratic term) was a better fit compared to the linear model ($dAICc = 12.25$; Appendix S5: Table S1), and the quadratic term was significant ($P < 0.001$), supporting a non-linear pattern in alkenylphenol concentration during development that peaked just before ripening. Developmental stage was also a significant predictor of alkenylphenol concentration across reproductive structure development ($P = 0.023$; Fig. 4). The average concentration of total alkenylphenols in ripe pulp was 1.8, 3.0, and 5.3 times higher compared to late, early, and developing flowers (stages 4, 5, and 6, respectively; Appendix S4: Table S3. However, the average concentration of total alkenylphenols in unripe pulp (stages 2

and 3) were 1.5 and 1.1 times higher compared to ripe pulp (Appendix S4: Table S3).
Concentrations of individual compounds are provided in Appendix S4.

Effects of alkenylphenols on fruit-associated fungi (antagonists)

The model that best fit the data was the LM that incorporated the interaction between fungal taxa and alkenylphenol concentration (dAICc = 11.4; Appendix S5: Table S2). The effect of alkenylphenol concentration varied based on fungal species ($F = 10.30$, $P = 0.0008$; two-way ANOVA of LM interaction). The growth of *Microdochium lycopodium* ($t = -7.03$, $P = 0.0002$, $R^2 = 0.88$; LM) and *Fusarium A* ($t = -5.21$, $P = 0.001$, $R^2 = 0.80$; LM) experienced clear negative effects when exposed to alkenylphenols; however, *Fusarium B* did not ($t = -1.00$, $P = 0.35$, $R^2 = 0.13$; LM). For every 1 mg/mL increase in alkenylphenol concentration, the average absorbance (growth) of *Microdochium lycopodium* and *Fusarium A* decreased by 0.50 OD and 0.43 OD, respectively (Fig. 5).

Effects of alkenylphenols on seed dispersers (mutualists)

During field observations, most infructescences of *P. sancti-felicitis* were removed at night (presumably bats; 67 infructescences, 91.8%). We found that diurnal removal events (presumably birds) were more likely to involve unripe infructescences (five unripe infructescences, 83.3%) whereas nocturnal events were more likely to involve ripe infructescences (59 ripe infructescences, 82.2%) ($\chi^2 = 14.609$, $df = 1$, $P = 0.00013$; chi-square test; Appendix S4: Table S5). During flight cage experiments, we found that alkenylphenols had a negative effect on bat feeding response ($t = 3.90$, $df = 15$, $P = 0.001$; paired t-test), but no detectable effect on birds ($t = 0.24$, $df = 9$, $P = 0.81$; paired t-test). Bats consumed an average of 2.4 times more control than treatment (Fig. 6a), whereas birds only consumed an average of 1.1 times more control than treatment (Fig. 6b).

DISCUSSION

Furthering our understanding of the chemical ecology of plant interactions requires integrative studies that incorporate the secondary metabolite structure determination, allocation, and ecological roles. Here, we present a case study of alkenylphenol compounds in a widespread tropical shrub. Using quantitative descriptions of intraplant variation combined with ecological experiments and observations, we put forth potential roles of these compounds in antagonistic and mutualistic interactions. We posited three hypotheses for fruit secondary metabolite allocation patterns: manipulation of mutualists, defense allocated by risk, and defense allocated by fitness (Table 1). We found that alkenylphenols in *P. sancti-felicitis* are dominant in the fruit pulp and flowers, but they were almost absent in leaves and seeds (with lower concentrations and diversity). By quantifying alkenylphenols across fruit development (from developing flower to ripe fruit pulp), we found that the secondary metabolite concentration follows a nonlinear trend, increasing through development until the fruit begins to ripen, then declining in the final ripening stage. This pattern is consistent with the hypothesis that fruit secondary metabolites function primarily as an adaptive defense and are allocated in fruits according to the fitness costs and benefits of consumption. Alternatively, the decline with ripening could be non-adaptive, and instead due to general enzymatic degradation during ripening (Brady 1987). However, the fungal and vertebrate bioassays also supported an adaptive explanation for the observed allocation patterns. Alkenylphenols had clear negative effects on two taxa of potentially pathogenic fungi associated with seeds (Fig. 5), but also had a negative effect on the feeding preferences of bats (specifically *C. perspicillata*; Fig. 6)—one of the main seed dispersers of *P. sancti-felicitis* (Maynard et al. 2019). Thus, alkenylphenols may represent a trade-off in fruits between defense against antagonists and

attraction of mutualists, and plants may downregulate these compounds during ripening to minimize these trade-offs.

Structure elucidation of alkenylphenols in Piper sancti-felicii

Alkenylphenols are relatively rare in the plant kingdom, and, aside from Piperaceae, have been reported in only three other plant families: Anacardiaceae (Franke et al. 2001), Myrsinaceae (Jiménez-Romero et al. 2007), and Myristicaceae (Gonzalez et al. 1996). Alkenylphenol compounds have been identified in seven other species of *Piper*: *P. hispidum* (Vieira et al. 1980), *P. gibbilimum* (Orjala et al. 1998), *P. obliquum* (Valdivia et al. 2008), *P. malacophyllum* (de Oliveira et al. 2012, Varela et al. 2017), *P. sarmentosum* (Yang et al. 2013), *P. dilatatum* and *P. diospyrifolium* (Yoshida et al. 2018). These previously described alkenylphenols have been found in several organs of species of *Piper*, including leaves (Orjala et al. 1998, Valdivia et al. 2008, de Oliveira et al. 2012), inflorescences (Valdivia et al. 2008), infructescences (Yoshida et al. 2018), roots (Yoshida et al. 2018), and aerial parts (Yang et al. 2013). However, this is the first study to find alkenylphenol concentration and diversity highest in *Piper* fruit pulp. Previously described alkenylphenols in *Piper* species have shown the compounds to exhibit antifungal, antimicrobial, and cytotoxic properties (Valdivia et al. 2008, Yang et al. 2013). However, to our knowledge, this is the first study to explore the adaptive significance of alkenylphenols with a variety of interactors, including the three most abundant fungal taxa found on the seeds (antagonists) and two taxa of the most frequent vertebrate frugivores (mutualists).

Variation of alkenylphenols across tissue types and stages of development

Our results showing alkenylphenols are found largely in fruits and flowers is a key piece of evidence suggesting these compounds function in reproductive structures. This pattern is in direct contrast to predictions that stem from the non-adaptive “physiological constraints”

hypothesis that suggests that certain secondary metabolites in fruits may be present due to strong selection for defense of leaves and other plant parts, combined with physiological constraints on their exclusion from fruit tissues (Swain 1977, Eriksson and Ehrlén 1998). Instead, as alkenylphenols were found primarily in pulp (Fig. 4), their presence in leaves and seeds may be due to physiological constraints on their exclusion from those tissues. This pattern of secondary metabolites restricted to non-vegetative plant parts is observed in other species. For example, in *Capsicum annuum*, fruits and placenta contain capsaicin but vegetative parts do not (Iwai et al. 1979). In *Piper reticulatum*, seeds have the highest concentrations and chemical diversity of amides compared to fruit pulp and leaves (Whitehead et al. 2013). And in *Vaccinium macrocarpon*, ripe berries contain anthocyanin but unripe berries, leaves, stems, roots, and seeds do not (Zhou and Singh 2002). The various classes of secondary metabolites likely play an adaptive role in flowers and/or fruits, which experience complex and often conflicting selection pressures as they serve to both attract mutualists (pollinators and seed dispersers) while deterring antagonists (predators and pathogens; Cazetta et al. 2008, Kessler and Halitschke 2009).

Our results showing developmental variation in alkenylphenols uncover more detailed information about the value and function of secondary metabolites. Of the three hypotheses posited in this case study (manipulation of mutualists, defense allocated by attack, and defense allocated by fitness), our results are consistent with the last. We found that fruit pulp had the highest concentration of alkenylphenols, and the concentrations were allocated non-linearly across ripening (increasing across development, then decreasing as fruit pulp ripened), which suggests that the fitness consequences of tissue loss may be a better predictor of allocation patterns than relative risk of attack (assuming the early developing tissues are indeed at higher risk of attack, which has not been explicitly shown in this system). However, we cannot rule out the second non-

adaptive hypothesis, where the reduction in the concentration of a compound during ripening could be due to a non-adaptive enzymatic degradation that occurs during fruit softening (Brady 1987). To explore this hypothesis, we would need to further understand the mechanisms behind the reduction of alkenylphenols during ripening, including the processes involved, the ultimate fate of the compounds, and any functional consequences of the products.

Effects of alkenylphenols on fruit-associated fungi (antagonists)

We found that alkenylphenols had clear antifungal activity against two (*Fusarium A* and *M. lycopodium*) of three fungal taxa, even at conservative doses relative to the concentrations found in fruit pulp (Fig. 5). This suggests that fruit defense against fungal pathogens may be at least one important function of alkenylphenols, similar to the role of other classes of secondary metabolites found in high concentration in fruits (Haak et al. 2012, Whitehead and Bowers 2014). Many fungal pathogens can destroy seeds, inhibit seed germination, and deter vertebrate seed dispersers (Whittaker and Feeny 1971, Janzen 1977, Gallery et al. 2010); thus, defense of seeds against fungi is critical for plant fitness. The fungal taxa used in our study were isolated from *P. sancti-felicitis* seeds. While this study did not directly test the pathogenic relationships between the fungi and *P. sancti-felicitis*, both fungal taxa have been documented in other systems as having pathogenic properties (Tedersoo et al. 2014, Blacutt et al. 2018). *Fusarium* is a genus that is well known for producing plant pathogens in many types of crops across ecosystems (Booth 1971, Summerell 2019). For instance, *Fusarium verticilloides* can act as a seed-borne fungus in maize (*Zea mays*), which causes seed, ear and stalk rot, among other seedling diseases (Marasas 2001). While one of the most common *Fusarium* pathogens, *Fusarium graminearum*, damages the reproductive structures of multiple different species of cereals and other crops in both temperate and semi-tropical habitats (Goswami and Kistler 2004). Other species of *Fusarium* are known

pathogens of *Piper* species: *P. betle* and *P. nigrum* (Shahnazi et al. 2012, Edward et al. 2013). Furthermore, *Microdochium* is a common pathogen of grasses (Hernández-Restrepo et al. 2016), but it can also act as a dark septate endophyte which colonizes the roots of grasses (Mandyam et al. 2012). In an experimental inoculation with *Microdochium* as a dark septate endophyte, colonization increased plant biomass or had no effect, depending on the species of grass (Mandyam et al. 2012). Taken together, these studies suggest that *Microdochium* can be beneficial, antagonistic, or have no effect on the host plant, depending on the context. Considering this variation in the outcomes of plant-fungal interactions, further work is necessary to understand the ultimate fitness consequences of antifungal alkenylphenols in *Piper* infructescences.

Given that one of our fungal taxa (*Fusarium* B) did not demonstrate a decline in growth with concentration of alkenylphenols (Fig. 5), it is possible that it has evolved greater tolerance to plant secondary metabolites that interfere with metabolism, which may be achieved through the use of alternative energy production enzymes (Kerscher et al. 1999, Marcet-Houben et al. 2009, O'Donnell et al. 2011). For instance, in the wild chili pepper (*Capsicum chacoense*), fungal tolerance to dihydrocapsaicin was found in 16 fungal pathogens from four distantly related genera (*Alternaria*, *Colletotrichum*, *Fusarium* and *Phomopsis*), which had alternative enzymes at different stages of the electron transport chain such as for NADH dehydrogenase and oxidase (Adams et al. 2019). Due to the phylogenetic breadth of this study, it suggests that alternative mechanisms which allow fungal pathogens to tolerate capsaicinoids may be common (Adams et al. 2019). Furthermore, it's interesting that one *Fusarium* taxon experienced a decline in growth while the other taxon did not. This might be caused by variations in alternative metabolism enzymes mentioned above, as this is a large genus which infects many different plant species. Alternatively, Peris et al. (2017) found that fungal infestations can boost fruit odors, thereby

increasing fruit removal by vertebrates. Thus, the fruit may be experiencing contradicting selective pressures to protect against fungal competition of resources while maintaining fungi to attract vertebrate seed dispersers.

Effects of alkenylphenols on seed dispersers (mutualists)

Our results suggest that alkenylphenols may also mediate *P. sancti-felicitis* interactions with seed dispersers. Most neotropical species of *Piper* are largely dispersed by bats (Fleming 2004), but many species are also consumed extensively by birds (Palmeirim et al. 1989, Theis and Kalko 2004), or occasionally by other small mammals (Leiser-Miller et al. 2019) or ants (Thies and Kalko 2004, Clemente and Whitehead 2019). Our study expanded on the natural history knowledge of seed dispersal in this system by quantifying nocturnal and diurnal fruit removal (Appendix S4: Table S5) and further documented the community of birds that utilize *P. sancti-felicitis* as a resource (Appendix S4: Fig. S2). We found that *R. passerini* was the bird species found most often using *P. sancti-felicitis* plants, and a total of six bird species were observed eating the infructescences of *P. sancti-felicitis* (Appendix S4: Fig. S2). Field observations revealed a strong difference in the amount and maturity of infructescences of *P. sancti-felicitis* taken by nocturnal and diurnal animals. Nocturnal consumers (presumably mostly bats) removed more infructescences and favored ripe over unripe infructescences, whereas diurnal consumers (presumably mostly birds) removed fewer infructescences and most were unripe (Appendix S4: Table S5). This makes sense considering our experimental results showing that birds were less deterred by alkenylphenols, which are present in higher concentrations in unripe fruit. A key unanswered question for determining the fitness consequences of bird interactions with *P. sancti-felicitis* is whether the seeds consumed in unripe infructescences contain viable seeds that are dispersed intact following bird consumption.

The stronger preference for ripe fruit in bats could also be in part due to alkenylphenol content, which we found to be higher in unripe than in ripe fruit pulp (Fig. 4). Our disperser preference trials indicated that alkenylphenol compounds decrease palatability but only for one group of dispersers: bats (Fig. 7). A similar scenario has been shown in other systems, where birds seem to have a higher threshold for secondary metabolites in fruits compared to small mammals (Tewksbury and Nabhan 2001, but see Karasov et al. 2012). However, in this case the deterrent effect is against the most frequent (and likely most effective) seed disperser. Quantifying the fitness consequences of this deterrent effect would require extensive field studies to track seeds and seedlings, but there are likely important costs associated with reduced bat preference. Even if most infructescences are removed, infructescences containing high concentrations of deterrent secondary metabolites could be rejected once bats begin to feed and dropped partially intact below a feeding roost (as is the case with amides, Whitehead et al. 2016), where competition and pathogen loads are likely high. In addition, less-preferred fruits may experience shorter dispersal distances if they are removed later in the evening once the peak hours of bat activity have passed (Baldwin et al. 2020). Thus, our overall findings are consistent with the defense trade-off hypothesis (Cipollini and Levey 1997)—alkenylphenols may defend against fungi but have costs associated with deterring an important group of seed dispersers. This scenario also provides a parsimonious explanation for our results showing nonlinearity of alkenylphenol concentration across development (Fig. 4)—the decrease upon ripening could be a product of selective pressure exerted on fruit chemistry by bat feeding preference.

Future directions

In *P. sancti-felicitis*, we were unable to definitively determine the double bond position and stereochemistry of the doubly unsaturated components for four of the 10 alkenylphenols (C, D, G,

and H). Future work may target the isolation and structure determination of the less abundant components as they may also have important biological roles. Although our study provides a strong foundation for understanding the ecological role of alkenylphenols in *P. sancti-felicitis*, we cannot definitively determine the *raison d'être* of these compounds. They may have a wide spectrum of potential functions (as seen with emodin; Izhaki 2002), and we did not address them all. To expand upon the current knowledge and further address questions posed in this study, future work may explore the potential multifunctionality of alkenylphenols for plants and how they improve plant fitness. To address the other hypotheses put forth by Cipollini and Levey (1997), studies may examine the effects of alkenylphenols on seed germination and establishment (seed germination inhibition hypothesis) and on vertebrate disperser digestion (gut retention time hypothesis). We determined that alkenylphenols are decreasing during ripening, but we did not determine the fate of the alkenylphenols over the ripening process. Future studies may examine whether the decrease in alkenylphenols across development is a result of biochemical activity, dilution, or translocation.

Conclusions

In this case study, we detail 10 previously undescribed alkenylphenol compounds in *P. sancti-felicitis*. We found that these alkenylphenols have greater concentrations and diversity in fruit pulp and flowers when compared to leaves and seeds. Furthermore, the compound concentration experiences a non-linear pattern across fruit development—increasing until fruit ripeness, when it decreases. This uneven allocation of concentration and diversity in fruit pulp and flowers, with the highest concentrations found in fruit pulp, suggests a role in fruit interactions. Although the compounds are protecting against potential fungal pathogens, they deter important nocturnal seed dispersers (bats). We suggest that these compounds are primarily defensive and allocated based on the fitness cost of tissue consumption. We hope this research will serve as a roadmap for using

intraplant allocation patterns to better understand the evolutionary ecology of plant chemical traits and as a case study demonstrating the important roles of secondary metabolites in seed dispersal and fruit defense.

ACKNOWLEDGEMENTS

We thank Orlando Vargas Ramírez, Danilo Brenes Madrigal, Joel Alvarado, Danielle Salcido, and Bernadette Rigley for their assistance at La Selva Biological Station. We thank Sherri Maynard for her illustrations. Funding for the chemical isolation and analysis was provided by the Hitchcock Center for Chemical-Ecology at UNR and the National Science Foundation (DEB-1145609 and DEB-1442103). We thank Jennifer L. McCracken for her assistance with the collection of the alkenylphenol spectroscopic data. Quantification of alkenylphenols at Virginia Tech was partially supported by NSF Grant DEB-1856766 to SRW. LDM was supported by a National Science Foundation Graduate Research Fellowship, OTS Graduate Research Fellowships, and Virginia Polytechnic Institute and State University, including start-up funds to SRW and the Graduate Student Assembly Graduate Research Development Program. HLS was supported by a Natural Sciences and Engineering Research Council Post-Graduate Doctoral Scholarship, the Mycological Association of America Backus Award, and the College of Biological Science at the University of Guelph.

LITERATURE CITED

Adams, C. A., K. Zimmerman, K. Fenstermacher, M. G. Thompson, W. Skyrud, S. Behie, and A. Pringle. 2019. Fungal seed pathogens of wild chili peppers possess multiple mechanisms to tolerate capsaicinoids. *Applied and Environmental Microbiology* 86:e01697-19.

617 Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment
618 search tool. *Journal of Molecular Biology* 215:403–410.

619 Baldwin, J. W., D. K. N. Dechmann, W. Thies, and S. R. Whitehead. 2020. Defensive fruit
620 metabolites obstruct seed dispersal by altering bat behavior and physiology at multiple
621 temporal scales. *Ecology* 101:e02937.

622 Baldwin, J. W., and S. R. Whitehead. 2015. Fruit secondary compounds mediate the retention
623 time of seeds in the guts of neotropical fruit bats. *Oecologia* 177:453–466.

624 Barnea, A., J. B. Harborne, and C. Pannell. 1993. What parts of fleshy fruits contain secondary
625 compounds toxic to birds and why? *Biochemical Systematics and Ecology* 21:421–429.

626 Barton, K. E., K. F. Edwards, and J. Koricheva. 2019. Shifts in woody plant defence syndromes
627 during leaf development. *Functional Ecology* 33:2095–2104.

628 Beckman, N. G. 2013. The distribution of fruit and seed toxicity during development for eleven
629 neotropical trees and vines in central Panama. *PLoS ONE* 8:e66764.

630 Blacutt, A. A., S. E. Gold, K. A. Voss, M. Gao, and A. E. Glenn. 2018. *Fusarium verticillioides* :
631 advancements in understanding the toxicity, virulence, and niche adaptations of a model
632 mycotoxigenic pathogen of maize. *Phytopathology* 108:312–326.

633 Booth, C. 1971. The genus *Fusarium*. Commonwealth Mycological Institute, Kew, Surrey, UK.

634 Brady, C. J. 1987. Fruit ripening. *Annual Review of Plant Physiology* 38:155–178.

635 Cappelletti, E. M., G. Innocenti, and G. Caporale. 1992. Possible ecological significance of
636 within-fruit and seed furocoumarin distribution in two *Psoralea* species. *Journal of*
637 *Chemical Ecology* 18:155–164.

638 Cazetta, E., H. M. Schaefer, and M. Galetti. 2008. Does attraction to frugivores or defense
639 against pathogens shape fruit pulp composition? *Oecologia* 155:277–286.

640 Charles-Dominique, P. 1986. Inter-relations between frugivorous vertebrates and pioneer plants:
641 *Cecropia*, birds and bats in French Guyana. Pages 119–135 in A. Estrada and T. H.
642 Fleming, editors. Frugivores and seed dispersal. Springer Netherlands, Dordrecht.

643 Cipollini, M. L. 2000. Secondary metabolites of vertebrate-dispersed fruits: evidence for
644 adaptive functions. *Revista Chilena de Historia Natural* 73:421–440.

645 Cipollini, M. L., and D. J. Levey. 1997. Secondary metabolites of fleshy vertebrate-dispersed
646 fruits: adaptive hypotheses and implications for seed dispersal. *The American Naturalist*
647 150:346–372.

648 Clemente, S. R., and S. R. Whitehead. 2019. Ant seed removal in a non-myrmecochorous
649 neotropical shrub: Implications for seed dispersal. *Biotropica* 00:1–11.

650 D’Abrosca, B., M. Lavorgna, M. Scognamiglio, C. Russo, V. Graziani, C. Piscitelli, A.
651 Fiorentino, and M. Isidori. 2017. 2D-NMR investigation and in vitro evaluation of
652 antioxidant, antigenotoxic and estrogenic/antiestrogenic activities of strawberry grape.
653 *Food and Chemical Toxicology* 105:52–60.

654 Dyer, L. A., C. D. Dodson, J. Beihoffer, and D. K. Letourneau. 2001. Trade-offs in antiherbivore
655 defenses in *Piper cenocladum*: ant mutualists versus plant secondary metabolites. *Journal*
656 *of Chemical Ecology* 27:581–592.

657 Dyer, L. A., J. Richards, and C. D. Dodson. 2004. Isolation, synthesis, and evolutionary ecology
658 of *Piper* amides. Pages 117–139 in L. A. Dyer and A. D. N. Palmer, editors. *Piper*: a
659 model genus for studies of phytochemistry, ecology, and evolution. Kluwer
660 Academic/Plenum Publishers, New York, NY.

661 Edward, E. J., W. S. King, S. L. C. Teck, M. Jiwan, Z. F. A. Aziz, F. R. Kundat, O. H. Ahmed,
662 and N. M. A. Majid. 2013. Antagonistic activities of endophytic bacteria against

663 *Fusarium* wilt of black pepper (*Piper nigrum*). International Journal of Agriculture and
 664 Biology 15:291–296.

665 Eriksson, O., and J. Ehrlén. 1998. Phenological adaptations in fleshy vertebrate-dispersed fruits
 666 of temperate plants. Oikos 82:617–621.

667 Fleming, T. H. 2004. Dispersal ecology of neotropical *Piper* shrubs and treelets. Pages 58–77 in
 668 L. A. Dyer and A. D. N. Palmer, editors. *Piper: a model genus for studies of*
 669 *phytochemistry, ecology, and evolution*. First edition. Kluwer Academic/Plenum
 670 Publishers, New York, NY.

671 Fox, J., S. Weisberg, B. Price, D. Adler, D. Bates, G. Baud-Bovy, B. Bolker, S. Ellison, D. Firth,
 672 M. Friendly, G. Gorjanc, S. Graves, R. Heiberger, P. Krivitsky, R. Laboissiere, M.
 673 Maechler, G. Monette, D. Murdoch, H. Nilsson, D. Ogle, B. Ripley, W. Venables, S.
 674 Walker, D. Winsemius, A. Zeileis, and R-Core. 2019. car: Companion to applied
 675 regression.

676 Fujiwake, H., T. Suzuki, and K. Iwai. 1982. Intracellular distributions of enzymes and
 677 intermediates involved in biosynthesis of capsaicin and its analogues in *Capsicum* fruits.
 678 Agricultural and Biological Chemistry 46:2685–2689.

679 Galinis, D. L., and D. F. Wiemer. 1993. Villiramulins A and B: new phenol derivatives from
 680 *Piper villiramulum*. The Journal of Organic Chemistry 58:7804–7807.

681 Gallery, R. E., D. J. P. Moore, and J. W. Dalling. 2010. Interspecific variation in susceptibility to
 682 fungal pathogens in seeds of 10 tree species in the neotropical genus *Cecropia*. Journal of
 683 Ecology 98:147–155.

684 Gentry, A. H. 1990. Four neotropical forests. Yale University Press, New Haven, C.T., U.S.A.

685 Goswarmi, R. S., and H. C. Kistler. 2004. Heading for disaster: *Fusarium graminearum* on

686 cereal crops. *Molecular Plant Pathology* 5:515–525.

687 Greig, N. 2004. Introduction. Page in L. A. Dyer and A. D. N. Palmer, editors. *Piper: a model*
688 genus for studies of phytochemistry, ecology, and evolution. Kluwer Academic/Plenum
689 Publishers, New York, NY.

690 Haak, D. C., L. A. McGinnis, D. J. Levey, and J. J. Tewksbury. 2012. Why are not all chilies hot?
691 A trade-off limits pungency. *Proceedings of the Royal Society B: Biological Sciences*
692 279:2012–2017.

693 Hall, R. D., M. A. Holden, and Michael. M. Yeoman. 1987. The accumulation of
694 phenylpropanoid and capsaicinoid compounds in cell cultures and whole fruit of the chilli
695 pepper, *Capsicum frutescens* Mill. *Plant Cell, Tissue and Organ Culture* 8:163–176.

696 Hernández-Restrepo, M., J. Z. Groenewald, and P. W. Crous. 2016. Taxonomic and phylogenetic
697 re-evaluation of *Microdochium*, *Monographella* and *Idriella*. *Persoonia* 36:57–82.

698 Iwai, K., T. Suzuki, and H. Fujiwake. 1979. Formation and accumulation of pungent principle of
699 hot pepper fruits, capsaicin and its analogues, in *Capsicum annuum* var. *annuum* cv.
700 Karayatsubusa at different growth stages after flowering. *Agricultural and Biological*
701 *Chemistry* 43:2493–2498.

702 Izhaki, I. 2002. Emodin: a secondary metabolite with multiple ecological functions in higher
703 plants. *The New Phytologist* 155:205–217.

704 Janzen, D. H. 1977. Why fruits rot, seeds mold, and meat spoils. *The American Naturalist*
705 111:691–713.

706 Karasov, W. H., E. Caviedes-Vidal, B. H. Bakken, I. Izhaki, M. Samuni-Blank, and Z. Arad.
707 2012. Capacity for absorption of water-soluble secondary metabolites greater in birds
708 than in rodents. *PLoS ONE* 7:e32417.

709 Kerscher, S. J., J. G. Okun, and U. Brandt. 1999. A single external enzyme confers alternative
710 NADH:ubiquinone oxidoreductase activity in *Yarrowia lipolytica*. *Journal of Cell*
711 *Science* 112:2347–2354.

712 Kessler, A., and R. Halitschke. 2009. Testing the potential for conflicting selection on floral
713 chemical traits by pollinators and herbivores: predictions and case study. *Functional*
714 *Ecology* 23:901–912.

715 Kessler, A., and A. Kalske. 2018. Plant secondary metabolite diversity and species interactions.
716 *Annual Review of Ecology, Evolution, and Systematics* 49:115–138.

717 Kõljalg, U., K.-H. Larsson, K. Abarenkov, R. H. Nilsson, I. J. Alexander, U. Eberhardt, S.
718 Erland, K. Høiland, R. Kjøller, E. Larsson, T. Pennanen, R. Sen, A. F. S. Taylor, L.
719 Tedersoo, and T. Vrålstad. 2005. UNITE: a database providing web-based methods for
720 the molecular identification of ectomycorrhizal fungi. *New Phytologist* 166:1063–1068.

721 Kolniak-Ostek, J. 2016. Chemical composition and antioxidant capacity of different anatomical
722 parts of pear (*Pyrus communis* L.). *Food Chemistry* 203:491–497.

723 Kulkarni, A. 2005. Chemical changes and antioxidant activity in pomegranate arils during fruit
724 development. *Food Chemistry* 93:319–324.

725 Kursar, T. A., and P. D. Coley. 1991. Nitrogen content and expansion rate of young leaves of
726 rain forest species: implications for herbivory. *Biotropica* 23:141–150.

727 Leiser-Miller, L. B., Z. A. Kaliszewska, D. Villalobos-Chaves, and S. E. Santana. 2019. A
728 pygmy rice rat eats a peppery snack. *Frontiers in Ecology and the Environment* 17:369–
729 369.

730 Lenth, R., H. Singmann, J. Love, P. Buerkner, and M. Herve. 2019. emmeans: estimated
731 marginal means, aka least-squares means.

732 Levey, D. J. 1987. Seed size and fruit-handling techniques of avian frugivores. *The American*
 733 *Naturalist* 129:471–485.

734 Linden, M. J. M. and portions of code contributed by D. 2019. AICcmodavg: model selection
 735 and multimodel inference based on (Q)AIC(c).

736 Mandyam, K., C. Fox, and A. Jumpponen. 2012. Septate endophyte colonization and host
 737 responses of grasses and forbs native to a tallgrass prairie. *Mycorrhiza* 22:109–119.

738 Marasas, W. F. O. 2001. Discovery and occurrence of the fumonisins: a historical perspective.
 739 *Environmental Health Perspectives* 109:239–243.

740 Marcet-Houben, M., G. Marceddu, and T. Gabaldón. 2009. Phylogenomics of the oxidative
 741 phosphorylation in fungi reveals extensive gene duplication followed by functional
 742 divergence. *BMC Evolutionary Biology* 9:295.

743 Maynard, L. D., A. Ananda, M. F. Sides, H. Burk, and S. R. Whitehead. 2019. Dietary resource
 744 overlap among three species of frugivorous bat in Costa Rica. *Journal of Tropical*
 745 *Ecology* 35:165–172.

746 McCall, A. C., and J. A. Fordyce. 2010. Can optimal defence theory be used to predict the
 747 distribution of plant chemical defences? *Journal of Ecology* 98:985–992.

748 Moore, B. D., R. L. Andrew, C. Külheim, and W. J. Foley. 2014. Explaining intraspecific
 749 diversity in plant secondary metabolites in an ecological context. *New Phytologist*
 750 201:733–750.

751 Nguyen, N. H., Z. Song, S. T. Bates, S. Branco, L. Tedersoo, J. Menke, J. S. Schilling, and P. G.
 752 Kennedy. 2016. FUNGuild: an open annotation tool for parsing fungal community
 753 datasets by ecological guild. *Fungal Ecology* 20:241–248.

754 Nilsson, R. H., K.-H. Larsson, A. F. S. Taylor, J. Bengtsson-Palme, T. S. Jeppesen, D. Schigel,

755 P. Kennedy, K. Picard, F. O. Glöckner, L. Tedersoo, I. Saar, U. Kõljalg, and K.
 756 Abarenkov. 2019. The UNITE database for molecular identification of fungi: handling
 757 dark taxa and parallel taxonomic classifications. *Nucleic Acids Research* 47:D259–D264.
 758 Nilsson, R. H., M. Ryberg, E. Kristiansson, K. Abarenkov, K.-H. Larsson, and U. Kõljalg. 2006.
 759 Taxonomic reliability of DNA sequences in public sequence databases: a fungal
 760 perspective. *PLoS ONE* 1:e59.
 761 O'Donnell, A., L. M. Harvey, and B. McNeil. 2011. The roles of the alternative NADH
 762 dehydrogenases during oxidative stress in cultures of the filamentous fungus *Aspergillus*
 763 *niger*. *Fungal Biology* 115:359–369.
 764 de Oliveira, A., J. T. Mesquita, A. G. Tempone, J. H. G. Lago, E. F. Guimarães, and M. J. Kato.
 765 2012. Leishmanicidal activity of an alkenylphenol from *Piper malacophyllum* is related
 766 to plasma membrane disruption. *Experimental Parasitology* 132:383–387.
 767 Orjala, J., P. Mian, T. Rali, and O. Sticher. 1998. Gibbilibols A-D, cytotoxic and antibacterial
 768 alkenylphenols from *Piper gibbilimum*. *Journal of Natural Products* 61:939–941.
 769 OTS. 2020. Organization for Tropical Studies database: la flora digital de La Selva.
 770 <https://sura.ots.ac.cr/florula4/>.
 771 Palmeirim, J. M., D. L. Gorchov, and S. Stoleson. 1989. Trophic structure of a neotropical
 772 frugivore community: is there competition between birds and bats? *Oecologia* 79:403–
 773 411.
 774 Peris, J. E., A. Rodríguez, L. Penã, and J. M. Fedriani. 2017. Fungal infestation boosts fruit
 775 aroma and fruit removal by mammals and birds. *Scientific Reports* 7:1–9.
 776 R Core Team. 2020. R: A language and environment for statistical computing. R Foundation for
 777 Statistical Computing. Vienna, Austria.

778 Rodríguez, A., B. Alquézar, and L. Peña. 2013. Fruit aromas in mature fleshy fruits as signals of
779 readiness for predation and seed dispersal. *New Phytologist* 197:36–48.

780 Schoch, C. L., K. A. Seifert, S. Huhndorf, V. Robert, J. L. Spouge, C. A. Levesque, W. Chen,
781 and F. B. Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region
782 as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of*
783 *Sciences* 109:6241–6246.

784 Schupp, E. W., P. Jordano, and J. M. Gómez. 2010. Seed dispersal effectiveness revisited: a
785 conceptual review. *New Phytologist* 188:333–353.

786 Shahnazi, S., S. Meon, G. Vadamalai, K. Ahmad, and N. Nejat. 2012. Morphological and
787 molecular characterization of *Fusarium* spp. associated with yellowing disease of black
788 pepper (*Piper nigrum* L.) in Malaysia. *Journal of General Plant Pathology* 78:160–169.

789 Summerell, B. A. 2019. Resolving *Fusarium*: current status of the genus. *Annual Review of*
790 *Phytopathology* 57:323–339.

791 Swain, T. 1977. Secondary compounds as protective agents. *Annual Review of Plant Physiology*
792 28:479–501.

793 Tedersoo, L., M. Bahram, S. Polme, U. Koljalg, N. S. Yorou, R. Wijesundera, L. V. Ruiz, A. M.
794 Vasco-Palacios, P. Q. Thu, A. Suija, M. E. Smith, C. Sharp, E. Saluveer, A. Saitta, M.
795 Rosas, T. Riit, D. Ratkowsky, K. Pritsch, K. Poldmaa, M. Piepenbring, C. Phosri, M.
796 Peterson, K. Parts, K. Partel, E. Otsing, E. Nouhra, A. L. Njouonkou, R. H. Nilsson, L. N.
797 Morgado, J. Mayor, T. W. May, L. Majuakim, D. J. Lodge, S. S. Lee, K.-H. Larsson, P.
798 Kohout, K. Hosaka, I. Hiiesalu, T. W. Henkel, H. Harend, L. -d. Guo, A. Greslebin, G.
799 Grelet, J. Geml, G. Gates, W. Dunstan, C. Dunk, R. Drenkhan, J. Dearnaley, A. De
800 Kesel, T. Dang, X. Chen, F. Buegger, F. Q. Brearley, G. Bonito, S. Anslan, S. Abell, and

801 K. Abarenkov. 2014. Global diversity and geography of soil fungi. *Science*
802 346:1256688–1256688.

803 Tewksbury, J. J. 2002. Fruits, frugivores and the evolutionary arms race. *New Phytologist*
804 156:137–139.

805 Tewksbury, J. J., and G. P. Nabhan. 2001. Directed deterrence by capsaicin in chillies. *Nature*
806 412:403–404.

807 Thies, W., and E. K. V. Kalko. 2004. Phenology of neotropical pepper plants (Piperaceae) and
808 their association with their main dispersers, two short-tailed fruit bats, *Carollia*
809 *perspicillata* and *C. castanea* (Phyllostomidae). *Oikos* 104:362–376.

810 Thies, W., E. K. V. Kalko, and H.-U. Schnitzler. 1998. The roles of echolocation and olfaction in
811 two neotropical fruit-eating bats, *Carollia perspicillata* and *C. castanea*, feeding on
812 *Piper*. *Behavioral Ecology and Sociobiology* 42:397–409.

813 Tohge, T., S. Alseekh, and A. R. Fernie. 2014. On the regulation and function of secondary
814 metabolism during fruit development and ripening. *Journal of Experimental Botany*
815 65:4599–4611.

816 Valdivia, C., N. Marquez, J. Eriksson, A. Vilaseca, E. Muñoz, and O. Sterner. 2008. Bioactive
817 alkenylphenols from *Piper obliquum*. *Bioorganic and Medicinal Chemistry* 16:4120–
818 4126.

819 Varela, M. T., M. L. Lima, M. K. Galuppo, A. G. Tempone, A. de Oliveira, J. H. G. Lago, and J.
820 P. S. Fernandes. 2017. New alkenyl derivative from *Piper malacophyllum* and analogues:
821 antiparasitic activity against *Trypanosoma cruzi* and *Leishmania infantum*. *Chemical*
822 *Biology & Drug Design* 90:1007–1011.

823 Vieira, P. C., M. A. D. Alvarenga, O. R. Gottlieb, and H. E. Gottlieb. 1980. 4-

824 Hexadecenylphenol and flavonoids from *Piper hispidum*. *Planta Medica* 39:153–156.

825 Whitehead, S. R., and M. D. Bowers. 2013. Evidence for the adaptive significance of secondary
826 compounds in vertebrate-dispersed fruits. *The American Naturalist* 182:563–577.

827 Whitehead, S. R., and M. D. Bowers. 2014. Chemical ecology of fruit defence: synergistic and
828 antagonistic interactions among amides from *Piper*. *Functional Ecology* 28:1094–1106.

829 Whitehead, S. R., C. S. Jeffrey, M. D. Leonard, C. D. Dodson, L. A. Dyer, and M. D. Bowers.
830 2013. Patterns of secondary metabolite allocation to fruits and seeds in *Piper reticulatum*.
831 *Journal of Chemical Ecology* 39:1373–1384.

832 Whitehead, S. R., M. F. Obando-Quesada, and M. D. Bowers. 2016. Chemical tradeoffs in seed
833 dispersal: defensive metabolites in fruits deter consumption by mutualist bats. *Oikos*
834 125:927–937.

835 Whittaker, R. H., and P. P. Feeny. 1971. Allelochemicals: chemical interactions between species.
836 *Science* 171:757–770.

837 Wickham, H., W. Chang, L. Henry, T. L. Pedersen, K. Takahashi, C. Wilke, K. Woo, H. Yutani,
838 and RStudio. 2019. ggplot2: create elegant data visualisations using the grammar of
839 graphics.

840 Yang, S.-X., Q.-Y. Sun, F.-M. Yang, G.-W. Hu, J.-F. Luo, Y.-H. Wang, and C.-L. Long. 2013.
841 Sarmentosumols A to F, new mono- and dimeric alkenylphenols from *Piper*
842 *sarmentosum*. *Planta Med* 79:693–696.

843 Yoshida, N. C., A. M. Benedetti, R. A. dos Santos, C. S. Ramos, R. Batista, L. F. Yamaguchi,
844 and M. J. Kato. 2018. Alkenylphenols from *Piper dilatatum* and *P. diospyrifolium*.
845 *Phytochemistry Letters* 25:136–140.

846 Zeileis, A., F. Cribari-Neto, B. Gruen, I. Kosmidis, A. B. S. (earlier version by), and A. V. R.

847 (earlier version by). 2019. betareg: beta regression.

848 Zgoda, J. R., and J. R. Porter. 2001. A convenient microdilution method for screening natural
849 products against bacteria and fungi. *Pharmaceutical Biology* 39:221–225.

850 Zhang, Y., P. Li, and L. Cheng. 2010. Developmental changes of carbohydrates, organic acids,
851 amino acids, and phenolic compounds in ‘Honeycrisp’ apple flesh. *Food Chemistry*
852 123:1013–1018.

853 Zhou, Y., and B. R. Singh. 2002. Red light stimulates flowering and anthocyanin biosynthesis in
854 American cranberry. *Plant Growth Regulation* 38:165–171.

Table 1. Hypothesized patterns of secondary metabolite concentrations during fruit development.

<i>Increasing linear</i> Manipulation of mutualists	<i>Decreasing linear</i> Defense: allocated by risk	<i>Nonlinear</i> Defense: allocated by fitness
Hypothesized function: Secondary metabolites function in ripe fruits to manipulate mutualists and increase the effectiveness of dispersal services. Thus, the concentration is highest in fully developed fruit that is ready to be dispersed from the parent plant.	Hypothesized function: Secondary metabolites function in unripe fruits to defend against antagonists and are allocated according to risk of attack. Thus, the concentration is highest in young tissues that are not yet physically defended.	Hypothesized function: Secondary metabolites function in unripe fruits to defend against antagonists and are allocated according to fitness consequences of consumption. Thus, the concentration is highest just prior to seed maturity when the fitness costs of loss would be highest.
Predicted trend: Secondary metabolites increase linearly with fruit development.	Predicted trend: Secondary metabolites decrease linearly with fruit development.	Predicted trend: Secondary metabolites increase initially with development then decrease as fruits ripen.

FIGURE LEGENDS

Figure 1. *Piper sancti-felicis* reproductive structures developing successively along a branch.

From left to right: developing inflorescence, two inflorescences, two unripe infructescences, and one ripe infructescence. Illustration by Sherri Maynard.

Figure 2. Secondary metabolites (alkenylphenols) isolated from the infructescences of *Piper sancti-felicis* (A-J).

Figure 3. Alkenylphenol concentration (proportion dry weight) differed across plant tissues.

Pulp, including ripe and late unripe, had higher concentrations of alkenylphenols compared to all other tissue types. Late flowers had higher concentrations than leaves and seeds. Box margins indicate the 25th and 75th percentiles, whiskers the 5th and 95th percentiles, solid lines within the boxes the median, and points individual data observations of total alkenylphenol concentrations from $N = 21$ plants. Letters indicate significant differences from post-hoc pairwise comparisons among tissue types. Ripe pulp ($N=20$), late unripe pulp ($N=21$), late flowers ($N=21$), mature leaves ($N=4$), and seeds ($N=6$).

Figure 4. The concentration of alkenylphenols follows a nonlinear trend over reproductive structure stage of development that peaks just before ripening. Stage of development ($z = 2.27$, $P = 0.023$; beta regression) was significant in predicting alkenylphenol concentration. Colored lines are individual plants, and the bold black line is the nonlinear fit of the data with the gray band indicating 95% confidence intervals. Ripe pulp ($N=20$), late unripe pulp ($N=21$), early

unripe pulp ($N=19$), late flowers ($N=21$), early flowers ($N=15$), and developing flowers ($N=11$). Concentrations of alkenylphenols calculated as internal standard equivalents (mg/ml).

Figure 5. The effect of alkenylphenols on hyphal growth of three types of fungi harvested from unsterilized seeds of *Piper sancti-felicis*. Hyphal growth was measured as the difference in optical density (or absorbance) at 72h minus 0h. Points are individual observations, lines are linear fits of the data with gray bands indicating 95% confidence intervals. Alkenylphenols had antifungal effects for two of the three naturally occurring fungi (*Fusarium* A and *Microdochium lycopodium*) but not *Fusarium* B. Concentrations of alkenylphenols are estimated as internal standard equivalents (mg/ml).

Figure 6. Overall effect of alkenylphenols on bat (*Carollia perspicillata*; a) and bird (*Ramphocelus passerinii*; b) feeding responses. Treatment diets contained approximately 14.6 mg infructescence-extracted alkenylphenols in 3 g of diet, a concentration that mimicked the average concentration found in a ripe infructescence of *P. sancti-felicis* (0.0049 proportion wet weight). Amount of control and treatment diets eaten were averaged for each individual (bats $N = 16$, birds $N = 10$) for all trials (bat trials $N = 58$, bird trials $N = 27$). Alkenylphenols had a negative effect on bat feeding response ($t = 2.20$, $df = 15$, $P = 0.044$; paired t-test) but no detectable effect on birds ($t = 0.26$, $df = 9$, $P = 0.80$; paired t-test). Bats consumed an average of 2.4 times more control than treatment, whereas birds only consumed an average of 1.1 times more control than treatment. Box and whisker plots show the median, 25th and 75th percentile, and range of average amount of each dish consumed, and points are the average amount consumed across trials for each individual.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6