

ARTICLE

Fruit secondary metabolites alter the quantity and quality of a seed dispersal mutualism

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Abstract

Plant secondary metabolites are key mechanistic drivers of species interactions. These metabolites have primarily been studied for their role in defense, but they can also have important consequences for mutualisms, including seed dispersal. Although the primary function of fleshy fruits is to attract seed-dispersing animals, fruits often contain complex mixtures of toxic or deterrent secondary metabolites that can reduce the quantity or quality of seed dispersal mutualisms. Furthermore, because seeds are often dispersed across multiple stages by several dispersers, the net consequences of fruit secondary metabolites for the effectiveness of seed dispersal and ultimately plant fitness are poorly understood. Here, we tested the effects of amides, nitrogen-based defensive compounds common in fruits of the neotropical plant genus *Piper* (Piperaceae), on seed dispersal effectiveness (SDE) by ants, which are common secondary seed dispersers. We experimentally added amide extracts to *Piper* fruits both in the field and lab, finding that amides reduced the quantity of secondary seed dispersal by reducing ant recruitment (87%) and fruit removal rates (58% and 66% in the field and lab, respectively). Moreover, amides not only reduced dispersal quantity but also altered seed dispersal quality by shifting the community composition of recruiting ants (notably by reducing the recruitment of the most effective disperser by 90% but having no detectable effect on the recruitment of a cheater species that removes fruit pulp without dispersing seeds). Although amides did not affect the distance ants initially carried seeds, they altered the quality of seed dispersal by reducing the likelihood of ants cleaning seeds (67%) and increasing their likelihood of ants redispersing seeds outside of the nest (200%). Overall, these results demonstrate that secondary metabolites can alter the effectiveness of plant mutualisms, by both reducing mutualism quantity and altering mutualism quality through multiple mechanisms. These findings present a critical step in understanding the factors mediating the outcomes of seed dispersal and, more broadly, demonstrate the importance of considering how defensive secondary metabolites influence the outcomes of mutualisms surrounding plants.

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KEYWORDS

ants, chemical ecology, fruit defense, mutualism, plant secondary metabolites, seed dispersal effectiveness, seed dispersal quality, seed dispersal quantity

INTRODUCTION

Plants are embedded in complex ecological communities, and their survival and reproduction require both defense against antagonists and the attraction of mutualists. These conflicting requirements are mediated in large part through chemistry. Plants produce an incredible diversity of secondary metabolites that play a central role in shaping biotic and abiotic interactions (Kessler & Kalske, 2018; Wetzel & Whitehead, 2020). Secondary metabolites are most widely recognized for their role in plant defense and coevolution with herbivores, influencing the ecology and evolution not only of plants but also their consumers and surrounding food webs (Kessler & Kalske, 2018; Mithöfer & Boland, 2012). However, the same chemical compounds that defend plants against attack can also carry the cost of deterring effective mutualists. For instance, both constitutive and induced defenses have been shown to reduce pollinator (Kessler et al., 2011) and seed disperser preferences (Whitehead et al., 2016; Whitehead & Poveda, 2011). However, compared to their role in plant defense, our understanding of how secondary metabolites shape mutualisms, and especially seed dispersal mutualisms, is still in its infancy, limiting our ability to fully understand the adaptive significance of these traits.

Defensive secondary metabolites in fruits can strongly alter animal-mediated seed dispersal. Because plants are rooted in place, they often rely on animals to disperse their seeds. It is estimated that 52% of all seed-producing plants are dispersed by animals (Rogers et al., 2021), with the prevalence sometimes exceeding 80% in the tropics (Howe & Smallwood, 1982). One of the primary ways in which plants attract seed dispersers is by producing fleshy fruits containing attractive chemical compounds and nutritional rewards (Nelson & Whitehead, 2021). Yet, fruits also contain complex mixtures of secondary metabolites that can be toxic or deterrent (Nelson & Whitehead, 2021; Whitehead et al., 2016), sometimes even at higher concentrations and diversities than in leaves or other plant tissues (Whitehead et al., 2013, 2022; Whitehead & Bowers, 2013). This is perplexing, given that the purpose of fruits is to attract rather than deter seed dispersers. Numerous hypotheses have been proposed to explain the occurrence of secondary metabolites in fruits, including that they play important roles in defending fruits against seed predators and pathogens, even if this comes at the cost of simultaneously deterring effective seed dispersers (Cipollini, 2000;

Cipollini & Levey, 1997; Nelson & Whitehead, 2021). However, the ecological consequences of fruit secondary metabolites in interactions surrounding fruits are complex and poorly understood, limiting our ability to determine their adaptive significance and net effects on plant fitness.

Mutualism effectiveness is a widely used conceptual framework for examining the factors that influence the outcome of mutualisms (Schupp et al., 2017). This framework has been especially useful for understanding seed dispersal effectiveness (SDE) and can be used as a tool for evaluating the effects of plant secondary metabolites on seed dispersal mutualisms (Nelson & Whitehead, 2021; Schupp, 1993; Schupp et al., 2010). SDE depends on both the total number of seeds dispersed (dispersal quantity) and the success of those seeds (dispersal quality), and it is calculated as quantity multiplied by quality. Fruit secondary metabolites can influence all stages of the seed dispersal process, with important effects on seed dispersal success (Nelson & Whitehead, 2021). Chemical compounds in fruits not only affect individual components of SDE, such as when toxins reduce seed dispersal quantity by deterring dispersers (Maynard et al., 2020; Whitehead et al., 2016), but also carry the potential for multiple synergistic or contrasting effects on different components of SDE. For instance, when fruit secondary metabolites reduce the gut retention time of seeds, this can reduce seed dispersal quality by decreasing the distance that seeds are dispersed (Baldwin et al., 2020) but also increase dispersal quality by reducing the likelihood of seeds being damaged in the gut (Traveset et al., 2007). Yet, due to the difficulties of tracking seeds across all stages of the dispersal process, no previous study has comprehensively assessed the effects of fruit secondary metabolites on both seed dispersal quantity and quality, limiting our ability to determine the net effects on SDE and ultimately plant fitness.

Most studies have used the SDE framework to compare the variable contributions of vertebrate frugivores (e.g., birds, bats, and primates) or granivores (e.g., rodents) to plant fitness (Nogales et al., 2017; Zwolak, 2018). However, plants are also frequently dispersed by a broad diversity of invertebrates (e.g., ants and dung beetles), which can be equally if not more important than vertebrates (e.g., Anjos et al., 2020; Ness et al., 2009; Pérez-Ramos et al., 2013). For instance, ants can act as primary dispersers (removing fruits directly from parent plants) or secondary dispersers (moving fruits or

seeds that have been previously handled by other animals or dispersed some distance abiotically). Ant-dispersed plants include species with lipid-rich appendages (“elaiosomes”) adapted to ant dispersal (Giladi, 2006; Lengyel et al., 2010) as well as species with fleshy fruits that are dispersed by both vertebrate frugivores (as primary dispersers) and ants (as primary or secondary dispersers; Byrne & Levey, 1993; Clemente & Whitehead, 2020; Levey & Byrne, 1993). Seeds are not typically dispersed far by ants (only several meters) before they are either dropped or brought into the nest, and those brought into nests are often “redispersed” by foragers that subsequently carry seeds back outside of the nest to deposit them in refuse piles (Figure 1; e.g., Canner et al., 2012). Seeds dispersed by ants can be deposited in

microsites beneficial for seed survival and germination (Giladi, 2006). A meta-analysis of 62 published studies found that ant dispersal benefits plants because ants deposit seeds in high nutrient soils (46% of studies) and predator-free space within their nests (81% of studies; Giladi, 2006). Yet, seeds deposited within ant nests may also be buried too deep to germinate. For this reason, redispersal outside of the nest could be beneficial, if it does not put seeds at greater risk of predation or reduced nutrient availability. Because ant foraging strongly depends on chemical cues (Nelson et al., 2019, 2020), defensive fruit secondary metabolites may alter the SDE of ants, presenting an ideal system for investigating the roles of plant secondary metabolites in shaping mutualisms surrounding plants.

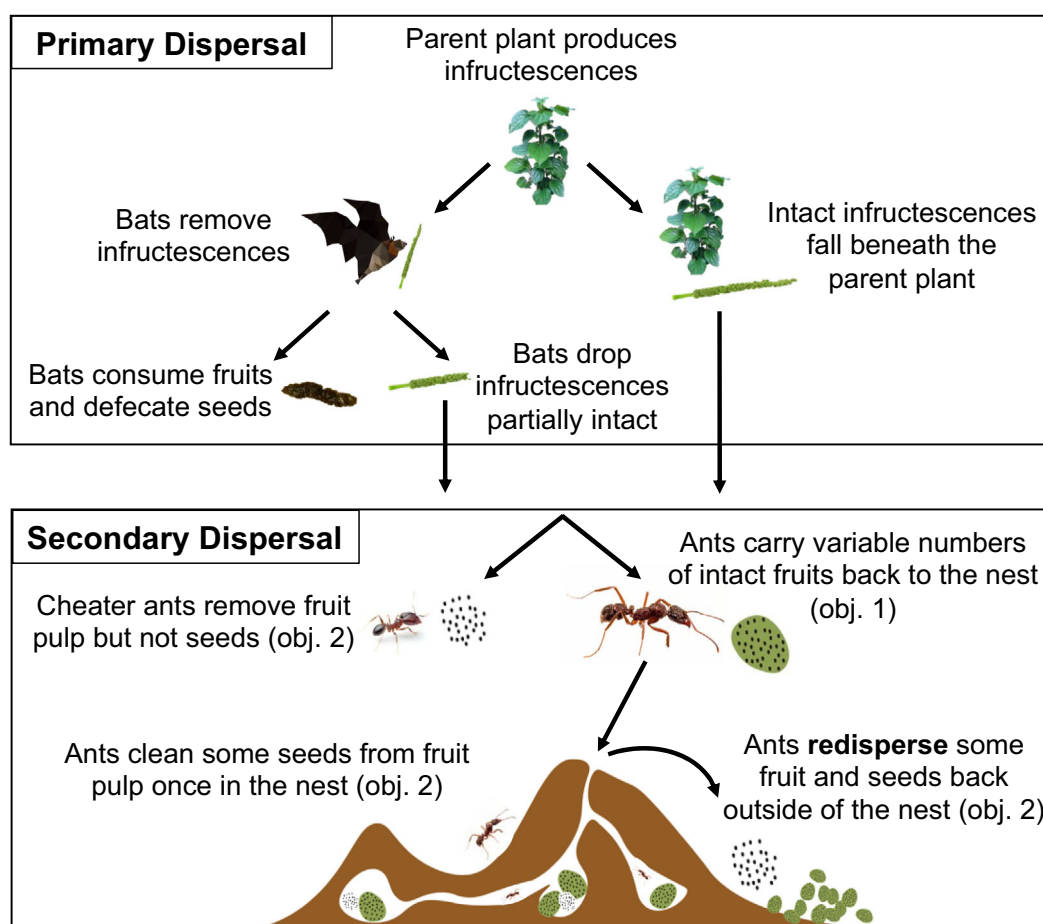


FIGURE 1 Diagram of the stages of primary (top box) and secondary seed dispersal (bottom box) of *Piper reticulatum*. Seeds undergo primary dispersal by bats when fruits are removed from the parent plant and either consumed and later defecated or dropped prior to consumption. Seeds can alternatively undergo primary dispersal via gravity by simply falling to the ground directly beneath the parent plant or via ants that remove fruits directly from the plant (not pictured). Fruits that fall to the ground or are dropped by bats intact can undergo secondary dispersal by ants, if they are not first attacked by cheater (low quality partner) ant species that remove fruit pulp without dispersing seeds. Here, we tested whether secondary metabolites in fruit influence seed dispersal quantity (obj. 1), which is measured as the number of seeds or fruits removed by ants. We also tested for effects of secondary metabolites on seed dispersal quality (obj. 2), which depends on both how ants handle seeds (including whether they attack fruits or effectively disperse them, as well as whether they clean seeds from fruit pulp), as well as how far and where they deposit them. If intact fruits or seeds are initially stored within the nest but later removed and deposited in refuse piles outside the nest, they are considered “redispersed”.

Here, we investigated the effects of amides—nitrogen-based defense compounds common across *Piper* (Piperaceae) plant species—on the effectiveness of a seed dispersal mutualism. In *Piper reticulatum*, amides are well-characterized and have been found to occur across all tissues, and at especially high concentrations and diversity in seeds and fruit pulp (Whitehead et al., 2013). The bioactivity of amides has been extensively studied, with past work demonstrating that they can influence vertebrate seed dispersal (Whitehead et al., 2016), invertebrate herbivory (Cosmo et al., 2021; Dyer et al., 2003; Richards et al., 2010; Whitehead & Bowers, 2014), and microbial pathogen attack (Whitehead & Bowers, 2014; Zarai et al., 2013). Ants serve as important seed dispersers of *Piper reticulatum* (Figure 1; see data on ant recruitment and fruit removal in Appendix S1: Section S1), yet the effects of amides on the total effectiveness of seed dispersal provided by ants is unknown. Here, we asked two main questions: (1) Do fruit secondary metabolites affect seed dispersal quantity through effects on ant recruitment and fruit removal rates?, and (2) Do fruit secondary metabolites affect seed dispersal quality through effects on how ants handle (e.g., whether they clean or destroy seeds) and where they deposit seeds (e.g., how far seeds are carried and whether they are deposited within or outside of the nest)? By addressing these questions, this study provides some of the first evidence that defensive plant secondary metabolites can mediate both the quantity and quality of a seed dispersal mutualism, resulting in complex trade-offs between seed dispersal and fruit defense.

MATERIALS AND METHODS

Study site and system

This study was conducted from October to December 2021 at La Selva Biological Station in Sarapiquí, Heredia Province, Costa Rica (10.422° N, 84.015° W). La Selva is a 1600-ha lowland tropical wet forest that receives approximately 4 m precipitation annually and consists of primary and secondary rain forest as well as abandoned farmlands (McDade et al., 1994). *Piper* (Piperaceae) is one of the largest genera (>1000 species globally) of flowering plants and is particularly diverse in the neotropics (Gentry & Guzman-Teare, 1990). More than 60 *Piper* species occur at La Selva and are most common in early- to mid-successional sites (Gentry & Guzman-Teare, 1990; OTS, 2022).

Piper reticulatum is one of the most abundant *Piper* species at La Selva. This species produces spike-shaped infructescences that contain hundreds of individual

fruits (Dyer & Palmer, 2004). A variety of secondary metabolites occur across *Piper* species (e.g., amides, alkaloids, terpenes, and alkenylphenols; Dyer et al., 2004; Maynard et al., 2020; Uckele et al., 2021; Whitehead et al., 2013). In *P. reticulatum*, amides are particularly abundant and diverse (Whitehead et al., 2013) and important for defense (Dyer et al., 2004; Whitehead & Bowers, 2014). Although infructescences take a month or more to mature (Whitehead et al., 2016; Whitehead & Bowers, 2014), they rapidly soften and swell during the final ripening period, typically beginning in the afternoon and becoming fully ripe by dusk (McAtee et al., 2013; Thies & Kalko, 2004; Whitehead et al., 2016). Most *Piper* spp. infructescences ($\geq 90\%$) are removed by *Carollia* spp. bats the evening that they ripen (Fleming, 2004; Maynard et al., 2020), but amides reduce the likelihood of bats removing infructescences and also increase the likelihood that bats will only partially consume infructescences once removed, dropping the remaining fruits below a feeding roost (Whitehead et al., 2016).

Fruits that are not removed by bats and instead fall directly below the parent plant, or fruits that bats drop below feeding roosts, are available to be dispersed by ants (Figure 1). In this study, at least six species of ants at La Selva recruited to ripe or overripe infructescences (but not unripe fruits or seeds removed from fruit pulp). When we removed intact *P. reticulatum* infructescences from the parent plant and placed them on the ground, 54% of all ripe and overripe infructescences attracted ants within 4 h (as secondary seed dispersers; see Appendix S1: Section S1). Ants also occasionally recruited to infructescences directly on the plant (as primary seed dispersers; for simplicity, however, we refer to ants exclusively as “secondary dispersers” throughout the rest of the paper). Of the ants that recruited, *Ectatomma ruidum* was the only species observed to strip and carry away entire fruits from the infructescence. In laboratory experiments (see below), *E. ruidum* was never observed to destroy seeds, and likely does not produce enough mandibular force to do so (Ruzi & Suarez, 2022), suggesting that it serves as an effective seed disperser, rather than seed predator, of *P. reticulatum*. In contrast, the other ant species (primarily *Pheidole* spp.) that commonly recruited to *P. reticulatum* fruits appeared to serve as ineffective seed dispersers or potential cheaters. By removing chunks of fruit pulp but not dispersing seeds, these species could reduce the recruitment of *E. ruidum* (via a reduction in fruit pulp as a reward) and thus reduce the dispersal of seeds. However, this may not always be detrimental, if fruit pulp removal promotes seed survival and germination, as has been shown in other ant seed dispersal systems (e.g., when ant seed cleaning prevents fungal pathogen attack; Ohkawara & Akino, 2005). Thus, secondary metabolites

could alter the effectiveness of seed dispersal by altering the preferences and behavior of not only *E. ruidum* but also the entire community of recruiting ants.

Experimental design overview

We conducted a series of manipulative experiments to examine the effects of amides on the quantity (obj. 1) and quality (obj. 2) of seed dispersal of *P. reticulatum* by ants. In all experiments, we supplemented fruits with either commercially purchased amides (piperine, piplartine, or piperine and piplartine combined) or amides extracted from ripe *P. reticulatum* fruits, which contain up to 23 unique compounds (Whitehead et al., 2013). Although the amides available for purchase (piperine and piplartine) are not known to occur in any *Piper* species at La Selva, they do occur in at least one *Piper* species (*P. tuberculatum*) in Costa Rica (de O. Chaves et al., 2003; Whitehead et al., 2016) and are common across the genus (Parmar et al., 1997). By supplementing fruits with these pure amides, we were thus able to distinguish between both the individual and combined effects of two compounds known to have widespread bioactivity and ecological effects on earlier stages of the seed dispersal process (i.e., fruit defense and primary dispersal by bats; Whitehead et al., 2016; Whitehead & Bowers, 2014). In contrast, although extracts from ripe *P. reticulatum* fruits are chemically complex mixtures and thus do not allow us to directly link observed effects to any particular amides within the mixture, extracts closely approximate the overall chemical composition of the fruits and are more ecologically realistic. Amide extracts were prepared following similar methods as Whitehead et al. (2013), with full methods for bulk amide extraction and quantification described in Appendix S1: Section S2. Briefly, amides were extracted from the pulp of 31 ripe *P. reticulatum* fruits overnight in ethanol, and extracts were dried and partitioned three times against chloroform. Chloroform fractions containing the amides were dried, and amide concentrations were quantified using gas chromatography/mass spectrometry (GC/MS).

In all experiments, we presented ants with fruits in amide-addition and control treatments. For experiments that did not rely on tracking the fate of individual seeds over space and time (i.e., obj. 1 experiments measuring seed dispersal quantity in terms of ant recruitment and fruit removal rate, as well as obj. 2 experiment measuring seed dispersal quality in terms of the distance entire chunks of fruit were carried; detailed below), amide treatments were applied onto the surface of infructescences of another *Piper* species, *P. sancti-felices*, in field seed depot experiments. We used infructescences of *P. sancti-felices* because this species commonly co-occurs throughout the

site and attracts a similar community of ants (Appendix S1: Section S1; Clemente & Whitehead, 2020), but does not contain detectable amides at a limit of 0.01% dry weight (Whitehead et al., 2016). Although *P. sancti-felices* fruits contain other secondary metabolites that do not occur in *P. reticulatum* (e.g., alkenylphenols) and can influence seed dispersal and fruit defense (Maynard et al., 2020), these compounds have no detectable effect on seed dispersal by ants (Clemente & Whitehead, 2020). Thus, supplementing *P. sancti-felices* infructescences with amides, as has been done in previous studies (Whitehead et al., 2016), allowed us to test for the effects of amides while eliminating any existing background variation in amide content that would naturally occur in fruits of *P. reticulatum*.

For experiments relying on tracking the fate of individual seeds (i.e., obj. 2 experiments measuring seed dispersal quality in terms of how ants handle and where they deposit seeds), amide extracts were applied onto the surface of individual *P. reticulatum* fruits (carefully separated from the rachis of the infructescences) and offered to captive ant colonies. Although *P. reticulatum* fruits contain variable background levels of amides, and it thus would have been more ideal to test for the effects of amide supplementation to amide-free *P. sancti-felices* fruits, the seeds of *P. sancti-felices* are not large enough to accurately count or track their fate individually. Moreover, because it was not possible to observe patterns of seed deposition and handling by ants in the field without destructively sampling their colonies, we instead used captive ant colonies in the lab, which could be more readily observed. These experiments were conducted using amide extracts only (and not commercially purchased amides), as pure amides were found to have minimal effects in seed dispersal quantity experiments (see the [Results](#) section below). Below, we describe each experiment in more detail.

Effects of secondary metabolites on ant recruitment and fruit removal rates (obj. 1)

We conducted baiting in the field across three sampling dates (one date for pure amide additions and two dates for amide extract additions) and measured the effects of amides on ant recruitment and fruit removal rates (i.e., seed dispersal quantity). Using a clean razor blade, we cut each *P. sancti-felices* infructescence into quarters. In experiments testing for the effects of pure amides, we added approximately 2.5 mg of piperine, piplartine, and 1:1 piperine:piplartine to three randomly selected quarters of the same infructescence by dissolving amides in ethanol at a concentration of 20 mg/mL and pipetting 0.125-mL aliquots onto the surface of the fruit. As a control,

we pipetted 0.125 mL of ethanol onto the last quarter of the same infructescence. In experiments testing for the effects of amide extracts, we similarly added approximately 2.5 mg amides to one randomly selected quarter of an infructescence. Ethanol was added as a control to two other randomly selected quarters of the same infructescence, with ants allowed access to one but excluded from the other to control for any potential effects of evaporation on mass loss rate. The last infructescence quarter was not used and instead simply discarded. Assuming all of the solution was absorbed, the amount we added is approximately the same as natural concentrations of amides previously reported in *P. reticulatum* fruits (~2% dry mass; Baldwin & Whitehead, 2015; Whitehead et al., 2013). We allowed the solvent to evaporate for at least 1 h prior to experiments.

All *P. sancti-felicitis* infructescence quarters were weighed and then placed in open 50 mL centrifuge tubes on the edge of the secondary forest floor along a different transect on each day. We established 10–16 bait stations per transect, each separated by a minimum of 10 m to avoid repeatedly sampling ants from the same colonies. Bait stations consisted of one infructescence quarter in each treatment (four treatments for pure amides, two treatments for amide extracts), each placed in separate centrifuge tubes within 10 cm of each other. Beginning between 9:30 and 10:30 am under similar ambient conditions (e.g., similar temperatures, no rain) on each morning, we counted the number of ants in each tube and collected voucher specimens for later identification of species. Counts were conducted every hour for 4–6 h (depending on ant activity), to maximize our ability to detect any potential differences among treatments, and data were summarized for each bait as the average number of ants observed per count. Because each treatment was represented within each bait station, treatment effects were not confounded with differences in sampling effort among transects. At the end of the sampling period, infructescences were reweighed to determine mass loss rate.

Effects of secondary metabolites on how ants handle and where they deposit seeds (obj. 2)

We first tracked where and how far ants carried chunks of *P. sancti-felicitis* fruits in the field to examine the effects of amides on seed dispersal distance (i.e., one aspect of seed dispersal quality). One infructescence quarter from each of two amide treatments (amide extract supplementation or control) was placed in a Petri dish and coated in fluorescent powder (pink or green; alternating color

assignments between stations) to distinguish between treatments. Baits were deployed at 8:00 am at 12 stations along a transect, each separated by a minimum of 10 m. We repeatedly visited each station for several hours, and any ants observed removing fruits in either treatment were followed until they either dropped or carried the fruit into their nest. The distance between the Petri dish and the deposition or nest entry location was recorded as the dispersal distance.

We then used captive colonies of the ant *Ectatomma ruidum* (Hymenoptera: Formicidae), which was the most common seed-dispersing ant at this site and is estimated to occur at densities of up to one colony m^{-2} (Clemente & Whitehead, 2020; McGlynn et al., 2010), to test for the effects of amides on how ants handle and where they ultimately deposit the seeds that they bring into the nest (i.e., several aspects of seed dispersal quality). We located 22 *E. ruidum* colonies, which are monogynous (i.e., contain a single queen) and monodomous (i.e., occupy a single nest), by following foragers back to the nest entrance (Breed et al., 1990). Entire colonies were dug out of the ground, and after confirming that each contained a live queen, we counted the total number of workers, larvae, and pupae (range 25–203 workers, 6–60 larvae, and 0–36 pupae). Colonies were maintained under ambient temperature and light conditions in plastic boxes ($36.2 \times 21.0 \times 12.4$ cm), which each contained a Petri dish covered with red cellophane as a nest chamber. Chambers were partially filled with plaster mixed with methylparaben (~0.13% dry mass; an antifungal agent), with four holes evenly drilled around the base of the dish to allow ants entrance and exit (García-Robledo & Kuprewicz, 2009). Except during experiments, ants were continuously fed a maintenance diet of dried mealworms, pecan sandies cookies, and an artificial diet modified from Kay et al. (2012) (recipe in Appendix S1: Section S3).

During the first lab experiment, we presented colonies with 10 *P. reticulatum* fruits (five amide-supplemented and five control fruits), which we placed in a clean Petri dish (hereafter “feeding dish”). In these experiments, we provided each captive colony with 10 individual *P. reticulatum* fruits, sourced from adjacent locations along the same infructescence to minimize any potential background variation in amide levels that may occur across fruits. Five of the fruits were supplemented with amide extracts (6.25 μL of 20 mg/mL solution of amide extracts in ethanol, which was enough to fully coat all fruits), and five were treated as a control (6.25 μL ethanol). To be able to later identify them, fruits of a given treatment were painted either white or pink (alternating color assignments between ant colonies) with an acrylic paint pen. After 48 h, we counted the number of fruits in the two treatments that had been removed from the

feeding dish. We first examined whether patterns of fruit removal by the captive *E. ruidum* colonies aligned with observed patterns of fruit removal in the field by testing for the effects of amide extracts on the proportion of fruits dispersed. We also examined the role of any potential background variation in amides on total seed removal (of all 10 seeds per colony) by storing all remaining untreated fruits from the same infructescence in the freezer for subsequent chemical analyses (detailed in Appendix S1: Section S1).

In a follow-up experiment, we similarly provided each captive ant colony with five amide-supplemented and five control *P. reticulatum* fruits, painted either white or pink to distinguish between treatments. However, instead of placing them in a feeding dish as above, we placed them directly into the nest chamber, as all dispersed seeds were observed to be taken directly into ant nests in the field (see [Results](#)). After 48 h, we recorded the number and identity of seeds that had been cleaned from fruit pulp (versus left in intact fruit), destroyed, or redispersed outside of the nest.

Statistical analysis

All data analyses were performed in R version 4.1.1 (R Core Team, 2021). We constructed a series of linear mixed effects models (LMERs) to examine the effects of pure amide or amide extract supplementation (obj. 1) on ant recruitment to and removal of fruits in the field. Models of the effects on ant recruitment included the mean number of ants per observation (summarized across four to six repeated observations per bait and $\ln + 1$ transformed to improve normality of residuals) as the response variable, and models of the effects on fruit removal included the mass loss rate ([bait start mass – end mass]/time elapsed; square root transformed to improve normality of residuals) as the response. Each LMER included amide treatment as a fixed effect, bait station as a random intercept, and for data collected across multiple days, sampling date as a fixed effect. To determine whether differences in ant identity and recruitment rate predicted fruit and seed mass loss, we constructed an additional set of LMERs in which, in addition pure amide or amide extract treatment (obj. 1), we also included the recruitment rates (number of foragers per observation) of the three most abundant ants (*Ectatomma ruidum*, *Pheidole* sp. 1, and *Pheidole subarmata*; >90% of all individuals in each experiment) as separate fixed effects.

To test whether pure amide or amide extract treatments (obj. 1) influenced the community composition of ants that recruited to fruits or seeds, we assembled ant recruitment data for all species (mean number of ants per

observation) into matrices. We then used non-metric multidimensional scaling (NMDS) with the Bray–Curtis dissimilarity index to plot the ordinations. Due to the low frequency and abundance of several species, we only included species that occurred in >20% of the bait stations for these analyses. As a result, these analyses were based on only the three most abundant ant species (*E. ruidum*, *Pheidole* sp. 1, and *Pheidole subarmata*; >90% of all individuals in each experiment). To test for statistically significant differences among treatments, we constructed PERMANOVAs with 999 permutations, in which the mean number of ants per observation of each of the three most common species was the response, and treatment and bait station were fixed effects.

We tested for the effects of amide extract treatment on seed dispersal distance and the proportion of seeds dispersed, cleaned from fruit pulp, and redispersed outside of the nest (obj. 2) using separate LMERS. Because seeds were never observed to be destroyed by the lab colonies, we did not construct a model for the proportion of seeds destroyed. Each LMER included the main effect of amide extract treatment and the random intercept of either bait station (for the field experiment to measure seed dispersal distance) or ant colony (for laboratory experiments to measure seed cleaning and redispersal).

LMERs were constructed using the “lmer()” function in the “lme4” package (Bates et al., 2015), and NMDS ordinations and PERMANOVAs were conducted using the “metaMDS()” and “adonis2()” functions (respectively) in the “vegan” package (Oksanen et al., 2020). For tests of statistical significance for linear models, we used the “anova()” function in the “stats” package to conduct *F*-tests with Satterthwaite’s degrees of freedom (R Core Team, 2021). When main effects with more than two levels were significant, we used the “glht()” function in the “multcomp” package to conduct Tukey tests for multiple comparisons (Hothorn et al., 2008). To determine effect sizes based on estimated marginal means, we used the “emmeans()” function in the “emmeans” package (Lenth, 2021).

RESULTS

Effect of secondary metabolites on ant recruitment and fruit removal rates (obj. 1)

Pure amides had a significant effect on ant recruitment rate to *P. sancti-felicitis* fruits (LMER: $F_{3,45} = 3.60$, $p = 0.020$). Post hoc analyses revealed ant recruitment rate to fruits with both 1% piperine and 1% pipartine was 45% lower than to fruits without amides ($z = 3.13$, $p = 0.010$), although there were no other significant

pairwise differences (Figure 2a; Appendix S1: Table S2). Ordination analysis did not demonstrate any clear separation in ant communities based on pure amide treatment (Appendix S1: Figure S6), and although ant community composition significantly differed across bait stations (PERMANOVA: $p = 0.001$), there was no effect of amide treatment (PERMANOVA: $p = 0.101$). Moreover, the reduction in ant recruitment to 1% piperine and 1% piplartine fruits did not translate to changes in fruit consumption, as there were no detectable differences in mass loss across amide treatments (LMER: $F_{3,45} = 2.16$, $p = 0.106$; Figure 2b).

In contrast, amide extracts from *P. reticulatum* fruits reduced ant recruitment rate by 87% (LMER: $F_{1,19} = 43.82$, $p < 0.001$), and such effects were consistent across sampling dates (LMER: $F_{1,18} = 0.78$, $p = 0.388$; Figure 3a). Although there was no clear visual separation in ant communities in two-dimension ordination plots

(Appendix S1: Figure S7), we detected a statistically significant effect of amides on community composition (PERMANOVA: $p = 0.010$). Further analyses of the separate responses of the three most common ant species (>97% of all observed foragers) revealed that amides reduced the recruitment rate of *E. ruidum* by 90% (LMER: $F_{1,19} = 17.76$, $p < 0.001$) and of *Pheidole* sp. 1 by 87% (LMER: $F_{1,19} = 11.84$, $p = 0.003$), but there was no detectable effect on *Pheidole subarmata* recruitment (LMER: $F_{1,19} = 2.20$, $p = 0.154$; Appendix S1: Figure S8).

Fruit mass loss rate also significantly depended on amide treatment (LMER: $F_{2,38} = 7.96$, $p = 0.001$) after accounting for the effect of sampling date (LMER: $F_{1,18} = 6.85$, $p = 0.017$; Figure 3b). Post hoc analyses demonstrated that amide extracts reduced mass loss by 58% (Amides vs. ethanol: $z = 3.81$, $p < 0.001$), such that it was no greater than expected due to evaporation alone (Amides vs. evaporation control: $z = 0.88$, $p = 0.652$).

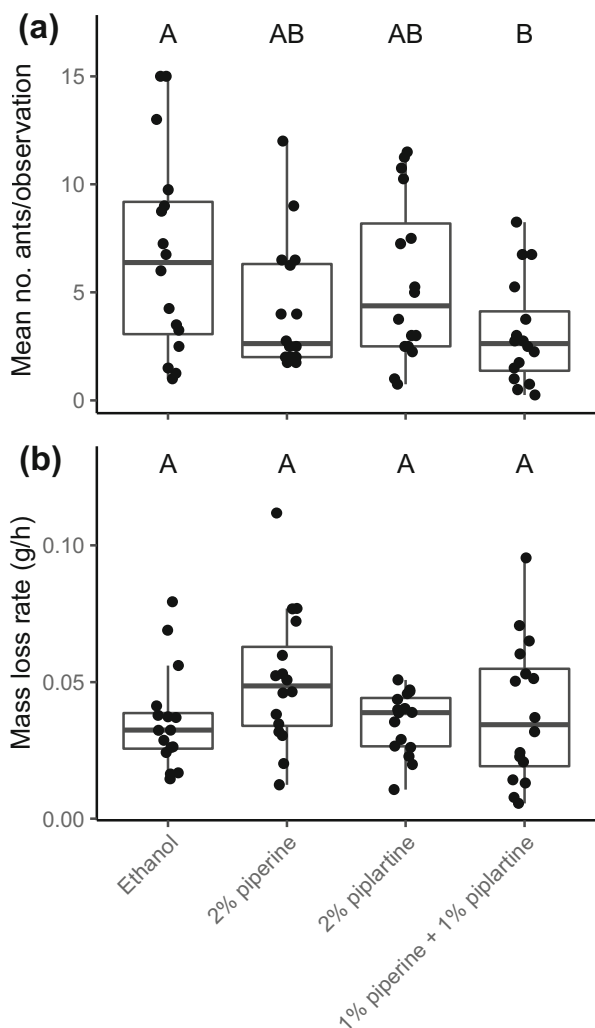


FIGURE 2 Effect of pure amides on (a) the number of ants per observation recruited and (b) mass loss rate (g h^{-1}) from fruits. Different letters indicate significant differences between groups.

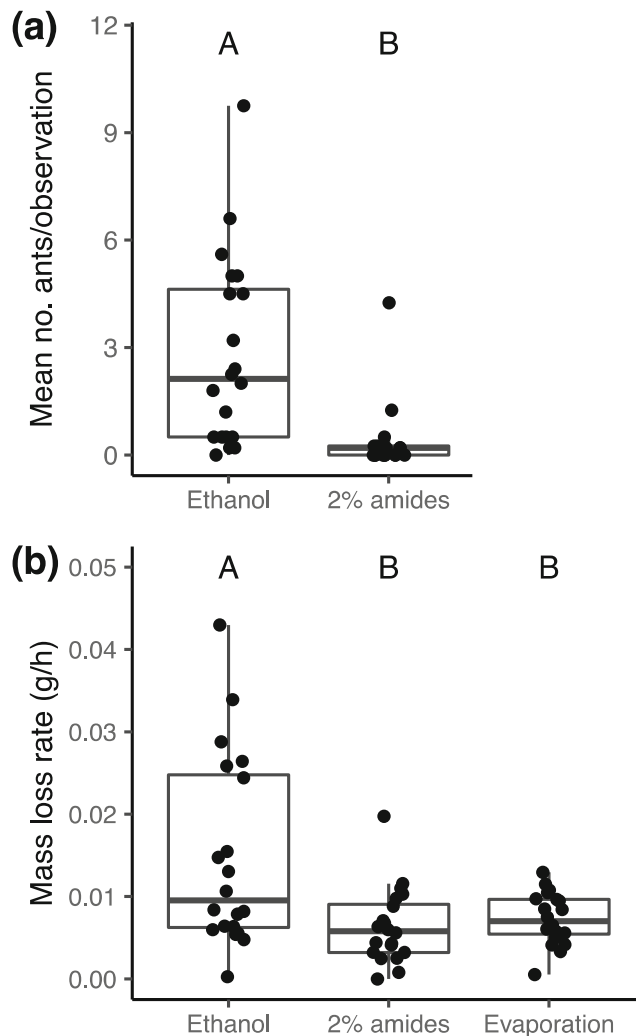


FIGURE 3 Effect of amide extracts on (a) the number of ants per observation recruited and (b) mass loss rate (g h^{-1}) from fruits. Different letters indicate significant differences between groups.

Mass loss rate was positively correlated with the recruitment of *E. ruidum* (LMER: $F_{1,34} = 7.06$, $p = 0.012$) but not *Pheidole* sp. 1 (LMER: $F_{1,31} = 0.66$, $P = 0.424$) or *P. subarmata* (LMER: $F_{1,34} = 0.01$, $p = 0.927$; Appendix S1: Figure S9). After accounting for differences in ant recruitment, the effect of amide extract treatment on mass loss rate was no longer significant (LMER: $F_{1,29} = 2.74$, $p = 0.109$), suggesting that differences in *E. ruidum* recruitment were the main drivers of patterns of fruit mass loss.

Effects of secondary metabolites on how ants handle and where they deposit seeds (obj. 2)

Despite amide extracts reducing the mass of fruits ants removed, they had no detectable effect on the distance that ants dispersed seeds in the field (LMER: $F_{1,50} = 0.35$, $p = 0.557$; Figure 4a). All observed instances of seed dispersal were by the ant *E. ruidum*, which carried seeds directly into their nest (rather than dropping them) in 100% of observations ($N = 21$ amide extract-supplemented and $N = 41$ unsupplemented fruits). Experiments with captive *E. ruidum* lab colonies revealed that the addition of amide extracts to *P. reticulatum* fruits caused a 66% reduction in the proportion of fruits removed by ants within 48 h (LMER: $F_{1,21} = 43.49$, $p < 0.001$; Figure 4b). Moreover, although there was no detectable effect of the total amide concentration in seeds on removal rate (LM: $F_{1,17} = 0.04$, $p = 0.841$), there was a non-significant trend for the total amide concentration in fruit pulp to be negatively associated with seed removal (LM: $F_{1,20} = 3.09$, $p = 0.094$; Appendix S1: Figure S5). Seeds were never observed to be destroyed by the captive ant colonies, but amides altered the quality of seed handling by ants by reducing the proportion of seeds cleaned from fruit pulp by 67% (LMER: $F_{1,21} = 14.93$, $p < 0.001$; Figure 4c) and increasing the likelihood of seeds (that had either been cleaned or left in intact fruits) being redispersed outside of the ant nest chamber by 200% (LMER: $F_{1,21} = 69.30$, $p < 0.001$; Figure 4d).

DISCUSSION

Secondary metabolites are ubiquitous in plant reproductive structures, but their consequences for mutualisms are poorly understood. We found that secondary metabolites in fruits both reduce the quantity and alter the quality of seed dispersal by ants, with the potential for strong negative effects on SDE and plant fitness. The application of two pure amides to *Piper* fruits had no effect on seed dispersal rate when tested individually, but

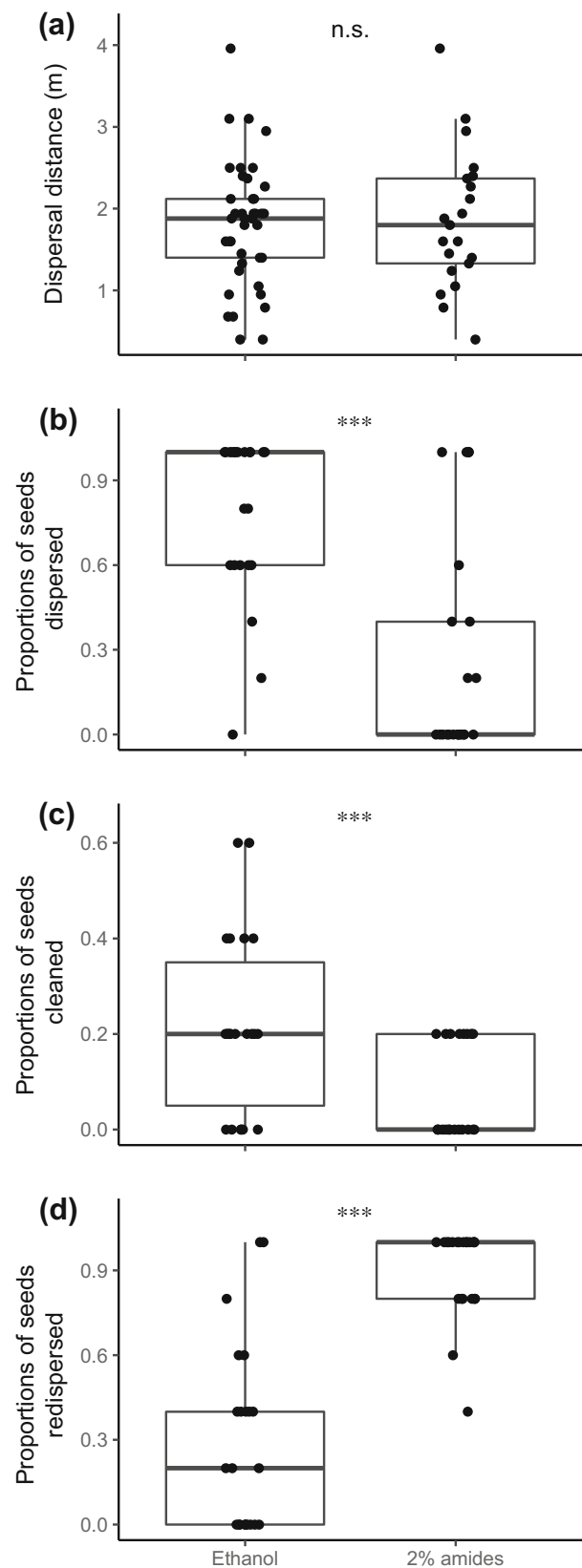


FIGURE 4 Effect of amide extracts on seed handling by ants, measured as (a) dispersal distance in the field and (b) the proportions of seeds dispersed, (c) cleaned from fruit pulp, and (d) redispersed out of the nest chamber within 48 h in lab colonies. Asterisks (***) indicate significant differences among groups at the $p < 0.001$ level.

in combination they significantly reduced ant recruitment. Moreover, amide extracts from ripe *P. reticulatum* fruit (containing a mixture of 20+ amides) reduced ant recruitment by 87% and fruit mass loss by 58%. Amide extracts not only decreased dispersal quantity but also altered seed dispersal quality by shaping the community composition of ants recruiting to fruits (90% decrease in the recruitment rate of the effective disperser *E. ruidum* but not of the cheater *P. subarmata*, which removed fruit pulp but not seeds). Although amides had no detectable effect on seed dispersal distance, they further altered seed dispersal quality by reducing the likelihood of ants cleaning seeds by 67% and increasing the likelihood of ants redispersing seeds outside of the nest by 200%. These results, combined with our past work, suggest that while amides can reduce fruit attack by insect herbivores and pathogens (Whitehead & Bowers, 2014), this comes at the cost of also reducing the quantity and quality of seed dispersal mutualisms with not only bats (Whitehead et al., 2016) but also ants (Figures 2–5).

Despite ants strongly recruiting to ripe and overripe fruits, amides present in *P. reticulatum* fruits and seeds deter ants and, as a result, reduce seed dispersal quantity. In the field, the addition of amide extracts to amide-free

fruits reduced ant recruitment by 87% and fruit mass loss by 58%. These results were paralleled in the lab, where the total amide concentration naturally present in fruit pulp showed a non-significant negative association with seed dispersal rate by *E. ruidum*, and the application of amide extracts significantly reduced the proportion of seeds dispersed by 66%. Together, these results suggest that the amounts of amides typical in *Piper* fruits reduce ant seed dispersal quantity by approximately 58%–66%. These findings are perhaps unsurprising, given that ant foraging is strongly driven by chemistry (Nelson et al., 2019, 2020) and amides have previously been found to deter other ant species from *Piper* leaves (Dyer et al., 2003). Yet, one previous study that examined the effects of fruit secondary metabolites (alkenylphenols in another *Piper* species) on seed dispersal quantity by ants found no detectable effect on ant recruitment to or removal of seeds, despite these compounds having known antimicrobial and cytotoxic effects (Clemente & Whitehead, 2020). Compared to alkenylphenols, amides are more broadly deterrent and toxic and occur at a higher diversity within the *Piper* fruits studied, potentially explaining why we found evidence for stronger effects of fruit secondary metabolites on seed dispersal by ants in this study. In fact, to our knowledge, this is the first study to clearly demonstrate that the seed

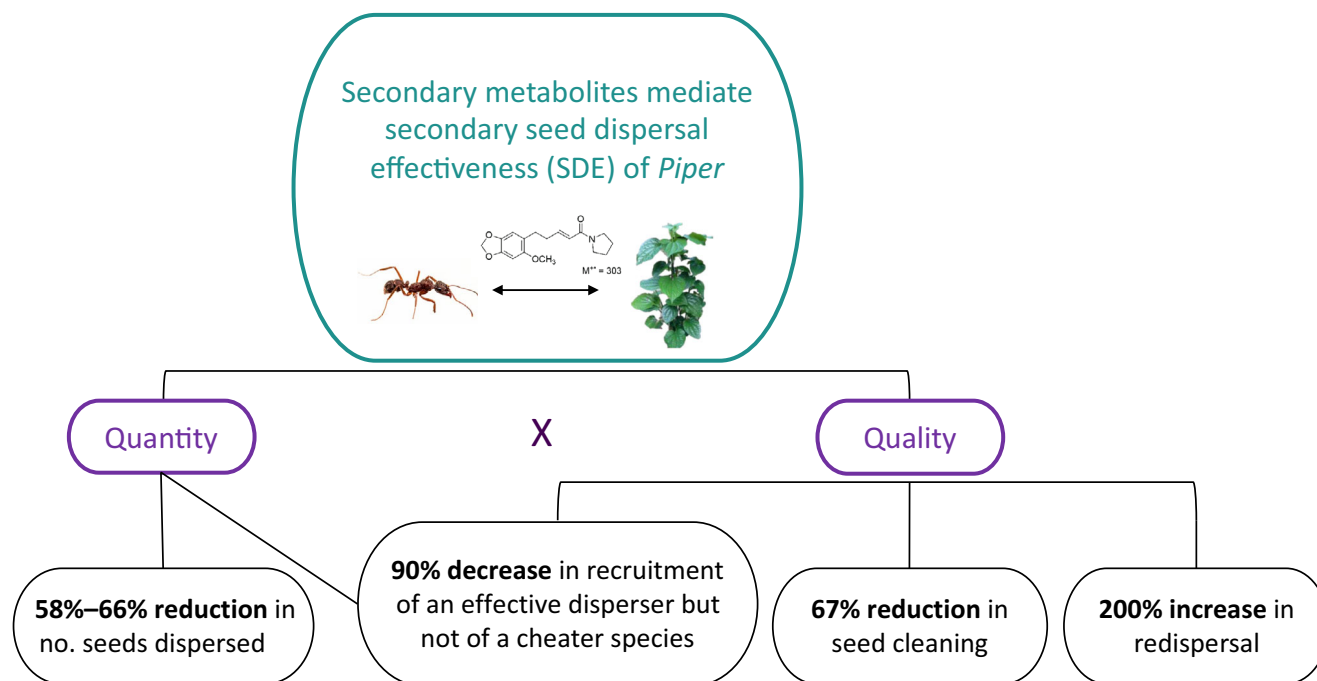


FIGURE 5 Net effect of amides on the seed dispersal effectiveness (SDE) of *P. reticulatum* by ants, calculated based on the changes in seed dispersal quantity multiplied by quality. Amides result in a net 58%–66% reduction in seed dispersal quantity. Net effects on seed dispersal quality are uncertain, as future studies must examine the proportional change in seed survival and germination due to changes in ant community composition, seed cleaning behavior, and redispersal outside of the nest. Note that because shifts in ant community composition not only alter seed dispersal quality but also determine seed dispersal quantity (contributing to the net 58%–66% reduction), we depict it as linked to both quality and quantity.

dispersal quantity by this key group of invertebrate seed dispersers can be strongly reduced by secondary metabolites in fruits.

While we did not find evidence that pure amides alone or in mixture reduced seed dispersal quantity, reductions in ant recruitment to fruits with piperine and piperidine applied in combination suggest a potential synergistic effect. In previous studies, amides have likewise been found to have (even stronger) synergistic effects on ant recruitment in other contexts (e.g., leaf cutting of other *Piper* species; Dyer et al., 2003), and this likely explains why the amide extracts used in this study (containing a complex mixture of 20+ compounds) had strong deterrent effects. It is also possible that reductions in seed dispersal quantity are driven by particular amides within the extracts, presenting a promising area for future investigation.

In addition, this study uncovers multiple simultaneous mechanisms through which fruit secondary metabolites alter seed dispersal quality. First, by altering the community composition of ants recruiting to *P. reticulatum* fruits, amides not only decreased seed dispersal quantity (by reducing the recruitment of *E. ruidum*, the most effective seed-dispersing ant, by 90%) but also altered seed dispersal quality. Although amides decreased the recruitment of *Pheidole* sp. 1 by 87%, there was no detectable change in *P. subarmata* recruitment. Both species were observed to remove fruit pulp but not seeds from the infructescence and thus serve as ineffective dispersers and potential cheaters. It is possible that the loss of the most effective ant partner (*E. ruidum*), in combination with the continued recruitment of one of the two less effective partners (*P. subarmata*), further strengthens the negative effects of amides on seed dispersal. By continuing to remove fruit pulp (but not seeds), *P. subarmata* reduces the rewards available to attract more effective dispersers, thus compounding the negative effects of amides on *E. ruidum* recruitment. Yet, for seeds that were nonetheless dispersed by *E. ruidum*, we did not detect an effect of amides on seed dispersal quality measured in terms of dispersal distance, as foragers were always observed to carry fruits directly into their nests (mean 1.82 m; range 0.4–3.96 m). This suggests that despite amides having a strong influence in the initial decision by ants to remove fruits, amides do not influence the distance that ants carry them. For vertebrates, fruit secondary metabolites can either increase or decrease dispersal distance by mediating the retention time of seeds in the gut (Baldwin & Whitehead, 2015; Wahaj et al., 1998). However, seed retention by ants is not driven by similar physiological effects, because while ants consume the fruit flesh, they do not consume the seeds and instead only carry them.

Once seeds are brought into the nest, amides strongly mediate seed dispersal quality through changes in seed

handling and deposition patterns. Like past studies demonstrating that toxic or deterrent secondary metabolites in fruit can reduce consumption by vertebrate frugivores (Samuni-Blank et al., 2012; Steele et al., 1993; Whitehead et al., 2016), we found that ants were 67% less likely to remove and consume the fruit pulp surrounding seeds with amide extracts experimentally added. This likely causes a strong reduction in seed dispersal quality, as seeds embedded within fruit pulp are typically inhibited from germinating (Fricke et al., 2019; Meyer & Witmer, 1998) and more susceptible to attack by microbial pathogens and seed predators (Fricke et al., 2013), especially when seed cleaning by ants has an anti-fungal effect (Offenberg & Damgaard, 2019; Ohkawara & Akino, 2005). Moreover, amides increase the likelihood of seeds (both cleaned and in intact fruit pulp) being redispersed outside of the ant nest within 48 h by 200%, likely due to their toxic or deterrent effects, or because ants are actively preventing the accumulation of pathogens in the nest, which may grow on fruit pulp that is left uneaten (due to being high in amides and therefore less attractive to ants). Ants frequently redisperse seeds outside of their nest within the first several days of collection, depositing them in refuse piles or scattered around nearby (Canner et al., 2012; Connell et al., 2016; Gorb et al., 2000). While it is possible that additional seeds would have been redispersed over a longer period of observations, this is the first study to demonstrate that differences in fruit traits predict the probability of redispersal. Redispersal could have variable effects on seed dispersal quality, as seeds left in ant nests may be buried too deep to germinate, especially for seeds buried up to 40 cm deep in *Ectatomma* nests (Renard et al., 2010; Ruzi & Suarez, 2022). However, remaining in the nest can be beneficial because the soil in and near nests is sometimes more nutrient rich (Farji-Brener & Werenkraut, 2017), potentially (but not necessarily) promoting germination (Giladi, 2006; Horvitz & Schemske, 1986; Sondej & Domisch, 2022). Seeds deposited in ant nests are also better protected against seed predators (e.g., rodents; Heithaus, 1981). Therefore, the net effects on plant fitness depend on the relative effects of burial depth, nutrient availability, and seed predation risk on seed survival and germination success, presenting a promising area for future investigation.

Altogether, by reducing seed dispersal quantity and altering seed dispersal quality, amides likely result in an overall reduction in the SDE of *P. reticulatum* by ants (Figure 5). However, additional in-depth studies examining the relative importance of changes in seed dispersal quantity and quality under various scenarios are needed to determine the net effects on SDE and plant fitness. The findings from this study parallel those found

previously with bats, in which amides in *P. reticulatum* fruits not only reduce the number of infructescences removed by bats but also alter seed dispersal quality by reducing the number of fruits bats consume (versus drop on the ground) (Whitehead et al., 2016), the retention time of seeds in the gut (Baldwin & Whitehead, 2015), and ultimately seed dispersal distance (Baldwin et al., 2020). Thus, it appears that amides reduce the SDE of *P. reticulatum* by multiple disperser groups (vertebrate frugivores and invertebrates), although this may not be detrimental to the plant in all contexts. For example, because fleshy fruit pulp serves as the primary reward for ants, ants do not strongly recruit to *Piper* seeds in bat feces (Clemente & Whitehead, 2020). Amides could therefore increase the relative proportion of seeds dispersed by ants by reducing total fruit removal and consumption by bats. This could improve overall SDE of *P. reticulatum* if bats and ants provide complementary dispersal services, resulting in a positive effect of seed disperser diversity, as has previously been found for plants dispersed by both birds and ants (Christianini & Oliveira, 2010). Whereas bats provide long distance dispersal, benefits associated with gut passage, and directed dispersal to favorable microhabitats beneath roosting sites (Baldwin et al., 2020; Salazar et al., 2013), ants provide shorter distance but directed dispersal to microhabitats in and surrounding ant nests, where seeds may experience a different set of benefits (Giladi, 2006).

Overall, this study provides evidence for several previously undocumented consequences of secondary metabolites for mutualistic interactions. It also significantly advances our holistic understanding of a model system for the chemical ecology of seed dispersal. Amides clearly have many complex and interacting effects on all stages of the seed dispersal process as well as fruit defense. From this and past studies, we know that amides have negative effects not only on the SDE of vertebrate and invertebrate seed dispersers, but also on microbial pathogens and invertebrate seed predators, resulting in a trade-off between multiple components of seed dispersal and defense (Whitehead et al., 2016; Whitehead & Bowers, 2014). To some degree, amides likely also cause a trade-off between dispersal by bats and ants, as seeds prevented from being consumed by bats are more likely to be dispersed by ants (Clemente & Whitehead, 2020). It is thus clear that amides could serve multiple adaptive functions in mediating these interactions, though additional work is needed to understand the net effects of amides on plant fitness under different ecological contexts, such as under variation in seed predation pressure and seed disperser availability. Another fascinating area for future work is to examine the costs and benefits of variation in amide production within a landscape context. For example, the relative degree of variation in amides within versus

among *P. reticulatum* individuals may play a strong role in determining plant fitness. It would perhaps be beneficial for an individual plant to have high variability in amide content across fruits, as it is likely that some fruits would be heavily attacked, but others would be dispersed by bats or ants, improving the probability that at least some seeds would survive and germinate. By demonstrating that fruit secondary metabolites alter the effectiveness of a seed dispersal mutualism through multiple complex mechanisms, this study opens a new area for future research essential for understanding the adaptive significance of secondary metabolites in fruits.

AUTHOR CONTRIBUTIONS

Annika S. Nelson and Susan R. Whitehead led project design, Annika S. Nelson and Estefania Morales-M. collected the data, Annika S. Nelson and Mariana Gelambi conducted chemical extractions and analyses, and Annika S. Nelson conducted data analyses and writing, with input from all other authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data, metadata, and R code (Nelson et al., 2023) used to generate results and figures is available in Dryad at <https://doi.org/10.7280/D1R39K>.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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