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Fruit secondary compounds mediate the retention time of seeds in the guts of Neotropical fruit bats

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Abstract Plants often recruit frugivorous animals to transport their seeds; however, gut passage can have varying effects on plant fitness depending on the physical and chemical treatment of the seed, the distance seeds are transported, and the specific site of deposition. One way in which plants can mediate the effects of gut passage on fitness is by producing fruit secondary compounds that influence gut-retention time (GRT). Using frugivorous bats (*Carollia perspicillata*: Phyllostomidae) and Neotropical plants in the genus *Piper*, we compared GRT of seeds among five plant species (*Piper colonense*, *Piper peltatum*, *Piper reticulatum*, *Piper sancti-felicii*, and *Piper silvivagum*) and investigated the role of fruit amides (piperine, pipartine and whole fruit amide extracts from *P. reticulatum*) in mediating GRT. Our results showed interspecific differences in GRT; *P. reticulatum* seeds passed most slowly, while *P. silvivagum* and *P. colonense* seeds passed most rapidly. Pipartine and *P. reticulatum* amide extracts decreased GRT, while piperine had no effect. In addition, we examined the effects of GRT on seed germination success and speed in laboratory conditions. For germination success, the effects were species specific; germination

success increased with GRT for *P. peltatum* but not for other species. GRT did not influence germination speed in any of the species examined. Plant secondary compounds have primarily been studied in the context of their defensive role against herbivores and pathogens, but may also play a key role in mediating seed dispersal interactions.

Keywords Fruit chemistry · Seed dispersal · Amides · *Carollia* · *Piper*

Introduction

Seed dispersal is a critical ecological process that influences plant population dynamics, community structure, gene flow, and the maintenance of biodiversity (Howe and Smallwood 1982; Schupp et al. 2010). Particularly in the tropics, this process is often mediated by animals, which consume fruits for their nutritional rewards (van der Pijl 1982). For example, in one tropical lowland wet forest in Costa Rica, 85 % of canopy trees and 98 % of sub-canopy trees have fruit traits that suggest adaptation for seed dispersal by animals (Frankie et al. 1974). However, frugivorous animals can vary greatly in their effectiveness as dispersers (McKey 1975). Their contribution to the recruitment of new individuals to plant populations depends on factors such as fruit handling behavior, retention time in disperser guts, seed breakage, and disperser defecation patterns (Schupp et al. 2010). Thus, plants should experience strong selective pressure for fruit traits that maximize dispersal effectiveness.

Of the factors that influence seed dispersal, the amount of time that seeds remain in the gut of a disperser can have particularly strong fitness consequences for fruit-bearing plants (Traveset 1998). In some cases, longer gut-retention

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time (GRT) can facilitate germination and raise seed viability by cleaning pulp from the seeds or abrading the seed coat to allow for higher permeability of gases and water (Traveset 1998). Alternatively, high GRT can reduce seed viability if the seeds do not survive the proteolytic environments of frugivore digestive tracts (Janzen 1981; Cosyns et al. 2005; Tewksbury et al. 2008a). In addition to these effects on seed viability, GRT can affect dispersal distance and patterns of gene flow as dispersers migrate or forage over long distances, as well as the potential for interspecific composition among seedlings if generalist frugivores deposit seeds of multiple species in single droppings (Loiselle 1990; Murray et al. 1994; Shilton et al. 1999). Thus, depending on the ecological context, plant fitness may be increased through fruit traits that either speed up or slow down the retention time of seeds (Cipollini and Levey 1997).

One primary way in which plants may mediate the retention time of seeds in animal guts is through the production of secondary compounds in fruit pulp (Cipollini and Levey 1997; Cipollini 2000). These compounds can influence seed passage rates, either shortening or prolonging GRT, and influence seed viability (Murray et al. 1994; Wahaj et al. 1998; Tewksbury et al. 2008a). However, the overall effects of secondary compounds on seed dispersal may vary considerably depending on the particular class of compound, plant tissue in which the compounds are located, species of plant, and disperser involved. The few studies that have examined the role of fruit secondary compounds in GRT have focused on birds (Murray et al. 1994; Wahaj et al. 1998; Tewksbury et al. 2008a) but we know of no ecological studies that have examined the role of fruit secondary compounds in mediating the GRT of seeds in mammalian frugivores. There is strong evidence that plant-derived secondary compounds can influence mammalian feeding preferences and physiology (Freeland et al. 1985; Bodmer 1991), and that these effects may differ considerably from the effects that secondary compounds have on birds (Howe and Smallwood 1982; Freeland et al. 1985; Struempf et al. 1999; Mason et al. 1991; Tewksbury and Nabhan 2001; Whitehead 2013).

Among frugivorous mammals, bats are particularly important seed dispersers because they deposit large quantities of seeds in diverse sites, including open areas that are avoided by other animals, influencing patterns of forest structure and playing critical roles in forest regeneration and succession (Fleming and Heithaus 1981; Galindo-Gonzalez et al. 2000; Estrada-Villegas et al. 2007; Muscarella and Fleming 2007). Although the GRT of seeds in frugivorous bats is often short, on the order of 1 h or less (Fleming 1988; Bizerril and Raw 1998), some species (e.g., *Cynopterus sphinx*: Pteropodidae) can transport seeds for over 12 h, dispersing them hundreds of kilometers away (Shilton

et al. 1999). Because this large variation in GRT is likely to have important consequences for plant fitness, bat-dispersed plants may experience strong selection for traits that either speed up or slow down GRT, depending on the effects of the gut environment on seed viability, the success of seeds deposited at different distances from the parent plant, and seedling community composition.

We address the following questions about the relationship of fruit secondary compounds and frugivore-mediated dispersal, using the diverse tropical plant genus *Piper* and its primary seed dispersers in the Neotropics, bats in the genus *Carollia*, as a model system:

1. Does the GRT of seeds in *Carollia* vary among different species of *Piper*?
2. Can amides, a diverse class of secondary compounds produced in the fruits of many *Piper* species, affect the GRT of seeds?
3. Does the variation in GRT influence seed germination?

Due to the large interspecific variation in *Piper* fruit chemistry in terms of both types and levels of fruit secondary compounds (Kato and Furlan 2007), we hypothesize that GRT will differ among *Piper* species. We also hypothesize that fruit secondary compounds can alter disperser GRT, although we do not predict whether GRT will be shortened or prolonged, since both effects have been shown in other systems (e.g., Murray et al. 1994; Wahaj et al. 1998). Finally, we hypothesize that GRT will influence seed germination, but again make no specific predictions as to the direction of the effect, since both positive and negative effects of GRT have been shown in other systems (e.g., Murray et al. 1994; Tewksbury et al. 2008a).

Materials and methods

Study system and site

This study was conducted from June until July 2011 and in September 2012 during the wet season at La Selva Biological Station, Heredia Province, Costa Rica. La Selva is classified as lowland tropical wet forest (Holdridge 1967) with an annual rainfall of 4,000 mm (McDade et al. 1994). Plants of the diverse pantropical genus *Piper* (Piperaceae) are readily found throughout the reserve, with more than 50 species co-occurring (Gentry 1990). They are important early and mid-successional plants, growing up to 4 m tall and comprising large amounts of understory biomass (Dyer et al. 2004a). Fruits are borne on distinct spike-shaped infructescences containing hundreds to thousands of individual fruits. In the Neotropics, seeds are dispersed primarily by frugivorous bats, although many species are also

consumed by a wide variety of birds (Fleming and Heithaus 1981; Palmeirim et al. 1989; Fleming 2004). Among the more than 65 species of bats that are found at La Selva (Timm 1994), three species in the genus *Carollia* (*Carollia sowelli*, *Carollia castanea*, and *Carollia perspicillata*) feed preferentially on *Piper* fruits (Lopez and Vaughan 2004). They locate ripe *Piper* primarily by odor (Thies et al. 1998), remove entire infructescences in flight (Online Resource 3), and carry them to nearby feeding roosts for consumption. We focused on Seba's short-tailed fruit bat (*Carollia perspicillata*), which is the most amenable to captive studies. Field experiments with *Carollia* were conducted in accordance with Costa Rican law and University of Colorado Institutional Animal Care and Use Committee protocols.

Piper species harbor a wide variety of secondary compounds, and many species are particularly rich in amides, a large group of N-containing compounds that have known defensive functions in *Piper* leaves (Dyer et al. 2004b). Amides are also widely found in *Piper* fruits (Yang et al. 2002; de O Chaves et al. 2003; Siddiqui et al. 2005; Whitehead et al. 2013), e.g., piperine, an amide found in approximately one-fifth of all *Piper* species investigated thus far, is most well known from the fruits of *Piper nigrum*, which are the source of black pepper (Parmar et al. 1997; Vasques da Silva et al. 2002; Dyer et al. 2004a). Amides have been shown to decrease *Carollia*'s preference for *Piper* fruits (Whitehead 2013), but their effects on bat physiology and seed germination are unknown. The pharmaceutical literature reports that *Piper* amides strongly affect humans and other mammals in laboratory trials, showing antihypertensive, hepatotoxic, cytotoxic, anxiolytic, sedative, anti-convulsant, antinociceptive or bioavailability-enhancing effects (Nerurkar et al. 2004; Felipe et al. 2007; Sharma and Mishra 2007; Rodrigues et al. 2009; Hlavackova et al. 2010; Liu et al. 2010; Mishra et al. 2011).

Field capture of *C. perspicillata*

We captured bats at La Selva using mist-nets (38-mm mesh, 9 × 2.6 m; Avinet) from 16 until 23 June 2011 and from 9 until 14 September 2012. We recorded each individual's mass (60-g spring scale; Pesola, Switzerland), forearm and tibia length, sex, age, and reproductive status. We released reproductive females, and transferred males and non-reproductive females either directly to solitary 2 × 1 × 1.5-m feeding cages or to a large 10 × 10 × 3-m holding cage. All experiments were conducted in the solitary cages, and bats kept in the holding cage were always transferred to the solitary cages at least 24 h prior to the start of the experiments. Prior to the start of the feeding trials, bats were fed a maintenance diet of bananas, papayas, and water ad libitum, allowing acclimation to the flight cage and ensuring that the gut was void of *Piper*. This food was removed on

the afternoon before the feeding trial, ensuring that *Piper* fruits were the first meal the bats received on the night of the experiment.

Experiment 1: variation in GRT of seeds from *Piper* spp.

To quantify the variation in GRT of seeds from different *Piper* species, we conducted a series of feeding trials in 2011 with four readily available species of *Piper* (*Piper colonense*, *Piper peltatum*, *Piper reticulatum*, and *Piper silvaginum*). Fruits of *P. reticulatum* are known to contain a diverse mixture of amides (Whitehead et al. 2013), but the chemistry of the other three species has not been investigated. We offered ripe infructescences of the four species in random order across four successive nights to 14 males and four non-reproductive females of *C. perspicillata*. Each afternoon, we collected fresh infructescences to use in the feeding trials that night and weighed the fruit that was to be presented to each bat. Ripe infructescences were distinguished from unripe ones by checking for fruit softness and swelling, and we used only infructescences that had ripened on the day of collection (2-day-old infructescences are rarely found because they are generally removed on the first night they are ripe, and those that are found are much softer and often rotten-smelling). Furthermore, as hard unripe infructescences are only dislodged with great difficulty from the plant, we were able to collect ripe fruits from hard-to-reach branches by shaking *Piper* shrubs and gathering the fallen soft infructescences from the ground. On each successive night of the trials, we presented bats with infructescences from one species of *Piper* at 1800 hours. To begin the feeding trial, one or two ripe infructescences were placed in the feeding cage, standing vertically to mimic their natural presentation on *Piper* plants. The total mass of the fruit we offered to the bat averaged 2.43 g ($n = 100$, $SE = 0.0676$) and was most often contained in a single infructescence, but varied between 0.9 and 3.8 g due to varying sizes of infructescences and limited availability of some species. To satisfy the daily demand for fruit, we repeatedly harvested ripe infructescences from six plants of *P. colonense*, seven plants of *P. peltatum*, seven plants of *P. reticulatum* and three plants of *P. silvaginum*. We harvested between one and eight infructescences from each plant over the course of the study.

We observed the bats within the feeding cages during feeding and defecation. We covered the feeding cage floor using a plastic sheet with a grid to localize and recover feces. Immediately following fruit presentation and consumption, we continuously checked the cages to observe when bats began eating, finished eating and produced green, seed-filled defecations by checking with a flashlight at least every 5 min or listening: when *Carollia* finishes eating a *Piper* infructescence, it audibly drops the rachis that

has been stripped of individual fruits, and its defecations were equally loud on the plastic sheets. We discarded data from nights when bats ate less than half the presented fruit in the first feeding bout. Over all feeding observations, the time elapsed from the onset of a feeding bout to its end was short (mean = 1.865 min, SE = 0.135, $n = 193$). For each discernible defecation we additionally recorded its passage time (GRT) and location of fecal sample on the grid, which allowed us to later match individual fecal and seed samples with GRT. Trials continued for up to 6 h or until most bats had consumed and defecated *Piper* fruit. Bats that had not consumed the presented infructescences within 8 h were given the maintenance diet for the remainder of the night and tested again on subsequent evenings. We calculated GRT by subtracting the finish time of fruit consumption from the time at first defecation. After every trial, the maintenance diet was replaced for the rest of the evening and all remaining *Piper* infructescences were removed.

Experiment 2: effects of *Piper* fruit amides on GRT

We examined the effects of *Piper* fruit amides on GRT by adding amides that were either purchased commercially or extracted from *P. reticulatum* to infructescences of *Piper sancti-felicitis*, a species which contains no detectable amides (Whitehead 2013). Feeding trials with these amide-supplemented infructescences were conducted in the same manner as above.

First, we conducted a set of trials in 2011 using the same bats captured for experiment 1. Bats were offered ripe infructescences of *P. sancti-felicitis* that originated from three plants that we repeatedly harvested. We supplemented these infructescences with either piperine (Sigma-Aldrich, St Louis, MO) or piplartine (Indofine Chemical, Hillsborough, NJ), two amides that are found in many species throughout the genus *Piper* (Parmar et al. 1997). Bats were also offered unsupplemented infructescences as controls. We supplemented infructescences with 10 mg amide/infructescence (~2 % dry mass), a relatively low concentration of amides compared to levels found in the fruits of many *Piper* species (de O Chaves et al. 2003). To supplement the infructescences, amides were dissolved in ethanol at a concentration of 20 mg/mL, and 0.5-mL aliquots were pipetted onto the fruit surface. We treated unsupplemented control infructescences in the same manner using ethanol only, and all infructescences were dried for at least 1 h to allow evaporation of the solvent. We presented each bat with the piperine-supplemented, piplartine-supplemented, and control infructescences on three successive evenings in random order. Because these trials were conducted in the same time frame and with the same bats used in experiment 1, we also included control-treated *P. sancti-felicitis* fruits from this experiment in the analysis of interspecific differences in GRT.

Next, we conducted an additional set of trials in 2012 where we offered four male and seven female bats ripe *P. sancti-felicitis* infructescences that were supplemented with varying levels of amide extracts from *P. reticulatum*, the ripe fruits of which can contain up to 23 individual amides at an average total concentration of 0.71 % dry mass (Whitehead et al. 2013). Amide extracts were prepared as described in Whitehead et al. (2013). Briefly, 78 g of ground fruit material (containing pulp and seeds) from *P. reticulatum* was extracted in ethanol, filtered, partitioned between 3:1 water:ethanol and chloroform, and the chloroform fraction (containing the amides) was evaporated to dryness. The extracts were then re-suspended in 41 mL ethanol and divided into four vials with 3.5, 6.5, 12.5 and 18.5 mL, respectively. The ethanol in each vial was evaporated, and each vial's extract was then re-suspended in 6.5 mL ethanol. These final extract solutions were pipetted in 0.5-mL aliquots onto individual ripe *P. sancti-felicitis* infructescences that we had harvested from 12 plants. Based on the mass of dry starting material, this procedure would have produced *P. sancti-felicitis* infructescences with a maximum of half, one, two and three times the concentration of amides of a single *P. reticulatum* infructescence. We conducted feeding trials as above with these supplemented infructescences and with ethanol-supplemented control infructescences. During all trials in 2012, we additionally measured the infructescence length and the length of the infructescence that was consumed to calculate the proportion of fruit consumed. Multiplying this proportion with the fruit mass yielded the consumed fruit mass. Multiplying the proportion with the amide concentration yielded the consumed amide dose. While in 2011, bats that did not eat in one night were tested in subsequent nights, in 2012, when bats had not fed after 8 h, they were captured and fed while holding them in the hand (21 of 54 GRTs were obtained from hand-fed bats). Holding the fruit in front of its nose was sufficient to initiate a bat's feeding behavior, and we noted whether the bat had been hand-fed in this manner or not.

Experiment 3: effects of GRT on seed germination

In 2011 seeds were removed from the defecations collected during experiments 1 and 2, rinsed, and air-dried. Seeds were also manually removed from the pulp of uneaten ripe fruits of each *Piper* species except for *P. reticulatum* (due to limited availability of ripe fruits), rinsed, and air-dried. Three months later, in November 2011, we sowed a total of 12,601 seeds from all five *Piper* species: 10,548 seeds originated from 74 defecations that had experienced a range of GRTs from 8 to 119 min and 2,053 seeds originated from fresh fruits (GRT = 0). We sowed seeds into a 2 % agar and 10^{-4} mM nitrate medium, keeping seeds

Table 1 Model output evaluating interspecific differences in gut-retention time (GRT) among five species of *Piper* accounting for the influence of fruit mass (FM)

Fixed effects	Random effects	\hat{c}	AICc	Δ AICc relative to set best	Akaike weight
<i>Piper</i> species \times FM	BatID/PlantID	1.020	490.3	5.0	0.0653
<i>Piper</i> species + FM	BatID/PlantID		485.3	0	0.7897
<i>Piper</i> species	BatID/PlantID		494.5	9.2	0.0078
FM	BatID/PlantID		489.4	4.1	0.1017
None	BatID/PlantID		491.5	6.2	0.0354

The response variable for each model is GRT in minutes. \times implies fixed effects and their interactions

AICc Akaike information criteria corrected for small sample sizes, ID identity

Table 2 Model output evaluating the influence of pure amide (piperine or piplartine) treatment on GRT

Fixed effects	Random effects	\hat{c}	QAICc	Δ QAICc relative to set best	Akaike weights
AT \times FM	BatID/PlantID	1.676	228.9	4.2	0.0737
AT + FM	BatID/PlantID		224.7	0	0.6142
AT	BatID/PlantID		226.1	1.4	0.3099
FM	BatID/PlantID		237.1	12.5	0.0012
None	BatID/PlantID		237.6	12.9	<0.001

The response variable for each model is GRT in minutes

QAICc quasi-AIC, AT amide treatment; for other abbreviations and symbols, see Table 1

from each defecation together in an individual petri dish. We placed the dishes in a temperature-controlled chamber set at 26° C with a light source on a 12/12-h light/dark cycle. The light radiated an intensity of 1.5–3.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and had a red:far-red ratio of 1.37. These germination conditions represent successful artificial germination conditions for *P. peltatum* and ecologically similar species of Central American *Piper* (Daws et al. 2002). We counted the number of germinated seeds and non-germinated seeds in each defecation every 2 days for 23–37 days, until germination success on each plate had plateaued.

Statistical analysis

To examine differences in GRTs among *Piper* species, we used a generalized linear mixed model (GLMM) with a Poisson distribution (O'Hara and Kotze 2010). We used GRT as the response variable, *Piper* species identity (ID), fruit mass, and their interaction as fixed effects, and individual bat ID and individual plant ID as partially crossed random effects. This accounted for repeated measures of individual bats and repeated harvest of fruits from plants. Residuals were checked for overdispersion using $\hat{c} > 1.5$ (for the most complex model) as a cutoff (Zuur et al. 2009). For hypothesis testing, we compared models with increasingly simplified fixed effect structures and a null model with only random effects using Akaike information criteria corrected for small sample sizes (AICc) according to Bolker

et al. (2009). We considered fixed effects to be strongly supported if comparisons with simplified models showed a $\Delta\text{AICc} > 10$, moderately supported if they showed $2 < \Delta\text{AICc} < 10$, and not supported if they had a $\Delta\text{AICc} < 2$ (Bolker 2008; Bolker et al. 2009). To examine the relative importance of each fixed effect and to obtain a parameter estimate for the continuous effect of fruit mass, we performed model averaging using the MuMIn package in R on a subset of models that were within $\Delta 10$ AICc scores of the best model (Burnham and Anderson 1998; Bartón 2013). After finding moderate support for an effect of *Piper* species on GRT (see “Results”; Table 1), we performed post hoc Wald Z-tests to compare mean GRTs among species using the multcomp package (Hothorn et al. 2014).

To determine the effects of piperine and piplartine on GRT in *P. sancti-felicitis*, we used a Poisson GLMM with GRT as the response variable, amide treatment (piperine, piplartine, or control), fruit mass, and their interaction as fixed effects, and individual bat ID and individual plant ID as partially crossed random effects (Table 2). We performed model simplification, model averaging and hypothesis testing as above, in this case using quasi-AICc (QAICc) for model comparison because $\hat{c} > 1.5$ (Bolker et al. 2009). In addition, we a priori specified two post hoc tests to compare the mean GRT of piperine- and piplartine-supplemented fruits to control fruits, which we performed using Wald Z-tests in the multcomp package in R (Hothorn et al. 2014).

Table 3 Model outputs evaluating the influence of *Piper reticulatum* amide treatment on GRT

The response variable for each model is GRT in minutes
CPrA Consumed *P. reticulatum* amides, *CIM* consumed infructescence mass, *F* hand-fed; for other abbreviations, see Table 1

Fixed effects	Random effects	\hat{c}	AICc	Δ AICc relative to set best	Akaike weight
CPrA + CIM + F	BatID/PlantID	1.20406	411.2	0	0.9890
CPrA + CIM	BatID/PlantID		420.9	9.7	0.0078
CPrA + F	BatID/PlantID		423.6	12.4	0.0020
CPrA	BatID/PlantID		424.6	13.4	0.0012
CIM + F	BatID/PlantID		465.0	53.7	<0.001
CIM	BatID/PlantID		482.6	71.4	<0.001
F	BatID/PlantID		485.0	73.8	<0.001
None	BatID/PlantID		488.2	77.0	<0.001

Table 4 Model outputs evaluating the effect of *P. reticulatum* amide treatment (*PrAT*) on the decision to hand-feed a bat

Fixed effects	Random effects	\hat{c}	AICc	Δ AIC relative to set best	Akaike weights
PrAT	BatID/PlantID	1.000093	74.7	2.3	0.242
None	BatID/PlantID		72.4	0	0.758

The response variable for each model is GRT in minutes. For other abbreviations, see Table 1

To determine the effects of *P. reticulatum*-extract concentration on GRT, we used a Poisson GLMM with GRT (rounded to the nearest integer) as a response variable. Consumed amide dose, consumed fruit mass and hand-feeding were included as fixed effects, and individual bat ID and individual plant ID were included as partially crossed random effects (Table 3). Model simplification, model averaging, and hypothesis testing were performed using AICc as described above. After finding a correlation between two of the fixed effects, consumed fruit mass and hand-feeding (see “Results”), we compared consumed fruit mass among hand-feeding groups with a *t*-test. To ensure that hand-feeding was not biasing our conclusions regarding the effects of amide treatment, we also tested whether hand-feeding was equally distributed across amide treatments using a binomial GLMM with hand-feeding status as a response variable, *P. reticulatum* amide treatment as a fixed effect, and individual bat ID and individual plant ID as partially crossed random effects. This model was compared to a null model with only random effects (Table 4).

To determine the effects of GRT on seed germination, we first fit the germination data (expressed as proportional germination success at each time step) to a Richards’ curve (Richards 1959) of the form below.

$$P(t) = \frac{\text{Max}}{\sqrt[1]{1 + v * e^{-B*(t-\text{Mid})}}}$$

$P(t)$ is the proportional germination success of an individual defecation at time t . Max is the final proportional germination success, B is the slope of the logistic regression, v is a sloping parameter, and Mid represents the time point at which $P(t)$ is half the value of Max. Using this model for each defecation we estimated the final

germination success (Max), as well as the time to reach 50 % of the final germination success (Mid). To average out counting errors, we estimated the number of seeds that successfully germinated by multiplying the average total seed count of each defecation by Max and rounding the result to the nearest integer. The number of seeds that failed to germinate was obtained by subtracting the estimated number of successfully germinated seeds from the average total seed count of each defecation and rounding the result to the nearest integer.

Next, we analyzed the effect of GRT on both the germination speed and the germination success. For germination speed, we focused on the number of days needed to reach 5, 50 % (the time point Mid), and 95 % of the final germination success Max, as estimated from the Richard’s curve described above. We fit separate linear mixed models (LMMs) for each number of days, where the times to reach 5, 50, or 95 % of the final proportional germination success were the response variables. *Piper* species, GRT, and their interaction were included as fixed effects while bat ID and plant ID were included as partially crossed random effects (Table 5). For each germination speed, we performed model simplification, hypothesis testing, and model averaging as above, in this case using QAICc scores for model comparisons (Bolker et al. 2009) and using model averaging to obtain parameter estimates for the continuous effect of GRT. To analyze the influence of GRT on germination success, we used a GLMM with binomial errors and the logit link function. The number of germinated and non-germinated seeds was the response variable (as estimated from the Richard’s curve described above). *Piper* species, GRT, and their interaction were included as fixed effects and bat ID and plant ID were included as partially crossed random

Table 5 Model outputs evaluating the influence of GRT on germination speed among five species of *Piper*

Fixed effects	Random effects	\hat{c}	QAICc	Δ QAICc relative to set best	Akaike weight
Germination speed T5 %					
<i>Piper</i> species \times GRT	BatID/PlantID	2.255	245.1	8.9	0.009
<i>Piper</i> species + GRT	BatID/PlantID		238.6	2.4	0.233
<i>Piper</i> species	BatID/PlantID		236.2	0	0.759
GRT	BatID/PlantID		258.6	22.4	<0.001
None	BatID/PlantID		256.5	20.3	<0.001
Germination speed T50 %					
<i>Piper</i> species \times GRT	BatID/PlantID	2.683	210.3	9.9	0.006
<i>Piper</i> species + GRT	BatID/PlantID		202.8	2.3	0.237
<i>Piper</i> species	BatID/PlantID		200.5	0	0.757
GRT	BatID/PlantID		222.8	22.3	<0.001
None	BatID/PlantID		220.9	20.4	<0.001
Germination speed T95 %					
<i>Piper</i> species \times GRT	BatID/PlantID	3.407	182.7	10.6	0.0039
<i>Piper</i> species + GRT	BatID/PlantID		174.4	2.3	0.236
<i>Piper</i> species	BatID/PlantID		172.1	0	0.76
GRT	BatID/PlantID		186.3	16.3	<0.001
None	BatID/PlantID		188.4	14.2	<0.001

For abbreviations and symbols, see Tables 1 and 2

Table 6 Model output evaluating the influence of GRT on germination success

Subset	Fixed effects	Random effects	Type	\hat{c}	AIC	Δ AIC relative to set best	Akaike weights
All species	<i>Piper</i> \times GRT	BatID/PlantID	AICc	1.446	908.0	0	1
	<i>Piper</i> + GRT	BatID/PlantID	AICc		938.4	30.4	<0.001
<i>Piper colonense</i>	GRT	BatID/PlantID	AICc	0.894	131.9	0.5	0.43
	None	BatID/PlantID	AICc		131.4	0	0.57
<i>Piper peltatum</i>	GRT	BatID/PlantID	QAICc	2.166	110.4	0	1.0
	None	BatID/PlantID	QAICc		125.1	14.7	<0.001
<i>Piper reticulatum</i>	GRT	BatID/PlantID	AICc	1.308	109.8	2.4	0.23
	None	BatID/PlantID	AICc		107.3	0	0.77
<i>Piper sancti-felices</i>	GRT	BatID/PlantID	AICc	1.442	240.0	2.7	0.21
	None	BatID/PlantID	AICc		237.3	0	0.79
<i>Piper silvivagum</i>	GRT	BatID/PlantID	AICc	1.070	172.0	1.3	0.34
	None	BatID/PlantID	AICc		170.7	0	0.66

For abbreviations and symbols, see Table 1

effects (Table 6). We detected an interaction between *Piper* species and GRT (see “Results”; Table 6), so we fit binomial GLMMs for each species’ data separately, including only GRT as a fixed effect. We then performed model simplification, model averaging, and hypothesis testing as above, using either AICc or QAICc for model comparison based on varying values of \hat{c} for each set of models (Table 6).

All GLMMs and LMMs were fit with the Laplace approximation. All statistical analyses were performed with R Statistical Software version 3.0.2. using the packages lme4, multcomp, MuMIN, and bbmle (Bates et al. 2014; R Development Core Team 2013; Bolker and R Development Core Team 2014; Bartón 2013; Hothorn et al. 2014). All plots

were created with the R package ggplot2 (Wickham 2009). To ensure reproducibility, our analysis code and output are available as supplementary material (Online Resources 1, 2), which we produced with the R packages markdown and knitr (Allaire et al. 2014; Xie 2014) and our raw data are available on Dryad Digital Repository (Baldwin and Whitehead 2014).

Results

Interspecific differences in GRT

GRT of *Piper* seeds in *C. perspicillata* was short on average (23.3 min, SE = 12.03) and highly variable, ranging

from 8 to 71 min across all species of *Piper* (Fig. 1). Model comparisons examining the effects of *Piper* species, fruit mass, and their interaction on GRT indicated that a model that included *Piper* species and fruit mass (but no interaction) was the most supported ($\Delta\text{AICc} = 6.2$ from a null model; Table 1); however, there was considerable uncertainty among candidate models (all possible subsets of the full model with $\Delta\text{AICc} < 10$; Table 1). Comparisons of the best model to simplified versions indicated moderate support for an effect of fruit mass on GRT ($\Delta\text{AICc} = 9.2$) and moderate, but weaker support for an effect of *Piper* species on GRT ($\Delta\text{AICc} = 4.1$). Model averaging revealed a strong negative effect of fruit mass on GRT (relative importance = 0.96, $\beta = -0.2904$, $P = 0.00562$) and supported an overall effect of *Piper* species (relative importance = 0.86). Post hoc tests comparing *Piper* species showed that the GRT of *P. reticulatum* was significantly longer than the GRTs of both *P. silvavagum* (Wald $Z = -3.438$, $n = 26$, $P = 0.0053$) and *P. colonense* (Wald $Z = 2.782$, $n = 24$, $P = 0.0428$), and that the GRT of *P. peltatum* was significantly longer than that of *P. silvavagum* (Wald $Z = -2.824$, $n = 28$, $P = 0.0379$; Fig. 1).

The role of amides in determining GRT

Pipltartine, but not piperine, reduced GRT (Fig. 2). Model comparisons examining the effects of amide treatment, fruit mass, and their interaction on GRT indicated that the model that included amide treatment and fruit mass (but no interaction) was the most supported (ΔQAICc to null model = 12.9; Table 2). However, the model that included amide treatment only was nearly indistinguishable from the best model ($\Delta\text{QAICc} = 1.4$), and the model that included both fixed effects and their interaction was also reasonably well supported ($\Delta\text{QAICc} = 4.2$). Comparisons of the null model to more complex versions indicated strong support for an effect of amide treatment on GRT ($\Delta\text{QAICc} = 11.5$; Fig. 2) and did not support an effect of fruit mass on GRT ($\Delta\text{QAICc} = 0.4$). However, model averaging of the three best-fitting candidate models suggested that both amide treatment (relative importance = 1.00) and fruit mass (relative importance = 0.92, $\beta = -0.280$, $P = 0.0089$) were important explanatory variables and showed a negative effect of fruit mass. Using a priori specified post hoc tests comparing piperine and pipltartine-supplemented fruits to controls, we found that piperine supplementation had no significant effect on GRT (Wald $Z = -1.226$, $n = 28$, $P = 0.359$), while pipltartine supplementation significantly reduced GRT, from 22.0 to 13.7 min (Wald $Z = -5.108$, $n = 27$, $P = 0.0059$).

Amide supplementation with *P. reticulatum* extracts decreased GRT (Fig. 3). Model comparisons examining the effects of consumed *P. reticulatum* amide dose,

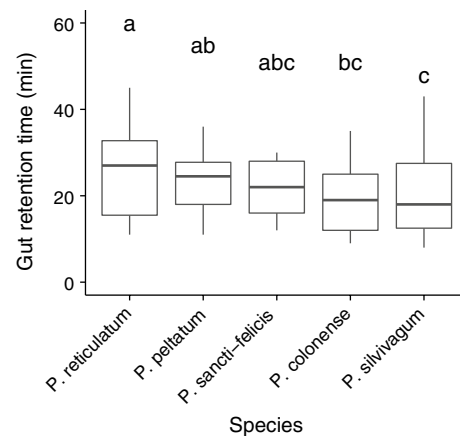


Fig. 1 Gut retention time (in min) of ripe *Piper* fruits in *Carollia perspicillata* varies among five species. Boxes display mean, 25th and 75th percentiles, and whiskers indicate 1.5 times the interquartile range. Means were compared in a generalized linear mixed model (GLMM) that included amide treatment and fruit mass as fixed effects and bat identity (ID) and plant ID as random effects. Levels that do not share letters above box plots are significantly different from one another in Wald Z post hoc tests at $P < 0.05$

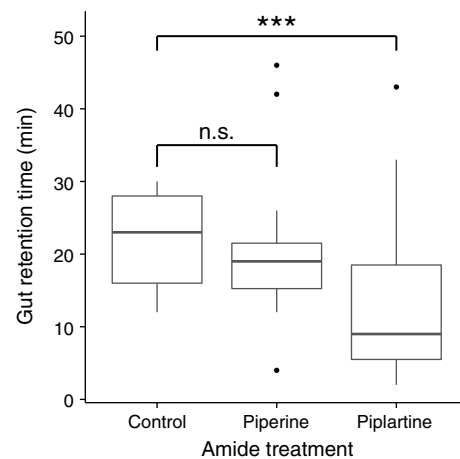


Fig. 2 Gut retention time (in min) of *Piper sancti-felices* seeds in *C. perspicillata* is reduced by adding pipltartine, and not changed by adding piperine, to fruits. Boxes display mean, 25th and 75th percentiles, and whiskers indicate 1.5 times the interquartile range. Means were compared in a GLMM that included amide treatment and fruit mass as fixed effects and bat ID and plant ID as random effects. Asterisks indicate significance levels from Wald Z post hoc tests (*** $P = 0.0059$)

consumed infructescence mass and hand-feeding on GRT indicated that the model that included all fixed effects was the most supported (ΔAICc to null model = 77.0; Table 3). Model comparison also suggested strong support for the effect of consumed *P. reticulatum* amide dose (ΔAICc to null model = 64.2; Table 3). Model averaging using the best two models revealed negative effects of consumed infructescence mass (relative importance = 1.0,

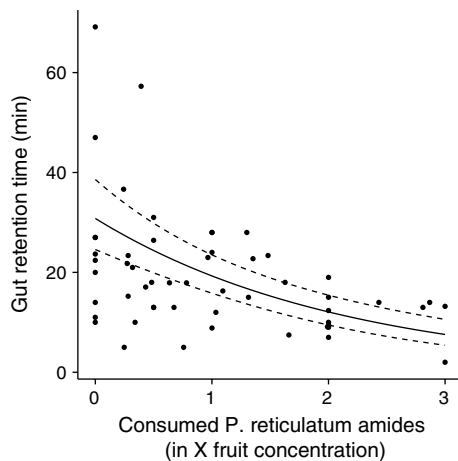


Fig. 3 Gut retention time (in min) of *Piper sancti-felicis* seeds in *C. perspicillata* is reduced as bats consume more amide extracts from *Piper reticulatum* fruits. The units on the x-axis represent the amount of *P. reticulatum* amides consumed by bats, presented in multiples of the amount that was extracted from a single *P. reticulatum* infructescence. The solid line indicates predicted retention times from model-average coefficients, and the dashed lines indicate the 95 % confidence interval

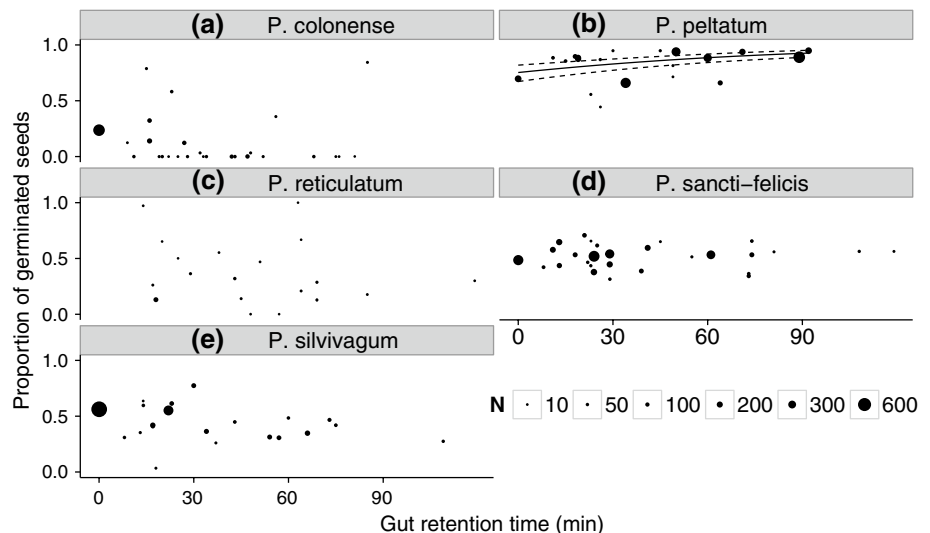
$\beta = -0.406$, $P = 0.00052$) and consumed *P. reticulatum* amide dose (relative importance = 1.0, $\beta = -0.468$, $P < 0.00001$) as well as a significant effect of hand-feeding (relative importance = 0.99, $\beta = -0.466$, $P = 0.00124$). Model comparison also suggested moderate support for hand-feeding (ΔAICc to best model = 9.7; Table 3), but hand-feeding and consumed infructescence mass were confounded, as hand-fed bats consumed significantly less fruit mass than non-hand-fed bats (mean hand-fed = 1.268 g, mean not hand-fed = 1.898 g; student's $t = 3.9568$, $df = 51.843$, $P = 0.00023$). *P. reticulatum* amide treatment did not influence whether or not bats had to be hand-fed, as

the model that included *P. reticulatum* amide treatment as a fixed effect was less supported than the model with no fixed effects ($\Delta\text{AICc} = 2.3$; Table 4).

The effect of GRT on germination

The influence of GRT on germination speed was not strongly supported (Table 5), but there were some species-specific effects of GRT on germination success (Table 6; Fig. 4). The variation in time to reach 5 % of P_{\max} was explained best by a model that included only *Piper* species as a fixed effect (ΔQAICc to null model = 20.3); however, a model containing *Piper* species and GRT was also moderately supported (ΔQAICc to best model = 2.4) as was a model containing *Piper* species, GRT and their interaction (ΔQAICc to best model = 8.9). Model averaging among the three best models indicated a strong effect of *Piper* species (relative importance = 1.00) and a weak effect of GRT ($\beta = 0.0006$, relative importance = 0.28, $P = 0.915$). Similarly, the variation in time to reach 50 % of P_{\max} was explained best by a model that included only *Piper* species as a fixed effect (ΔQAICc to null model = 20.4); however, a model containing *Piper* species and GRT was also moderately supported (ΔQAICc to best model = 2.3) as was a model containing *Piper* species, GRT and their interaction (ΔQAICc to best model = 9.9). Model averaging among the three best models indicated a strong effect of *Piper* species (relative importance = 1.0) and a weak effect of GRT ($\beta = -0.0583$, relative importance = 0.27, $P = 0.803$). Lastly, the time to reach 95 % of P_{\max} was explained best by a model that included only *Piper* species as a fixed effect (ΔQAICc to null model = 14.2). However, a model containing *Piper* species and GRT was also moderately supported (ΔQAICc to best model = 2.3). Model averaging among the two best models indicated a strong effect

Fig. 4a–e Germination success of seeds passed through *C. perspicillata* increases with gut-retention time for *Piper peltatum*. Points represent the mean germination success of each defecation and point size indicates the number of seeds/defecation (n). For *P. peltatum*, the solid line indicates predicted germination success, and the dashed lines indicate the 95 % confidence interval



of *Piper* species (relative importance = 1.0) and a weak effect of GRT ($\beta = -0.0014$, relative importance = 0.25, $P = 0.660$).

Model comparisons examining the effects of *Piper* species, GRT, and their interaction on germination success indicated a strong interaction between *Piper* species and GRT ($\Delta\text{AICc} = 30.4$ between full model and model without interaction term; Table 6), thus we analyzed the effects of GRT on germination success separately for each *Piper* species (Table 6; Fig. 4). For *P. colonense*, *P. reticulatum*, *P. sancti-felicitis* and *P. silvivagum* model comparisons indicated that the best model was the null model without any effect of GRT; yet models containing GRT were also reasonably well supported (*P. colonense*, $\Delta\text{AICc} = 0.5$, relative importance = 0.48, $\beta = -0.0055$, $P = 0.130$; *P. reticulatum*, $\Delta\text{AICc} = 2.4$, relative importance = 0.23, $\beta = -0.0012$, $P = 0.427$; *P. sancti-felicitis*, $\Delta\text{AICc} = 2.7$, relative importance = 0.21, $\beta = 0.0183$, $P = 0.9419$; *P. silvivagum*, $\Delta\text{AICc} = 1.3$, relative importance = 0.34, $\beta = -0.0013$, $P = 0.212$; Table 6).

For *P. peltatum*, model comparisons indicated that the best model included GRT, and comparisons to the null model indicated strong support for an effect of GRT on germination success ($\Delta\text{QAICc} = 14.7$). Parameter estimates from the model showed a weak positive effect of GRT on germination ($\beta = 0.0156$, relative importance = 1, $P = 0.0002$; Fig. 3b).

Discussion

One important mechanism through which plants may influence seed dispersal success and their own reproductive fitness is through fruit traits that mediate the GRT of seeds in frugivores. This study examines whether secondary compounds produced in fruits can mediate the GRT of seeds consumed by *C. perspicillata*, providing a rigorous test of the gut-retention hypothesis outlined by Cipollini and Levey (1997) in a mammalian frugivore. We detected interspecific differences in GRT of seeds among five species of *Piper* consumed by *C. perspicillata* (Fig. 1), and showed that certain amides produced in fruits can significantly reduce GRT (Figs. 2, 3). Moreover, GRT had species-specific effects on germination success, although they were generally weak (Fig. 4).

Mean GRT of seeds from the five *Piper* species was rapid, ranging from 19.4 to 29.4 min (Fig. 1). GRT of *P. reticulatum* was higher than for *P. colonense* and *P. silvivagum*, and GRT of *P. peltatum* was higher than for *P. silvivagum* (Fig. 1). It is unclear from our results why GRT varied among species, but many possible fruit traits, such as chemical differences (Murray et al. 1994) or seed/pulp ratio (Traveset 1998), could have influenced GRT. *Piper* is

exceptionally chemically diverse (Parmar et al. 1997; Kato and Furlan 2007), so different species may or may not contain amides, and may additionally contain a diversity of secondary metabolites in fruits, e.g., alkenylphenols, phenylpropanoids or terpenes (Chaves and Santos 2002; Rali et al. 2007; Morandim et al. 2010). Different secondary metabolites in the same class or across classes, originating from fruits of the same or different species, could act synergistically or antagonistically in the gut (Scott et al. 2002; Dyer et al. 2003; Richards et al. 2010; Whitehead and Bowers 2014), so GRT may vary considerably depending on the specific diet composition and foraging rhythm of individual bats. However, species-specific traits leading to consistent interspecific differences in GRT likely play an important role in determining patterns of seed rain and the distribution of *Piper* species across different habitats and microsites.

Certain *Piper* amides decreased GRT (Figs. 2, 3), similar to the effects of some fruit secondary compounds on avian frugivores (Murray et al. 1994; Wahaj et al. 1998; Izhaki 2002), but unlike the amide capsaicin, which has been shown to prolong GRT in some bird species (Tewksbury et al. 2008a). The amide-induced GRT decreases we observed are fairly substantial and occurred only for certain amides (from 22.0 to 13.7 min due to 10 mg pipartine/fruit; from 27.6 to 14.8 min due to three times *P. reticulatum* amides). However, fruit mass consistently reduced GRT as well. Fruit traits and the mass of ingested fruit likely interact, as amides have been shown to deter consumption by bats (Whitehead 2013; Online Resource 2) and could thus decrease the mass of ingested fruit. Thus, in natural settings, GRT is likely driven by the mass of ingested fruit in addition to fruit and disperser traits.

There are a number of important ways through which changes in GRT can influence seed dispersal success and plant fitness, either through changes in seed viability or changes in the spatial patterns of seed deposition. We tested the effects of GRT on seed viability and found that GRT can influence germination success, but under the germination conditions we used, the effects were species specific (Fig. 4). Seeds of *P. peltatum* had higher germination success at higher GRTs and seeds of *P. colonense*, *P. reticulatum*, *P. sancti-felicitis* and *P. silvivagum* appeared to germinate independently of GRT. This variation among species may be due to many potential differences in seed morphology that were not measured in this study, such as seed-coat thickness, hardness, shape, and surface structure. Another possibility is that GRT in *Carollia* has generally benign effects on *Piper* seed coats, but benefits certain species by cleaning seeds of associated fungi that inhibit germination. We did observe many fungal infections in germinating seeds, and one potential explanation for our results is that seeds with thin seed coats (such as those of *P. peltatum*, of

which seeds were very small; diameter <1 mm, personal observation) could benefit the most from longer gut-passage times that reduce fungal pathogen load. Yet, the potential for complex interactions involving plants, dispersers and pathogens (Tewksbury et al. 2008b) requires further study in *Piper*. It is also notable that different *Piper* species varied considerably in their germination success over the course of the study (Fig. 4), and any potential effects of GRT may have been harder to detect for species such as *P. reticulatum* and *P. colonense* that had relatively low germination success overall.

In addition to their potential effects on seed viability, changes in GRT may alter plant fitness through their effects on seed deposition patterns. Because *Carollia* forages on different species of *Piper* over the course of a night, its defecations often contain seeds of multiple species of *Piper*, and interspecific competition among *Piper* seedlings can be strong (Loiselle 1990). If a decrease in GRT could change the species composition inside defecations (resulting in more homogeneous loads), *Piper* may benefit from shorter GRTs. *Piper* could also benefit from short GRTs if short GRTs cause seeds to be defecated nearby in the same habitat as the parent plant. Many *Piper* species have strict habitat requirements (e.g., early, mid or late successional sites) and require specific germination niches (Daws et al. 2002). However, because bat foraging patterns are complex and variable, understanding how GRT affects seed deposition patterns and plant fitness is difficult. For example, seeds from some *Piper* species are less likely to germinate under feeding roosts than when they are dropped into open fields (Galindo-Gonzalez et al. 2000), so depending on bat feeding rhythms (i.e., how long bats spend at feeding roosts after fruits are consumed) shorter GRTs could either increase or decrease the probability that seeds are defecated in flight and land in open areas. Although GRT is integral to modeling seed shadows, how GRT influences dispersal kernels in bats has rarely been investigated (Tsoar et al. 2011). Further work integrating dynamic GRT and forager behavior to produce spatially explicit seed shadows is required to understand whether amide-induced GRT decreases could potentially increase plant fitness by altering seed deposition.

Our results provide strong evidence that fruit chemical traits can influence GRT, which for some plant species can influence seed germination. However, it is unclear whether fruit amides are an adaptive trait in the sense that they evolved specifically to mediate GRT. Fruit secondary compounds are diverse, and can include compounds unique to fruit as well as compounds found throughout the plant (Levey et al. 2007; Whitehead and Bowers 2013). These compounds likely assume a variety of functions in fruits (Cipollini and Levey 1997), and in particular may play a key role in the defense of fruits against insects and

fungal pathogens (Dyer et al. 2003, 2004b; Tewksbury et al. 2008b; Richards et al. 2010; Whitehead and Bowers 2014). However, even compounds that evolved primarily as a defense may be maintained in plants in part due to their multifunctionality (Izhaki 2002), and their occurrence in fruits can be best understood in a whole-plant context that takes into account the variety of interactions in which plants are simultaneously involved (Whitehead and Bowers 2013). Thus, whether or not *Piper* amides evolved specifically to mediate GRT or other aspects of interactions with seed dispersers, our results show that they can influence disperser physiology and suggest a mechanism through which fruit chemical traits may influence *Piper* seed shadows and species distributions.

In addition to their role in seed dispersal and reproductive fitness of *Piper*, amides may also have important consequences for the physiology and ecology of *Carollia* bats. Amides have been shown to have overall negative effects on the foraging and feeding preferences of *Carollia* bats (Whitehead 2013; Online Resource 2), potentially leading to a tradeoff between the costs of reduced frugivore preferences and the benefits of defense or any increase in seed dispersal success accrued by lower gut-retention times. Although frugivorous bats have rapid metabolisms (Amitai et al. 2010), *Carollia*'s ability to assimilate nutrients may be compromised by amide-mediated GRT decreases. If *Carollia* bats have low net energy intake while foraging on amide-rich fruits with low GRT, they may compensate by increasing their foraging effort to meet their energy budgets, or alternatively choosing low-amide high-GRT fruits when possible. Amides may even be toxic to bats, as terrestrial mammalian frugivores are deterred by amides produced by chilies (Tewksbury and Nabhan 2001), and some *Piper* amides are known to be hepatotoxic (Nerurkar et al. 2004) or cytotoxic (Liu et al. 2010; Mishra et al. 2011). Additional evidence of the potential toxicity of amides to *Carollia* comes from two observations of individual bats regurgitating piplartine- and *P. reticulatum* amide-supplemented fruits during our experiments, and a previous study suggesting that *Carollia* engages in geophagy in order to detoxify harmful plant secondary metabolites (Voigt et al. 2008). Further research on how *Piper* amides are processed or detoxified by *Carollia* through physiological or behavioral mechanisms may be fruitful.

This study provides strong evidence that amides, which have known roles in the defense of both leaves and fruits, can also influence GRT and seed germination. This adds a new component to the growing body of literature that secondary compounds can have multiple ecological functions, especially in fruits, where they can function in both antagonistic and mutualistic interactions. The *Piper/Carollia* system provides an excellent model for future work on how plant chemical traits influence multiple aspects of

the dispersal process, as well as pre- and post-dispersal processes.

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