

# Chemical ecology of fruit defence: synergistic and antagonistic interactions among amides from *Piper*

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## Summary

1. Although ripe, fleshy fruits function primarily to attract seed dispersers, they must also be defended against diverse communities of seed predators and pathogens. For some plants, the concentration and diversity of secondary metabolites in fruits can exceed that of leaves and other plant parts, but little is known about the functional significance of the suites of compounds found in fruits. Fruit secondary metabolites may function in defence, or they may play a variety of other roles in seed development and dispersal.

2. In this study, we conducted a series of experiments to test the effects of amides, a highly diverse class of secondary metabolites found in *Piper* fruits, on a variety of antagonistic fruit pests, including an insect seed predator (*Sibaria englemanni*, Pentatomidae) and three unidentified species of fungi isolated from ripe *Piper reticulatum* (Piperaceae) fruits. We tested the effects of amide-rich extracts from unripe and ripe fruits of *P. reticulatum* and the effects of two pure compounds, piperine and pipartine, presented alone and in combination.

3. Amide-rich extracts from unripe and ripe fruits had no effect on insect feeding preferences, but strong negative effects on fungal growth rates. A comparison of the relative bioactivity of unripe and ripe fruit extracts, controlling for concentration, showed that the specific composition of compounds in unripe fruits provides a more effective defence than that of ripe fruits against two of the three fungal species tested.

4. Pure amides had variable effects on insect feeding preferences and strong negative effects on fungal growth rates. Tests of the bioactivity of two pure amides, presented alone and in combination, showed that the same two compounds can interact either synergistically or antagonistically in mixtures depending on the particular consumer involved.

5. Together, these results suggest that the secondary metabolites in fruits may be a key characteristic contributing to fruit defence and plant reproductive success. Specifically, our results emphasize: (i) the potential for slight changes in the composition of mixtures to alter the efficacy of defence; and (ii) the potential for complex interactions among compounds in mixtures that can alter the bioactivity of secondary metabolites differentially among different consumers.

**Key-words:** chemical diversity, fruit secondary metabolites, *Piper reticulatum*, Plant–herbivore interactions, Plant–microbe interactions, seed predation, *Sibaria englemanni*, synergy

## Introduction

Plants produce an enormous diversity of secondary metabolites that are thought to function primarily as a defence against herbivores and pathogens (Rosenthal & Berenbaum 1991; Bennett & Wallsgrove 1994). Decades of research have shown that the variation in chemical defence traits within and between individuals can have important

and complex consequences for structuring ecological communities (reviewed in Coley & Barone 2001; Iason, Dicke & Hartley 2012). However, most of our understanding of the ecology and evolution of plant defence has come from studies that examine the role of one or a few major compounds in leaves. We still know very little about the diversity and functional significance of secondary metabolites produced in other plant parts (Adler 2000; Harborne 2001; Tewksbury 2002). In particular, relatively few studies have examined the role of secondary metabolites in fleshy fruits

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(Tewksbury 2002; Levey *et al.* 2007). Because fleshy fruits function primarily to attract animal consumers, it is often assumed that secondary metabolites in these tissues are ecologically costly – their presence best explained as a pleiotropic consequence of the defence of leaves and other plant parts (Ehrlen & Eriksson 1993; Eriksson & Ehrlen 1998; Whitehead & Poveda 2011). Yet, plant defence theory provides several reasons to predict that there should be strong independent selection for the defence of fleshy fruits: (i) fruits have high fitness value due to their direct link to developing seeds; (ii) both fruit pulp and seeds are at high risk of attack due to their high nutritional content; and (iii) both fruit pulp and seeds are often exposed to enemies over a long period of time during development and post-ripening persistence (McKey 1974, 1979; Rhoades & Cates 1976; Zangerl & Rutledge 1996).

Many fleshy fruits do contain deterrent or toxic secondary metabolites at levels comparable to or exceeding those in vegetative plant parts (Herrera 1982; Cipollini & Levey 1997b; Whitehead & Bowers 2013a), and these compounds may be found in unripe and ripe fruit pulp as well as in seeds (Barnea, Harborne & Pannell 1993; Beckman 2013; Whitehead *et al.* 2013). In some cases, concentrations in pulp may exceed what is found in seeds (Barnea, Harborne & Pannell 1993; Beckman 2013), and in other cases, the opposite may be true (Ehrlen & Eriksson 1993; Whitehead *et al.* 2013). Although these metabolites may influence seed dispersal in a variety of ways, including various effects on mutualistic seed dispersers (Cipollini & Levey 1997b), increasing evidence has suggested that their primary role is in the defence of fruits and seeds against antagonists, such as insect seed predators and fungal pathogens (Cipollini & Levey 1997a; Cazetta, Schaefer & Galetti 2008; Schaefer, Rentzsch & Breuer 2008; Tewksbury *et al.* 2008). The chemical defence of fleshy fruits may lead to fitness trade-offs (the defence trade-off hypothesis; Cipollini & Levey 1997b), where fruits that are the most defended are also the least preferred by mutualist seed dispersers (Cipollini & Levey 1997b; Schaefer, Schmidt & Winkler 2003; Cipollini *et al.* 2004). Alternatively, plants may minimize the negative effects of secondary metabolites on seed dispersal through a variety of mechanisms, such as the compartmentalization of fruit secondary metabolites into seeds (Ehrlen & Eriksson 1993; Whitehead *et al.* 2013), which are not intended as part of the reward for mutualists, or the production of metabolites that are bioactive against specific pests, but not mutualist seed dispersers (the microbe-pest specificity hypothesis, *sensu* Cipollini & Levey 1997b; Struempf, Schondube & Del Rio 1999; Tewksbury *et al.* 2008). Another important way that plants may minimize any negative effects of secondary metabolites on seed dispersal is through changes in fruit chemistry with ripening. These changes may include a reduction in the overall concentration of metabolites or an alteration in the chemical composition that specifically reduces deterrent effects on mutualists. Changes in fruit chemistry with ripening are common (e.g. Pearce, Ryan & Liljegren 1988; Tsahar,

Friedman & Izhaki 2002; Whitehead & Bowers 2013b), but little is known about how these changes affect seed dispersal or fruit defence. In addition, because most past work on fruit secondary metabolites has focused on testing the effects of a single compound on a particular consumer (reviewed in Levey *et al.* 2007), we still know very little about the chemical diversity of fruits, how the composition and relative concentrations of compounds change with ripening and the potential for specificity in the bioactivity of fruit metabolites in interactions with seed dispersers and/or different classes of fruit pests.

In the few studies that have provided quantitative comparisons of secondary metabolites in wild fleshy fruits and leaves, evidence has shown that fruit pulp and seeds can be highly diverse chemically and contain a variety of compounds that never occur in leaves (Kliebenstein, Gershenzon & Mitchell-Olds 2001; Torres *et al.* 2002; Whitehead & Bowers 2013a; Whitehead *et al.* 2013). This chemical diversity may play a key role in fruit defence (Berenbaum & Zangerl 1996; Castellanos & Espinosa-García 1997). Although it is difficult to disentangle the effects of individual compounds when they occur in mixtures, correlative evidence has suggested that different individual compounds from fruits may be effective against different classes of consumers (e.g. insects vs. microbes) and that in some cases the most bioactive compounds in mixtures are minor components in terms of concentration (Whitehead & Bowers 2013a). Furthermore, when complex suites of compounds occur together, there is strong potential for synergistic or antagonistic interactions between individual compounds (Berenbaum 1985; Nelson & Kursar 1999). Increasing evidence has shown that synergy between defensive metabolites is a common and widespread occurrence and may play a key role in determining the outcome of species interactions (Berenbaum 1985; Berenbaum & Neal 1985; Scott *et al.* 2002; Dyer *et al.* 2003; Richards *et al.* 2010, 2012). However, despite the high diversity of secondary metabolites in fruits, we know of only a few studies that have tested for synergistic effects of fruit secondary metabolites in mutualistic and antagonistic fruit–frugivore interactions (Cipollini & Stiles 1992; Cipollini & Levey 1997a,c).

One group of plants that produces fruits with high concentrations and diverse mixtures of secondary metabolites is the tropical genus *Piper* (Morikawa *et al.* 2004; Siddiqui *et al.* 2005; Whitehead *et al.* 2013). In particular, many *Piper* species are rich in amides, a group of secondary metabolites that play an important ecological role in the defence of leaves against herbivores (Dyer *et al.* 2001, 2004; Fincher *et al.* 2008; Richards *et al.* 2010), but have not been examined in the context of fruit–frugivore interactions. In laboratory studies, amides have a broad range of bioactivity against insects, fungi and molluscs (Bernard *et al.* 1995; da Silva *et al.* 2002; Yang *et al.* 2002; Navickiene *et al.* 2003; Siddiqui *et al.* 2005; Marques *et al.* 2010; Morandim *et al.* 2010), and thus may provide defence against a variety of fruit antagonists. In a recent study describing the amides in different plant parts of *Piper*

*reticulatum* (Fig. 1a), a common and widespread Neotropical species, amide diversity was higher in fruit pulp and seeds than in vegetative plant parts and fruit chemical diversity, but not concentration, was negatively correlated with levels of seed damage in natural populations (Whitehead *et al.* 2013). Together, these results suggest that amide diversity *per se* may be one of the most important aspects of the chemical defence of fruits in this species. Several past studies have shown that amides can function synergistically in leaf defence (Scott *et al.* 2002; Dyer *et al.* 2003; Richards *et al.* 2010), and, considering the complex mixtures of up to 25 individual amides detected in *P. reticulatum* fruits (Whitehead *et al.* 2013), the potential for interactions among compounds is particularly high. Furthermore, because the fruits of *P. reticulatum* are attacked by a number of antagonistic frugivores and pathogens, complex suites of secondary metabolites could provide simultaneous defence against different consumers. These factors emphasize the need to conduct controlled experiments with multiple species that interact with fruits and to consider the combined effects of suites of defensive compounds.

In this study, we conducted a series of experiments that addressed the role of amides in fruit defence of *P. reticulatum* and other *Piper* species. Because amides occur in complex mixtures, we focused on the bioactivity of fruit extracts that contain suites of compounds. We examined the effects of fruit extracts on two important classes of fruit antagonists, insects and fungi, and for both groups, we compared the bioactivity of extracts from unripe and ripe fruits. In addition, to provide a more general test of the bioactivity of amides from the genus *Piper*, we conducted identical experiments with two pure amides that are common to many *Piper* species, piperine and piplartine. Although these two compounds do not occur in *P. reticulatum*, they are among the most commonly detected amides in the genus (Parmar *et al.* 1997) and are available

commercially in pure form. The effects of the pure compounds were tested alone and in combination, which also allowed us to explicitly test for interactive effects (synergy or antagonism) between the two compounds that may alter the effectiveness of fruit defence. Specifically, we addressed the following four questions:

Q1: Do amide-rich extracts from unripe and ripe *P. reticulatum* fruits exhibit bioactivity against *Sibaria englemanni* (Pentatomidae; Fig. 1b), a common insect seed predator on *Piper* species, and/or against three species of naturally occurring fruit-associated fungi?

Q2: If so, how do the changes in amide profile that occur with fruit ripening influence the bioactivity against these different consumers?

Q3: Are these same consumers also deterred by two individual amides common to many other *Piper* species, piperine and piplartine?

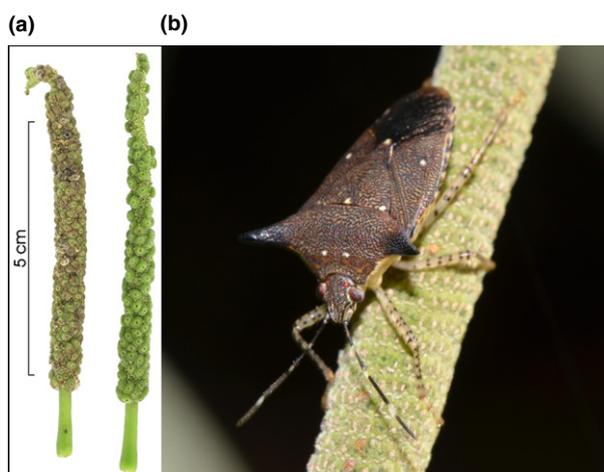
Q4: Can piperine and piplartine function synergistically to increase bioactivity and therefore the effectiveness of defence against these consumers?

## Materials and methods

### STUDY SITE AND SYSTEM

All sample collections and field experiments were conducted at La Selva Biological Station, Heredia Province, Costa Rica. La Selva is managed by the Organization for Tropical Studies and includes 1600 hectares of protected area that consists of primary premontane and tropical wet forest (*sensu* Holdridge 1967), as well as secondary forest and abandoned agricultural areas (McDade *et al.* 1994). The site is a high centre of diversity for *Piper*, with 50+ species co-occurring (Gentry 1990; OTS 2012).

The genus *Piper* is one of the most species-rich and dominant members of Neotropical forests and includes small trees, shrubs and vines (Gentry 1990; Dyer & Palmer 2004). Fruits are borne on distinct spike-shaped infructescences that can contain anywhere from 100 to 3000 tiny individual fruits, each with a single seed (Fleming 1985; Greig 1993). A small group of bats (*Carollia* spp.) in the family Phyllostomidae are the primary dispersers of *Piper* seeds in the Neotropics, although some species are also consumed by birds (Palmeirim, Gorchov & Stoleson 1989; Fleming 2004; Thies & Kalko 2004). Immature fruits are also attacked heavily by insects; in a comparative study of six *Piper* species, up to 87% of seeds were lost to insect seed predators (Greig 1993). A single hemipteran species (*S. englemanni*) is by far the most abundant piercing-sucking insect feeding on *Piper* fruits at the site (Greig 1993; S. R. Whitehead, pers. observ.). Past studies have classified this species as an insect seed predator (Greig 1993), and the common occurrence of damaged *Piper* seeds that essentially appear to be hollow seed coats (S. R. Whitehead, pers. observ.) provides some additional indirect evidence that *S. englemanni* does feed on seeds. However, many pentatomids feed on fruit pulp, seeds or both, and the feeding strategy of *S. englemanni* is not definitively known. Their impact on seed viability may be due to the direct effects of seed predation or the indirect effects of damage to the fruit surface that leads to increased risk of pathogen attack (Tewksbury *et al.* 2008). In *Piper*, pathogen risk appears to increase sharply upon ripening, as evidenced by the long maturation time of fruits (*c.* 1 month) and relatively short period of time (*c.* 24 h) that ripe fruits persist before they begin to rot (Thies & Kalko 2004; S. R. Whitehead, pers. observ.).



**Fig. 1.** Mature and rotting infructescences of *Piper reticulatum* (a) and *Sibaria englemanni* feeding on an immature infructescence of *Piper sancti-felicitis* (b). Photo credits: Steven Paton, Smithsonian Tropical Research Institute (a) and Susan Whitehead (b).

*Piper reticulatum* is a large understorey shrub 3–7 m in height that occurs throughout Central and South America as far south as Bolivia (Tropicos 2012). It is one of the most common species of *Piper* found in secondary forest and along trails at La Selva. Unripe fruits are heavily attacked by *S. englemanni* and a variety of other insects and fungal pathogens (S. R. Whitehead, pers. observ.). Mature trees can produce hundreds of infructescences that ripen sequentially, with anywhere from 1 to 20 infructescences maturing per day over a period of several weeks. Bats remove entire infructescences in flight, usually on the first evening that they are ripe. Those that are not removed on the first night start to appear mushy and rotten-smelling on the following day and usually fall to the ground within 24 h (S. R. Whitehead, pers. observ.).

*Piper* species are rich in a broad range of amides, phenylpropenoids, lignans, terpenes, benzoic acids, chromenes, alkenylphenols and steroids (Parmar *et al.* 1997; Dyer, Richards & Dodson 2004; Kato & Furlan 2007). Amides in particular are abundant in this genus and have known ecological and economic importance (e.g. the amide piperine is responsible for the spiciness of black pepper-corns, which are the dried fruits of *Piper nigrum*; Parmar *et al.* 1997). They are especially diverse in *P. reticulatum*, where we detected a combined total of 40 individual amides across different plant parts (Whitehead *et al.* 2013). The composition of amides in unripe fruit pulp, ripe fruit pulp and seeds of *P. reticulatum* is similar and includes a number of major and minor components that never occur in vegetative plant parts (Whitehead *et al.* 2013). Total concentrations of amides are highest in seeds, intermediate in unripe fruit pulp and lowest in ripe fruit pulp (Whitehead *et al.* 2013).

#### EXTRACTIONS OF AMIDES FROM *P. RETICULATUM* FRUITS

Large numbers of fruits were collected in bulk from 10 to 15 individuals of *P. reticulatum* growing along trails and in open areas surrounding the field station. Fruits were separated into ripe and unripe on the basis of softness and swelling, and we used only ripe fruits that had ripened on the day of collection (day-old ripe fruits are easily distinguished as they quickly become mushy). Due to the limited numbers of fruits that ripened per day, collections were made repeatedly from the same individuals over a several week period. All fruits were brought immediately back to the La Selva laboratory, where they were dried at 50 °C for 48 h, and entire fruits (including pulp and seeds) were ground to a fine powder in a coffee grinder. Pulp and seeds were not separated for the extracts for several reasons: (i) the composition of amides in pulp and seeds is similar; (ii) both pulp and seed chemistry can be considered ecologically relevant for insect seed predators and fruit-associated fungi; and (iii) the separation of pulp and seeds for large-scale extractions of amides was impractical. To prepare large-scale extracts for bioassays, we used a scaled-up version of extraction and quantification procedures as described in Dyer, Richards & Dodson (2004). For each fruit type (unripe or ripe), 52.5 g of dry material was placed in a 1-L Erlenmeyer flask with 500 mL of ethanol and left on a stir-plate for overnight extraction. The ethanol was then decanted through a Buchner funnel with No. 2 Whatman filter paper, and another 500 mL of ethanol was added to the plant material for a second overnight extraction. This process was repeated again for a third overnight extraction. The three filtered extracts were combined, evaporated to dryness and then resuspended in 250 mL 3 : 1 water : ethanol. This solution was transferred to a separatory funnel and partitioned three times against equal volumes of chloroform. The water fraction was discarded, and the combined chloroform fractions (containing the amides) were evaporated to dryness. The resulting extracts were resuspended in 52.5 mL ethanol and partitioned among seven scintillation vials for use in the bioassays described below. This

extraction procedure results in an extract that contains c. 78–84% amides (see below for quantification methods). Small aliquots of 500 µL from each extract were evaporated to dryness and transported to the University of Colorado for analysis using gas chromatography combined with mass spectrometry (GC-MS).

#### IDENTIFICATION AND QUANTIFICATION OF AMIDES IN FRUIT EXTRACTS USING GC-MS

Methods for GC-MS analysis were modified from previously described methods (Dyer, Richards & Dodson 2004) and are described in detail in Whitehead *et al.* (2013). Briefly, the extract aliquots were dissolved in dichloromethane, and piperine (an amide which does not occur in *P. reticulatum*) was added as an internal standard at a concentration of 0.75 mg mL<sup>-1</sup>. Samples were then injected onto an HP Agilent 6890N GC coupled with an Agilent 5975C MS and equipped with a DB-5MS capillary column (30 m × 0.25 mm i.d., 0.5-µm film thickness; Agilent Technologies, Santa Clara, CA, USA). Data were recorded and processed using MSD ChemStation software, version D.02.00.275 (Agilent Technologies). We estimated the quantities of individual and total amides in the extracts based on the known concentration of the internal standard (piperine). Compounds were identified based on matches of retention times and mass spectral data in a user-created library for amides in *P. reticulatum*. Full structural elucidation of major amide components in this species was carried out in a previous study (Whitehead *et al.* 2013).

#### Q1 AND Q2: EFFECTS OF *P. RETICULATUM* EXTRACTS ON INSECTS AND FUNGI

To determine whether *P. reticulatum* extracts can affect feeding behaviour of *S. englemanni*, we conducted a series of paired choice experiments in 2011–2012 that tested the effects of *P. reticulatum* fruit extracts on insect preference. The extracts were added to unripe fruits of *Piper sancti-felicis*, another commonly occurring species of *Piper* at the field site. Unripe *P. sancti-felicis* fruits are commonly consumed by *S. englemanni* (S. R. Whitehead, pers. observ.), but contain no detectable amides at a detection limit of c. 0.01% dry weight (S. R. Whitehead, pers. observ.). Although *P. sancti-felicis* may contain a variety of other secondary metabolites that could potentially affect the feeding preferences of *S. englemanni*, we found that *S. englemanni* would not consistently accept an artificial diet in preliminary trials and using natural fruits ensured that the insects would exhibit typical feeding behaviours. We minimized any potential variation among *P. sancti-felicis* fruits that could confound our comparisons between amide-treated and control fruits by pairing two halves of the same *P. sancti-felicis* infructescence as the treatment and control fruit in each choice trial. To supplement fruits with amides, we prepared serial dilutions of the *P. reticulatum* extracts dissolved in ethanol, where each successive dilution was 90% strength of the preceding solution. The unripe fruit extract had a starting total amide concentration of 17.9 mg mL<sup>-1</sup> and the ripe fruit extract had a starting concentration of 14.75 mg mL<sup>-1</sup> (see Results), and we prepared 50 dilutions of each, thus the dilutions ranged from 0.10 to 17.9 mg mL<sup>-1</sup> for unripe extracts and 0.085 to 14.75 mg mL<sup>-1</sup> for ripe extracts. Infructescences of *P. sancti-felicis* were cut in half, and the halves were randomly assigned to treatment and control groups. The treatment halves were supplemented with a single concentration of extract by pipetting aliquots of 200 µL of solution into a 150-mm glass petri dish, and rolling the infructescences in the dish until all of the solution was absorbed by the fruit, and the surface was evenly coated. Control infructescences were treated in the same manner with ethanol only, and both halves were then left for several hours to allow evaporation of the solvent. The

paired treatment and control halves from a single infructescence were then placed on opposite sides of a clean 100-mm petri dish. The wide range of concentrations of amides used in this and all subsequent experiments was chosen to extend well above and below the natural concentrations encountered in fruits. The amount of amides added to infructescences receiving the highest concentration treatment for unripe extracts (*c.* 3.58 mg added to a half-infructescence of *c.* 0.28 g dry weight) would have yielded a concentration roughly equivalent to the average concentration in *P. reticulatum* unripe fruit pulp (1.22% dry weight; Whitehead *et al.* 2013) if the compounds had diffused evenly throughout the pulp and seeds of the infructescence. However, because the compounds were applied only to the surface, the highest concentration treatment is likely well above the range typically experienced by insects feeding on natural fruits.

All *S. englemanni* individuals used in the study were collected opportunistically from *P. sancti-felicis* plants growing near the field station, and included a mix of adults and juveniles. Voucher specimens of the species were deposited at the University of Colorado Natural History Museum, and their identity was confirmed by Donald B. Thomas (USDA Research Entomologist). Insects were held in vials for 8–16 h after collection and prior to beginning the feeding trials. To begin the experiment, a single individual was placed in the centre of a petri dish between the treatment and control fruits, and the dish was monitored every 10 min for a period of 2 h, and every hour thereafter for a total of 24 h. Insects that did not feed after 24 h were scored as 'no-choice' and excluded from the data analysis. A choice was recorded as soon as an insect was observed feeding, that is, with its stylet fully inserted into the fruit. Insects were often observed walking on fruits and probing fruits with their mouthparts prior to initiating feeding; however, we did not record these behaviours as a choice. We conducted two sets of trials (each set involving one trial at each of the 50 concentrations) for each extract treatment (ripe or unripe), using a naïve individual of *S. englemanni* and a fresh *P. sancti-felicis* infructescence for each trial. Because some of the insects did not feed within the 24-h period and had to be excluded from analysis, sample sizes varied among treatments (see Results).

To investigate the effects of amides on fruit-associated fungi, we conducted a series of bioassays using three strains of fungi isolated from field-collected ripe *P. reticulatum* fruits. Infructescences were collected from 10 individual plants, surface-sterilized using a 3% bleach solution, cut in half with a sterile blade and placed pulp side down on an agar growth medium. This procedure was conducted in sterile conditions at the La Selva laboratory using a UV-sterilized laminar flow hood. The agar medium was prepared to mimic the nutrient composition of *Piper* fruit (Kelm *et al.* 2008), following methods in Cipollini & Stiles (1993). In 250 mL deionized water, we added 5.0 g agar, 1.25 g soy protein powder, 1.91 g fructose, 1.73 g glucose, 0.38 g oil (1 : 1 corn oil : peanut oil), 0.88 g cellulose and 0.29 g pectin. We sorted the resulting fungal cultures by morphology and chose three commonly occurring morphotypes for use in bioassays (denoted as F1, F2 and F3), all of which had distinct, radial hyphal growth for ease of comparative measurement.

For each fungal strain, we tested the effects of unripe and ripe fruit extracts by adding extracts to the agar growth medium at 50 different concentrations using the same serial dilutions described above for the insect bioassays. Solutions were added to the fruit-mimic agar in 60 mm petri plates by pipetting 200- $\mu$ L aliquots over the surface of the agar, spreading evenly using a sterile glass rod and allowing the plate to stand open under a sterile laminar flow hood for 1 h to allow evaporation of the ethanol. Control plates were also prepared for each fungal species using ethanol only. Each plate contained *c.* 0.46 g dry material; thus, the amount added to the plate in the highest concentration treatment would have yielded a concentration in the agar *c.* 64% of that typical of *P. reticulatum* unripe fruit pulp if the compounds had

diffused evenly throughout the agar. However, the extracts visibly remained on the surface of the agar once the solvent evaporated, and diffusion into the lower part of the plate was likely negligible. Thus, the highest concentration treatment is likely well above the range typically experienced by fungi growing on natural fruits. Supplemented plates were inoculated with fungi by placing small (1 cm  $\times$  1 cm) agar plugs from the pure cultures in the centre of the plate. All plates were then placed in an incubator at 28 °C for a period of 24–36 h depending on the fungal strain. For each fungal strain, the radial growth of hyphae was measured in three locations on the plate using calipers, roughly chosen to represent the longest, shortest and average distance of hyphal growth. These three measurements were averaged to obtain one measurement of hyphal growth for each treatment at each concentration.

### Q3 AND Q4: EFFECTS OF PIPERINE AND PIPLARTINE ON INSECTS AND FUNGI

We tested the effects of two commercially available pure amides, piperine (purchased from Sigma-Aldrich, St. Louis, MO, USA) and piplartine (purchased from Indofine Chemical Company, Hillsborough, NJ, USA) on *S. englemanni* and the same three fungal species isolated for the experiments with *P. reticulatum* extracts. Although piperine and piplartine do not occur in *P. reticulatum* (Whitehead *et al.* 2013), both have been isolated from fruits and leaves of a number of *Piper* species (Parmar *et al.* 1997; Dyer, Richards & Dodson 2004) and often occur together in the same plant (Parmar *et al.* 1997). We prepared serial dilutions of 50 concentrations for each compound in the same manner as above, but with a starting concentration of 10 mg mL<sup>-1</sup> and ranging to a low concentration of 0.01 mg mL<sup>-1</sup>. To test for potential synergy or antagonism between the two compounds, we also prepared a serial dilution from a combination solution that contained the two compounds in a 1 : 1 ratio (5 mg mL<sup>-1</sup> piperine and 5 mg mL<sup>-1</sup> piplartine). Using these solutions in place of the extracts, we conducted bioassays with *S. englemanni* and the three species of fungi in the same manner as above.

### STATISTICAL ANALYSES

To test whether amide extracts and/or pure compounds are bioactive against insects and/or fungi (Q1 and Q3), we fit dose–response curves to the data from each bioassay experiment using base functions and the package 'drc' in the R environment for statistical computing (R Development Core Team 2012; Ritz & Streibig 2012). For insect bioassays, we used a two-parameter logistic regression model with insect choice (0 = control, 1 = treatment) as the response variable and the amount of amides added to the treatment fruit as the predictor variable. This was carried out with the 'glm' function in the R base package, using the binomial distribution and the logit link function. Amide amounts were base-10 log-transformed prior to analysis. We used the 'logi.hist.plot' function in the package 'popbio' to visualize these data (Stubben, Milligan & Nantel 2012; Fig. 4). For fungal bioassays, we used a three parameter log-logistic model of the form:

$$f(x) = 0 + \frac{d - 0}{1 + \exp(b(\log(x) - \log(e)))} \quad \text{eqn 1}$$

which describes a sigmoidal curve where the lower limit of the curve is fixed at zero,  $d$  = the upper limit of the curve,  $e$  = the inflection point of the curve and  $b$  = the relative slope around  $e$ . This was carried out using the 'drm' function in the package 'drc' (Tallarida 2000; Ritz & Streibig 2012). Because we conducted the bioassays simultaneously and in identical laboratory conditions for the fruit extracts and then for the pure compounds for each fungal species, we fit the dose–response curves simultaneously for

fruit extracts and then for pure compounds and specified a common parameter estimate for the upper limit of the curve [i.e. the estimated maximum growth of a particular fungal species on control plates (amide dose = 0) was the same across amide treatments]. For both the insect and fungal assay data, we assessed the goodness-of-fit of the regression models using Hosmer–Lemeshow tests and ensured that observed values were not significantly different from predicted ( $P > 0.05$ ) (Tallarida 2000). We then examined the regression coefficients ( $\beta$ ) and associated  $P$ -values, and slopes that differed significantly ( $P < 0.05$ ) from zero were taken as evidence for an effect of amide treatment.

To test for differences in the effectiveness of unripe fruit extracts vs. ripe fruit extracts in fruit defence (Q2), we employed a common metric that is used to compare the efficacy of two drugs in pharmacology studies: the potency ratio  $R$  (Tallarida 2000). Although we have found no examples of this method being implemented in chemical ecology research, it provides a number of advantages over ANOVA or other methods based on linear models because it does not depend on the distribution of data (binomial, normal, Poisson), makes no assumptions about the shape of the compounds' dose–response curves (which are rarely linear) and yields a value for  $R$  that depends only on the relative efficacies of the compounds being tested, independent of the effects of concentration (Tallarida 2000). To calculate  $R$ , we first used the regression models described above to determine the effective dose (ED) of the extracts necessary to reduce insect preference or fungal growth by 50% from the level expected based on no effect, commonly referred to as ED50 values in toxicology and pharmacology studies. For fungi, the ED50 value was estimated directly as a model parameter,  $e$ , which is the inflection point about which the curve is symmetric and the point where fungal growth is reduced by half relative to controls. For the insect bioassays, the baseline expectation based on no effect was that insects would choose control fruits 50% of the time and treatment fruits 50% of the time, thus the ED50 value represented the dose where insects chose treatment fruits 25% of the time. This value was calculated using inverse prediction of the dependent variable using the model generated by the logistic regression. Once the ED50 values were determined, we then calculated the potency ratio as  $R = (A/B)$ , where  $A$  and  $B$  represent the ED50 values for extracts A and B. Values of  $R > 1$  indicate that A is more potent (e.g. has a stronger negative effect on fungal growth), values of  $R < 1$  indicate that B is more potent. Methods for calculating the standard error and confidence intervals for this metric are described in detail in Tallarida (2000). Because we used the estimated total quantities of amides in the unripe and ripe fruit extracts to describe the dose–response curves for each, a value of  $R$  significantly different from one (i.e. with 95% confidence intervals that do not cross one) indicates that there are differences in the effectiveness of unripe and ripe fruit extracts that are not explained by differences in the total concentration of amides, but rather are due to changes in the composition or relative concentrations of compounds with ripening.

To test for the presence of synergistic or antagonistic interactions between piperine and piplartine (Q4), we used another metric that is common in pharmacology and toxicology research: the interaction index  $\gamma$  (Tallarida 2000, 2002). This method is also rarely implemented in chemical ecology studies (but see Richards *et al.* 2012), despite the fact that its potential utility for providing rigorous tests of interactions among plant defence compounds has been discussed in detail (Nelson & Kursar 1999). The interaction index is calculated as  $\gamma = Z/(p_A A + p_B B)$ , where  $Z$  is the observed ED50 value (or any other specified level of response) for the mixture, and the denominator ( $p_A A + p_B B$ ) represents the expected ED50 based on an additive relationship between two compounds.  $A$  and  $B$  represent the ED50 values for compounds A and B when tested individually, and  $p_A$  and  $p_B$  represent the proportions of the two compounds in the mixture (in our case  $p_A = 0.5$  and  $p_B = 0.5$ ). Values of  $\gamma < 1$  indicate synergy and values of  $\gamma > 1$

indicate antagonism between the two compounds. Methods for calculating the standard error and confidence limits for this metric are described in detail in Tallarida (2000).

## Results

### AMIDES IN *P. RETICULATUM* EXTRACTS

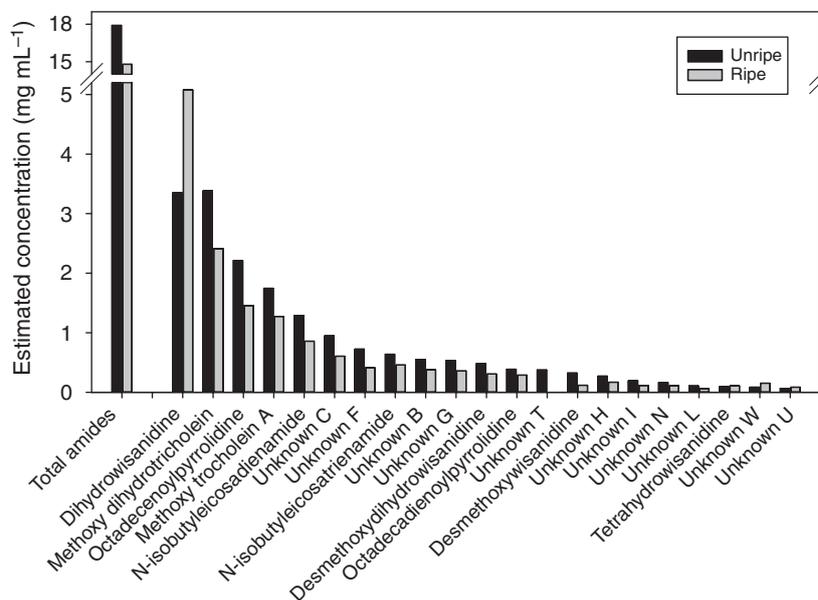
Extracts of both unripe and ripe fruits of *P. reticulatum* contained diverse mixtures of amides, the most abundant of which were dihydrowisanidine and methoxy dihydrotricholein (Fig. 2). These and eight other compounds were identified based on MS and NMR data in a previous study (Whitehead *et al.* 2013). An additional 11 compounds were also detected in extracts and classified as amides based on characteristic fragmentation patterns in MS data as described in Whitehead *et al.* (2013), but were unidentified (Fig. 2). Total quantities of amides were estimated as 1.79% dry weight for unripe fruits and 1.48% dry weight for ripe fruits. For individual compounds, most amides had higher estimated concentrations in unripe fruits than in ripe, with the exception of dihydrowisanidine and amide AA (Fig. 2), which had higher concentrations in ripe fruits than unripe.

### Q1 AND Q2: EFFECTS OF *P. RETICULATUM* EXTRACTS ON INSECTS AND FUNGI

Insect feeding preferences (control vs. treatment) were not affected by the concentration of unripe ( $P = 0.30$ ; Table 1) or ripe fruit extracts ( $P = 0.95$ ; Table 1) applied to the treatment fruit, and thus, we did not test for differences between the two extracts. In contrast, the growth rates of all three fungal species were negatively affected by both unripe and ripe fruit extracts ( $P < 0.0001$  for all analyses; Table 2). For two of three fungal species, unripe fruit extracts were significantly more effective at reducing growth than the ripe fruit extracts (Table 3; Fig. 3). Specifically, the chemical profile of unripe extracts was over four times more potent against fungus F1 ( $R = 4.81$ ; Table 3; Fig. 3) and over 12 times more potent against fungus F3 ( $R = 12.82$ ; Table 3; Fig. 3). There were no differences in potency for fungus F2 (Table 3; Fig. 3).

### Q3 AND Q4: EFFECTS OF PIPERINE AND PIPLARTINE ON INSECTS AND FUNGI

Insect feeding preferences were reduced by piperine and by the combination of piperine and piplartine, but were not affected by piplartine alone (Table 1; Fig. 4). Specifically, as the concentration of piperine doubled, the odds of the insects choosing the treatment fruit decreased by a factor of 0.50 (Odds Ratio = 0.50;  $P = 0.028$ ; Table 1). The combination of piperine and piplartine also reduced insect preference (Odds Ratio = 0.54;  $P = 0.041$ ; Table 1), but there was no effect of piplartine when tested alone (Odds Ratio = 0.84,  $P = 0.53$ ). Because there was no effect of



**Fig. 2.** Estimated concentrations ( $\text{mg mL}^{-1}$ ) of total amides and of individual compounds in unripe and ripe fruit extracts from *Piper reticulatum*. Letter designations for unknown amides follow Whitehead *et al.* (2013).

**Table 1.** Logistic regression results showing effects of amides on insect feeding preferences

Amide treatment	$\beta$ †	OR‡	$z$	$P$
Unripe extract	-0.39	0.67	-1.03	0.30
Ripe extract	-0.03	0.97	-0.07	0.95
Piperine	-0.69	0.50	-2.19	0.028
Piplartine	-0.16	0.85	-0.63	0.53
Combination	-0.61	0.54	-2.04	0.041

†Regression coefficient.

‡Odds Ratio represents the factor by which the odds of insects choosing treatment fruits decreases with each unit increase in concentration.

piplartine, the expected ED<sub>50</sub> for the piperine + piplartine combination based on an additive response was calculated simply as  $A/p_A$  (Tallarida 2000). The ED<sub>50</sub> for piperine ( $A$ ) was  $1.27 \text{ mg mL}^{-1}$  (Table 4), giving an expected value of  $2.55 \text{ mg mL}^{-1}$ . However, the observed ED<sub>50</sub> for the combination was  $8.61 \text{ mg mL}^{-1}$ , providing evidence for an antagonistic relationship between the two compounds ( $\gamma = 3.38$ ; Table 4).

The growth of all three fungal species was strongly reduced by all pure compound treatments, including piperine, piplartine and the combination ( $P < 0.0001$  for all

analyses; Table 2; Fig. 5). For fungi, the evidence for interaction between the two compounds was inconsistent between the three fungal strains. For F1, we found evidence for an antagonistic interaction between piperine and piplartine, where the combination was less effective than expected based on additive effects ( $\gamma = 1.72$ ; Table 4, Fig. 5). For F2, we found evidence for a synergistic interaction, where the combination was more effective than expected based on additive effects ( $\gamma = 0.36$ ; Table 4, Fig. 5). There was no evidence of interaction between the two compounds for F3 (Table 4).

## Discussion

The ecological and evolutionary roles of plant secondary metabolites have been studied primarily in leaves, but increasing evidence has shown that secondary metabolites can also serve a variety of important roles in fruits, including effects on seed dispersal and fruit defence (Levey *et al.* 2007; Whitehead & Bowers 2013a). This study focuses on the role of secondary metabolites in fruit defence and adds an important new component to our understanding of how fruit secondary metabolites influence interactions with the broad range of antagonists that feed on fruit tissues. We tested the effects of amides from *Piper* fruits on an

**Table 2.** Results from a log-logistic regression showing strong effects of amides on fungal growth

Amide treatment	Fungus 1			Fungus 2			Fungus 3		
	$\beta^*$	$t$ -stat	$P$	$\beta^*$	$t$ -stat	$P$	$\beta^*$	$t$ -stat	$P$
Unripe extract	0.64	8.3	<0.0001	0.47	10.67	<0.0001	0.47	8.87	<0.0001
Ripe extract	1.85	5.14	<0.0001	0.46	10.02	<0.0001	0.4	5.18	<0.0001
Piperine	1.64	3.87	<0.0001	0.74	6.78	<0.0001	0.76	4.03	<0.0001
Piplartine	0.44	5.51	<0.0001	1.49	7.22	<0.0001	1.13	7.49	<0.0001
Combination	1.55	5.44	<0.0001	0.54	9.79	<0.0001	0.73	6.18	<0.0001

\*Regression coefficient for slope of dose-response curve.

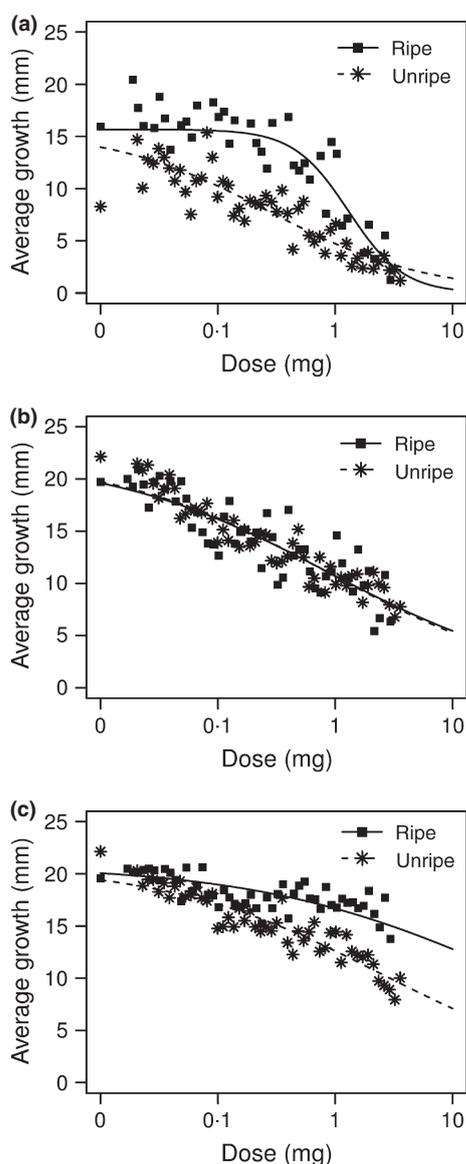
**Table 3.** Relative potency ratios for unripe and ripe fruit extracts

Fungal species	ED50† (mg) unripe	ED50† (mg) ripe	Relative potency ( $R$ )‡	95% CI for $R$
Fungus 1	0.27	1.30	4.81	2.81–6.82*
Fungus 2	0.86	0.88	1.02	0.45–1.58
Fungus 3	2.46	31.55	12.82	2.94–22.70*

\*Confidence intervals that do not cross one indicate a significant difference in potency between unripe and ripe extracts at  $P < 0.05$ .

†ED50 values represent the dose at which fungal growth is reduced by 50%, see text for details.

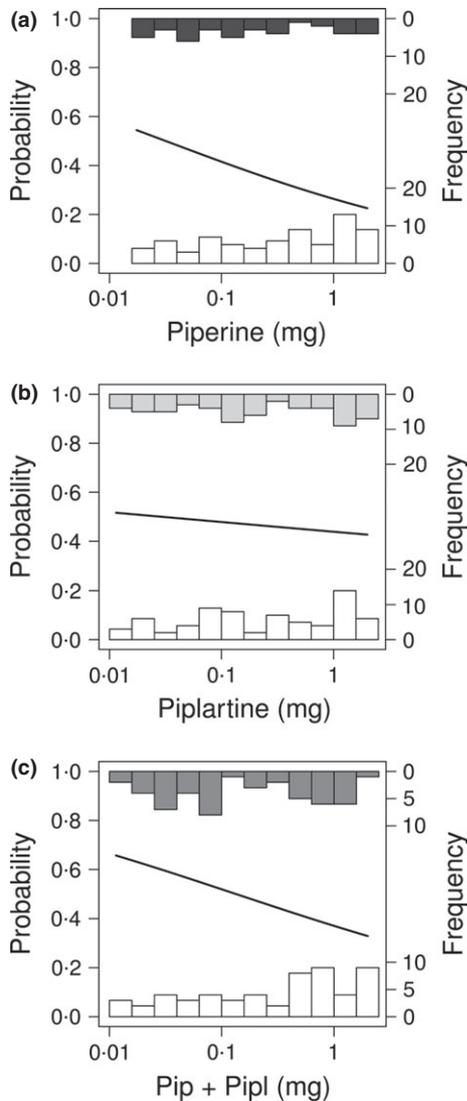
‡Values of  $R < 1$  indicate ripe extracts are more potent, and values of  $R > 1$  indicate unripe extracts are more potent.



**Fig. 3.** Negative effects of *Piper reticulatum* fruit extracts on the growth of fungus F1 (a), F2 (b) and F3 (c). The ED50 is the value for dose ( $\text{mg mL}^{-1}$ ) that corresponds to a 50% reduction in fungal growth from the maximum [7.84 mm in (a); 11.06 mm in (b); and 10.43 mm in (c)]. For (a) and (c), the ED50 values were significantly higher for unripe extracts than ripe extracts, providing evidence that the suites of compounds in unripe extracts were more effective in reducing fungal growth.

insect seed predator and several strains of fruit-associated fungi, and found that the specific effects were variable depending on the combination of amide treatment and consumer tested. Fruit extracts from *P. reticulatum* did not affect insect preferences, but strongly reduced fungal growth (Fig. 3). For two of the three strains of fungi tested, the suites of compounds in unripe fruits had stronger antifungal effects than those in ripe fruits (Table 3). Two amides common to many *Piper* species (but not found in *P. reticulatum*), piperine and pipartine, had variable effects on insect preferences and also strongly reduced fungal growth (Figs 4 and 5). Interestingly, we found that these two compounds can either interact antagonistically or synergistically depending on the consumer involved (Table 4). Together, our results suggest that the diverse suites of metabolites found in *Piper* fruits likely have complex adaptive roles in defence, especially against fungi, and that the effects of compounds in mixtures cannot be explained by simple additive models.

For an insect seed predator, *S. englemanni*, we found that only one individual amide, piperine, appeared to reduce feeding preference. However, even for piperine, the effects were not absolute – at the highest concentrations tested a small percentage of insects still fed on treated fruits (Fig. 4). Although amides applied to the fruit surface may have had only a limited effect on the amounts of compounds actually consumed by the insects, especially if *S. englemanni* feeds exclusively on seeds, fruit surface chemistry is likely an important cue in fruit choice. We regularly observed *S. englemanni* engaged in typical plant surface exploration behaviour (Backus 1988), during which the insect repeatedly dabs the surface of the fruit with its mouthpart prior to initiating feeding. Surface chemistry is also known to be an important determinant of hemipteran feeding choice in agricultural systems, where surface sprays are commonly used to repel seed predators (e.g. Kamminga *et al.* 2009). Thus, the lack of strong effects of amides on fruit choice in this study suggests that *S. englemanni* is not deterred by amides and may have the ability to tolerate or detoxify these compounds. This species can be considered a genus specialist on *Piper*; we have observed it feeding on at least 10 *Piper* species that co-occur at the study site, and at least four other *Piper* species are reported as host plants in the literature (Greig 1993).



**Fig. 4.** Negative effects of piperine (a), null effects of piplartine (b) and negative effects of the combination (c), on the feeding preferences of *Sibarida englemanni*, a hemipteran seed predator. Bars show frequency distributions for the number of insects choosing treatment fruits (grey bars, top) and control fruits (white bars, bottom) at each concentration. Lines represent model predictions from a logistic regression showing the probability that insects will choose the treatment fruits at varying concentrations. The ED<sub>50</sub> is the value for dose ( $\text{mg mL}^{-1}$ ) that corresponds to a model-predicted probability of 0.25 (representing a 50% reduction in insect preference) and is  $1.27 \text{ mg mL}^{-1}$  for piperine (a) and not estimated for piplartine due to a lack of significant treatment effects. The higher than expected value for ED<sub>50</sub> of the combination ( $8.61 \text{ mg mL}^{-1}$ ) shown in (c) provides evidence for an antagonistic interaction between the two compounds.

Although the chemistry of the fruits from these different *Piper* hosts is highly diverse and includes amides, alkenylphenols, phenylpropanoids and terpenes (Parmar *et al.* 1997; S. R. Whitehead, pers. observ.), amides are among the most commonly detected compounds in the genus (Parmar *et al.* 1997; de Nascimento *et al.* 2012), and therefore, *S. englemanni* may have specific adaptations that allow it to feed on amide-rich fruits. We focused on

*S. englemanni* in this study because of its abundance at the site and important impacts on seed production in *Piper* (Greig 1993), but it is possible that additional experiments with other non-adapted generalist species may show that amides play a stronger role in insect defence than was detected here.

In contrast to the variable effects of amides on the insect species examined, there were universally negative effects of amides on fungal growth. These results corroborate past work suggesting that defence against fungal pathogens may be one of the most important adaptive benefits of secondary metabolites in fruits (Herrera 1982; Cipollini & Levey 1997a; Cazetta, Schaefer & Galetti 2008; Schaefer, Rentzsch & Breuer 2008; Tewksbury *et al.* 2008). Metabolites that reduce fungal growth may benefit plants directly by protecting seeds from rot or damage that can reduce viability, or indirectly by increasing the persistence time of fruits and their attractiveness to mutualist seed dispersers (Herrera 1982; Cipollini & Stiles 1993). Although we cannot be certain that the specific fungal species we isolated from fruits are important as pathogens (many fungi that occur on plants do not have negative effects on plant fitness; Rodriguez *et al.* 2009), the fact that there were universal strong negative effects across all fungal species tested suggests that amides are likely effective against a broad range of potential fungal pathogens that attack fruits.

Our results showing differences in the effectiveness of unripe and ripe fruit compound mixtures for two of the three fungal species (Fig. 3) also suggest there are important interactive effects of mixtures and/or differences in the relative toxicity of different compounds. Although the qualitative composition of major compounds was similar between unripe and ripe fruit extracts, the relative abundances of compounds differed (Fig. 2). In particular, unripe fruits had a lower proportion of dihydroiswanidine and a higher proportion of methoxy dihydrotricholein, octadecenylpyrrolidine, methoxy tricholein A and N-isobutyleicosadienamide compared with ripe fruits. Because even closely related compounds can vary greatly in their biological activity (e.g. Gbewonyo, Candy & Anderson 2006; Pandey *et al.* 2013), it is likely that the chemical changes that occur with ripening have important consequences for fruit defence. The changes in relative abundances in *P. reticulatum* fruits occur concurrently with a reduction in the total concentrations of compounds; thus, these combined effects likely indicate that ripe fruits are much more susceptible to attack than unripe. This is apparent in natural populations of *P. reticulatum*, where unripe fruits are often persistent on the plant over a period of development that can last for a month or more, while ripe fruits appear rotten, fall to the ground, and are often covered in fungal hyphae within a few days of maturation. However, because the large majority of fruits are removed by seed dispersing bats on the same night of ripening, a short persistence time once ripe may not have any negative fitness consequences in this species. Rather, plants may

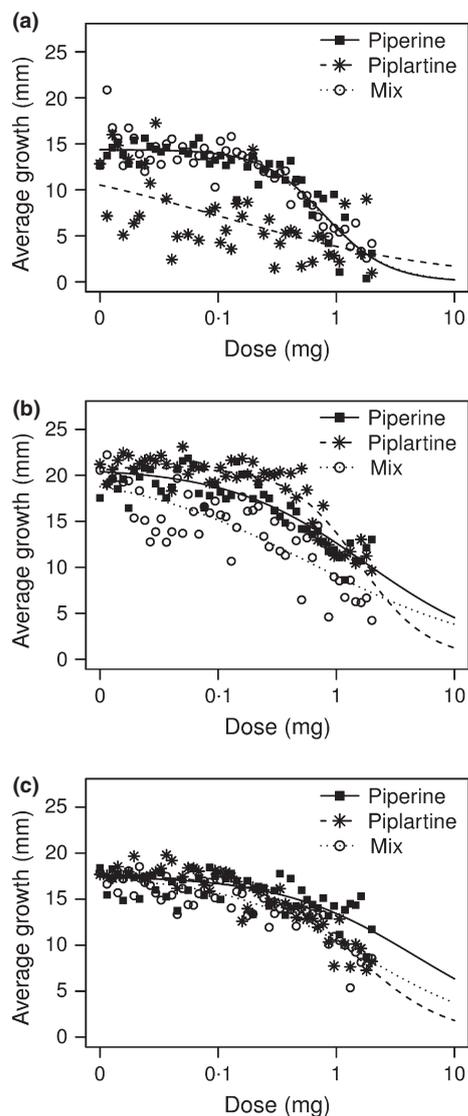
**Table 4.** Interaction indices for piperine and piplartine

Species	ED50† (mg) piperine	ED50† (mg) piplartine	ED50† (mg) combination	Interaction index ( $\gamma$ )‡	95% CI for $\gamma$
<i>Sibaria englemanni</i>	1.27	–	8.61	3.38	1.12–5.63*
Fungus 1	0.8	0.1	0.78	1.72	1.13–2.32*
Fungus 2	1.79	1.56	0.62	0.36	–0.02 to 0.76*
Fungus 3	4.72	1.48	1.69	0.55	–1.21 to 2.31

\*Confidence intervals that do not cross one indicate a significant difference in potency between unripe and ripe extracts at  $P < 0.05$ .

†ED50 values represent the dose at which insect preference or fungal growth is reduced by 50%, see text for details.

‡Values of  $\gamma < 1$  indicate synergy, and values of  $\gamma > 1$  indicate antagonism.



**Fig. 5.** Negative effects of piperine, piplartine and the combination on the growth of fungus F1 (a), F2 (b), and F3 (c). The ED50 is the value for dose ( $\text{mg mL}^{-1}$ ) that corresponds to a 50% reduction in fungal growth from the maximum [7.19 mm in (a); 10.42 mm in (b); and 8.79 mm in (c)]. An additive effect of piperine and piplartine would be demonstrated by an ED50 for the mixture that was intermediate between piperine and piplartine. For (a) the ED50 of the mixture was higher than expected, providing evidence for an antagonistic interaction, and for (b) the ED50 of the mixture was lower than expected, providing evidence for a synergistic interaction between piperine and piplartine.

maximize fitness by reducing the concentrations of compounds that could have negative effects on the feeding preferences of mutualists during the final period of ripening.

For both insects and fungi, there was evidence that the effects of combinations of compounds cannot be explained by a simple additive model. Notably, our results show that the same two compounds (piperine and piplartine) can function either synergistically or antagonistically depending on the target organism. While the potential for interactions among plant defensive compounds has received increasing interest over the last decade (Gershenzon *et al.* 2012), there are still only a limited number of studies that have provided empirical evidence for synergistic interactions, and we know of only one previous ecological study that has reported antagonistic interactions (Diawara *et al.* 1993). This may be in part due to the limited number of ecological studies that have used rigorous methods for detecting and analysing compound interactions (Nelson & Kursar 1999). Considering the enormous diversity of compounds that occur in plants (Wink 2010), an appreciation for the fundamental role of compound synergy and/or antagonism in determining the outcome of species interactions may provide important new insights into the ecology and evolution of plant defence. Our results showing different interactive effects of the same two compounds on different organisms emphasize the need for integrative approaches to understanding the costs and benefits of suites of secondary metabolites in the diversity of interactions in which plants are involved.

Fruit secondary metabolites play a key role in the defence of fruits against a variety of antagonistic consumers and therefore may be more important determinants of plant fitness than is generally appreciated. In this study, we found large differences in the bioactivity of unripe extracts and ripe extracts due to small changes in composition of compounds, as well as evidence for interactions between individual compounds, emphasizing the potential importance of chemical diversity and composition in fruits in the efficacy of defence. In addition, large differences in the bioactivity of amide mixtures against different consumers suggest that the importance of fruit secondary metabolite diversity cannot be understood based on simple tests of the effects of particular compounds on particular organ-

isms. Because fruit secondary metabolites can have a variety of role in fruits, often simultaneously affecting interactions with both mutualists and antagonists (Cipollini & Levey 1997b; Izhaki 2002; Levey *et al.* 2007), future work should focus on understanding the complex costs and benefits of suites of fruit secondary metabolites in interactions with different classes of fruit pests and mutualistic seed dispersers. This integrative approach could provide important new insights that can improve theories of both seed dispersal and plant defence.

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## Data accessibility

All data associated with the study are deposited in the Dryad repository: <http://doi.org/10.5061/dryad.2m5c4> (Whitehead & Bowers 2014).

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