Herbivore-induced changes in fruit–frugivore interactions

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Summary

1. Herbivore attack can induce dramatic changes in plant chemical defences. These responses protect plants against future herbivory, but can also have important physiological and ecological costs. Ecological costs of defence have received recent theoretical attention; however, many proposed costs have not yet been demonstrated empirically. In particular, field data are lacking as to whether induced responses in leaves can lead to correlated changes in fruit palatability that reduce fruit removal by mutualist seed dispersers.

2. Using the tropical shrub, *Hamelia patens* (Rubiaceae), we examined changes in fruit removal, palatability and maturation time following various treatments to the subtending leaves, including herbivory, mechanical damage and/or application of methyl jasmonate (MeJA).

3. Fewer fruits were removed from herbivory- and MeJA-treated branches than from controls, and results from three bioassays with ants and fungi suggested that this response was mediated by changes in fruit palatability. In addition, fruits from MeJA-treated branches matured more quickly than those from control branches.

4. *Synthesis.* Taken together, our results provide novel evidence that induced responses to herbivory can affect fruit–frugivore interactions through two mechanisms: changes in fruit palatability and changes in fruit development time. This highlights the importance of physiological linkages between leaf and fruit traits in determining the overall costs of plant defence and the fitness outcomes of multispecies interactions.

Key-words: ecological cost of defence, frugivory, fruit–frugivore interactions, *Hamelia patens*, induced resistance, plant–herbivore interactions, seed dispersal, toxic fruit

Introduction

Plants employ a remarkable armoury of chemical resistance traits as defences against herbivores and pathogens. These defences are generally divided into constitutive defences (always present in plant tissue) and induced defences (produced in response to damage) (Kessler & Baldwin 2002). Induced defences are thought to reduce the costs associated with continuous production of defences during times when herbivores and pathogens are not present (Karban & Baldwin 1997), but numerous studies have shown that even induced defences can result in significant costs in terms of plant fitness (Koricheva 2002; Strauss *et al.* 2002). Fitness costs can include allocation costs, when investment in defence reduces allocation of resources to growth and reproduction, and ecological costs,

when reduced fitness is due to changes in interactions with other organisms (Strauss *et al.* 2002).

Ecological costs of induced defence have been the subject of several reviews (Heil 2002; Strauss *et al.* 2002; Cipollini, Purrington & Bergelson 2003; Walters & Heil 2007) and may include reduced resistance to other enemies, deterrence of mutualists or reduced competitive ability. For example, induced responses to leaf herbivory can increase deterrent compounds in nectar and pollen, thereby altering visitation rates and/or nectar and pollen removal by pollinators (e.g. Herrera *et al.* 2002; Adler *et al.* 2006; Kessler & Halitschke 2009). The fitness implications of this effect may depend greatly on ecological context (e.g. Gegear, Manson & Thomson 2007), but, at least in some cases, pollinator preferences can create a selective advantage for less-defended plants (Strauss *et al.* 1999).

Although the recognition of ecological costs has improved our ability to detect overall costs of defence and had a strong

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impact on plant defence theory (Koricheva 2002; Strauss *et al.* 2002), many proposed ecological costs have not yet been demonstrated empirically. It has been suggested that costs parallel to those demonstrated for pollination mutualisms may also exist for seed dispersal mutualisms (Strauss *et al.* 2002), and there is some evidence of correlations between leaf and fruit resistance traits (Redman, Cipollini & Schultz 2001; McCall & Karban 2006). In addition, induced responses to herbivory have been correlated with changes in fruit maturation time (Redman, Cipollini & Schultz 2001), which has the potential to impose additional fitness costs depending on ecological context. However, there is still no experimental evidence that induced plant resistance traits can affect fruit chemistry, fruit–frugivore interactions or seed dispersal.

A surprisingly large proportion of ripe, fleshy fruits contain potentially deterrent or toxic compounds (Herrera 1982), and it has been debated whether these compounds should be explained adaptively (Cipollini & Levey 1997) or as a physiological cost of producing defensive compounds in leaves and other tissues (Eriksson & Ehrlen 1998). There is growing evidence that constitutive deterrent compounds in fruits are functionally important in defence, but may simultaneously impose costs in terms of reduced fruit removal by mutualists (e.g. Izhaki 2002; Schaefer, Schmidt & Winkler 2003; Cazetta, Schaefer & Galetti 2008; Tewksbury *et al.* 2008). However, in addition to selection from mutualistic and antagonistic frugivores, there may be important constraints on the expression of fruit chemical traits due to the complex selective environment imposed by herbivores, pathogens, pollinators and competitors. We have very little information on how the expression of fruit chemical traits may vary depending on the larger ecological context.

In this study, we conducted a series of short experiments to test the hypothesis that induced responses to leaf herbivory can alter fruit palatability and thereby diminish fruit removal by potential seed dispersers. Since methyl jasmonate (MeJA), an important hormone involved in plant responses to herbivory, also plays a fundamental role in fruit development (Creelman & Mullet 1997), we also tested how induced responses in leaves affect fruit maturation. To our knowledge, these are the first manipulative experiments that explore the potential for ecological costs of plant responses to leaf herbivory in terms of changes in fruit–frugivore interactions.

Materials and methods

STUDY SITE AND SYSTEM

All experiments were conducted between February 2008 and January 2010 at La Selva Biological Station, Heredia Province, Costa Rica. The area consists of tropical wet forest and receives *c.* 4000 mm rain year^{-1}, spread relatively evenly throughout the year. Average monthly temperatures range between 24.7 °C and 27.1 °C (McDade *et al.* 1994).

Hamelia patens Jacq. (Rubiaceae) is a 2- to 6-m tall shrub with a distribution ranging from Mexico to Bolivia (Croat 1978). A variety of herbivores feed on the leaves of *H. patens*; observations at our study site include the sawfly *Waldheimia interstitialis* (Cameron) (Hymenoptera: Tenthredinidae), the leaf-cutter ant *Atta cephalotes*

(Linnaeus) (Hymenoptera: Formicidae) and at least 12 species of Lepidoptera from seven families (Dyer & Gentry 2002; S.R. Whitehead, pers. obs.). Only the sawfly, *W. interstitialis*, was located in sufficient numbers for experimentation during the course of this study. A large diversity of birds, including toucans, warblers, honeycreepers, thrushes, flycatchers and tanagers, feed on *H. patens* fruits, which are produced continuously throughout the year on infructescences containing anywhere from 15 to 50 fruits (Leck 1972; Croat 1978; Levey 1987). Individual fruits remain green for 2 months until they enter a final ripening period, lasting *c.* 6 days and characterized by a sequential colour change from green to cream to pink to red to maroon to black (Levey 1987).

EFFECTS OF HERBIVORY AND SIMULATED HERBIVORY ON FRUIT REMOVAL

To investigate how induced responses to herbivory can affect fruit removal by potential seed dispersers, we used natural populations of *H. patens* shrubs located in the La Selva arboretum and open areas surrounding the station. Four infructescences per shrub, matched for the approximate numbers of fruits in different ripening stages, were randomly assigned to one of the following treatment groups: (i) herbivory—two *W. interstitialis* larvae feeding for 3 days on the leaves immediately subtending the infructescence, (ii) MeJA—1 μL of 10 μg μL^{-1} MeJA in lanolin paste applied to the petioles of the two leaves immediately subtending the infructescence (Halitschke *et al.* 2001), (iii) lanolin control—1 μL of pure lanolin paste applied in the same manner as above as a control for the MeJA treatment, (iv) absolute control—branches handled in a similar manner but without treatment applied as a control for the herbivory group. Since we were limited by the number of herbivores we could find in the environment, we had seven replicates of the herbivory and absolute control treatments, and 22 replicates of the MeJA and lanolin control treatments. All infructescences were enclosed in mesh bags for 3 days, after which the bags were removed and the fruits were exposed for 2 days to consumers. We counted the number of fruits before and after the 2-day period, and assumed the majority of the missing fruits had been removed by birds, since we found no fruits on the ground below shrubs, no fruits that had fallen in the mesh bags, and no evidence of other vertebrate consumers of *H. patens* fruits.

EFFECTS OF SIMULATED HERBIVORY ON FRUIT PALATABILITY

To investigate whether leaf herbivory can potentially lead to correlated changes in fruit chemistry, we simulated herbivory to *H. patens* leaves using mechanical damage and/or treatment with MeJA, and, using a series of bioassays, examined whether there were changes in the palatability of adjacent fruits to ants and fungi. Ants and fungi were chosen as bioassay test organisms because they are abundant and easily manipulable, and have been successfully used to assay changes in plant chemistry in other studies (e.g. Kessler & Baldwin 2007; Fincher *et al.* 2008; Liu *et al.* 2009). In addition, these organisms interact with *H. patens* in natural populations; ants (in particular *Ectatomma ruidum*) visit extrafloral nectaries located on the distal end of *H. patens* fruits (S.R. Whitehead, pers. obs.), and the fungal strain that we used was isolated from rotting fruits collected from *H. patens* trees at our study site. Thus, the responses of ants and fungi in bioassays can provide ecologically relevant information about the potential for plant–herbivore interactions to affect various other interactions in which plants are involved.

First, for the ant bioassays, we chose two comparable infructescences on each of ten shrubs that were randomly assigned to MeJA or lanolin control treatments as described above and enclosed in mesh bags to prevent fruit removal. After 3 days we removed the bags and harvested the infructescences for use in two identically designed bioassays with two species of ants. In order to minimize the potentially confounding effects of differences among fruits of different ripening stages, we used only those fruits from the infructescence that were maroon in colour. Fruits only remain at this colour stage for *c.* 24 h; thus all fruits used in our bioassays were of similar maturity at the time of harvest.

We modelled our ant bioassays after those described in Kessler & Baldwin (2007). We first prepared a 12.5% sucrose solution in distilled water. This sugar concentration is within the range typically found in ripe, bird-dispersed fruits and has been successfully used to attract a diversity of ants in other studies (Witmer 1998; Kessler & Baldwin 2007). We placed one maroon fruit from each infructescence in a 1.5-mL Eppendorf vial, added 1 mL of the sugar solution, and macerated the fruit inside the vial using a glass stirring rod. Vials were filled to the top with additional sugar solution and homogenized with a vortex mixer. A third group of positive control vials was filled with sugar solution only. Thus, each set of vials consisted of a paired set of sugar solutions containing MeJA-treated or lanolin control fruits taken from the same shrub, along with a vial containing sugar solution only. Ten sets of vials were prepared for each of two species of ant: *E. ruidum* (Roger), a common ground forager in lowland wet forests, and *Paratrechina longicornis* (Latreille), a non-native household and agricultural pest that recruits in large numbers. Ten feeding stations per ant species were established in different locations at least 50 m apart, along the forest edge for *E. ruidum* and around the La Selva laboratory for *P. longicornis*. At each station a set of three vials was buried in the soil 15 cm apart so that the openings were even with the soil surface. After 1 h we closed the vial lids to trap all ants that were currently foraging inside and returned them to the laboratory to count the number of recruited individuals using a stereoscope.

In a third bioassay conducted several months later, we examined growth rates of a fungus we isolated and cultured in the La Selva laboratory from rotting *H. patens* fruits and tentatively identified as *Mucor sp.* (Mucoraceae). We chose fifteen shrubs and assigned four infructescences per shrub to the following treatments: (i) mechanical damage, (ii) MeJA, (iii) lanolin control and (iv) absolute control. These treatments were all identical to those described above, with the exception of mechanical damage, which we added as a substitute for herbivory since we were unable to locate herbivores in sufficient numbers for experimentation. For this treatment we inflicted six rows of puncture damage on the two leaves subtending the infructescence using a pattern wheel (Baldwin & Schmelz 1994). Three days after the treatments, one fresh maroon fruit from each of the four infructescences was macerated with a glass rod in a test tube and soaked in 5 mL methanol for 24 h. The resulting extracts were filtered, and 200-μL aliquots were distributed evenly over the surface of a potato dextrose agar plate using a flame-sterilized glass spreader. The plates were left uncovered on a sterile laminar-flow bench for 30 min to allow evaporation of the solvent and then inoculated with a 0.5-cm^2 plug of agar from a stock culture of the fungus. An additional fifteen control plates were prepared in the same manner using methanol only. Inoculated plates were incubated at ambient temperatures (20–25 °C), and the radial growth of hyphae was measured after 48 h.

EFFECTS OF SIMULATED HERBIVORY ON FRUIT RIPENING

To investigate whether fruit ripening rates may also be influenced by induced responses to herbivory, we treated two infructescences on each of ten shrubs as either MeJA or lanolin control as described above. Fruits were enclosed in mesh bags to prevent removal, and the numbers of fruits in four ripening categories (green, cream/pink, red/maroon and black) were counted on each infructescence at the time of treatment and again after 3 days. Since individual fruits were not marked, a conservative estimate of the number of fruits that were actively ripening during this time was taken by adding the number of fruits entering the black category and the number of fruits leaving the green category. Based on our own observations, we assumed that no fruits could have changed from green to black during the 3-day period; therefore this method underestimates the number of actively ripening fruits by ignoring the fruits changing from cream/pink to red/maroon categories.

STATISTICAL ANALYSES

To test whether herbivore- or MeJA-induced responses affect fruit removal, we used a GLMM with a binomial distribution and the logit link function, run using the lme4 package (Bates & Maechler 2010) of the statistical software R ver. 2.11.1 (R Development Core Team, 2010). Treatment was specified as a fixed effect and shrub was included as a random effect, and the model was fit by the Laplace approximation. Residuals did not reveal any outliers or indicate overdispersion. For hypothesis testing, we used Akaike Information Criterion corrected for small sample size (AIC$_C$), since likelihood ratio tests are unreliable for small sample sizes in GLMMs (Bolker *et al.* 2009). We took a conservative approach and considered a ΔAIC$_C$ > 10 between the full model and a null model that included only shrub as a random effect as support for our hypothesis. Pairwise contrasts of MeJA to lanolin control and herbivory to absolute control were specified *a priori*, and tested using Wald Z-statistics.

To test preferences of *E. ruidum* and *P. longicornis* ants to various sugar solutions, we used nonparametric Friedman's ANOVAs, blocked for feeding station, followed by Wilcoxon–Nemenyi–McDonald–Thompson tests for multiple comparisons (Hollander & Wolfe 1999). Fungal growth rates were compared using a one-way analysis of variance, followed by a Tukey's HSD test for multiple comparisons. To test the ripening rates of fruits on MeJA-treated vs. control infructescences, the average proportion of maturing fruits per infructescence was compared using a paired t-test. Data were arcsin square-root transformed prior to analysis to fit assumptions of normality. Analyses were performed in JMP (2009) or R Version 2.11.1 (R Development Core Team 2010).

Results

LEAF HERBIVORY REDUCES FRUIT REMOVAL

Blue-grey Tanagers (*Thraupis episcopus*), Passerini's Tanagers (*Ramphocelus passerinii*) and Collared Araçaris (*Pteroglossus torquatus*) were observed feeding on *H. patens* fruits during this study. In our experiment examining the effects of herbivory and treatment with MeJA on fruit removal rates (Fig. 1), the model that included the effects of treatment provided a much better fit to the data than the null model that included shrub only (ΔAIC$_C$ = 18.2). Pairwise contrasts showed a significant
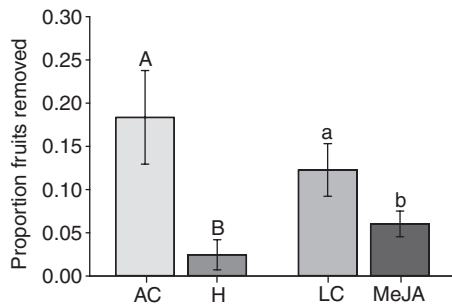
Fig. 1. Average (± SE) proportion of fruits removed from *Hamelia patens* infructescences receiving the following treatments: herbivory on subtending leaves (H), similar handling but no treatment applied (AC: absolute control), methyl jasmonate in lanolin paste applied to subtending leaves (MeJA) or lanolin paste only applied to subtending leaves (LC: lanolin control). Different letters indicate differences from pairwise contrasts of herbivory to absolute control and MeJA to lanolin control.

reduction in removal rates in herbivory treatments as compared to absolute controls (Wald $Z = 3.417$, $N = 7$, $P = 0.0006$), as well as a significant reduction in removal rates in MeJA treatments as compared to lanolin controls (Wald $Z = 3.272$, $N = 22$, d.f. $= 1$, $P = 0.001$).

SIMULATED HERBIVORY REDUCES FRUIT PALATABILITY

In the bioassays using *E. ruidum*, there was an overall effect of sugar solution on ant recruitment ($\chi^2 = 7.19$, d.f. $= 2$, $P < 0.028$), and *post hoc* comparisons showed fewer individuals recruited to solutions made from MeJA-treated fruits as compared to lanolin control fruits (Fig. 2a). For bioassays using *P. longicornis*, there was also an overall effect of sugar solution ($\chi^2 = 17.18$, d.f. $= 2$, $P < 0.00019$), and here *post hoc* tests showed sugar-only solutions had the highest recruitment, followed by lanolin control fruit and then MeJA-treated fruit (Fig. 2b).

In the fungal growth bioassay, the growth of hyphae was significantly affected by growth medium treatment ($F_{5,70} = 12.00$, $P < 0.0001$; Fig. 2c). Specifically, *post hoc* tests showed that fungi grew faster on methanol only, absolute control and lanolin control plates than on plates treated with fruit extracts from MeJA and mechanically damaged groups.

SIMULATED HERBIVORY INCREASES FRUIT RIPENING RATE

MeJA-treated infructescences had significantly more actively ripening fruits relative to infructescence size than control infructescences (paired $t = -2.39$, d.f. $= 9$, $P = 0.04$) (Fig. 3).

Discussion

Induced defences are important physiological responses to herbivory that can protect plants against further damage. However, these responses can lead to correlated changes in the
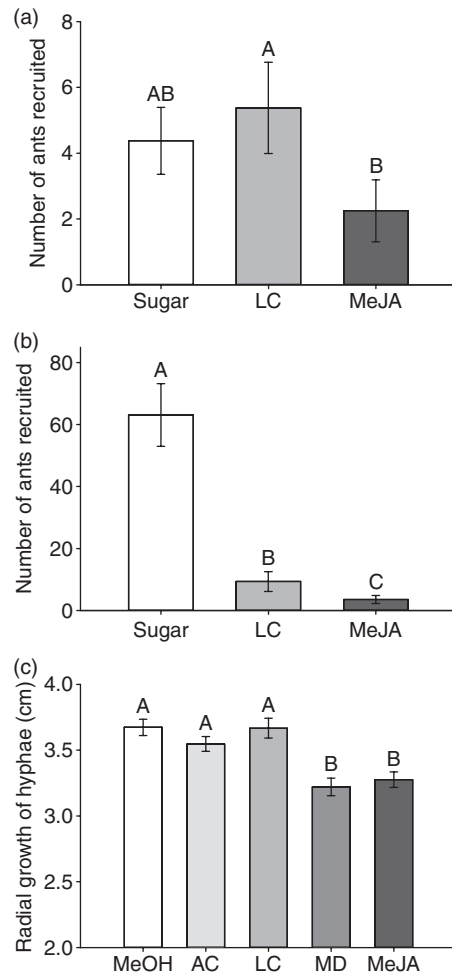
Fig. 2. Three bioassays testing the effects of induced responses to herbivory on fruit palatability. We compared the average number (± SE) of *Ectatomma ruidum* (a) and *Paratrechina longicornis* (b) ants recruited to control sugar solutions (Sugar), sugar solutions with fruits from lanolin control branches (LC), and sugar solutions with fruits from methyl jasmonate treated branches (MeJA). We also compared the average (±SE) growth rates of fungi (c) on potato dextrose agar supplemented with methanol only (MeOH) or methanol extracts of *Hamelia patens* fruits from absolute control (AC), lanolin control (LC), mechanical damage (MD), or methyl jasmonte-treated groups (MeJA). Different letters indicate differences ($P < 0.05$) from *post hoc* comparisons of data by Wilcoxon–Nemenyi–McDonald–Thompson tests (a, b) and a Tukey's HSD test (c).

expression of other traits and thus the alteration of interactions with non-target organisms. Our results provide the first documentation of an ecological cost of plant responses to herbivory in the currency of fruit removal by seed dispersers. Leaf herbivory or MeJA application to leaf petioles led to reduced fruit removal on adjacent infructescences, and results from several bioassays with ants and fungi suggested that this response was mediated by a chemical change that reduced fruit palatability to these organisms. In addition, MeJA-mediated responses in leaves affected fruit development, providing evidence for an additional mechanism through which leaf herbivory can affect fruit–frugivore interactions and seed dispersal.
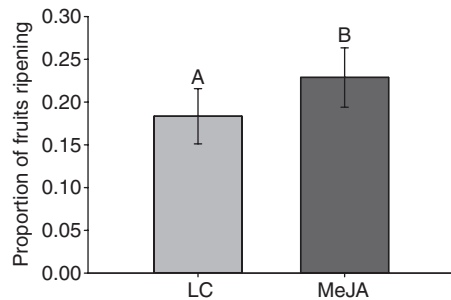
Fig. 3. The average proportion (± SE) of fruits that were actively ripening (estimated as the total number of fruits that were either initiating ripening or entering final ripening stages) from *Hamelia patens* infructescences from lanolin control (LC) and methyl jasmonate (MeJA) treatment groups. Different letters indicate differences from a paired *t*-test on transformed data, and data were back-transformed for the figure.

A plausible mechanistic explanation for our results is that the reduced removal rates and palatability of induced fruits to our bioassay test organisms were due to an increase in deterrent compounds in fruits after leaf herbivory. Although chemical analyses of fruits were not performed, we used three separate bioassay organisms that bridged broad taxonomic groups (two ant species and one fungal strain), and all of these showed a negative response to fruits from induced branches in comparison to controls. Birds were also seemingly deterred from feeding upon induced fruits, and prior work has shown that birds, in particular those, such as tanagers, that masticate fruits prior to ingestion, can detect even small changes in the chemical composition of fruits (Levey 1986). The leaves of *H. patens* are rich in pentacyclic indole alkaloids, the most abundant of which is isopteropodine (Reyes-Chilpa *et al.* 2004), and these same alkaloids may also be present in fruits (unpublished data, referenced in Levey 1987).

Our results showing faster ripening of fruits on MeJA-treated branches also corroborate our hypothesis of chemical changes in fruits following leaf herbivory. Given the importance of MeJA in mediating multiple physiological pathways, it could be argued that the effect of herbivory on fruit removal might be an artefact of changes in fruit development. In a study by Redman, Cipollini & Schultz (2001), the expression of induced resistance traits *decreased* fruit ripening rates in tomato plants (*Lycopersicon esculentum*), but this is the opposite of what we found in our plants. Our results show that MeJA increased fruit ripening rates, and, assuming birds or other consumers are more likely to remove ripe fruits, we would have expected that fruits of MeJA-treated plants would be removed faster than control fruits. Instead these fruits were removed more slowly; thus any potential increase in the availability of ripe fruits on induced branches either did not increase removal or did not increase it enough to counteract the effects of any potential changes in fruit chemistry following simulated herbivory.

Although the negative effects of herbivore addition or simulated wounding on fruit removal and palatability to our bioassay test organisms are clear, the overall effect of this change in terms of individual plant fitness requires further investigation.

Two important considerations come to mind. First, the relationship between fruit removal from the parent plant and the eventual establishment of reproductive offspring is undoubtedly complex and involves stochastic processes that can happen over long time scales (Wang & Smith 2002). Induced changes in fruit chemistry could affect multiple aspects of the process, e.g. seed germination or seedling defence (Cappelletti, Innocenti & Caporale 1992; Agrawal 2002). Secondly, since the fungal strain used in one of our bioassays was isolated from rotting *H. patens* fruits found still attached to the plant, this experiment also provides ecologically relevant information about the potential defensive role of fruit compounds against fungal pathogens. Although it is not clear from the present study whether the *Mucor* fungus had any causal role in fruit rot, fungi in this genus have been shown to speed up fruit deterioration in other systems (Okwulehie & Alfred 2010). Thus, a reduction in its growth rate or that of other fungal species involved in rot could potentially provide some adaptive benefit to the plant by reducing seed damage or increasing the persistence time of ripe fruits. The optimal expression of defensive compounds in fruits likely reflects a balance between the costs in terms of reduced fruit removal and the benefits in terms of defence against pests, as suggested by the defence trade-off hypothesis for the presence of deterrent compounds in fruit (Herrera 1982; Cipollini & Levey 1997). However, our results clearly suggest that, in addition to the multiple selection pressures on fruit chemical traits from mutualistic and antagonistic frugivores, there can also be important physiological constraints on these traits depending on the larger ecological context.

Overall, our study provides strong initial evidence of correlations between the expression of leaf and fruit chemical traits, and suggests two mechanisms through which induced responses to herbivory may impose costs in terms of alteration of fruit–frugivore interactions: changes in fruit palatability and changes in fruit ripening rates. Future work in this and other systems should focus on providing quantitative analysis of leaf and fruit chemistry in response to damage, and a thorough examination of how changes in fruit palatability translate to effects on plant fitness. Since plants interact simultaneously with both mutualists and antagonists, integrative studies of the fitness effects of correlated plant traits are necessary if we are to understand complex selective forces and constraints on the evolution of plant chemical traits.

Acknowledgements

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