Evidence for the Adaptive Significance of Secondary Compounds in Vertebrate-Dispersed Fruits

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abstract: Although the primary function of fleshy fruits is to attract seed dispersers, many ripe fruits contain toxic secondary compounds. A number of hypotheses have been proposed to explain this evolutionary paradox, most of which describe the potential adaptive role that secondary compounds may play in seed dispersal. However, some authors have argued that fruit secondary compounds may be nonadaptive and instead explain their occurrence as a pleiotropic consequence of selection for defense of leaves and other tissues. We address these alternative evolutionary hypotheses through a comparative examination of iridoid glycosides in the leaves, unripe fruits, and ripe fruits of *Lonicera × bella* (Belle's bush honeysuckle), combined with an examination of fruit damage and removal in natural populations. We provide several lines of evidence that fruit secondary compounds cannot be explained solely as a consequence of foliar defense, including higher concentrations and more individual compounds in fruits compared to leaves and a negative relationship between iridoid glycoside concentration and fruit damage. However, we also show that the compositions and concentrations of secondary compounds in leaves and fruits are not entirely independent, emphasizing that selection in different plant parts is intrinsically linked. We conclude that the adaptive significance of chemical traits is best considered in a whole-plant context that includes fruit-frugivore interactions.

Keywords: fruit secondary metabolites, iridoid glycosides, *Lonicera*, optimal defense theory, phenotypic correlation, seed dispersal.

Introduction

Because the ultimate function of fleshy fruits is to facilitate seed dispersal by mutualist animals, ripe fruits should theoretically be attractive and nutritious. However, in addition to nutritional rewards, fleshy fruits commonly contain secondary compounds that are distasteful or even highly toxic (Herrera 1982). For example, the ripe fruits of *Atropa belladonna* (Solanaceae) are so toxic that the consumption of even a few berries can be fatal to humans (Caksen et

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al. 2003). Although various fruit-feeding animals may be able to tolerate or detoxify fruit secondary compounds (e.g., Struempf et al. 1999; Tewksbury and Nabhan 2001), the occurrence of toxic fruits represents an evolutionary paradox: Why would a ripe fruit, which functions primarily to attract seed dispersers, contain toxic secondary compounds?

The evolutionary paradox of toxic fruit was noted in the scientific literature as early as the 1920s (Heim de Balsac 1928), yet there are still only a few systems in which the evolutionary ecology of fruit secondary compounds has been examined in an integrative manner, and theoretical progress in the field remains relatively slow (reviewed in Levey et al. 2007). A number of adaptive hypotheses have been proposed to explain how fruit secondary compounds may enhance seed dispersal success through mechanisms such as inhibiting seed germination in intact fruits, reducing the length of animal foraging bouts, regulating gut retention time of seeds, deterring less efficient seed dispersers, and defending fruits against seed predators and pathogens (Herrera 1982; Cipollini and Levey 1997a; Cipollini 2000). Most of these hypotheses have not been adequately tested, but a few have gained some empirical support. In particular, a number of studies have emphasized the role of secondary compounds in defense against fruit antagonists and/or supported the idea of trade-offs in fruit traits between defense and attraction of seed dispersers (Cipollini and Stiles 1993; Cipollini and Levey 1997b; Tsahar et al. 2002; Schaefer et al. 2003; Tang et al. 2005; Cazetta et al. 2008; Tewksbury et al. 2008; Haak et al. 2012).

Despite the evidence that fruit secondary compounds can, in some cases, confer an adaptive benefit, in other cases, secondary compounds in fruits may be nonadaptive (i.e., provide no fitness benefits) in the context of fruit-frugivore interactions (Ehrlen and Eriksson 1993; Eriksson and Ehrlen 1998). Plants are under strong selection for the defense of leaves and other vegetative tissues (reviewed

in Schoonhoven et al. 2005), and thus fruit secondary compounds may be best explained by physiological or pleiotropic constraints on the exclusion of these compounds from fruit tissue. Constraints on fruit chemistry are likely, since defensive traits in different plant parts can be linked through shared genetics, hormonal regulation, biosynthetic pathways, and metabolism (Herrera et al. 2002; Adler et al. 2006). For example, recent evidence suggests that induced plant responses to leaf herbivory can lead to corresponding changes in fruit chemistry and reduced fruit removal rates by birds (Whitehead and Poveda 2011). In such cases, fruit secondary compounds may best be explained as an ecological cost of defense of vegetative tissues (Heil 2002; Strauss et al. 2002; Cipollini et al. 2003). A similar explanation has been invoked for the occurrence of secondary compounds in nectar (reviewed in Adler 2000), and a number of studies have supported this nonadaptive hypothesis with evidence that floral secondary compounds can be costly in terms of reduced pollination success and are often correlated with the compounds found in leaves (Strauss et al. 1999; Adler et al. 2006; Gegear et al. 2007; Kessler and Halitschke 2009; Manson et al. 2012). However, despite the importance of trait linkages among different plant parts in the ecology and evolution of plant-animal interactions, we know of only one study that has explored quantitative covariation in secondary compounds that occur in both leaves and fruits of a fleshy-fruited species (Cipollini et al. 2004).

Regardless of their evolutionary raison d'etre, fruit secondary compounds are likely to play a key role in structuring interactions between plants and multiple classes of seed-dispersing and non-seed-dispersing frugivores. Thus, it is surprising that there is so little information on the qualitative and quantitative variation in fruit chemical traits in natural populations. Rigorous examination of plant secondary chemistry has played a central role in developing theories of plant-herbivore interactions (reviewed in Schoonhoven et al. 2005) and, more recently, in studies of plant-pollinator interactions (Strauss et al. 1999; Adler 2000; Irwin and Adler 2008). An increased emphasis on the role of secondary chemistry in fruit-frugivore interactions could contribute greatly to our understanding of seed dispersal mutualisms, evolutionary trade-offs, and plant defense theory. In particular, to address the importance of fruit chemical traits in plant/animal interactions, we need more information on chemical variation in natural populations at the intraspecific scale, since it is this variation that provides the basis for natural selection (Izhaki et al. 2002; Tewksbury 2002).

In this study, we examined patterns of individual-level variation in one group of plant secondary compounds, the iridoid glycosides (IGs), in the leaves, unripe fruits, and ripe fruits of a hybrid bush honeysuckle (*Lonicera × bella*

Zabel, Caprifoliaceae). In addition, we monitored fruit damage by insects and microbes as well as fruit disappearance (presumably due to birds and other potential dispersers) in natural populations over two growing seasons to determine whether IG concentrations are correlated with patterns of fruit damage and removal. These data were used to address two alternative hypotheses: (1) fruit secondary compounds provide an adaptive benefit in the context of fruit-frugivore interactions or (2) fruit secondary compounds are nonadaptive and their presence is best explained as a consequence of foliar defense. Specifically, we considered the evidence for the following patterns, which, if found, would provide support for an adaptive role of fruit secondary compounds: (1a) certain compounds are unique to fruits, (1b) quantities of secondary compounds in fruits are higher than those in leaves, (1c) quantities of secondary compounds in fruits are independent of those in leaves, and (1d) fruit secondary compounds are negatively correlated with levels of fruit damage or positively correlated with levels of fruit removal by dispersers in natural populations. Alternatively, if fruit secondary compounds are a consequence of foliar defense, then the following patterns might be observed: (2a) fruit secondary compounds represent a subset of those found in leaves, (2b) quantities of secondary compounds in leaves are higher than those in fruits, (2c) quantities of secondary compounds in fruits are correlated with those in leaves, and (2d) fruit secondary compounds have no effect on fruit/frugivore interactions or are negatively correlated with fruit removal rates by dispersers.

Methods

Study System and Site

Lonicera × bella is a hybrid bush honeysuckle that can be an aggressive invader in much of the United States (USDA-PLANTS 2011). Its parental species were both introduced to the country as ornamentals—*Lonicera tatarica* from Russia in 1752 and *Lonicera morrowii* from Japan in 1854 (Barnes and Cottam 1974). The hybrid species was first described in 1889 and is thought to have arisen repeatedly wherever the two parental species co-occur (Rehder 1903; Hauser 1966; Barnes and Cottam 1974). Although all three species have long been problematic invaders in the eastern and central United States (Ingold and Craycraft 1983; Woods 1993), their establishment in western states has been relatively recent (Sperger 2003; USDA-PLANTS 2011). In our study area of Colorado, herbarium records indicate *L. morrowii* was well established in 1973 (COLO 2011); however, based on our own comparative examination of local specimens and specimens of the parental species collected from their native

ranges (obtained from the Arnold Arboretum of Harvard University; BG-BASE 2011), we believe that most local specimens likely have some degree of hybrid origin. For the purposes of this study, we considered any individual with pink flowers and sparsely pubescent leaves and peduncles to be of hybrid origin (Hauser 1966), and our identifications were confirmed by two local authorities (T. Hogan and D. Clark, COLO Herbarium, University of Colorado Museum of Natural History). Voucher collections from each study population are available at the University of Colorado Natural History Museum (COLO; accession numbers 543191, 543192, and 543193).

Our study populations of *L. × bella* were located at three sites in Boulder County, Colorado: Bluebell Canyon (39.99135N, −105.28568W), Gregory Canyon (39.99727N, −105.2940W), and Skunk Canyon (39.98611N, −105.27660W). All three sites can be described as Foothills Riparian, with a mosaic of *Pinus ponderosa* woodland and open areas dominated by shrubs, grasses, and forbs. *Lonicera × bella* is well established in these areas, particularly along streams. It fruits in abundance in late summer, and fruits are often persistent on the plant into the fall and winter. The seeds are thought to be dispersed primarily by birds but are also consumed by mammals such as white-tailed deer (Vellend 2002; Drummond 2005; Bartuszevige and Gorchov 2006; McCay et al. 2009; S. R. Whitehead, personal observation). Fruits are damaged by a variety of insect seed predators, primarily hemipterans such as the box-elder bug *Boisea trivittata* (Rhopalidae) and several species in the family Pentatomidae (S. R. Whitehead, personal observation).

Leaves and fruits of *Lonicera* contain two important classes of secondary compounds, phenolics and iridoid glycosides (Ikeshiro et al. 1992; Zadernowski et al. 2005). Here we focus on IGs, which have well-documented antifeedant activity toward insect herbivores, as well as antimicrobial effects (Bowers 1991; Marak et al. 2002*a*, 2002*b*). Our previous research has shown that *L. × bella* inherited a diversity of IGs from both parental species and that these compounds occur in both leaves and fruits (Whitehead and Bowers 2013*b*). However, past work on the ecological role of IGs has focused primarily on leaves (e.g., Peñuelas et al. 2006; Jamieson and Bowers 2010; Quintero and Bowers 2011), and to our knowledge their importance in mediating seed dispersal and fruit defense is unexplored.

Field Observations

Ten individuals of *L. × bella* from each of the three study populations described above (*N* = 30 plants) were monitored from June to September in 2007 and 2008. Twenty-four individuals were monitored throughout both seasons, and six individuals were replaced in 2008 due to poor regrowth or damage by trail maintenance activities. On each shrub, we marked two branches during early fruit development that were approximately 25 cm in length and contained 20–150 fruits. We visited the same plants every 1–3 weeks until most fruits had either disappeared or rotted. Each plant in the study population was visited on six occasions during 2007 and on 13 occasions during 2008. At each visit, we recorded the number of maturing unripe and ripe fruits remaining on marked branches, the number that were aborted, and the number of maturing fruits that were visibly affected by two categories of fruit pests: insects (primarily piercing/sucking hemipterans that left visible feeding scars) and microbes (causing discoloration or fruit rot). For the purposes of our study, we assumed that most ripe fruits that disappeared from the branch were removed by potential seed dispersers. Although a small proportion of the fruits may have fallen off the shrub, we believe that fruit senescence during the monitoring period was rare for three reasons: (1) we regularly searched the ground below plants and found no fallen fruits, (2) we never observed fruits falling while we were handling the shrub to count fruits, and (3) fruits that were aborted by the plant remained attached to the branch for extended periods (sometimes months) and were not included in our counts of fruit disappearance.

Collection of Plant Material for Chemical Analysis

We collected samples for chemical analysis from each plant in our study population once in 2007 and once in 2008. We harvested two branches from each shrub, and each branch was separated into leaves, unripe fruits, and ripe fruits (for a total of 360 samples). Because one of our main objectives was to compare the chemical traits of different plant parts, we collected branches when fruits were at midripening, and leaves, unripe fruits, and ripe fruits could be obtained from the same branch at the same time. Ripening times were variable among shrubs; thus, we collected branches between mid-July and late August depending on the individual. Samples were stored in a cooler until we returned to the laboratory (always within 3 h of collection). All plant material was then weighed and oven-dried at 50°C to constant mass (IGs are thermally stable at this temperature; see Gardner and Stermitz 1988; Lampert and Bowers 2011; Mraja et al. 2011). Dry fruits were first ground through a fine-mesh screen that allowed us to remove all seeds from the sample, leaving only pulp and skin, followed by further grinding to a fine powder in a mortar and pestle. Leaves were ground to a fine powder using only a mortar and pestle.

Quantification of Iridoid Glycosides

Methods for quantification of IGs were modified from previously described methods (Gardner and Stermitz 1988; Bowers and Stamp 1993) and are described in detail in Whitehead and Bowers (2013*b*). Briefly, 25–50-mg aliquots of plant material were extracted in methanol for 24 h, filtered, and partitioned between water and ether to remove hydrophobic compounds. Phenyl-β-D-glucopyranoside was added as an internal standard, and IGs were derivatized to their trimethylsilyl analogues using Tri-Syl-Z (Pierce Chemical, Rockford, IL). Quantities were determined based on total ion current using an HP Agilent 6890N gas chromatograph (GC) coupled with an Agilent 5975C mass spectrometer (MS). We quantified six IGs by comparison with authentic reference standards (loganin, secologanin, loganic acid, sweroside, secoxyloganin, and morroniside), which together represented ~88% of the total IG content. One other major component (kingiside) and six minor compounds were also identified as IGs based on MS profiles (Inouye et al. 1976; Popov and Handjieva 1983) and their quantities estimated by assuming a response factor equivalent to our internal standard.

Statistical Analysis

First, to examine whether secondary compounds in unripe and/or ripe fruits can be unique (prediction 1a) or represent a subset of those found in leaves (prediction 2a), we used qualitative (presence or absence) comparisons of IG occurrence, combined with an examination of multivariate chemical similarity among plant parts using nonmetric multidimensional scaling (NMDS). NMDS is an ordination technique that first calculates a distance matrix for each observation and then uses an iterative process to find a spatial configuration of the data points that best matches the rank similarities in the distance matrix (Zuur et al. 2007). NMDS is better than other ordination methods at preserving high-dimensional structure with a few axes, thus providing a simple graphical output in which points that are close to one another are more similar than those that are farther apart (Zuur et al. 2007). For the purpose of the NMDS, we included only the mean concentration of each individual IG for each plant (averaging between branches and between years). For those individuals that were monitored in only one year, we included only the average for that year, resulting in a total sample size of $N = 36$ individuals for this analysis. The three-dimensional NMDS ordination was based on the Bray-Curtis dissimilarity index and used 200 replicates with random starting coordinates (Minchin 1987). This was conducted using the vegan package in R (Oksanen et al. 2010; R Development Core Team 2012).

Next, to determine whether IG quantities were higher in fruits (prediction 1b) or in leaves (prediction 2b), we compared total IG quantities among plant parts using a linear mixed model fitted by maximum likelihood (Crawley 2007). IG quantities (as a proportion of dry weight) were first logit transformed to meet linear modeling assumptions (Warton and Hui 2011). Plant part was specified as a fixed effect, and random effects accounted for both the nested effects of plant parts within individuals within populations and the effect of sampling year (2007 or 2008). A number of samples had to be excluded due to insufficient material for chemical analysis, resulting in a total of $N = 316$ samples from 58 individuals for this analysis. To test for overall differences in IG concentrations among plant parts, we used a likelihood ratio test to compare the model that included plant part as a fixed effect to a null model that included the random effects only (Crawley 2007). We also used this analysis to examine the variance structure of our chemical data, that is, the proportion of total IG variation that can be attributed to each random effect. To examine pairwise differences among plant parts, we followed this analysis with a Tukey's HSD post hoc test. These analyses were conducted using the lme4 and multcomp packages in R (Bates and Maechler 2010; Hothorn et al. 2011; R Development Core Team 2012).

Third, to determine whether IG concentrations in leaves and fruits were independent (prediction 1c) or correlated (prediction 2c), we tested for correlations in IG concentrations between leaves and unripe fruits, leaves and ripe fruits, and unripe fruits and ripe fruits using nonparametric Kendall's rank correlations (individual IG concentration data were not transformable to fit linear modeling assumptions). These were conducted separately for the total IG concentration as well as for all individual IG components that occurred in both leaves and fruits (for a total of 33 separate correlation analyses). To account for multiple inferences in this family of tests, we controlled for false discovery rate at $\alpha = 0.05$ using methods described in Benjamini and Hochberg (1995). These analyses were conducted using JMP, version 9 (SAS Institute, Cary, NC).

To determine whether fruit IG concentrations were associated with patterns of fruit damage and disappearance in natural populations (predictions 1d and 2d), we used a two-step analysis. First, because our field observations involved repeated measures during each of two fruiting seasons, we created composite damage and disappearance scores for each individual plant in each year. To do this, we analyzed the levels of fruit damage and disappearance on each individual plant relative to all other plants measured in that year using generalized linear mixed models (GLMMs). These analyses were conducted separately for insect damage, microbe damage, and fruit disappearance

for both 2007 and 2008. Our response variables were binomial counts of the number of damaged or removed fruits on a branch and the number of not damaged or removed fruits, which we modeled using the binomial distribution with the logit link function. In all models, individual was specified as a fixed effect, and random effects included the nested effects of branches within individuals within populations and the continuous effect of sampling date (repeated measures on each branch). We specified a priori contrast coefficients to compare each individual to the overall mean for all other individuals in that year. Thus, the output of the GLMMs gave us effect size estimates for each individual plant that can be interpreted as relative fruit damage or disappearance scores: negative effect sizes indicated plants with lower than average rates of fruit damage or disappearance, while positive effect sizes indicated plants with higher than average rates of fruit damage or disappearance. These analyses were conducted using the lmer package in R (Bates and Maechler 2010; R Development Core Team 2012).

In part two of the analysis, we examined the relationships between total and individual IG concentrations and the relative damage and disappearance scores using mixed model regressions. To obtain a representative measure of IG concentration for each plant in each year, we first averaged our IG data from the two replicate branches collected from each individual. We then considered the effects of unripe fruit IGs on both insect and microbe damage as well as the effects of ripe fruit IGs on microbe damage and fruit disappearance. We did not consider the effects of ripe fruit IGs on insect damage because almost all insect damage occurred in unripe fruits. A number of samples were excluded from these analyses due to insufficient field data or insufficient material for chemical analysis, resulting in sample sizes of $N = 56$ for insect damage, $N = 46$ for microbe damage, and $N = 55$ for removal. The sample size for microbes was relatively low due to insufficient field data in 2007; monitoring was less frequent in 2007, and we excluded all data points for which there were not at least two time periods in which microbe damage was observed. To examine the relationship between total IGs and fruit damage/disappearance scores, we used linear mixed models that included IG concentration as a fixed effect and individual plant as a random effect (to control for repeated measures on individuals in 2007 and 2008). We did not include population as a random effect in these models due to very low levels (<3%) of variation explained by population-level effects (see "Results").

Using the same data set, we also examined the multivariate effects of the 12 individual IGs on fruit damage/disappearance scores using principal component (PC) regressions, which can provide a more robust alternative to multiple regressions or separate individual regressions

when predictor variables are highly correlated (Graham 2003), as was the case with individual IGs (correlations among compounds are reported in tables A1, A2; tables A1–A5 available online). In PC regressions, orthogonal PC axes are calculated for the predictor variables (i.e., the 12 quantified IGs) independently of the response variable (i.e., fruit damage or disappearance scores), and the PCs are then used as the predictor variables in multiple regressions. The PC analyses provided 10 orthogonal axes of variation for unripe fruits (explaining 98.74% of the IG variation) and nine orthogonal axes for ripe fruits (explaining 96.6% of the IG variation; eigenvalues, percent of variance explained by each PC, and axis loadings are shown in tables A3, A4; fig. A1, available online). This portion of the analysis was conducted using JMP, version 9 (SAS Institute, Cary, NC). To identify which PCs were important predictors of fruit damage or removal scores, we used an information theoretic approach (Burnham and Anderson 2002; Grueber et al. 2011; Hegyi and Garamszegi 2011). We first defined a global model that included all of the significant PCs as fixed predictor variables and then ranked all possible subsets of the model using corrected Akaike's Information Criterion (AICc) based on maximum likelihood. Individual was included as a random effect in all models to control for the nonindependence of multiple measures from each plant (one from 2007 and one from 2008). From all possible submodels, we retained a candidate set that included all models with ΔAICc < 2 (a complete list of all candidate models is provided in table A5). We then used model averaging based on Akaike weights for all candidate models to estimate coefficients and confidence intervals for all retained predictor variables. The significance of the predictor variables was determined using z statistics. These analyses were conducted using the MuMIn package in R (Bartoń 2011; R Development Core Team 2012).

Results

Support for Prediction 1a: Certain Compounds Are Unique to Fruits

Our GC/MS analyses revealed a total of 12 IGs in the fruits and leaves of *Lonicera × bella* (fig. 1). Of these compounds, nine occurred in both fruits and leaves, and three were unique to fruits (morroniside, kingiside, and unknown F). We did not detect any IGs that were unique to leaves. The NMDS analysis examining overall chemical similarity among plant parts revealed that the chemical profile of leaves is distinct from that of fruits, but there is substantial overlap in the chemical profiles of unripe fruits and ripe fruits (fig. 2). Data underlying the NMDS
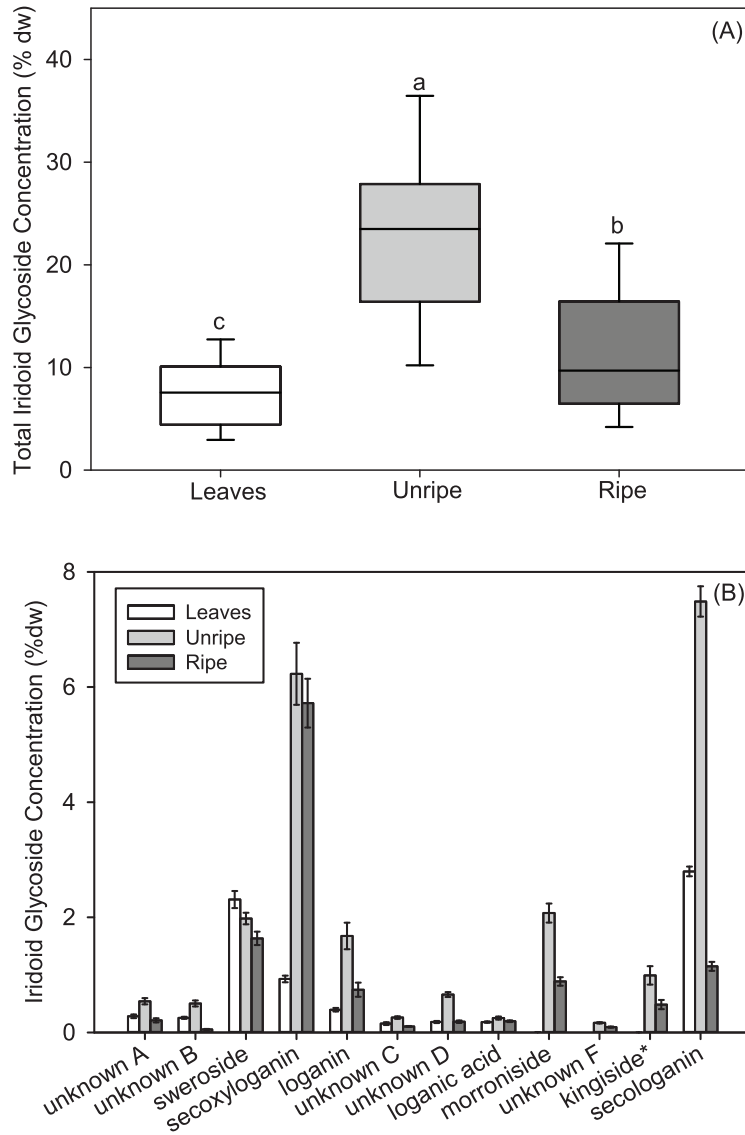
Figure 1: Average iridoid glycoside (IG) concentrations (percentage of dry weight [dw]) in leaves, unripe fruits, and ripe fruits of *Lonicera* × *bella*. *A*, Box-and-whisker plots show the median, twenty-fifth and seventy-fifth percentiles, and range of total IG concentrations from $N = 30$ plants. Letters represent significant differences from Tukey's HSD post hoc comparisons among plant parts. *B*, Mean ($\pm$ SE) concentration is shown for all detected IGs. The asterisk represents tentative identification.

are available from the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.29560 (Whitehead and Bowers 2013*a*).

Support for Prediction 1b: IG Concentrations Are Higher in Fruits than in Leaves

Plant part was a significant predictor of total IG concentration ($\chi^2 = 95.57$, df $= 2$, $P < .0001$), and post hoc comparisons among leaves, unripe fruits, and ripe fruits showed all three plant parts were significantly different from each other, with the highest concentrations in unripe fruits, lower concentrations in ripe fruits, and lowest concentrations in leaves (fig. 1*A*). The random effects in our mixed model showed that 2.43% of the variation in total IG content was explained by population, 22.22% was explained by individual, 22.52% was explained by plant part, 7.64% was explained by year, and 45.19% was residual variation. Data underlying the plant part comparisons are available from the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.29560 (Whitehead and Bowers 2013*a*).
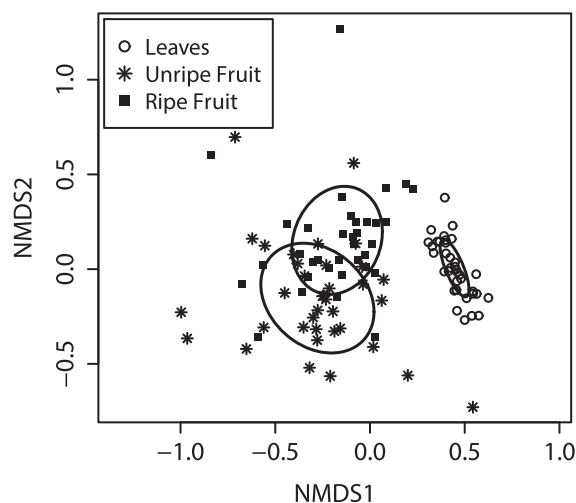
Figure 2: Nonmetric multidimensional scaling (NMDS) ordination plot of overall chemical similarity among plant parts (three-dimensional final stress = 12.67, R^2 = 0.59). Ellipses represent 95% confidence intervals for group centroids (± 1 SD).

Support for Predictions 1c and 2c: Certain IGs Are Correlated between Fruits and Leaves, and Others Are Independent

Total IG concentrations were correlated between leaves and unripe fruits, not correlated between leaves and ripe fruits, and strongly correlated between unripe fruits and ripe fruits (table 1). These patterns were variable for the individual compounds examined; 6 of 12 IGs were correlated between leaves and unripe fruits, 3 of 12 IGs were correlated between leaves and ripe fruits, and 12 of 12 IGs were correlated between unripe fruits and ripe fruits (table 1). Data underlying the IG correlations are available from the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.29560 (Whitehead and Bowers 2013a).

Support for Prediction 1d: Fruit IGs Are Associated with Reduced Fruit Damage

The relative insect damage score tended to decrease with increasing total IG concentration, but the effect was not significant ($F_{1,38.9}$ = 2.85, P = .0996; fig. 3A). In our examination of the effects of unripe fruit individual IGs (combined as PCs) on fruit damage from insects, model selection using AICc resulted in a candidate set of eight models with ΔAICc < 2 (table A5). The top model had an Akaike weight of ω_i = 0.20 and included PC1 (explaining 32.24% of IG variation), PC2 (explaining 16.84% of IG variation), and PC4 (explaining 10.24% of IG variation) as predictor variables; however, model averaging among the entire candidate set indicated that only PC1

(z = 2.692, P = .0071) and PC2 (z = 3.072, P = .0021) were significant predictor variables, both of which had a negative effect on insect damage (table 2; fig. 3B, 3C). Data underlying this analysis are available from the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.29560 (Whitehead and Bowers 2013a).

Total IGs in ripe fruits had a significant negative effect on the relative microbe damage score for each plant ($F_{1,40.5}$ = 5.37, P = .0256; fig. 4A). In our examination of the effects of ripe fruit individual IGs (combined as PCs) on fruit damage from microbes, model selection using AICc resulted in a candidate set of eight models with ΔAICc < 2 (table A5). The top model had an Akaike weight of ω_i = 0.23 and included PC3 (explaining 13.13% of IG variation), PC6 (explaining 6.68% of IG variation), and PC7 (explaining 5.59% of IG variation) as predictor variables; however, model averaging among the entire candidate set indicated that only PC3 (z = 1.989, P = .0467) had a significant negative effect on microbe damage score (table 2; fig. 4B). Data underlying this analysis are available from the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.29560 (Whitehead and Bowers 2013a). Total IGs in unripe fruit had no effect on microbe damage score ($F_{1,40.2}$ = 0.13, P = .72), and model selection in the principal component regression resulted in a candidate set of 11 models with ΔAICc < 2. The top model had an Akaike weight of ω_i = 0.16 and included PC4 (explaining 10.69% of IG variation) and PC6 (explaining 6.68% of the IG variation) as predictor variables. However, model averaging among the entire candidate set indicated that neither of these variables had a significant effect on microbe damage score (data not shown).

Total IGs in ripe fruits had no effect on relative fruit disappearance scores ($F_{1,51.8}$ = 0.108, P = .743). In our examination of the effects of ripe fruit individual IGs (combined as PCs) on fruit disappearance, model selection using AICc resulted in a candidate set of eight models with ΔAICc < 2 (table A5). The top model had an Akaike weight of ω_i = 0.21 and included PC4 and PC6 as predictor variables; however, model averaging among the entire candidate set indicated that only PC4 (z = 2.200, P = .0278) had a significant positive effect on fruit disappearance (table 2; fig. 5). Data underlying this analysis are available from the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.29560 (Whitehead and Bowers 2013a).

Discussion

Many vertebrate-dispersed fruits contain high levels of secondary compounds (Herrera 1982), and increasing evidence has shown that these compounds can have important effects on both seed dispersers and fruit predators/

Table 1: Nonparametric Kendall's rank correlation coefficients and *P* values examining relationships among iridoid glycosides (IGs) in different plant parts

Compound ID	Leaves and unripe fruits		Leaves and ripe fruits		Unripe and ripe fruits	
	τ	Prob > $\|\tau\|$	τ	Prob > $\|\tau\|$	τ	Prob > $\|\tau\|$
Unknown A	.2347	.0011**	.22	.0023**	.4068	<.0001**
Unknown B	.1559	.0316*	−.0314	.6594	.3869	<.0001**
Sweroside	.2278	.0016**	.1813	.0061*	.5325	<.0001**
Secoxyloganin	.0655	.3658	.0951	.1534	.5214	<.0001**
Loganin	.1796	.0127*	.0772	.2437	.4008	<.0001**
Unknown C	.0329	.6499	−.0067	.9203	.4534	<.0001**
Unknown D	.1891	.0087*	.2267	.0007**	.3498	<.0001**
Loganic acid	−.0596	.4107	.0837	.2075	.2037	.0035*
Morroniside	...	...	...	...	.5789	<.0001**
Unknown F	...	...	...	...	.3902	<.0001**
Kingiside[a]	...	...	...	...	.642	<.0001**
Secologanin	.2405	.0009**	.0454	.5015	.3619	<.0001**
Total IGs	.1898	.0085*	.1278	.0533	.2831	<.0001**

Note: Asterisks for significance are based on a Holm's sequential Bonferroni correction for multiple inferences.
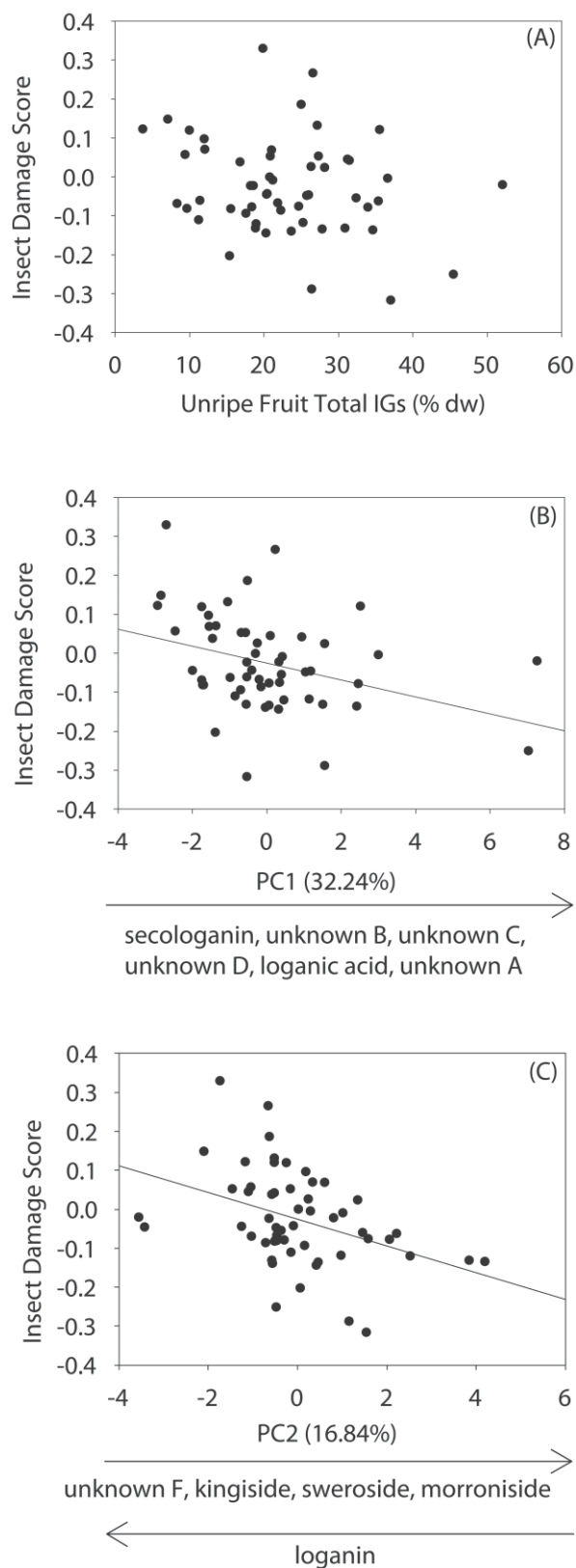
[a] Tentative identification.

* Significant ($P \leq .0316$).

** Highly significant ($P \leq .003$).

pathogens (reviewed in Levey et al. 2007). However, there is little evidence available to address the fundamental question of whether the occurrence of these compounds is the result of selective pressures in fruits or primarily a pleiotropic consequence of defense of other plant tissues (Cipollini and Levey 1997a; Eriksson and Ehrlen 1998). Our study represents the first detailed examination of quantitative chemical variation and covariation among leaves, unripe fruits, and ripe fruits in natural populations of a vertebrate-dispersed species, allowing us to explicitly test these alternative hypotheses. We provide multiple lines of evidence that fruit secondary compounds cannot be explained solely as a consequence of foliar defense: (1) of 12 individual IGs occurring in *Lonicera × bella*, three are unique to fruits, and none are unique to leaves; (2) most IGs are present in higher concentrations in fruits than in leaves; (3) concentrations of most IGs in ripe fruits are independent of concentrations in leaves; and (4) total IG concentration (for microbes) or most individual IG concentrations (for insects) are negatively correlated with fruit damage in natural plant populations. Together, these results strongly suggest that there has been selection for secondary compounds in fleshy fruits independent of selection in leaves and point to an adaptive role in fruit defense or seed dispersal. However, we also show that many compounds are shared between fruits and leaves and that, for a few compounds, the concentrations found in fruits and leaves are strongly correlated, emphasizing the existence of physiological linkages among different plant parts and the importance of considering the whole-plant context in chemical trait evolution.

Our results showing higher IG concentrations in fruits compared to leaves suggest that fruit defense may be a key factor in plant fitness. This supports the predictions of optimal defense theory (ODT), which suggests that plant parts with the highest fitness value, such as flowers and developing fruits, should be the most protected against herbivore attack (McKey 1974; Rhoades and Cates 1976; McCall and Fordyce 2010). The average total IG concentration in unripe fruits was extremely high (22.9% dry weight), approximately double the average concentration in ripe fruits (11.4% dry weight), and threefold higher than the average concentration in leaves (7.5% dry weight). In vertebrate-dispersed species, ODT also predicts that secondary compounds should disappear or diminish with ripening to allow for consumption by mutualistic animals, and this is commonly observed with fruit ripening in cultivated fruits (e.g., Friedman 2002). In accordance with this, our data show a marked reduction in total IG concentration with ripening; however, even in ripe fruits, IGs were present in higher concentrations than in leaves. It is also notable that the relative changes in IG concentration between unripe fruits and ripe fruits were variable among compounds (fig. 1*B*). Some major IG components (e.g., secoxyloganin) remained high even in ripe fruits; in fact, for roughly half of our samples, secoxyloganin concentration was higher in ripe fruits than in unripe fruits collected from the same branch at the same time. Thus, ripe fruit chemistry does not appear to be solely a consequence of leaf or unripe fruit chemistry but instead may be fine-tuned by selection for the quantities and ratios of

Figure 3: Effects of unripe fruit total iridoid glycosides (IGs; *A*) and significant predictor variables from a principal component (PC) regression of individual IGs (*B*, *C*) on the relative level of fruit insect damage. Insect damage scores represent a comparison of each individual plant to the average damage for all other plants monitored in that year, with negative scores representing lower than average damage and positive scores representing higher than average damage. *B*, *C*, The independent variables are PC axes based on 12 individual IGs. The percent variation in total IGs explained by each PC axis is indicated in parentheses, and the primary compounds loading on each axis are indicated by arrows below the *X*-axis label. Detailed PC analysis results are provided in tables A3, A4 and figure A1, available online.

compounds that maximize seed dispersal success while minimizing costs.

We show that the concentrations of most individual IGs in ripe fruits are independent of the concentrations in leaves. However, there were strong correlations for a few compounds, and many more were correlated between unripe fruits and leaves (table 1). IGs can be phloem mobile (Gowan et al. 1995); thus, the correlations among plant parts may be due to physiological linkages (i.e., the transport of compounds among plant parts) or to genetic linkages (i.e., correlations in the expression of chemical traits in different plant parts). These linkages do not preclude an adaptive role for secondary compounds in both leaves and fruits; because plants are under simultaneous selection from herbivores, pollinators, seed dispersers, competitors, and abiotic factors, an efficient strategy may be to produce compounds with multiple ecological functions that can be expressed throughout the plant (Izhaki 2002). However, our results showing the presence of compounds unique to fruits and the lack of correlation between leaves and fruits for certain compounds suggest that plants can control chemical trait expression in leaves and fruits independently in some cases.

One important factor that may influence both the qualitative and quantitative expression of IGs in different plant parts of *L.* × *bella* is the plant's evolutionary history as a hybrid species (Orians 2000; Cheng et al. 2011). Because *L.* × *bella* can readily backcross with parental species (Hauser 1966; Barnes and Cottam 1974) and various escaped cultivars may have contributed to our study population, the genetic background of our plants may be quite diverse. Our data do show high variation in IGs (fig. 1*A*), and it is unclear to what extent this variation may be due to hybridization. However, the general patterns of IG occurrence, with certain compounds unique to fruits and higher overall concentrations in fruits, holds for *L.* × *bella* and both its parental species (Whitehead and Bowers 2013*b*), suggesting that these patterns are shaped by past selection pressures in the parental species rather than being

Table 2: Estimated effects of individual iridoid glycosides (combined as principal components [PCs]) on fruit damage and disappearance scores based on multimodel averaging across a candidate set of top models with $\Delta AICc < 2$

| Response	Model-averaged coefficients	SE	z value	$\Pr(>\lvert z\rvert)$	Relative importance
Insect damage:					
Intercept	−.025731	.014482	1.715	.08628	
PC1	−.021779	.007432	2.692	.0071	1
PC2	−.034383	.010281	3.072	.00212	1
PC4	.020945	.013089	1.466	.1427	.52
PC3	−.016337	.012672	1.182	.23729	.25
PC5	.014717	.013442	1.004	.31557	.2
PC7	.019306	.021406	.827	.40838	.16
Microbe damage:					
Intercept	−.043331	.010556	3.959	<.0001	
PC3	−.020087	.008504	1.989	.0467	1
PC6	.023934	.011884	1.69	.091	.9
PC7	−.025266	.012984	1.632	.1026	.88
PC4	.011699	.0093	1.043	.2971	.14
PC8	−.016217	.014669	.916	.3595	.11
PC5	−.011258	.010891	.857	.3915	.1
PC1	−.005635	.005608	.833	.4049	.1
PC2	−.007082	.00775	.757	.4488	.09
Fruit disappearance:					
Intercept	−.06012	.02887	2.01	.0444	
PC4	.06048	.02551	2.2	.0278	1
PC7	.05987	.03479	1.594	.111	.64
PC6	.03413	.03099	1.02	.3076	.22
PC3	−.02651	.02313	1.062	.2883	.21
PC2	.0209	.02121	.911	.3622	.1
PC1	−.01163	.01523	.706	.4802	.08

Note: See table A5, available online, for candidate models.

an artifact of genetic recombination. Future research examining how fruit chemical traits correlate with patterns of genetic introgression and reproductive success in hybrids may provide important insights into the current selective pressures being exerted on *L. × bella* and the evolution of invasiveness in this genus.

With regard to the potential ecological role of secondary compounds in fruit defense, our data illustrated that higher levels of total IGs and/or particular combinations of individual IGs in unripe and ripe fruits were associated with lower levels of damage by insects and microbes (figs. 3, 4), supporting other studies that suggest an important defensive role of secondary compounds in fruits (e.g., Cipollini and Levey 1997*b*; Schaefer et al. 2008; Tewksbury et al. 2008). Because *L. × bella* is a nonnative plant that arrived relatively recently to our study area, most fruit damage likely comes from generalist fruit-feeding insects and pathogens that have a limited ability to tolerate or detoxify the high levels and diverse mixture of IGs we detected in fruits. The selective pressures exerted by these organisms may be very different from those in the native

ranges of the parental species, where there is a higher potential for coevolved specialists that may use IGs as feeding cues or even sequester IGs to provide protection against their own natural enemies (Bowers 1991). Thus, it is not clear from our results what the specific selective forces may have been that shaped fruit chemical traits in this species, only that IGs can influence interactions with the broad classes of generalist insects and pathogens that attack fruits. Understanding the specific effects IGs have on individual organisms and how this may relate to the evolutionary success of *L. × bella* will require further research, such as experimental bioassays that manipulate the concentrations of IGs in the diets of consumers. Our data suggest that the effects of individual compounds are complex and not necessarily limited to the most abundant compounds present (figs. 3, 4), and thus studies of this type should consider the combined effects of the suites of defensive compounds found in plants.

Our analyses of the effects of IGs on fruit disappearance revealed no clear trends. There was no relationship between total IGs and disappearance, and the four individual
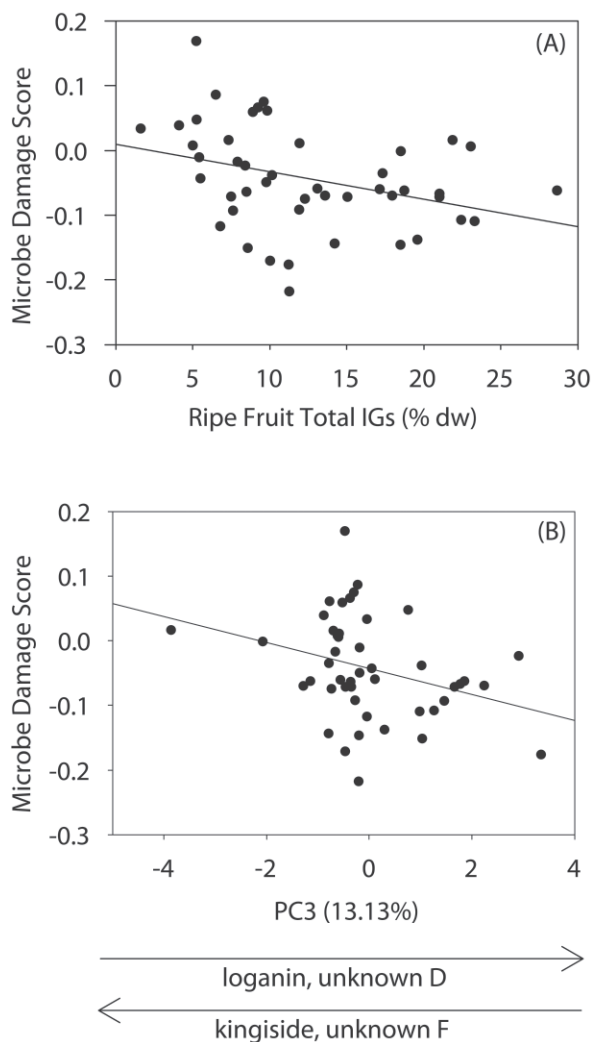
Figure 4: Effects of ripe fruit total iridoid glycosides (IGs; *A*) and significant predictor variables from a principal component (PC) regression of individual IGs (*B*) on the relative level of fruit microbe damage. Microbe damage scores represent a comparison of each individual plant to the average damage on all other plants monitored in that year, with negative scores representing lower than average damage and positive scores representing higher than average damage. *B*, The independent variable is a PC axis based on 12 individual IGs. The percent variation in total IGs explained by the axis is indicated in parentheses, and the primary compounds loading on the axis are indicated by arrows below the *X*-axis label. Detailed principal component analysis results are provided in tables A3, A4 and figure A1.

compounds (those loading on PC4) that appear to correlate with disappearance rates had mixed effects (fig. 5). It is possible that the IGs in ripe fruits may be able to defend fruits against antagonists with minimal negative effects on mutualist seed dispersers (the microbe-pest specificity model; sensu Cipollini and Levey 1997*a*). Alternatively, we may not have detected strong negative ef-

fects of IGs on fruit removal due to confounding variables, in particular the difficulty in relating fruit disappearance to removal by seed dispersers. Although we know of no studies that have examined the effects of IGs on frugivorous birds, there is evidence that predatory birds are deterred by insects that sequester IGs from their host plants (Bowers 1980), and therefore it is likely that at least some frugivores are deterred by IGs in fruits. It is important to note that the overall fruit disappearance rates were fairly low (averaging only 40% of the total fruit crop), and the majority disappeared late in the season. Past work on bird dispersal of *Lonicera* in the eastern United States has also shown that most fruits are removed late in the season once higher-quality native fruits have disappeared (White and Stiles 1992; Drummond 2005). Thus, regardless of frugivore preferences, one successful dispersal strategy for a nonnative shrub with uncertain dispersal opportunities may be to have more chemically defended fruits that remain available and relatively undamaged later in the season. Further elucidation of the specific role of IGs in the multifaceted aspects of seed dispersal will require an integrative approach to understanding frugivory that incor-
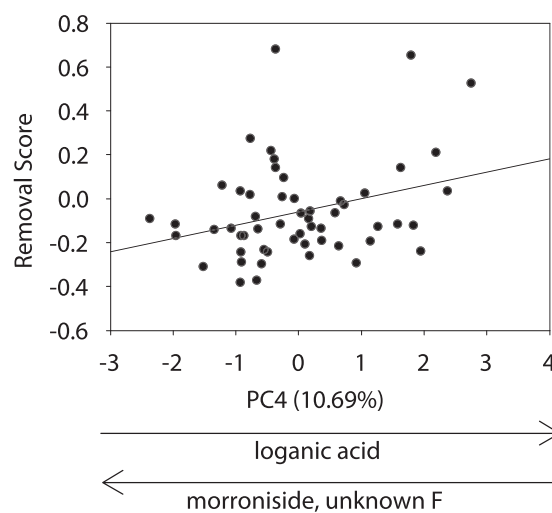
Figure 5: Effects of ripe fruit iridoid glycosides (IGs) on the relative level of fruit disappearance. Disappearance scores represent a comparison of fruit disappearance on each individual plant to the average disappearance on all other plants monitored in that year, with negative scores representing lower than average disappearance and positive scores representing higher than average disappearance. The *X*-axis shows PC4, which was the only significant predictor variable in a principal component (PC) regression based on 12 individual IGs (see main text). The percent variation in total IGs explained by the axis is indicated in parentheses, and the primary IGs loading on this axis are indicated by arrows below the *X*-axis label. Detailed principal component analysis results are provided in tables A3, A4 and figure A1.

porates fruit availability, feeding preferences, and community context.

An important consideration in the interpretation of the effects of individual IGs on fruit damage and disappearance in our study (figs. 3–5) is the shared biosynthetic pathways among different compounds. Past research on IGs in *Lonicera* has suggested a biosynthetic route from loganin and/or loganic acid to secologanin to other secoiridoids such as morroniside, sweroside, secoxyloganin, and kingiside (Takeda and Inouye 1976; Uesato et al. 1984). The fact that some of the quantified IGs in our study are the precursors to other more complex IGs could explain why some compounds are negatively correlated with each other (tables A1, A2; fig. A1) and thus may appear to have opposite effects on fruit damage/disappearance in PC regressions (figs. 3–5). Because the bioactivity and relative toxicity of different IGs along this biosynthetic route may vary considerably, it is possible that plants may convert existing IGs to modified forms as fruits develop in order to optimize seed dispersal success.

Finally, one other important factor that may increase the complexity of relationships among IGs and how they relate to patterns of damage/disappearance is that IGs are important as both constitutive and induced plant defenses and can vary in response to herbivory (Darrow and Bowers 1999; Fuchs and Bowers 2004; Peñuelas et al. 2006; Quintero and Bowers 2011; but see Jarzomski et al. 2000; Bennett et al. 2009). Induced defenses have traditionally been studied in leaves, and there is little information on the potential for induced defenses in vertebrate-dispersed fruits in response to direct fruit damage or leaf damage (Whitehead and Poveda 2011). While it is unknown whether IGs in fruits or leaves of *L.* × *bella* vary in response to damage, data from other studies have shown that induced changes in IG concentrations, when they are detectable at all, are small relative to constitutive levels, are localized on the plant, and last for a short period of time (Fuchs and Bowers 2004; Quintero and Bowers 2011). Thus, our sampling scheme of taking large numbers of fruits from each of two different branches on the shrub and sampling the same individual in 2007 and 2008 should have allowed us to capture consistent constitutive differences among individual plants. We measured fruit disappearance and damage over the entire season and created composite scores for each plant that encompassed the entire series of measurements; however, we captured only a snapshot of IG chemistry at a single point in time in each season. The fact that we did still find negative correlations between IGs and damage despite the potential for unexplained chemical variation due to induced responses is evidence that constitutive variation in IGs among individuals is important in determining susceptibility to insect and microbial attack. Additional controlled experiments

examining the effects of multiple feeding guilds on fruit secondary chemistry will be necessary to disentangle the relative importance of constitutive versus induced defense in different plant parts and how sequential damage to fruits over a growing season may alter the outcome of interactions with both antagonistic and mutualistic fruit consumers.

In conclusion, our results provide strong evidence that secondary compounds in fruits cannot be explained solely as a consequence of foliar defense but instead are likely to serve an adaptive function in the context of fruit/frugivore interactions. We emphasize that evidence for adaptive function does not preclude linkages among chemical traits in fruits and other plant parts and expect that many compounds present in fruits likely have multiple ecological roles. We hope this study will inspire further research that empirically addresses how fruit secondary compounds affect plant interactions with a broad range of antagonistic and mutualistic fruit consumers as well research on the linkages between leaf and fruit chemical traits in other systems. Studies of this nature are essential to understanding the true fitness costs and benefits of chemical traits and would have important implications for our understanding of the ecology and evolution of plant-animal interactions.

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Lonicera × *bella* with ripe fruits at one of the study sites in Colorado. Photograph by Susan R. Whitehead.