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Defense Trade-offs in the Evolution of the Fruits and Flowers of Genus Cornus

Danielle R. De La Pascua
University of Central Florida

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CHEMICAL DEFENSE TRADE-OFFS IN THE FRUITS AND FLOWERS OF
GENUS *CORNUS*

by

DANIELLE DE LA PASCUA

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Abstract

The optimal defense hypothesis predicts that the allocation of plant defenses across plant organs is proportional to the importance of a given organ to plant fitness. Despite this, much less work has been devoted to the study of reproductive defenses in plants relative to vegetative structures like leaves. This study examines the apparancy hypothesis and the resource availability hypothesis using a phylogenetic comparative approach within the genus *Cornus*. During the 2016 growing season, plants of 25 species of *Cornus* were tracked for flower and fruit phenology as well as sampled for floral and fruit tissue in a common garden experiment at the Arnold Arboretum of Harvard University. This tissue was used to quantify floral and fruit defensive chemistry (e.g. tannin activity, total phenolics, total flavonoids, titratable acidity), and fruit palatability traits (e.g. water, sugar, lipid, and protein content), and the color of reproductive structure using reflectance spectroscopy. Native habitat environmental data was obtained using digitized herbarium records and publicly available environmental data layers. Trait-trait and trait-environment relationships were assessed with phylogenetic generalized least squares regression. The evolution of later flowering phenology was correlated with increased floral phenolics and tannins, and the evolution of increased fruiting duration was correlated with increased fruit tannins, both supporting the apparancy hypothesis. Additionally, the evolution of higher fruit sugar content was correlated with higher fruit tannins, and a strong evolutionary trade-off between the production of tannins and the production of flavonoids was observed. With respect to habitat, floral and fruit flavonoids and tannins were consistently lower in species native to

warmer environments, while fruit phenolics and was higher in drier environments, which may

support the resource availability hypothesis.

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Introduction

Plants are subject to attack by a wide variety of natural enemies. Unlike animals, plants cannot move to flee these pressures. Though they must remain stationary, plants utilize a wide variety of both physical and chemical defenses. This has been observed to range widely throughout the plant kingdom. One of the most studied defense strategies is the use of secondary metabolites including phenolic, flavonoid, and tannin compounds, which have been hypothesized to make plants unpalatable or toxic to herbivores or to resist fungal and/or bacterial infection (Harborne and Turner 1984; Bhattacharya et al. 2010; Crozier et al. 2006; Ayman et al. 2013). Phenolics belong to a very large families of secondary metabolites, and contribute to resistance against microorganisms, herbivores, and insects (Ayman et al. 2013; Arif et al. 2009; Hammerschmidt 2005). Like phenolics, tannins are a class of constitutive secondary metabolites that contribute to plant defense in several ways, including forming complexes with proteins to inhibit protein assimilation (Cipollini and Levey 1997; Bernays et al. 1989; Butler 1989). Flavonoids are another class of secondary metabolites that are important for defense in addition to a wide number of other functions such as UV defense, anti-fungal activity, and pollinator and bird attraction through flower and fruit color (Moore et al. 2014; Halbwirth et al. 2009; Gould et al 2006; Harborne 1993). There are many hypotheses surrounding how plants allocate resources in light of diverse ecological strategies (Rhoades 1979; Coley et al. 1985; Stamp 2003). Two of the most prominent are termed the optimal defense hypothesis and the resource availability hypothesis.

The optimal defense hypothesis and the resource availability hypothesis

The optimal defense hypothesis addresses how the defensive needs of a plant contribute to the evolution of secondary metabolites, with costs of defense paid to maximize plant fitness (Rhoades 1979). The first part of this hypothesis states that organisms evolve and allocate resources in a way that maximizes individual fitness. This hypothesis has been under some scrutiny because of the apparent difficulty for researchers to be able to quantify and falsify the claim (Stamp 2003). The second part of this hypothesis states that plant defenses are costly because they divert resources from other needs such as reproduction, and can be broken up into four sub-hypotheses; (1) that plants evolve defenses proportional to their risk of predation (the apparency hypothesis), (2) that defenses are allocated in proportion to the value to the plants fitness and inversely to the cost of defense, (3) that defenses concentrations evolve to increase and decrease depending on the presence or absence of predators, and (4) that there is a tradeoff between investment in plant defense and other functions (Rhoades 1979, Stamp 2003). These hypotheses work together to predict why and how plant defense phenotypes evolve in relation to the biotic and abiotic environment.

All of the aspects of the optimal defense hypothesis involve consideration of the costs of plant defenses. These subhypotheses of the optimal defense hypothesis assume that selection acts on genetic variation among various defense methods such as classes of secondary metabolites, that herbivore and disease pressure is a major selective force for the production of these defense characteristics, and that defenses actually do reduce herbivory or infection. In general, there is sufficient data to support these assumptions (Stamp 2003; Hanh et al. 2019). Phylogenetically

explicit genus-wide studies have a special advantage in allowing for insight into the drivers of trade-offs among phenotypes in light of the evolutionary history of diversification among species.

Another proposed method for how plants allocate defenses is the resource availability hypothesis. The resource availability hypothesis states that the amount of resources in a habitat select for plant growth strategy, ranging from inherently fast-growing to inherently slow-growing, and that a species adapting to a low-resource environment will evolve a low inherent growth rate, and that a plant with a low inherent growth rate will tend to have the ability to invest more in chemical or physical defenses than a plant with a high inherent growth rate (Coley et al. 1985, Stamp 2003). This suggests that species occupying habitats with high available resource supply will tend select for a strategy that out-grows or compensates for herbivory rather than selecting for increased chemical defenses in their organs. This hypothesis has also been generally supported (Endara and Coley 2010). Thus, the resource availability hypothesis suggests that species that inhabit environments that are resource-rich in terms of water availability, nutrient availability, light availability, and conducive temperatures for growth should have lower observed levels of defense than environments that are more resource-poor (e.g., low rainfall, extreme temperatures, nutrient-poor soils).

The genus Cornus

The genus *Cornus* (Cornaceae) contains 40-50 species, known variously as dogwoods, cornels, and osiers, hereafter collectively referred to as dogwoods (Bailey 1977; Xiang et al.

2006). Dogwoods are widely spread within the temperate zones across Europe, North America, Africa, and Asia (Xiang 2005). These trees and shrubs bear an extremely wide range of morphological variation, especially among their reproductive structures (Feng et al. 2011). *Cornus* can be split into four distinct clades: the big bracted group, the blue or white fruited group, the cornelian cherries, and the dwarf dogwoods (Xiang et al. 2006, 2008). There has been evidence from multiple species of dogwood for a common whole genome duplication, taking place within the late Cretaceous period along with a dramatic increase in sea surface temperatures, which together may have led to the wide variety observed among species (Yu et al. 2017). The phylogenetic relationships among *Cornus* have been studied, and current relationships are reasonably well supported (Xiang et al. 2006). Dogwoods also have been observed to contain abundant anthocyanins, which are generally associated with attractive and colorful fruits, especially in species such as *Cornus mas* which is widely referred to as the Cornelian cherry (Seeram 2002). Like other reproductive structures, fruit phenotypes are highly varied across the genus, ranging from dry fruits to colorful, fleshy, and juicy fruits (Eyde 1988). Birds are the main disperser across the genus (Willson and Whelan 1993; Rossell et al 2001; Xiang 2005), suggesting that the more palatable and juicier a fruit is, the more attractive it is to a bird who will disperse seeds (Eyde 1988). However, these traits may also make the fruit more attractive to insect frugivores who do not contribute to seed dispersal, as well as more prone to fungal and bacterial infection. This sets up the potential for an evolutionary trade-off between these traits and defenses.

The questions asked in this study draw from the assumptions made by the optimal defense hypothesis and explore the evolution of *Cornus* reproductive defenses in relation to fruit

and flower phenology, fruit palatability, and fruit reflectance (color), as well as how all of these traits have evolved with native habitat environmental characteristics. We also specifically test whether the evolution of defenses in *Cornus* support the predictions of the optimal defense hypothesis and the resource availability hypothesis. The specific research questions addressed in this study are:

1. How do the concentrations of flower and fruit chemical defenses evolve in relation to flower and fruit phenology?

2. How do the concentrations of fruit chemical defenses evolve in relation to fruit palatability traits?

3. How has defense chemistry evolved within *Cornus* in relation to climate and soil fertility?

Methods

Sample collection

During the 2016 growing season, individuals of 25 different species of *Cornus* were tracked for flower and fruit phenology, as well as sampled for floral and fruit tissue at the Arnold Arboretum of Harvard University. Very similar climate and soil conditions within the Arboretum serve as an experimental common garden, minimizing the effect of environmental variation on species' trait differences relative to field sampling. Phenology was assessed weekly over six months from mid-April until mid-September, with plants categorized as vegetative (no reproductive structures present), flowering (specifically defined as post-anthesis with open flowers present), intermediate (i.e. post-flowering with developing fruits), or fruiting (defined as the presence of mature fruits).

From this data, the onset of flowering and the onset of fruiting were determined, and the length of the flowering and fruiting period were calculated. Samples of inflorescences and fruits were collected when first observed. Whole inflorescences were collected, including the bracts in the big bracted group. Out of the 25 species observed, flower samples were obtained for 22 species. No individuals of *C. walteri*, *C. glabrata*, or *C. alba* flowered during this study and were therefore not included in the analysis. Fruits were collected from 9 species and were obtained upon first sight of ripe fruit. Fruits were not present for sampling in some species either because fruits were rapidly removed by birds in the few days between ripening and weekly sampling, or

because plants did not produce fruit in the Arboretum, perhaps due to lack of adequate pollination.

Trait quantification

Inflorescence and fruit samples were weighed fresh and dried at 60°C to constant mass in a forced air-drying oven. After taking reflectance scans of the inflorescence, samples were ground using a mortar and pestle and used for the quantification of defense and fruit palatability traits. Fresh inflorescence and fruit samples were weighed to obtain fresh mass. Samples were then dried at 60°C for three days until constant mass and re-weighed to obtain dry mass. The difference between floral and fruit fresh and dry masses were used to calculate water content. Dried samples were then ground into a fine powder using a mortar and pestle for the quantification of defensive chemistry and palatability traits. On both tissue types, tannin activity was quantified using a radial diffusion assay (Graca and Barlocher, 2005) and expressed as tannic acid equivalents. Total phenolic content was estimated using the colorimetric Folin-Ciocalteau assay (Singleton, et al. 1999) and expressed as caffeic acid equivalents. Total flavonoid content was estimated using the colorimetric aluminum complexation assay (Pekal and Pyrzynska, 2014) and expressed as quercetin equivalents.

Fruit palatability traits were quantified from dried, ground fruit samples. Fruit palatability was defined in this study as traits that contribute to the consistency, flavor, and nutrition that would promote consumption by birds. The traits assessed were titratable acidity, lipid content, protein content, water content, and sugar content. Fruit titratable acidity was quantified using NaOH in an acid-base titration (Sadler and Murphy, 2010). Total lipid content was quantified

using the gravimetric method of Moles et al. (2005), using petroleum ether to extract lipids. Total fruit protein content was quantified using the colorimetric Bradford protein assay (Jones et al., 1989), which uses Bradford dye reagent added to plant extracts. Total fruit sugar content in sucrose equivalents was measured using the Brix method with a handheld refractometer (Kitinoja and Hussein 2005).

Digitized herbarium records were obtained from the Global Biodiversity Information Facility (http://www.gbif.org/, 2017) and Integrated Digitized Biocollections (http://www.idigbio.org/, 2017), describing the natural distributions of each species as occurrence data with geographic coordinates (latitude, longitude). Occurrence points were manually curated to remove non-native records, such as those from botanical gardens in continents not native to the species using natural species distribution ranges (Xiang 2005).

For each occurrence point, data was extracted from the WorldClim 2.0 interpolated global climate model (Fick and Hijman, 2017) at a resolution of 30-arcseconds, including mean annual temperature (℃), diurnal range (℃), isothermality (%), maximum temperature of the warmest and coldest month (℃), minimum temperature of the coldest month (℃), mean annual precipitation (mm), precipitation of the wettest and driest months (mm), precipitation of the warmest and coldest quarters (mm), and the frost free period (in months).

Soil data was extracted from SoilGrids250m interpolated global soil model (Hengl et al., 2017), including average soil bulk density (kg/m3), average soil cation exchange capacity (cmolc/kg), volumetric percentage of coarse fragment, average soil organic matter content (permille), average soil pH in water, soil silt percentage, soil sand percentage, and soil clay

percentage. Soil data was averaged across the seven available soil depths (0cm, 5cm, 15cm, 30cm, 60cm, 100cm, and 200cm) to cover the rooting zone of these trees and shrubs. Principal components analysis was used to assess which variables most captured source site environmental variation out of autocorrelated variables in temperature, precipitation, and soil composition.

Trait-trait correlations

Species means for all traits and environmental data were taken across sample points curated from the different databases used. Trait-trait correlationsand were assessed with phylogenetic generalized least squares (PGLS) regression using the matK-ITS phylogeny of the genus (Xiang 2006), pruned to include the study species (Figure 1). The R package *Rphylopars* was used to estimate trait covariance while accounting for missing trait data (Goolsby 2017). Pairwise trait-trait PGLS regression coefficients were generated for phenology and fruit/flower defense chemistry (Table 1). Ancestral state reconstruction was performed to visualize trait-trait relationships across the genus for floral and fruit phenology and defense trait correlations (Figure 2) and for fruit defense and palatability traits (Figure 3).

Results

Fruit and floral phenology and defense traits

      The timing of flowering and fruiting varied strongly across the phylogeny (Figure 1). The Cornelian cherry group was observed to flower at the very start of the growing season before leaf-out (mid-April), though only *C. mas* was observed to subsequently produce fruit. The big bracted clade bloomed at different times, *C. kousa* and *C. kousa ssp. chinensis* blooming about two weeks later in the season than *C. florida*. All three were observed to produce fruit later in the season, however *C. kousa* began fruiting about two weeks earlier than the other two in the big bracted clade. The blue and white clade flowered and fruited at a variety of times across the growing season.

Figure 1. The phenology of each species under study by Julian date observed, placed in relation to the Cornus phylogeny of Xiang et al. (2006).

There was a negative evolutionary correlation between flowering start date and flower flavonoid content ($R^2 = 0.43$), and a positive evolutionary correlation between flowering start date and floral tannin activity ($R^2 = 0.35$). In fruits, there was a positive evolutionary correlation between fruit start date and fruit phenolic content ($R^2 = 0.49$), and a negative evolutionary correlation between fruiting start date and titratable acidity ($R^2 = 0.34$). There was also a positive evolutionary correlation between the number of weeks fruiting and fruit content ($R^2 = 0.31$) as well as more weakly with fruit tannin activity ($R^2 = 0.16$).
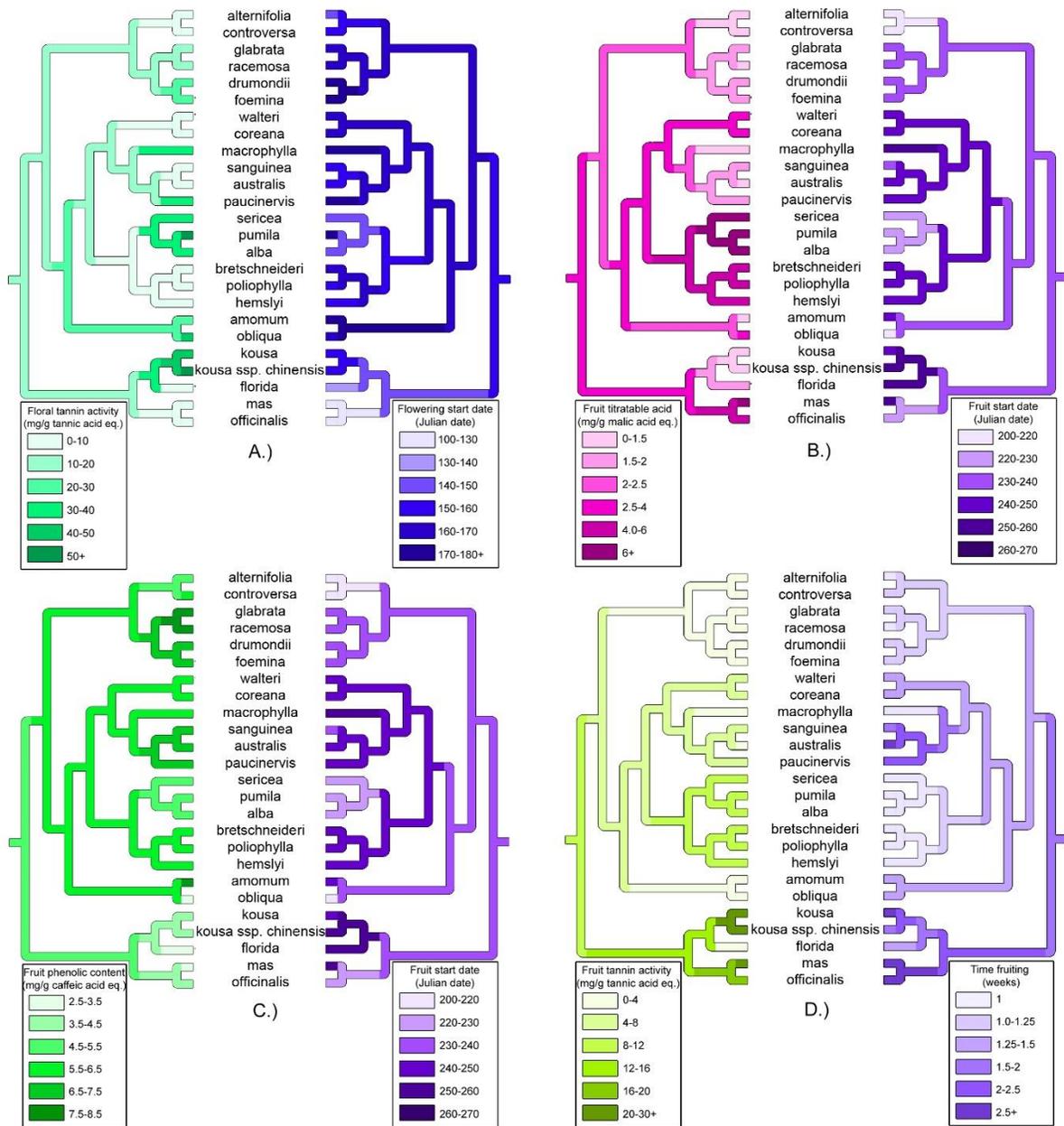
Figure 2.Ancestral state reconstruction of phenology and chemical defense across the genus Cornus: A.) positive evolutionary correlation between floral tannin activity and flowering start date (PGLS, $R^2 = 0.35$); B.) A negative evolutionary correlation between fruit titratable acidity and fruit start date (PGLS, $R^2 = 0.34$) C.) A positive evolutionary correlation between fruit phenolic content and fruit starting date (PGLS, $R^2 = 0.49$); D.) A positive evolutionary correlation between fruit tannin content and weeks fruiting (PGLS, $R^2 = 0.16$).

Fruit palatability and defense traits

Fruit defenses differed across the 11 species from the phylogeny. Phenolic content ranged from 1-8.3 mg/g (caffeic acid equivalents), flavonoid content ranged from 1.6-5.4 mg/g (quercetin equivalents), and tannin activity ranging from 0-31 mg/g (tannic acid equivalents). There was a strong evolutionary trade-off across the genus between fruit flavonoid content and tannin activity (R^2= 0.53, Table 1). There was also a positive evolutionary correlation between fruit sugar content and fruit tannin activity (R^2= 0.31, Table 1). Fruit lipid content ranged across species from 5.4-23.2 mg/g lipids. The species with the highest measured amount of tannin activity and sugar content were in the big bracted clade (excluding *C. florida*) and the cornelian cherry group. Fruit lipid content was observed to have a positive evolutionary correlation with fruit flavonoid content (R^2 = 0.39, Table 1). Several other weaker trait-trait relationships were observed. There were also weaker negative evolutionary correlations between fruit phenolic content and titratable acidity (R^2= 0.18, Table 1), flavonoid content (R^2= 0.19, Table 1), and lipid content (R^2= 0.21, Table 1). Fruit water content was weakly positively correlated with fruit phenolic content (R^2 = 0.16, Table 1).
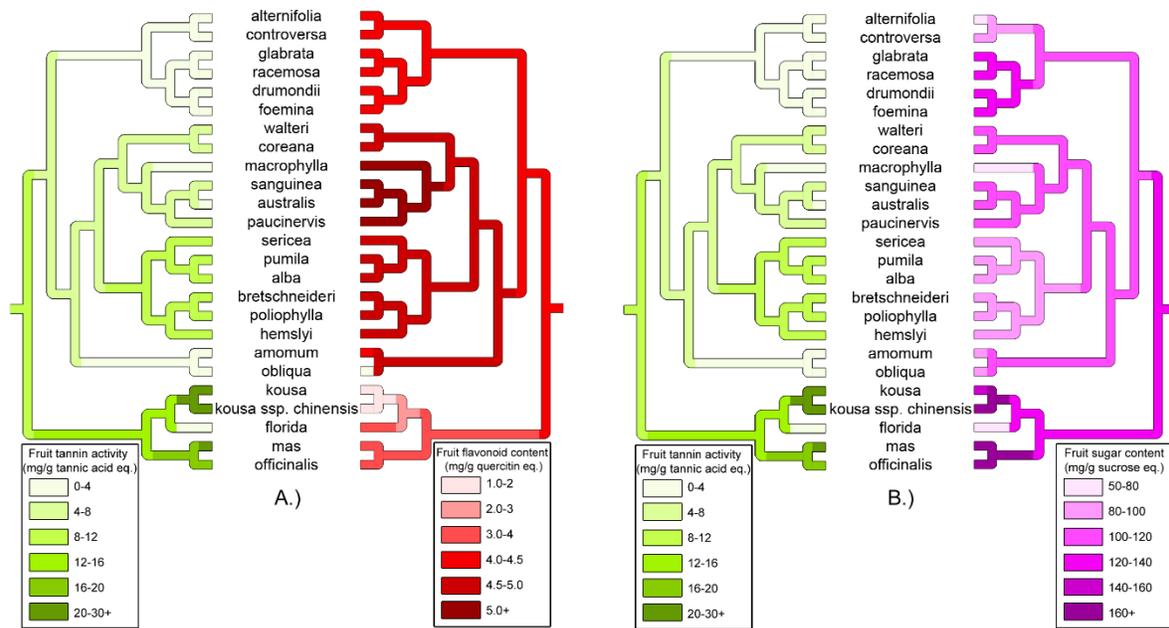
Figure 3. Ancestral state reconstruction of fruit palatability across the genus Cornus: A.) An evolutionary trade-off between fruit tannin activity and fruit flavonoid content (PGLS, R^2 = 0.53); B.) A positive evolutionary correlation between fruit tannin activity and fruit sugar content (PGLS, R^2 = 0.31).

Table 1. Macroevolutionary trait-trait correlations between fruit palatability and defense traits as assessed by phylogenetic generalized least squares regression. R^2 and directionality of significant correlations ($p<0.05$) are presented.

	Sugar content (sucrose eq. mg/g)	Fruit tannin activity (tannic acid eq. mg/g)	Fruit lipid content (mg/g)	Fruit water percentage	Fruit Flavonoid content (quercetin eq. mg/g)	Fruit Phenolic content (caffeic acid eq. mg/g)
Fruit titratable acid (malic acid eq. mg/g)	-	(+) 0.17	-	-	-	(-) 0.18
Fruit Phenolic content (caffeic acid eq. mg/g)	-	-	(-) 0.21	(+) 0.16	(-) 0.19	
Fruit Flavonoid content (quercetin eq. mg/g)	(-) 0.33	(-) 0.53	(+) 0.39	-		
Fruit water percentage	-	-	-			
Fruit lipid content (mg/g)	-	-				
Fruit tannin activity (tannic acid eq. mg/g)	(+) 0.31					

Defense traits and environmental data

Average soil organic matter was highly variable across the entire genus. Species soil organic matter was positively correlated across the phylogeny with floral flavonoid content ($R^2=$ 0.49, Table 2) and fruit phenolic content ($R^2 = 0.38$, Table 2). Soil organic matter was also negatively correlated with flowering start date across the phylogeny ($R^2 = 0.33$, Table 2). Soil sand, silt, and clay percentages were all highly correlated ($R^2 > 0.60$, Table 2), silt and clay were both negatively correlated with sand and positively correlated with one another. Habitat sand and silt soil percentages varied across the genus. Soil sand percentage ranged from 28.8-49.3%, and soil silt ranged from 30.7-45.5%. Soil sand percentage was weakly positively correlated with floral flavonoid content ($R^2= 0.27$, Table 2) and floral phenolic content ($R^2 = 0.27$, Table 2), and

strongly correlated with fruit phenolic content ($R^2 = 0.63$, Table 2) and fruit water percentage (R^2 = 0.61, Table 2). Soil silt percentage was weakly negatively correlated with floral flavonoid content (R^2= 0.29, Table 2) and floral phenolic content ($R^2 = 0.27$, Table 2), and strongly negatively correlated with fruit phenolic content ($R^2 = 0.58$, Table 2) and fruit water percentage ($R^2 = 0.65$, Table 2). Soil sand percentage was slightly negatively correlated with flowering start date ($R^2 = 0.17$, Table 2).

Species occupy habitats with a range of temperatures across the phylogeny. The maximum temperature in the warmest month was strongly negatively correlated across the phylogeny with floral flavonoids (R^2=0.81, Table 2) and weakly negatively correlated with fruit tannins ($R^2 = 0.22$, Table 2), while the maximum temperature in the coldest month was positively correlated across the phylogeny with floral flavonoids ($R^2 = 0.50$, Table 2). The species occupying the warmest climates with a maximum temperature during the warmest month above 30 °C were *C. florida, C. officinalis, C. foemina, C. drummondii.* Mean annual temperature was weakly negatively correlated across the phylogeny with fruit acidity ($R^2 = 0.23$, Table 2). The maximum temperature during the warmest month was positively correlated with flowering start date ($R^2 = 0.55$, Table 2).

Species occupying the wettest habitats are *C. macrophylla* and *C. controversa,* with mean annual precipitation above 150 cm, while species occupying the driest habitats are *C. kousa ssp. chinensis* and *C. bretschneideri* with mean annual precipitation below 60 cm. Precipitation during the driest quarter ranged from 1.3-22.8 cm among species. Fruit acidity and precipitation in the driest month had a strong negative correlation ($R^2 = 0.69$, Table 2), while fruit acidity and precipitation in the warmest quarter had a weaker negative correlation (R^2 =0.24, Table 2). Fruit

phenolic content was negatively correlated with precipitation during the driest month ($R^2 = 0.48$, Table 2). The precipitation during the warmest quarter ranged between 12.8-68.7 cm among species. Precipitation during the warmest quarter was weakly positively correlated with fruiting start date across the phylogeny ($R^2 = 0.24$, Table 2).

Table 2. Macroevolutionary correlations between fruit and floral traits and environmental characteristics traits as assessed by phylogenetic generalized least squares regression. R^2 and directionality of significant correlations ($p<0.05$) are presented.

	Floral Start date (Julian date)	Fruiting start date (Julian date)	Floral Flavonoid Content (mg/g)	Floral Phenolic Content (mg/g)	Fruit Tannin Activity (mg/g)	Fruit Titratable Acidity (mg/g)	Fruit Phenolic content (mg/g)	Fruit Water %
Soil Organic Matter	(-) 0.33	--	(+) 0.49	--	--	--	(+) 0.38	--
Soil Sand Percentage	(-) 0.17	--	(+) 0.27	(+) 0.27	--	--	(+) 0.63	(+) 0.61
Soil Silt Percentage	--	--	(-) 0.29	(-) 0.27	--	--	(-) 0.58	(-) 0.65
Mean Annual Temperature	--	--	--	--	--	(-) 0.23	--	--
Max Temp Warm Month	(+) 0.55	--	(-) 0.81	(-) 0.22	(-) 0.22	--	--	(-) 0.17
Min Temp Cold Month	(-) 0.17	--	(+) 0.50	(+) 0.25	--	--	--	--
Precip Dry Month	--	--	--	--	--	(-) 0.69	(-) 0.48	--
Precip. Warm Quarter	--	(+) 0.24	--	--	--	(-) 0.24	--	(-) 0.24

Discussion

Fruit and floral phenology and defense traits

We observed that across the phylogeny, a later starting date for flowering and fruiting led to a higher tannin activity and phenolic content, respectively. This indicates that later-blooming and later-fruiting species have evolved an increased level of chemical defense. One explanation for why this may be observed is due to the increase in insect abundance and pathogen prevalence as the growing season progresses (Bale et al. 2002; Wang et al. 2009; Evans et al. 2008; Huot et al. 2017, Hahn et al. 2019). While insect herbivory may increase later in the growing season, there is evidence that fungal infection tends to remain roughly the same throughout the year (Brody 1997). We found that there was a trade-off between floral flavonoid and flowering start date, meaning that species blooming earlier in the growing season have a higher concentration of flavonoids and a lower tannin activity, while species blooming in the later season have a higher tannin activity and a lower concentration of flavonoids. Interestingly, a significant overall trade-off between floral flavonoids and floral tannins was not observed. This may suggest that the reason for the evolution of increased tannin activity in the flowers of later blooming species may be due to an increase in the likelihood of herbivory through insects or herbivores later in the season. Additionally, flavonoid content and tannin activity both tended to be higher in inflorescences than in fruits (which did observe a trade-off between tannin activity and flavonoid content). This trade-off may be due to a selective advantage rather than a constraint of the phenylpropanoid pathway (Donovan et al. 2011, Schlichting and Pigliucci, 1998). This may strengthen the argument that floral phenology affects the chemical defense concentrations.

We have also observed a longer fruiting period to correlate with an increase in fruit tannin and sugar content, though this relationship is more difficult to interpret considering that fruit sugar and tannins have a positive correlation across the phylogeny. Willson and Whelan (1993) found that the chance of *C. drummondii* fruit being eaten by a seed predator or non-dispersing frugivore increased as the fruiting season went on. This suggests that species with longer fruiting periods will have a higher chance of predation, and therefore higher chance of not being dispersed. This may select for defenses in the fruits of those species, which the higher tannin activity in those species seems to suggest.

Fruit palatability and defense traits

Secondary metabolites often make up a significant portion of plant defenses (Harborne and Turner 1984; Crozier et al. 2007; Pais, Li, and Xiang 2018). These defenses are found in different concentrations within plant tissues. An evolutionary trade-off was observed within *Cornus* fruits between flavonoid and tannin concentrations. These secondary metabolites are a part of the phenylpropanoid pathway (Crozier 2007). In this pathway, tannins are made from a precursor compound flavan-3,4-diol, which is a flavonoid (Gutierrez-Gonzalez et al. 2010). Our observations may indicate that in some species a large amount of these flavonoid precursors are converted into tannins, while in others they are not. One hypothesis is that the amount of sugar in a fruit may have some influence over this process, indicated by the positive correlation between sugar content and fruit flavonoids. This increase in tannin activity when there is an increase in palatability among a species may support our prediction that higher palatability

predicts higher defenses within the fruits of genus *Cornus.* Another possible explanation as to why there is this observed positive correlation among species with fruit tannin activity and fruit sugars may be due to a presence of a tannin-carbohydrate complex with sugars, which reduces the effects of tannin activity in ripening and ripe fruits (Luthar and Tisler 1992; Cipollini and Levey 1997;Ozawa et al. 1987) which may suggest that the increase in total sugar content in the ripe fruits could be due to the increased tannin activity.

There was also a positive evolutionary correlation between fruit lipid content and fruit flavonoids. Interestingly, there was a trade-off between lipid and phenolic content as well. We have also observed an evolutionary trade-off within fruit flavonoid and phenolic content. A positive evolutionary correlation between fruit flavonoid content and lipid content may also support our predictions of higher defenses in more palatable fruits. High concentrations of lipids have been found in the pulp of some species of the genus such as *C. florida* (Rossell et al. 2001). Wilson and Whelon (1993) also found that *C. drummondii* fruits with higher lipid content had a higher probability of being eaten by birds than fruit with lower lipid content. Borowicz (1988) compared bird preference on fruits between *C. racemosa* (which had a higher lipid content) and *C. amomum* (which had a lower lipid content)*,* and found that some bird species selected the fruit with higher pulp lipid content over the fruit with lower pulp lipid content, while others showed no preference. This suggests that lipid content may be more dictated by fats and oils in the fruit rather than surface waxes, which would perhaps contribute more to fruit defense, and that lipid content may be a major determinant of palatability and preference in bird dispersers. There has also been evidence that some lipids in the acorns of *Quercus* have been found to reduce toxic effects of tannins (Cipollini and Levey 1997; Koenig 1991). While there is no significant

correlation between fruit tannin activity and fruit lipid content, the trade-off between fruit tannin activity and fruit flavonoid content may be influenced in part by lipid content, where flavonoid content may better defend fruits with higher lipid content.

Defense traits and environmental data

The *Cornus* genus contains species that have a wide range of distribution size ranging from transcontinental distributions in species such as *C. sericea,* which spans across most of North America and some of Central Europe, to species with very small distributions such as *C. kousa ssp. chinensis* which occur in smaller distributions in China and Taiwan. Concomitant with this variation in geographic distributions, *Cornus* species occupy ranges that differ across major environmental gradients, including temperature, precipitation, and soil characteristics. We find many strong correlations of these environmental factors with floral flavonoid content, fruit phenolic content, fruit water content, and fruit titratable acidity.

As species move into sandier habitats, we observe the evolution of higher floral and fruit phenolic content, floral flavonoid content, and higher fruit water content, while species that move into soils with higher silt percentages result in the evolution of lower values of all of these traits. Similar findings have been made in studies that test the resource availability hypothesis, where species that evolved in sandier soils (where nutrient supply was insufficient to support high inherent growth rates to compensate for herbivory) evolved higher flavonoid content and higher phenolic content relative to species occupying environments with less sand (Fine et al. 2004, 2006; Hahn et al. 2019). We also found that as species moved into soils with a higher organic matter content, species evolve higher floral flavonoid content and fruit phenolic content,

which appears to run counter to the resource availability hypothesis if organic matter content is assumed to predict soil fertility, however the availability of organic matter-derived nutrients is highly dependent on complex interactions of other factors, like temperature, water availability, and soil microbial activity.

Species occupying habitats with elevated precipitation in both the dry and warm seasons began fruiting later than other species, as well as containing lower tannin activity. This may support the resource availability hypothesis since access to more resources through increased rainfall may enable the species to evolve to select for growth to compensate for predation loss. Pezzola et al. found that in many natural populations of sagebrush, increased water availability led to increase in photosynthetic activity (2017). Higher precipitation has also been found to sustain elevated photosynthetic rates in other systems (Kawabata et al. 2001). These trends may support that the *Cornus* species occupying habitats with higher precipitation have experienced selection for increased growth rate and decreased defense chemistry, aligning with the resource availability hypothesis.

Warmer temperatures are an important environmental characteristic that allows plants to grow more rapidly (e.g. higher growing degree days), as well as typically providing a longer growing season. There is evidence that increased temperature also tends to correlate with increased populations of insects and pathogens, which may shape the evolution of plant defenses (Hout et al. 2017, Whang et al. 2010; Hanh et al. 2019). As *Cornus* species have diversified into warmer environments that may support an increase in growth rate, we observe lower floral defenses, which supports the resource availability hypothesis. Species that occupied habitats with a higher minimum temperature during the coldest month contained higher floral flavonoid

content, as well as higher floral and fruit phenolic content than those species who occupied habitats with lower minimum temperatures during the coldest month. This suggests that when species evolve into areas with warmer winters, they evolve higher defenses in terms of floral and fruit phenolic content and floral flavonoid content. One reason this may be occurring is due to the evolution of adaption to the absence of harsh frosts or prolonged cold periods, which typically kills a portion of insects and pathogens (Dukes et al. 2009, Ayres and Lombardero 2000, and Bale et al. 2002).

Conclusion

Our results provide support for the apparency hypothesis, a subhypothesis of the optimal defense hypothesis, through elevated chemical defenses in inflorescences that bloom later in the growing season and fruits that have evolved to have a longer fruiting period. We have also found an evolutionary trade-off in the flavonoid content and tannin activity in the fruits of the genus *Cornus*, which suggests a selective advantage of investing resources into one compound over the other. We also found a strong coupling of fruit tannin activity and fruit sugar content, as well as fruit flavonoid content and fruit lipid content. This may support our prediction of species evolving to have more defenses with the evolution of more palatable fruits. We also found mixed support for the resource availability hypothesis among species depending on the environmental variable considered. We found that species that diversified into sandier soils invested more highly in defenses than species that moved into soils with a higher silt and clay content, which supports the resource availability hypothesis inasmuch as sandy soils tend to have lower fertility. Species moving into soils that contained more organic matter content, however, invested in defenses more so than species from soils with lower organic matter content, which does not support the resource availability hypothesis. Species occupying warmer and wetter environments evolved to produce less chemical defenses than those in colder environments (which seems to support the resource availability hypothesis), however species that occupied environments with warmer winters evolved more defenses. Fruits and flowers play an integral part in determining plant fitness and shaping plant evolution. Despite this, work on leaf defense is disproportionately

high relative to the number of studies focusing on the defense of reproductive organs. Further research into fruit and floral defense is needed to remedy this imbalance. In addition, future studies wishing to better test the resource availability hypothesis in a phylogenetic context should explicitly incorporate data on plant inherent growth rate where possible alongside tissue defense chemistry and native source site environment to better define the energetic costs of defense investment.

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