

No simple sum: seasonal variation in tannin phenotypes and leaf-miners in hybrid oaks

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Abstract. Parent-hybrid asymmetries in the biosynthesis of defense chemistry are believed to affect the distribution of herbivorous insects within plant hybrid zones. While tannins are often produced as complex, dynamic mixtures, the ecological effects of biological and ontogenetic variation in tannin metabolism within hybrid zones are poorly understood. Here, we examine correlations between the seasonal variation of absolute and relative concentrations of individual ellagitannins and total proanthocyanidins in the *Quercus gambelii* × *Q. grisea* hybrid complex and a community of leaf-mining Lepidoptera (Gracillariidae, Heliozelidae, Nepticulidae, Tischeriidae), and examine the interactive effects between tannins on leaf-miner abundance. We found strong seasonal variation in oak tannin phenotypes, but two general phenotypes are characteristic of oaks of the *Q. gambelii* × *Q. grisea* complex, based upon biosynthetic variation in high molecular weight ellagitannins. Leaf-miner community structure was significantly correlated with the dominant tannin phenotypes in the *Q. gambelii* × *Q. grisea* hybrid zone in mid-summer (July, August). Additionally, leaf-miner density was significantly negatively correlated with the ratio of vescalagin-derivatives and total proanthocyanidins in August and September. Overall, hybridization affects biological and ontogenetic variation in ellagitannin and proanthocyanidin biosynthesis within the *Q. gambelii* × *Q. grisea* hybrid complex and this variation correlates with herbivore distribution and abundance. Future studies of phenolic chemistry in plant-herbivore interactions in hybrid zones should include a more rigorous quantification of tannin phenotypes as complex, temporally dynamic mixtures.

Keywords. Hybridization – *Quercus* – ellagitannin – leaf-miner – phenotype – defense

Introduction

Hybridization between plant species often produces natural variation in host-plant morphology and function, including plant defenses. Hybridization may affect the quantitative and qualitative distributions of tannins, putative plant defenses, through hybrid zones (Orians and Fritz 1995, Orians 2000, Fritz et al. 2001, O'Reilly-Wapstra et al. 2005). However, most studies have focused on summary measures of tannins (e.g. Schweitzer et al. 2004, Bailey et al. 2004) that may have limited applicability for ecological studies (Appel et al. 2001, Heil et al. 2002, Salminen 2003). A more accurate characterization of tannin phenotypes is needed to clarify the fine-scale spatial and temporal variation in tannins within hybrid zones and the potential effects for associated communities of consumers.

While tannins have historically been treated as a set of compounds with homogeneous ecological function, this class of phenolics represents a diverse assemblage of biochemicals with context-dependent biological functions (see Zucker 1981). Further, individual tannins have been shown to exhibit wide-ranging effects for different herbivores (Ayres et al. 1997). Moreover, there is a distinct lack of empirical evidence about how tannin phenotypes – the absolute and relative concentrations of individual tannins as a complex mixture – may affect herbivore distribution and abundance in natural populations. However, Barbehenn and colleagues (2006b) have recently demonstrated that at high pH, similar to that of insect digestive tracts, the oxidative capacity of individual ellagitannins is dependent upon structural characters, particularly the number of hydroxyl groups, and is me-

diated by the concentration of co-occurring proanthocyanidins at high pH *in vitro*. Consequently, the tremendous amount of taxonomic (Salminen et al. 2002, Lahtinen et al. 2006, Yarnes et al. 2006) and temporal variation (Salminen et al. 2001, 2004) in tannins may generate complex effects for herbivore communities.

Hybridization may facilitate host shifts in herbivorous insects (Floate and Whitham 1983) and accelerate the course of herbivore evolution (Schwarz et al. 2005, Stirman et al. 2005). Hybridization that results in the alteration of population and/or ontogenetic patterns of plant defenses may thereby increase the spatial and temporal availability of new hosts to herbivores. Alternatively, hybridization may disrupt the phenological timing of consumers on host plants. Surprisingly, the effect of non-equal ontogenetic patterns of tannin metabolism, even summary measures of tannins, within hybrid zones on herbivore communities has not been widely investigated (but see Fritz et al. 2001), despite well-documented seasonal variation in the metabolism of individual tannins (e.g. Salminen et al. 2001, Salminen et al. 2004) and the potential effects of variation in plant ontogeny on plant-herbivore interactions within hybrid zones (Floate et al. 1993, Lawrence et al. 2003).

Detailed molecular genetic studies have identified hybrids and backcrosses of *Q. gambelii* × *Q. grisea* at several sites in central New Mexico, U.S.A. (Howard et al. 1997, Williams et al. 2001). In this study, we examine correlations between the seasonal variation of absolute and relative concentrations of individual ellagitannins and total proanthocyanidins in the *Quercus grisea* × *Q. gambelii* hybrid complex and a community of leaf-mining Lepidoptera (Gracillariidae, Heliozelidae, Nepticulidae, Tischeriidae). We also examine the joint effects (ratios) of ellagitannins and proanthocyanidins (*sensu* Barbehenn et al. 2006b) on the distribution and abundance of leaf-miners within the *Q. gambelii* × *Q. grisea* hybrid zone. Taxonomic groups include parental species, *Q. gambelii* × *Q. grisea* hybrids, and their backcrosses, as identified through molecular genetic analysis (Howard et al. 1997).

Material and Methods

Study System

Quercus gambelii, Rocky Mountain White Oak, hybridizes with several oak species of the subgenus *Quercus* Section *Quercus* resulting in a number of multi-species hybrid complexes distributed throughout the southwestern United States. Hybrid progeny within these complexes are often colloquially referred to as "*Q. undulata*" (Tucker 1961). In the San Mateo Mountains of central New Mexico, a two-species hybrid complex exists between *Q. gambelii* and *Q. grisea* (Preszler and Boecklen 1994). The genetic identities of trees within the Monica Canyon Hybrid Complex (MCHC) have been previously described using RAPD molecular markers (Howard et al. 1997). Through the use of this data, five taxonomic classes were delineated (1) *Q. grisea*, (2) *Q. grisea* × hybrid backcross, (3) *Q. gambelii* × *Q. grisea* hybrid (parental hybrid), (4) *Q. gambelii* × hybrid backcross, (5) *Q. gambelii*.

Leaf and leaf-miner sampling

Ten trees of each taxonomic group were randomly selected from all trees of known genetic identity within the Monica Canyon hybrid zone. Most trees were < 1.5 m in height with relatively open canopies. Ten leaves from each tree were sampled for phytochemical analysis from the lower branches on June 9, July 1, August 10, and September 15 2004; obvious "sun" leaves (hard, waxy leaves) or any second-year leaves were avoided to minimize within-tree variation of samples (Feeny 1970). Leaves were placed in envelopes and transported on ice to the laboratory. Leaves were air-dried on a set of shelves in a ventilated fume hood. Air-drying is not the optimal drying method (Salminen 2003), but is known to alter levels of hydrolysable tannins and flavonoid glycosides only slightly in *Q. robur* (Salminen et al. 2004). Air-dried oak leaves were then ground to a fine powder using a ball mill (Wig-L-Bug, Reflex Analytical, New Jersey, U.S.A.) and pooled within trees. After grinding, phenolics were extracted three times from leaf tissue (~20 mg dry wt) with 70 % aqueous acetone containing 0.1 % ascorbic acid added to prevent oxidation of phenolics. This extraction solvent was found to provide better recovery over a wide range of aqueous solvents (methanol: 50 % MeOH, 70 % MeOH, 85 % MeOH; acetone: 50 % Me₂CO, 70 % Me₂CO, 85 % Me₂CO; Tuominen et al. 2005). Extracts were loosely covered with aluminum foil and placed in a closed fume hood overnight to remove the acetone fraction. The air-dried extracts were then dissolved in 1 ml water, the supernatants centrifuged (10 min at 5000 g), filtered through a 0.45 µm PTFE filter, and kept frozen at -20 °C until analysis with HPLC-DAD-ESI-MS.

The same trees were censused for late-instar leaf-miners on September 15. Five leaf-miner species were collected: *Cameraria* sp. (Gracillariidae), *Coptodisca* sp. (Heliozelidae), *Nepticula* sp. (Nepticulidae), *Phyllonorycter* sp. (Gracillariidae), and *Tischeria* sp. (Tischeriidae). Reference specimens are archived in the laboratory of William J. Boecklen at New Mexico State University.

Phytochemical analysis

Concentrations (mg/g dry weight) of the water-soluble tannins of oak leaves were determined using high performance liquid chromatography-diode array detection-electrospray ionization-mass spectrometry (HPLC-DAD-ESI-MS) as described by Yarnes et al. (2006). Leaf extracts were analyzed under negative electrospray ionization with a HPLC-DAD-ESI-MS system (Waters 2795 Separations module w/ 2996 DAD, Milford, MA U.S.A.; Micromass ZMD 2000, UK) at 280 nm. Two solvents were used: (A) 0.1 % formic acid (HCOOH) in H₂O and (B) 0.1 % HCOOH in acetonitrile (MeCN). The elution profile was: 0–3 min, 100 % A (isocratic); 3–30 min, 0–30 % B in A (linear gradient); 30–40 min 30–45 % B in A (linear gradient). Flow rate used was 1 ml/min. The HPLC column used was a Merck Superspher 100 RP-18 column (75 × 4.0 mm i.d., 4 µm, Darmstadt, Germany). The following conditions were used in negative-ion ESI-MS: capillary voltage, -2.75 kV; cone voltage, -43 V; extractor voltage, -5 V; desolvation temperature, 350 °C; desolvation gas flow rate, 550 L/hr. The DAD was operated at 280 nm.

Individual tannins were identified on the basis of UV and mass spectra, retention times, and NMR spectroscopy (Yarnes et al. 2006). Ellagitannins were quantified using penta-galloylglucose as an external standard. Pure compound peaks were quantified with DAD; in the case of overlapping compound peaks, they were quantified selectively with ion trace analysis of their deprotonated molecules as described in Salminen et al. (2001). Differences in the ionization between individual runs were standardized using 6-bromo-2-naphthyl-β-D-glucopyranoside as an internal standard.

Total proanthocyanidins were measured using the butanol-HCl assay as outlined in Ossipova et al. (2001) using a Hach DR/4000U UV-Vis spectrophotometer (Ames, IA U.S.A.). Measurements were quantified using a standard curve prepared using quebracho tannin (Tannin Corp., Peabody, MA U.S.A.). The use of this summary measure of proanthocyanidins was necessary due to extensive chromatographic

Table 1. Compound information for individual ellagitannins in oaks of the *Q. gambelii* × *Q. grisea* hybrid complex. R_t = retention time; M_r = molecular weight; UV = UV maximum and characteristic shoulders (sh.); m/z = characteristic fragments in ESI-MS.

Compound	R_t (min)	M_r (g mol ⁻¹)	UV	m/z
1 Vescavalonic acid	6.4	1102	205, 260–280sh.	1101, 1084, 1066
2 Castavalonic acid	7.5	1102	205, 260–280sh.	1101
3 Vescalagin	7.7	934	220, 260–280sh.	933, 915, 457
4 Mongolinin A	7.9	1374	208, 260–280sh.	1373, 1101, 933, 915, 289
5 Pedunculagin (anomeric mixture)	8.8, 11.3	784	215, 260–280sh.	783, 391, 301
6 Castalagin	9.5	934	220, 260–280sh.	933, 466
7 Acutissimin A/B	12.7	1206	206, 260–280sh.	1205, 933, 915, 289
8 Cocciferin D ₂	14.8	1870	206, 260–280sh.	1869, 934
9 Ellagitannin	17.6	1084	210, 260–280sh.	1083, 1067, 533, 301
10 Ellagitannin	12.3	1326	208, 260–280sh.	1326, 301

interference between proanthocyanidin polymers and the abundant ellagitannins in these oaks in normal-phase HPLC (Yarnes et al. 2006).

Statistical analysis

We used size and shape analysis (Mossiman 1970, Boecklen et al. 1991) to compare oak hybrids on the basis of absolute and relative concentrations of ellagitannins and total proanthocyanidins. First, we compared taxa with respect to \log_e+1 -transformed concentrations (size). We then created shape variables by subtracting from each \log_e+1 -transformed concentration the \log_e+1 geometric mean of the concentrations. Because the rank of the matrix of shape variables is one less than that for the matrix of size variables, we excluded the variable \log_e+1 (ellagitannin **10**)- \log_e+1 (geometric mean) from the analysis. This compound was chosen for exclusion because it was relatively invariant across hybrid taxa, was well correlated with acutissimin A/B (**7**; $r = 0.68$), and did not contribute heavily to the discriminant functions for size. This was done for the size and shape analysis of each sampling date.

For each date, we compared oak tannin chemistry with a one-factor multivariate analysis-of-variance (MANOVA) with class (oak taxonomic category) as the main effect and trees as replicates. We used this design for the 11 \log_e+1 -transformed concentrations and for the 10 shape variables.

We then compared absolute and relative concentrations of ellagitannins and total proanthocyanidins in the *Q. gambelii* × *Q. grisea* hybrid zone using discriminant function analysis (DFA). DFA indicated whether oak taxonomic categories differed in the absolute and relative concentrations of tannins and also provided information about how oak taxonomic categories differed. We analyzed the correlation coefficients between the absolute and relative concentrations of tannins and the canonical scores of trees to help interpret the weight of contribution of individual ellagitannins and total proanthocyanidins to the classification of hybrid zone taxa. We also used the ratio of the eigenvalues to determine the relative contribution of size (absolute conc.) and shape (relative conc.) variables to the classification of oak hybrid tannin chemistry.

Leaf-miner abundance was examined using a one-way analysis-of-variance (ANOVA) with oak hybrid category as a main effect; pairwise post-hoc comparisons were performed using Fisher's Least-Significant Difference (LSD). Canonical correlation analysis (CCA) was used as a global test to examine co-variation between the leaf-miner community (\log_e+1 -transformed density) and the absolute and relative \log_e+1 -transformed concentrations of ellagitannins and total proanthocyanidins (Table 1) throughout the growing season. Subsequently, we independently examined the relationship between the densities of individual leaf-miner species and the concentrations of ellagitannins and proanthocyanidins using multiple linear regression. Finally, the relationship between leaf-miner abundance and the ratios of the sum absolute concentration of castalagin derivatives (**2**, **8**) and vescalagin derivatives (**1**, **4**, **7**) (Fig. 1) to total proanthocyanidins (PA) was examined using correlation analysis. Analyses were carried out

using SYSTAT 10.2 (SPSS, Chicago, IL U.S.A.) and tests considered significant at the $\alpha = 0.05$ level.

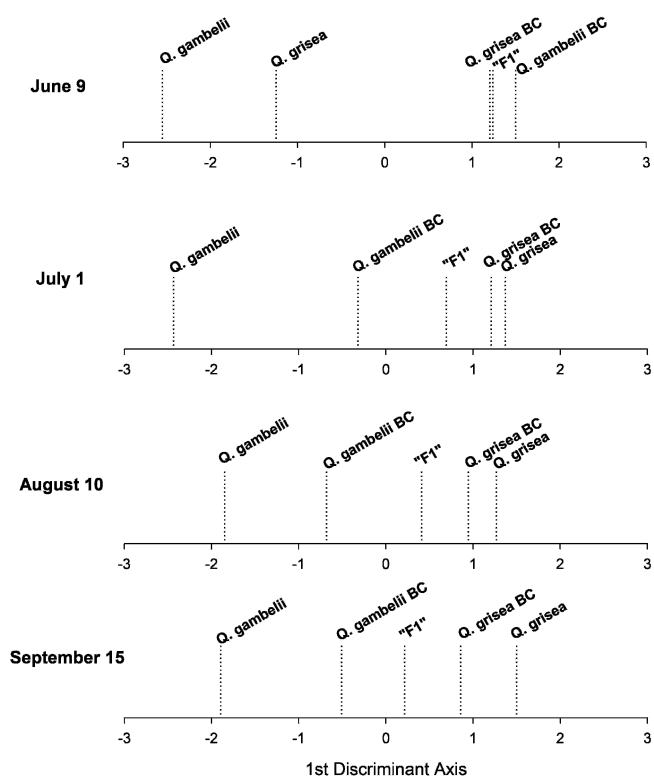


Figure 1. Classification of oak taxonomic categories based upon the absolute concentrations of individual ellagitannins and total proanthocyanidins. Ordination is based upon the canonical score of each category along the first discriminant axis from discriminant function analysis.

Results

Ellagitannins and total proanthocyanidins

We identified ten individual ellagitannins in oaks of the *Q. gambelii* × *Q. grisea* hybrid zone in Monica Canyon using negative ion HPLC-DAD-ESI-MS (Tables 1 & 2). Compounds were identified on the basis of their retention

times (R_t), UV spectra and mass spectral characteristics, for two compounds (**1**, **2**) NMR was also utilized (Yarnes et al. 2006). Ellagitannins in the *Q. grisea* × *Q. gambelii* complex include: vescavalonic acid (**1**), castavalonic acid (**2**), vescalagin (**3**), mongolinin A (**4**), pedunculagin (**5**), castalagin (**6**), acutissimin A (**7**), and cocciferin D₂ (**8**) (Ishimaru et al. 1988, Nonaka et al. 1990, König et al. 1994, Salminen et al. 1999, Quideau et al. 2003, Salminen et al. 2004). Two compounds (**9**, **10**) remained classified as ellagitannins, since their chemical structures could not be fully determined.

MANOVA indicates that *Q. gambelii* × *Q. grisea* oak hybrids and parental species contained significantly different absolute and relative concentrations of ellagitannins and proanthocyanidins from June–September (Tables 3 & 4).

Absolute dimensions of tannin phenotypes

Variation in the monthly absolute concentrations of ellagitannins and total proanthocyanidins among taxa within the *Q. gambelii* × *Q. grisea* hybrid zone produced four significant discriminant functions. The first two discriminant functions accounted for between 83–86 % of the monthly variation among hybrid zone taxa (Table 3), where the first discriminant axis accounted for 60–67 % of the variation in absolute concentrations of tannins (Fig. 1). The individual tannins important to the classification of hybrid oak taxa varied between dates. In June, variation between absolute dimensions of tannin phenotypes was largely described by decreasing absolute concentrations of mongolinin A (**4**) and increasing concentrations of cocciferin D₂ (**8**) and ellagitannin (**9**), and partitioned *Q. gambelii* and *Q. grisea* from hybrid taxa within the hybrid zone. In July, variation in tannin phenotypes was characterized by increasing absolute concentrations of vescalagin (**3**), pedunculagin (**5**), and cocciferin D₂ (**8**) and decreasing concentrations of mongolinin A (**4**). Absolute dimensions of tannin phenotypes in July primarily separated *Q. gambelii* and *Q. gambelii* backcrosses from the remaining taxa within the hybrid zone. In August, tannin phenotypes varied along decreasing absolute concentrations of mongolinin A (**4**), and increasing absolute concentrations of pedunculagin (**5**) and total proanthocyanidins (**PA**), and separated *Q. gambelii* and *Q. gambelii* backcrosses from other taxa within the hybrid zone. In September, absolute dimensions of tannin phenotypes were described by decreasing absolute concentrations of mongolinin A (**4**) and increasing concentrations of total proanthocyanidins (**PA**), and separated *Q. gambelii* and *Q. gambelii* backcrosses from other hybrid zone taxa (Fig. 1).

Table 2. Mean concentrations (mg/g dry weight) of ellagitannins and total proanthocyanidins in oaks of the *Q. gambelii* × *Q. grisea* hybrid zone.

Date	Taxonomic category	Compounds										
		1	2	3	4	5	6	7	8	9	10	PA
June 9	<i>Q. grisea</i>	7.34±0.18	1.73±0.05	4.89±0.13	0.05±0.01	2.04±0.03	1.86±0.05	1.09±0.06	6.78±0.08	3.07±0.26	0.89±0.05	11.84±1.06
	<i>Q. grisea</i> backcross	5.25±0.15	1.36±0.04	4.20±0.10	0.07±0.01	1.60±0.02	1.24±0.04	0.68±0.03	3.99±0.02	1.03±0.10	0.55±0.01	13.93±0.78
	<i>Q. gambelii</i> × <i>Q. grisea</i>	6.73±0.32	1.77±0.08	5.44±0.23	0.07±0.01	1.86±0.05	1.39±0.06	0.96±0.03	5.57±0.07	1.88±0.41	0.91±0.07	16.77±1.52
July 1	<i>Q. gambelii</i> backcross	6.76±0.17	1.97±0.05	14.59±2.53	0.03±0.01	2.76±0.06	2.01±0.06	1.02±0.06	5.89±0.06	1.63±0.27	0.95±0.05	12.32±0.81
	<i>Q. gambelii</i>	6.82±0.32	1.78±0.05	6.83±0.16	0.02±0.01	2.28±0.04	1.75±0.07	1.31±0.02	10.13±0.07	3.42±0.45	0.94±0.04	12.38±0.61
	<i>Q. grisea</i>	15.63±0.32	5.11±0.08	5.82±0.11	0.49±0.03	3.62±0.05	2.17±0.05	2.57±0.06	7.67±0.11	1.15±0.19	2.56±0.07	35.67±1.64
Aug. 10	<i>Q. grisea</i> backcross	15.76±0.22	5.31±0.03	5.87±0.12	0.74±0.04	4.60±0.06	2.35±0.06	2.48±0.02	9.78±0.08	1.08±0.20	1.84±0.06	52.17±3.01
	<i>Q. gambelii</i> × <i>Q. grisea</i>	14.29±0.27	5.50±0.20	6.14±0.12	0.41±0.02	4.00±0.06	1.87±0.04	1.98±0.02	8.56±0.07	0.79±0.27	2.12±0.10	42.06±1.59
	<i>Q. gambelii</i> backcross	17.29±0.30	5.87±0.10	7.40±0.14	0.32±0.03	5.36±0.11	2.36±0.11	2.40±0.06	10.17±0.11	1.08±0.33	2.61±0.10	44.21±2.81
Sept. 15	<i>Q. gambelii</i>	17.39±0.30	6.42±0.17	9.64±0.31	0.35±0.02	6.34±0.09	2.30±0.06	2.63±0.07	13.96±0.06	1.41±0.35	2.87±0.13	75.24±5.95
	<i>Q. grisea</i>	8.95±0.21	7.55±0.22	4.68±0.11	1.40±0.09	3.49±0.09	1.44±0.04	1.63±0.02	4.53±0.09	0.34±0.22	3.59±0.22	41.15±1.69
	<i>Q. grisea</i> backcross	11.42±0.23	10.09±0.22	5.29±0.14	1.94±0.11	4.72±0.07	1.78±0.05	1.85±0.02	6.23±0.08	0.38±0.21	3.72±0.13	59.09±2.68
Sept. 15	<i>Q. gambelii</i> × <i>Q. grisea</i>	10.20±0.31	10.91±1.12	6.97±0.65	2.49±0.36	3.99±0.07	1.52±0.04	1.34±0.03	5.94±0.05	0.34±0.24	3.46±0.34	55.69±2.03
	<i>Q. gambelii</i> backcross	10.60±0.20	8.99±0.29	6.25±0.21	1.21±0.06	4.92±0.12	1.55±0.04	2.12±0.02	5.70±0.13	0.35±0.19	4.19±0.14	65.76±3.31
	<i>Q. gambelii</i>	10.19±0.27	8.84±0.17	6.48±0.12	0.92±0.05	5.20±0.09	1.41±0.05	1.60±0.02	6.94±0.05	0.41±0.24	3.24±0.13	74.49±2.96
Sept. 15	<i>Q. grisea</i>	9.48±0.22	9.53±0.18	4.31±0.09	1.91±0.08	4.57±0.07	1.78±0.07	2.01±0.02	4.29±0.07	0.56±0.13	4.02±0.15	61.05±3.88
	<i>Q. grisea</i> backcross	14.60±1.53	10.02±0.22	4.38±0.09	2.65±0.10	8.21±0.56	2.81±0.27	3.22±0.11	8.14±0.28	0.87±0.87	3.77±0.16	80.26±2.48
	<i>Q. gambelii</i> × <i>Q. grisea</i>	9.29±0.20	10.28±0.23	4.90±0.15	2.12±0.08	5.43±0.08	1.60±0.04	1.91±0.01	5.41±0.07	0.54±0.20	3.42±0.20	76.61±2.94
Sept. 15	<i>Q. gambelii</i> backcross	9.73±0.16	10.20±0.18	5.01±0.12	1.64±0.07	5.52±0.09	2.00±0.08	2.36±0.02	5.09±0.07	0.54±0.17	4.13±0.16	103.26±2.88
	<i>Q. gambelii</i>	8.52±0.18	10.15±0.17	5.51±0.17	1.34±0.04	6.20±0.08	1.64±0.05	1.89±0.02	5.37±0.09	0.47±0.24	3.35±0.15	134.44±6.97

Table 3. Multivariate analysis-of-variance (MANOVA) and discriminant function analysis (DFA) of oak taxonomic categories in the *Q. gambelii* × *Q. grisea* hybrid zone based upon the absolute concentrations of ellagitannins and total proanthocyanidins.

MANOVA	Λ	F-Approx.	df	Significance				
June 9								
Wilks' Lambda	0.062	3.209	44, 132	$p < 0.001$				
July 1								
Wilks' Lambda	0.115	2.080	44, 120	$p < 0.001$				
August 10								
Wilks' Lambda	0.185	1.569	44, 124	$p = 0.028$				
Sept. 15								
Wilks' Lambda	0.202	1.511	44, 128	$p = 0.039$				
Compound	June 9		July 1		Aug. 10		Sept. 15	
	DF1	DF2	DF1	DF2	DF1	DF2	DF1	DF2
1	-0.123	0.121	-0.348	-0.113	-0.06	-0.152	0.237	-0.226
2	-0.058	0.365	-0.443	-0.068	-0.051	-0.249	-0.126	-0.089
3	-0.049	0.591	-0.812	0.013	-0.392	-0.127	-0.405	0.022
4	0.339	-0.496	0.373	-0.549	0.385	-0.294	0.522	-0.624
5	-0.094	0.563	-0.704	-0.329	-0.422	-0.106	-0.195	-0.355
6	-0.192	0.44	-0.113	-0.244	0.175	-0.09	0.175	-0.145
7	-0.253	0.194	-0.152	-0.138	-0.073	0.337	0.202	-0.159
8	-0.537	0.254	-0.639	-0.363	-0.343	-0.341	0.057	-0.404
9	-0.675	0.089	-0.382	-0.17	-0.08	-0.038	0.229	-0.204
10	-0.128	0.235	-0.322	0.337	-0.018	0.296	0.145	0.252
PA	0.069	-0.001	-0.303	-0.393	-0.455	-0.222	-0.712	-0.013
Eigenvalue	2.973	1.274	2.240	0.632	1.507	0.531	1.508	0.391
% Cum. Var.	60.3	86.1	64.8	83.1	62.1	83.9	66.8	83.6

^a The eigenvalues and proportion of variation accounted for by the first two discriminant axes are listed by date.

^b Correlation coefficients, r , are listed for the absolute concentrations of tannins and the canonical scores of individual trees along the first and second discriminant axis from the DFA.

^c Coefficients are listed in bold for those compounds that contribute strongly to the ordination of oak taxonomic categories.

Table 4. Multivariate analysis-of-variance (MANOVA) and discriminant function analysis (DFA) of oak taxonomic categories in the *Q. gambelii* × *Q. grisea* hybrid zone based upon the relative concentrations of ellagitannins and total proanthocyanidins.

MANOVA	Λ	F-Approx	df	Significance				
June 9								
Wilks' Lambda	0.075	3.286	40, 134	$p < 0.001$				
July 1								
Wilks' Lambda	0.155	1.959	40, 123	$p = 0.003$				
August 10								
Wilks' Lambda	0.213	1.600	40, 126	$p = 0.026$				
Sept. 15								
Wilks' Lambda	0.216	1.629	40, 130	$p = 0.021$				
Compound	June 9		July 1		Aug. 10		Sept. 15	
	DF1	DF2	DF1	DF2	DF1	DF2	DF1	DF2
1	0.004	0.026	0.112	0.328	0.132	-0.267	0.29	-0.212
2	0.316	-0.124	-0.035	0.078	0.089	-0.415	-0.161	0.343
3	0.24	0.259	-0.622	0.113	-0.347	-0.013	-0.383	0.424
4	0.217	-0.64	0.725	-0.216	0.578	-0.289	0.439	-0.316
5	0.224	0.389	-0.62	-0.297	-0.47	-0.338	-0.297	-0.374
6	0.114	0.269	0.299	0.127	0.427	0.037	0.16	-0.16
7	-0.173	-0.158	0.207	0.264	0.045	0.43	0.21	-0.27
8	-0.56	0.159	-0.548	-0.362	-0.36	-0.537	0.026	-0.445
9	-0.802	0.265	0.009	0.387	0.131	0.285	0.268	0.16
PA	0.114	-0.326	-0.154	-0.356	-0.408	-0.288	-0.719	-0.001
Eigenvalue	1.813	1.409	2.039	0.516	1.444	0.382	1.491	0.375
% Cum. Var.	44.6	79.3	69.6	87.3	65.7	83.3	67.8	84.8

^a The eigenvalues and proportion of variation accounted for by the first two discriminant axes are listed by date.

^b Correlation coefficients, r , are listed for the relative concentrations of tannins and the canonical scores of individual trees along the first and second discriminant axis from the DFA.

^c Coefficients are listed in bold for those compounds that contribute strongly to the ordination of oak taxonomic categories.

Relative dimensions of tannin phenotypes

Variation in the monthly relative concentrations of ellagitannins and total proanthocyanidins among taxa within the *Q. gambelii* × *Q. grisea* hybrid zone produced four significant discriminant functions. The first two discriminant functions accounted for 79–87 % of the variation among hybrid zone taxa (Table 4), where the first discriminant axis accounted for 45–68 % of the variation in the relative dimension of tannin phenotypes (Fig. 2). In June, relative dimensions of tannin phenotypes were primarily described by increasing relative concentrations of ellagitannin (9), and partitioned *Q. gambelii* and *Q. grisea* from the remaining taxa within the hybrid zone (Fig. 2). In July, tannin phenotypes were described by increasing relative concentrations of vescalagin (3) and cocciferin D₂ (8), and decreasing concentrations of mongolinin A (4) in *Q. gambelii* and *Q. gambelii* backcrosses compared to other taxa within the hybrid zone (Fig. 2). In August, decreasing relative concentrations of mongolinin A (4) and castalagin (6), and increasing relative concentrations of pedunculagin (5) and total proanthocyanidins (PA) separated *Q. gambelii* and *Q. gambelii* backcrosses from other taxa within the hybrid zone. In September, relative dimensions of tannin phenotypes corresponded to decreasing relative concentrations of mongolinin A (4) and increasing concentrations of total proanthocyanidins (PA), and separated *Q. gambelii* and *Q. gambelii* backcrosses from other taxa within the hybrid zone (Fig. 2).

The majority of the total variation among *Q. gambelii* × *Q. grisea* hybrid zone taxa in the log_e+1 concentrations of ellagitannins and total proanthocyanidins from June to September could be attributed to variation in the log_e+1 relative concentrations. The ratio of the sum of the eigenvalues of the first three discriminant functions for the absolute and relative concentrations indicates that between 76–98 % of the total variation in tannins between hybrid zone taxa was due to variation in relative concentrations (Tables 3 & 4). The proportion of variation in tannin phenotypes attributable to the relative concentration of tannins increased from June to September.

Leaf-miners in *Q. gambelii* × *Q. grisea*

The most abundant leaf-miners in the *Q. gambelii* × *Q. grisea* hybrid zone were *Cameraria* sp. and *Phyllonorycter* sp. (Fig. 3). All leaf-miner species were significantly distributed across the hybrid zone with lowest densities observed in *Q. grisea* (ANOVA; all species $p \leq 0.01$; Fig. 3). The distribution of *Cameraria* sp. and *Phyllonorycter* sp. were consistent with the hybrid intermediacy hypothesis (Aguilar and Boecklen 1992). However, less abundant leaf-miner species were not observed in all oak taxonomic categories and were distributed in a dominant fashion (Hjalten 1997) with highest abundances in *Q. gambelii* × *Q. grisea*, *Q. gambelii* backcrosses, and *Q. gambelii*.

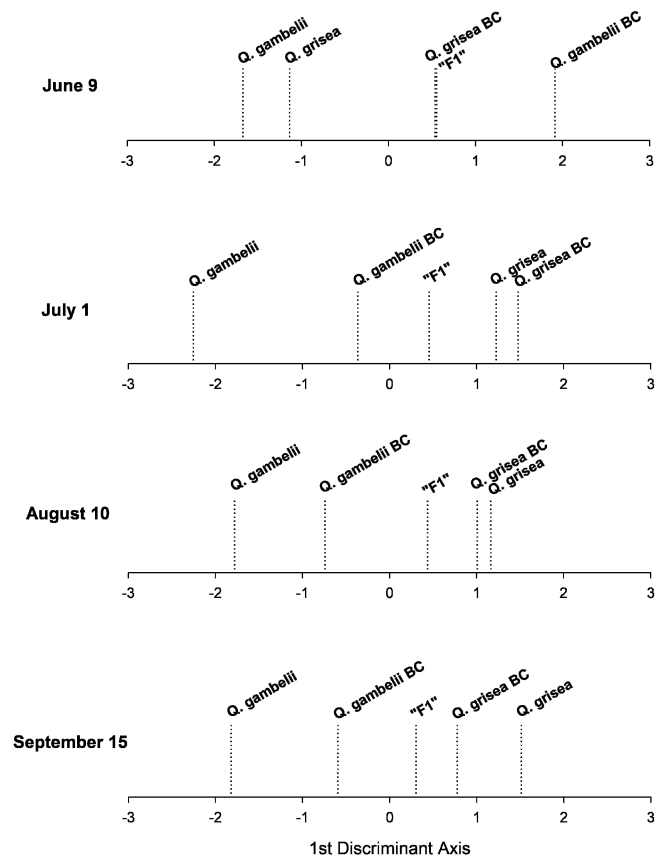


Figure 2. Classification of oak taxonomic categories based upon the relative concentrations of individual ellagitannins and total proanthocyanidins. Ordination is based upon the canonical score of each category along the first discriminant axis from discriminant function analysis.

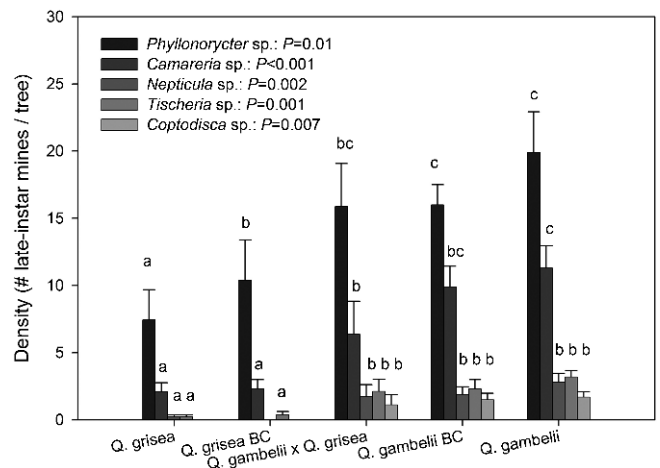


Figure 3. Densities of leaf-mining moths in the *Q. gambelii* × *Q. grisea* hybrid zone. Density = number of late-instar mines per tree (\pm SE).

Table 5. Canonical correlation analysis (CCA) and multiple regression analysis of absolute concentrations of ellagitannins and total proanthocyanidins and leaf-miner densities. Regression coefficients and *p*-values for the full regression model are listed for the significant CCA in July and August.

Absolute Concentrations		<i>Cameraria</i> sp.	<i>Coptodisca</i> sp.	<i>Nepticula</i> sp.	<i>Phyllonorycter</i> sp.	<i>Tischeria</i> sp.
July	1 Vescavalonic acid	0.478	0.189	0.103	0.448	-0.189
	2 Castavalonic acid	-0.111	-0.071	-0.091	-0.498	-0.009
	3 Vescalagin	0.578	0.206	0.536	0.683	0.284
	4 Mongolinin A	-0.439	-0.382	-0.335	-0.115	-0.567
	5 Pedunculagin	-0.42	0.329	-0.331	-0.056	-0.617
	6 Castalagin	-0.444	-0.176	-0.226	-0.427	0.153
	7 Acutissimin A/B	0.041	0.094	-0.052	0.22	0.159
	8 Cocciferin D ₂	0.353	-0.444	0.45	0.245	1.028
	9 Ellagitannin	-0.149	0.342	-0.231	-0.621	-0.387
	10 Ellagitannin	-0.216	-0.161	0.232	0.077	0.168
	Proanthocyanidins	0.341	0.093	0.203	0.145	0.036
Significance	0.015	0.273	0.006	0.058	0.001	
Rao's <i>F</i> - approx. = 1.673, <i>df</i> = 55, 133.2 <i>p</i> = 0.009						
		<i>Cameraria</i> sp.	<i>Coptodisca</i> sp.	<i>Nepticula</i> sp.	<i>Phyllonorycter</i> sp.	<i>Tischeria</i> sp.
August	1 Vescavalonic acid	0.405	-0.051	0.327	0.305	0.554
	2 Castavalonic acid	-1.035	-0.193	-0.732	-0.399	-1.065
	3 Vescalagin	0.346	0.389	0.533	-0.017	0.358
	4 Mongolinin A	-0.214	-0.346	-0.258	-0.447	-0.298
	5 Pedunculagin	0.041	-0.167	0.271	-0.033	0.367
	6 Castalagin	-0.357	-0.114	-0.221	-0.393	-0.196
	7 Acutissimin A/B	-0.217	0.217	-0.004	-0.173	-0.232
	8 Cocciferin D ₂	1.22	0.254	0.417	0.849	0.178
	9 Ellagitannin	-0.521	-0.244	-0.127	-0.444	-0.155
	10 Ellagitannin	0.236	0.091	-0.078	0.304	0.494
	Proanthocyanidins	0.03	0.285	0.116	0.165	0.316
Significance	<0.001	0.347	0.006	0.048	0.008	
Rao's <i>F</i> - approx. = 1.454, <i>df</i> = 55, 137.8 <i>p</i> = 0.042						
		<i>Cameraria</i> sp.	<i>Coptodisca</i> sp.	<i>Nepticula</i> sp.	<i>Phyllonorycter</i> sp.	<i>Tischeria</i> sp.
2004	1 Vescavalonic acid	-0.027	0.445	-0.057	0.065	-0.012
	2 Castavalonic acid	0.074	-0.260	0.405	-0.244	0.145
	3 Vescalagin	0.251	0.908	0.095	0.336	-0.175
	4 Mongolinin A	-0.564	-0.131	-0.661	-0.338	-0.683
	5 Pedunculagin	-0.245	-0.178	-0.423	0.041	-0.417
	6 Castalagin	-0.229	-0.560	-0.129	-0.677	0.109
	7 Acutissimin A/B	0.197	0.405	0.277	0.141	0.057
	8 Cocciferin D ₂	0.636	-0.277	0.569	0.335	0.827
	9 Ellagitannin	-0.245	0.095	-0.291	-0.139	-0.537
	10 Ellagitannin	0.052	-0.405	-0.163	0.023	0.275
	Proanthocyanidins	0.315	-0.019	0.344	0.192	0.348
Significance	<0.001	0.001	0.018	0.004	0.065	
Rao's <i>F</i> - approx. = 1.810, <i>df</i> = 55, 147.1 <i>p</i> = 0.003						

Community structure and tannins

Insect community structure was significantly correlated with the absolute concentrations of tannins in July and August (Table 5), but not in June (Rao's *F*-approx. = 1.017_{55,142.5}, *P* = 0.458) or September (Rao's *F*-approx. = 1.083_{55,133.2}, *P* = 0.35). Similarly, insect community structure was significantly correlated with the relative concentrations of tannins in July and August (Table 6), but not in June (Rao's *F*-approx. = 1.203_{50,144.7}, *P* = 0.458) or September (Rao's *F*-approx. = 1.13_{50,144.7}, *P* = 0.285). Insect community structure was signifi-

cantly related to both the annual mean absolute and relative concentrations of tannins in 2004 (Tables 5 & 6).

The abundances of all leaf-miner species were significantly correlated with the annual average of the absolute and relative concentrations of tannins in the oaks of the *Q. gambelii* × *Q. grisea* hybrid zone in 2004 (Tables 5 & 6). All leaf-miner species were negatively associated with increasing absolute and relative concentrations of mongolinin A (4), while four of the five species were also negatively associated with increased concentrations of castalagin (6) and positively associated with vescalagin

Table 6. Canonical correlation analysis (CCA) and multiple regression analysis of relative concentrations of ellagitannins and total proanthocyanidins and leaf-miner densities. Regression coefficients and p-values for the full regression model are listed for the significant CCA in July and August.

Relative Concentrations		<i>Cameraria</i> sp.	<i>Coptodisca</i> sp.	<i>Nepticula</i> sp.	<i>Phyllonorycter</i> sp.	<i>Tischeria</i> sp.
July	1 Vescavalonic acid	-0.062	0.121	-0.041	-0.159	-0.099
	2 Castavalonic acid	-0.022	0.036	0.097	0.08	-0.005
	3 Vescalagin	0.394	0.236	0.505	0.414	0.342
	4 Mongolinin A	-0.479	-0.473	-0.517	-0.399	-0.346
	5 Pedunculagin	0.362	0.218	0.42	0.335	0.178
	6 Castalagin	-0.193	-0.023	-0.153	-0.283	-0.273
	7 Acutissimin A/B	-0.011	-0.074	-0.136	-0.022	0.128
	8 Cocciferin D ₂	0.541	0.164	0.536	0.273	0.229
	9 Ellagitannin	0.061	-0.019	-0.009	-0.315	-0.14
	Proanthocyanidins	0.18	-0.065	0.175	0.116	0.134
Significance		0.001	0.297	< 0.001	0.006	0.01
Rao's <i>F</i> - approx. = 1.661, <i>df</i> = 50, 135.6 <i>p</i> = 0.011						
		<i>Cameraria</i> sp.	<i>Coptodisca</i> sp.	<i>Nepticula</i> sp.	<i>Phyllonorycter</i> sp.	<i>Tischeria</i> sp.
August	1 Vescavalonic acid	0.086	-0.04	0.099	0.309	0.476
	2 Castavalonic acid	-0.103	-0.066	-0.038	-0.411	-0.509
	3 Vescalagin	0.503	0.183	0.563	0.656	0.795
	4 Mongolinin A	-0.346	-0.642	-0.329	-0.108	-0.167
	5 Pedunculagin	-0.303	-0.28	-0.335	-0.222	-0.313
	6 Castalagin	-0.278	-0.067	-0.25	-0.432	-0.534
	7 Acutissimin A/B	0.07	0.01	-0.026	0.04	0.124
	8 Cocciferin D ₂	0.39	-0.033	0.412	0.221	0.053
	9 Ellagitannin	0.031	0.07	-0.003	-0.423	-0.238
	Proanthocyanidins	0.182	-0.034	0.226	0.119	0.256
Significance		< 0.001	0.157	0.001	0.015	0.014
Rao's <i>F</i> - approx. = 1.570, <i>df</i> = 50, 140.2 <i>p</i> = 0.021						
		<i>Cameraria</i> sp.	<i>Coptodisca</i> sp.	<i>Nepticula</i> sp.	<i>Phyllonorycter</i> sp.	<i>Tischeria</i> sp.
2004	1 Vescavalonic acid	0.069	0.221	-0.055	-0.033	0.042
	2 Castavalonic acid	0.216	-0.446	0.270	-0.141	0.324
	3 Vescalagin	0.352	0.657	0.059	0.292	-0.30
	4 Mongolinin A	-0.407	-0.163	-0.621	-0.304	-0.607
	5 Pedunculagin	0.014	-0.129	-0.282	-0.057	-0.319
	6 Castalagin	-0.189	-0.405	-0.051	-0.479	0.081
	7 Acutissimin A/B	0.403	-0.064	0.115	0.047	0.324
	8 Cocciferin D ₂	0.316	-0.125	0.376	0.048	0.448
	9 Ellagitannin	0.005	-0.025	-0.208	-0.042	-0.289
	Proanthocyanidins	0.356	-0.010	0.344	0.160	0.334
Significance		< 0.001	< 0.001	0.012	0.021	0.048
Rao's <i>F</i> - approx. = 2.162, <i>df</i> = 50, 149.3 <i>p</i> < 0.001						

(**3**), cocciferin D₂ (**8**) and total proanthocyanidins (Tables 5 & 6).

The abundances of four of the five leaf-miner species (*Coptodisca* sp. = n.s.) were significantly related to the absolute concentrations of ellagitannins and total proanthocyanidins in the *Q. gambelii* × *Q. grisea* hybrid zone in July and August (Table 5). These four species were all negatively associated with castavalonic acid (**2**), mongolinin A (**4**), castalagin (**6**) (except *Tischeria* sp.), and ellagitannin (**9**), and positively associated with absolute concentrations of vescalagin (**3**), cocciferin D₂ (**8**), and total proanthocyanidins in July (Table 5). In August, all leaf-miner species were negatively associated with castavalonic acid (**2**), mongolinin A (**4**), castalagin (**6**), and ellagitannin (**9**), and the four leaf-miner species whose

abundance was significantly related to tannin phenotypes were positively associated with absolute concentrations of vescalagin (**3**) (except *Phyllonorycter* sp.), cocciferin D₂ (**8**), and total proanthocyanidins.

While the overall leaf-miner community was not significantly correlated with the absolute concentrations of ellagitannins and total proanthocyanidins in June and September, there were significant multiple linear regressions for some individual species. In June, *Cameraria* sp. ($F_{10,35} = 2.2$, $P = 0.03$, $R^2 = 0.416$) and *Nepticula* sp. ($F_{10,35} = 2.517$, $P = 0.02$, $R^2 = 0.449$) were individually correlated with absolute concentrations of oak tannins, while *Cameraria* sp. ($F_{10,35} = 2.836$, $P = 0.01$, $R^2 = 0.494$) and was correlated with absolute concentrations of tannins in September.

Table 7 Correlation analysis of leaf-miner density and the ratio of ellagitannin products and total proanthocyanidins throughout 2004. Castalagin derivatives are the sum concentration of castavalonic acid (2) and cocciferin D₂ (8). Vescalagin derivatives are the sum concentration of vescalvalonic acid (1), mongolinin A (4), and acutissimin A/B (7).

		Castalagin derivatives: proanthocyanidins		Vescalagin derivatives: proanthocyanidins	
		r	p	r	p
June 9	<i>Cameraria</i> sp.	-0.031	0.837	-0.099	0.514
	<i>Coptodisca</i> sp.	0.011	0.944	-0.147	0.329
	<i>Nepticula</i> sp.	-0.072	0.632	-0.204	0.174
	<i>Phyllonorycter</i> sp.	0.141	0.349	0.018	0.903
July 1	<i>Tischeria</i> sp.	0.164	0.276	0.134	0.374
	<i>Cameraria</i> sp.	0.107	0.489	-0.209	0.173
	<i>Coptodisca</i> sp.	0.233	0.129	0.02	0.897
	<i>Nepticula</i> sp.	0.108	0.486	-0.139	0.369
August 10	<i>Phyllonorycter</i> sp.	0.125	0.418	-0.002	0.989
	<i>Tischeria</i> sp.	0.168	0.276	-0.083	0.594
	<i>Cameraria</i> sp.	-0.06	0.694	-0.233	0.123
	<i>Coptodisca</i> sp.	-0.184	0.227	-0.259	0.085
September 15	<i>Nepticula</i> sp.	-0.003	0.984	-0.226	0.136
	<i>Phyllonorycter</i> sp.	-0.21	0.166	-0.345	0.020
	<i>Tischeria</i> sp.	-0.104	0.498	-0.193	0.203
	<i>Cameraria</i> sp.	-0.113	0.466	-0.426	0.004
	<i>Coptodisca</i> sp.	-0.198	0.198	-0.273	0.073
	<i>Nepticula</i> sp.	-0.184	0.231	-0.458	0.002
	<i>Phyllonorycter</i> sp.	-0.144	0.350	-0.283	0.062
	<i>Tischeria</i> sp.	-0.106	0.494	-0.281	0.065

Abundances of four of the five leaf-miner species (*Coptodisca* sp. = n.s.) were also significantly correlated with the relative concentrations of ellagitannins and total proanthocyanidins in the *Q. gambelii* × *Q. grisea* hybrid zone in July and August (Table 6). These four species were all negatively associated with mongolinin A (4), castalagin (6), and ellagitannin (9), and positively associated with relative concentrations of vescalagin (3), cocciferin D₂ (8), and total proanthocyanidins in July (Table 6). In August, all leaf-miner species were negatively associated with castavalonic acid (2), mongolinin A (4), pedunculagin (5), and castalagin (6), and the four leaf-miner species whose abundance was significantly related to tannin phenotypes were positively associated with relative concentrations of vescalagin (3), cocciferin D₂ (8), and total proanthocyanidins.

While the overall leaf-miner community was not significantly correlated with the relative concentrations of ellagitannins and total proanthocyanidins in June and September, there were significant multiple linear regressions for some individual species. *Cameraria* sp. ($F_{10,35} = 2.166$, $P = 0.04$, $R^2 = 0.382$) and *Coptodisca* sp. ($F_{10,35} = 2.193$, $P = 0.04$, $R^2 = 0.385$) were individually correlated with relative concentrations of oak tannins in June, and *Cameraria* sp. ($F_{10,35} = 3.308$, $P = 0.004$, $R^2 = 0.486$) and *Phyllonorycter* sp. ($F_{10,35} = 2.329$, $P = 0.03$, $R^2 = 0.4$) were correlated with relative concentrations of tannins in September.

The ratio of castalagin-derivatives to total proanthocyanidins ratios was not significantly related to oak taxonomic category in June ($F_{4,44} = 1.483$, $P = 0.224$), July ($F_{4,39} = 1.048$, $P = 0.395$), August ($F_{4,40} = 0.368$, $P = 0.830$), or September ($F_{4,39} = 1.476$, $P = 0.228$). The ratio of

vescalagin-derivatives to total proanthocyanidins ratios was not significantly related to oak taxonomic category in June ($F_{4,44} = 1.082$, $P = 0.377$), July ($F_{4,39} = 1.260$, $P = 0.302$), or August ($F_{4,40} = 1.816$, $P = 0.145$), however *Q. gambelii* and *Q. gambelii* backcrosses contained lower vescalagin-derivative: proanthocyanidin ratios in September ($F_{4,39} = 4.726$, $P = 0.003$; Fig. 4). Densities of late-instar leaf-miners were not correlated with the castalagin-derivative: proanthocyanidin ratios in 2004 (Table 7). Densities of late-instar leaf-miners were also not related to vescalagin-derivative: proanthocyanidin ratios in June or July. *Phyllonorycter* sp. densities were negatively correlated with vescalagin-derivative: proanthocyanidin ratios in August, while *Coptodisca* sp. density was marginally lower with increasing vescalagin-derivative: proanthocyanidin ratios. In September, *Cameraria* sp. and *Nepticula* sp. were negatively correlated with vescalagin-derivative: proanthocyanidin ratios, while densities of *Coptodisca* sp., *Phyllonorycter* sp. and *Tischeria* sp. were only marginally related to the same ratios ($r \leq -0.25$, $P \leq 0.075$; Table 7).

Discussion

The production of ellagitannins within the *Q. gambelii* × *Q. grisea* hybrid zone typically peaked in July or August, while total proanthocyanidins steadily increased from June through September (Table 2). As such, tannin phenotypes varied seasonally both within and among oak taxonomic categories of the *Q. gambelii* × *Q. grisea* hybrid zone. The vast majority (>75 %) of variation in tannin phenotypes between taxonomic categories was contained

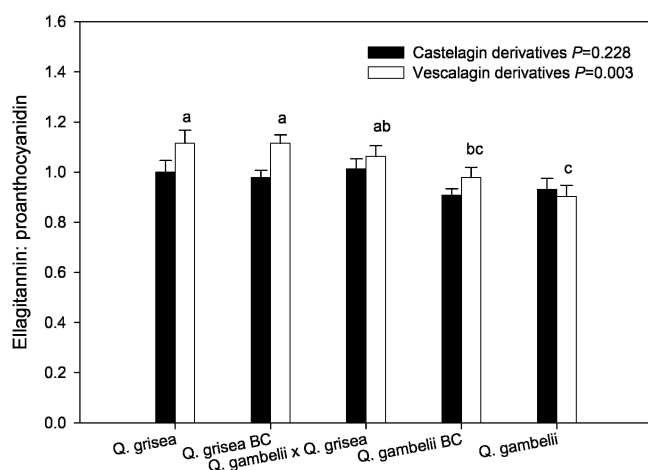


Figure 4. Ellagitannin: proanthocyanidin ratios of oak taxonomic categories in *Q. gambelii* × *Q. grisea* hybrid zone on September 14, 2004. Castelagin derivatives are the sum concentration of castavalonic acid (2) and cocciferin D2 (8). Vescalagin derivatives are the sum concentration of vescalvalonic acid (1), mongolinin A (4), and acutissimin A/B (7).

in differences between the relative concentrations of tannins. The importance of variation in relative concentrations to the overall variation in tannin phenotypes is consistent with previous studies in other Chihuahuan Desert oaks (Yarnes et al. 2006). The greatest variation in tannin phenotypes between oak taxonomic categories occurs between June 9 and July 1. In late summer (August–September), oaks intergraded taxonomically from *Q. gambelii* to *Q. grisea* based upon the ordination of tannin phenotypes.

In *Q. gambelii* × *Q. grisea* hybrid zone, *Q. grisea* exhibited most seasonal variability in tannin chemistry. *Q. grisea* backcrosses had the lowest concentration of nine of the ten ellagitannins on June 9, yet produced the highest concentration of seven of the ten ellagitannins on September 15. Relative to other hybrid oaks, the abnormal seasonal variation in *Q. grisea* backcrosses could potentially lead to poor oviposition choices by female leaf-miners in early summer if ellagitannins negatively affect larval growth primarily in late-summer. Recent work by Gripenberg et al. (2007) has found close correlations between the temporal consistency in *Q. robur* ellagitannin composition and leaf-miner survival (mine initiation and larval survival) in *Tischeria ekebladella*. Abnormal seasonal variation in the tannin chemistry of hybrid taxa may hinder either the transition to or relative use of an alternate, yet suitable, host species, i.e. *Q. grisea*. Notably, *Q. grisea* backcrosses hosted the fewest number of leaf-miner species.

Tannins that contributed most strongly to the classification of oak taxonomic categories were variable with sampling date. However, the relative concentrations of mongolinin A (4) and cocciferin D₂ (8) were consistently important from June–September. In combination with total proanthocyanidins from July–September, these high

molecular-weight ellagitannins predominantly determined the distribution of tannin phenotypes within the *Q. gambelii* × *Q. grisea* hybrid zone (Table 4). Indeed, two primary tannin phenotypes were found within the *Q. gambelii* × *Q. grisea* hybrid zone and were characterized by the relative biosynthesis of products of the isomeric ellagitannins, castalagin (6) and vescalagin (3) (Fig. 5). “*Q. gambelii*-type” oaks contained higher concentrations of the castalagin end-product, cocciferin D₂ (8), while “*Q. grisea*-type” oaks typically exhibited higher concentrations of vescalagin end-products, esp. mongolinin A (4). Further, “*Q. gambelii*-type” oaks produced higher vescalagin concentrations consistent with reduced biosynthetic activity in the latter portion of this pathway and accumulation of the intermediate compound. Conversely, “*Q. grisea*-type” oaks accumulated higher castalagin concentrations in August and September. The relative activity of proanthocyanidin polymerization may limit the amount of catechin monomers available for the synthesis of mongolinin A (4) and acutissimin A/B (7). Therefore, the higher concentrations of proanthocyanidins in “*Q. gambelii*-type” oaks may indicate substrate limitation for catechin and help account for the lower concentrations of mongolinin A (4) and acutissimin A/B (7). However, the higher concentrations of acutissimin A/B (7) in “*Q. gambelii*-type” oaks from June through July suggests that variation in the production of vescalagin-derivatives between the two tannin phenotypes is unlikely to be explained wholly by substrate limitation.

Leaf-miner community structure was significantly correlated with ellagitannins and total proanthocyanidins in July and August. In July, densities of all leaf-miner species were positively related to the absolute concentrations of vescalagin (3), pedunculagin (5), and cocciferin D₂ (8), and negatively associated with mongolinin A (4) and castalagin (6) (Table 6). These compounds closely mirror those that determine the two dominant tannin phenotypes within *Q. gambelii* × *Q. grisea*, as defined through DFA, and indicate a positive correlation with “*Q. gambelii*-type” oaks. In August, the correlation between oak phenotypes and leaf-miner densities was less clear. However, similar to July, all leaf-miner species were positively associated with vescalagin (3) and cocciferin D₂ (8), and negatively related to mongolinin A (4) and castalagin (6). As relative concentrations accounted for most of the total variation in tannin chemistry within the *Q. gambelii* × *Q. grisea* hybrid zone, patterns in the correlation of leaf-miner densities with the absolute concentrations of ellagitannins and proanthocyanidins followed closely, but not strictly, the relative concentrations of tannins (Table 5).

At the high pH found within herbivorous insect digestive tracts, ellagitannins produce numerous oxidation products (Barbehenn et al. 2006b) and oxidize at higher rates than other tannins (galloylglucoses, gallotannins, proanthocyanidins). Under these same conditions, proanthocyanidins reduce the oxidation products of ellagitannins in herbivores (Barbehenn et al. 2006b), which may

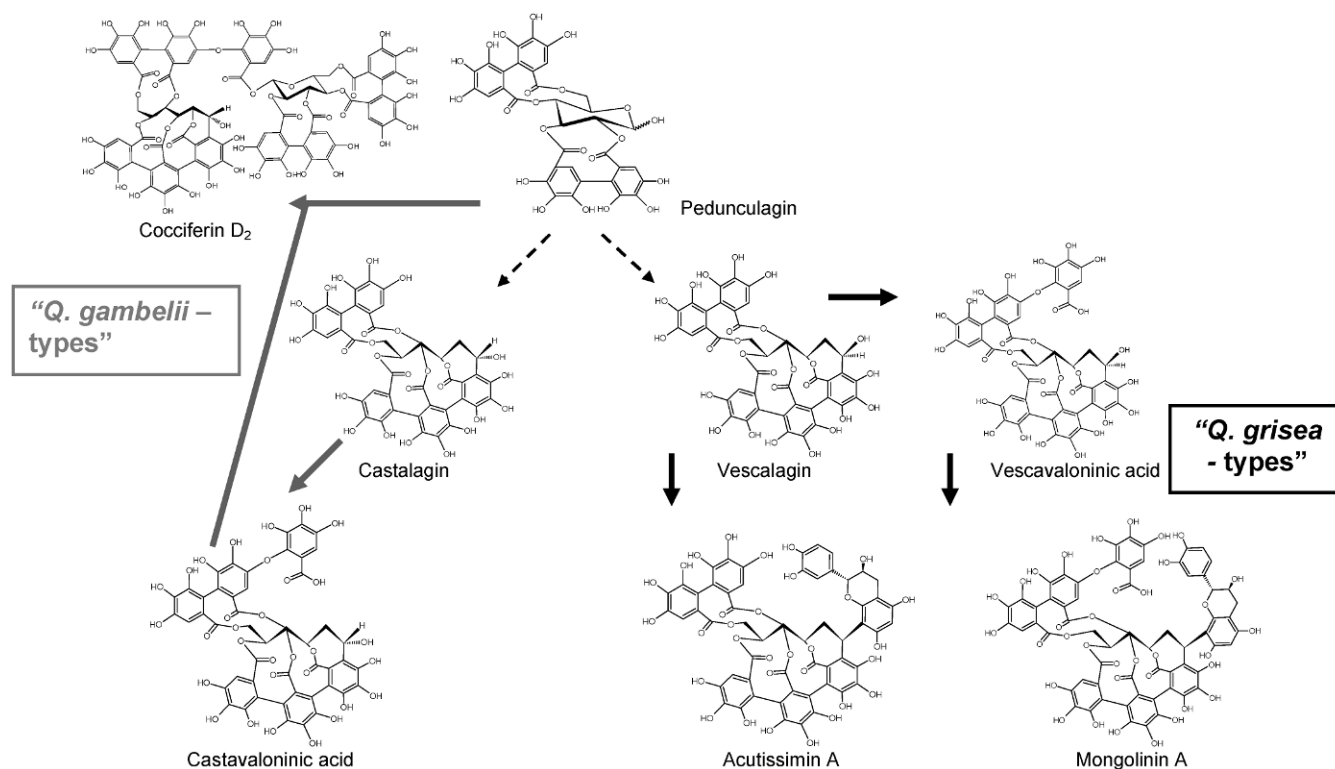


Figure 5. Biosynthetic pathway of tannins in *Quercus gambelii* x *Q. grisea* complex with special reference to ellagitannin biosynthesis. Dotted lines denote hidden metabolic intermediates not detected in this study (the isomers casuarictin and stachyurin).

partly explain the increased abundance of leaf-miners in *Q. gambelii* and its backcrosses (Fig. 3; Table 2). Densities of late-instar leaf-miners were negatively correlated with the ratio of vescalagin-derivatives to total proanthocyanidins in late summer. Further, the highest leaf-miner densities were on taxa with lowest vescalagin-derivative: proanthocyanidin ratios (Figs. 3 & 4).

Mid-summer (July/August) is an important period in the nutritional ecology of *Phyllonorycter* sp. larvae, as they undergo critical developmental changes in feeding behavior and most mortality likely occurs during this period (Yarnes and Boecklen 2005). Ellagitannins are primarily localized within cell walls and apoplastic spaces of foliar tissues (Grundhöfer et al. 2000). Because early-instar *Phyllonorycter* sp. larvae are sap-feeders, early-instars likely consume few ellagitannins. However, following the third-instar, *Phyllonorycter* sp. larvae mouthparts change in structure and orientation (R.W. Preszler, pers. comm., Preszler and Boecklen 1996) and the larvae begin chewing whole tissues rich in ellagitannins.

Evidence of both the direct and indirect effects of tannins on leaf-miner species is scarce, though probably due more to the difficulties of manipulating endophagous organisms. Further, these effects are likely to be complex. In *Q. emoryi*, leaves painted with tannin extracts increased *Cameraria* sp. mine predation, but decreased other sources of larval mortality (Faeth and Bultman 1986). Temporally, ellagitannins may initially have little effect on early-instar physiology or even provide a benefit

to young leaf-miner larvae through putative antimicrobial effects on pathogenic endophytic microbes; however, subsequent to the third-instar, ellagitannins would likely reduce the performance of late-instar leaf-miners once they begin to feed on parenchyma tissue. The negative correlation between late-instar leaf-miner densities and vescalagin-derivative: proanthocyanidin ratios in August and September in 2004 may reflect those developmental changes in exposure to ellagitannins, as modified by proanthocyanidin content. Further, it is possible that the current distribution of leaf-miners within the *Q. gambelii* x *Q. grisea* hybrid zone represents an ongoing host-shift. Morphological and physical components of hybridization may be facilitating the use of *Q. grisea* by leaf-mining herbivores of *Q. gambelii*, while remaining differences in ellagitannin and proanthocyanidins biosynthesis may still restrict significant use of *Q. grisea* and its backcrosses.

Data are plentiful concerning variation in herbivore community structure and community interactions across hybrid progeny (Whitham 1989, Boecklen and Spellenberg 1990, Aguilar and Boecklen 1992, Floate et al. 1993, Fritz et al. 1996, Preszler and Boecklen 1994). However, proposed mechanistic explanations of these patterns of herbivore distribution and abundance have suffered from a continued reliance on antiquated measurement techniques of tannins in hybrid plants. Most studies have focused on a limited number of compounds or an unreliable summary measure of tannins (e.g. Schweitzer et al. 2004, Bailey et al. 2006; see Appel et al. 2001, Salminen 2003)

and have generally ignored the potential for important biological action of tannins as complex mixtures.

The importance of structural and biosynthetic information about individual compounds and action as complex mixtures is critical to the further study of tannins in plant-herbivore interactions and cannot be underestimated. The use of size and shape analysis to examine complex phenotypic changes in ellagitannin metabolism through a natural hybrid zone has allowed for the identification of important characteristics of tannin biochemistry that correlate with herbivore community structure and trophic interactions in *Q. gambelii* × *Q. grisea* that would have been impossible without a compound-specific metabolic approach. The results of this study also emphasize the importance of evaluating temporal variation of individual compounds when examining phytochemical characteristics of plant-herbivore interactions within hybrid zones. This may be especially important regarding tests of the hybrid bridge hypothesis (Floate and Whitham 1993), where hybridization and introgression of parental traits, such as biosynthetic control of plant defenses, are thought to facilitate host shifts in herbivores. Failure to consider compound-specific, temporal, and spatial variation in phytochemistry limits the empirical content of phytochemical research in hybrid zones and may yield erroneous conclusions.

In summary, compound-level defense chemistry data can be used to construct statistically-useful biological phenotypes and reveal detailed aspects of co-variation between defense metabolism and herbivore communities in natural hybrid zones. This approach has the potential to illuminate physiological theories of plant defense metabolism and provide more detailed and biologically-meaningful information for which to anchor population genetic data. Combined with laboratory and experimental manipulations of tannin chemistry, the size and shape analysis of compound-level phytochemistry analyses approach represents a fruitful analytical approach that may help to clarify the role of phytochemistry in the evolutionary ecology of plant-herbivore interactions in hybrid zones.

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