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HYBRIDIZATION AFFECTS SEASONAL VARIATION OF PHYTOCHEMICAL PHENOTYPES IN AN OAK HYBRID COMPLEX (*QUERCUS GAMBELII* × *QUERCUS GRISEA*)

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The hybridization of plants can have significant consequences for the structure of consumer communities, and hybridization effects on plant defenses have been suggested to affect the distribution of consumers within hybrid zones. It is currently unknown how hybridization may affect ontogenetic patterns of phenolic biosynthesis. We describe the seasonal variation of absolute and relative concentrations of 18 individual phenolics, total proanthocyanidins, and nitrogen in the *Quercus grisea* × *Quercus gambelii* hybrid complex in central New Mexico. Expression patterns of individual compounds were most often equal between hybrid and parental taxonomic categories; nonequal patterns of expression were most often dominant (equal to one parent) toward *Q. gambelii*. These patterns of phenolic expression contrast with the more common additive patterns reported in hybrid zones of other tree species. *Quercus grisea* backcrosses displayed significant developmental instability in phenolic biosynthesis relative to other hybrid oaks. Importantly, the composition and structure of phenolic phenotypes were found to vary seasonally. Throughout the growing season, the majority (>93%) of variation between oak phenolic phenotypes was attributed to the relative concentrations of phenolics and nitrogen. The results of this study emphasize the importance of compound-specific evaluations of hybrid plant defense metabolites and metabolic variation in hybrid zones. These considerations are critical for the further empirical study of potential hybridization effects on the complex, dynamic mixtures that make up plant defense chemistry.

Keywords: hybrid, phenolic, *Quercus*, ellagitannin, seasonal, metabolism.

Online enhancements: tables.

Introduction

Hybridization among plant species is a commonly observed phenomenon that some botanists believe has contributed to the evolution of more than 70% of all angiosperms (Arnold 1997). Plant hybrid zones provide ecologists a unique opportunity for the study of the effects of natural biological variation in plant traits on herbivores (Whitham 1989; Boecklen and Spellenberg 1990; Aguilar and Boecklen 1992; Preszler and Boecklen 1994; Fritz et al. 1996), microbes (Gaylord et al. 1996; Preszler et al. 1996), and, potentially, ecosystems (Whitham et al. 2003; Bailey et al. 2004). A multitude of patterns in herbivory have been observed across hybrid zones (Boecklen and Spellenberg 1990; Aguilar and Boecklen 1992, Floate et al. 1993; Fritz et al. 1996; Hjalten 1997). Variability in host plant quality and defense between parental species and hybrids is often hypothesized as an explanation of these patterns in herbivory. However, several important ecological components of phytochemical variation have still not been accounted for within hybrid zones. While phytochemists have long emphasized the quantification of metabolic variation in plant defenses through compound-specific techniques, ecologists studying hybridization effects on plant defense have primarily utilized summary measures of compound types or analyzed a select few defense compounds. If the goal is to understand how genetic variation produced by hybridization affects plant defenses and ultimately herbivory, a more comprehensive examination of metabolic variation in individual phytochemicals and the potential interactive effects (synergistic or antagonistic) between individual compounds as a biologically dynamic mixture is critical (e.g., Barbehenn et al. 2006*a*, 2006*b*).

Biochemical changes in plant foliage throughout the year can also have significant effects on herbivore population dynamics and communities (Haukioja 2003; Tikkanen and Julkunen-Titto 2003; Riipi et al. 2004; Yarnes and Boecklen 2005). Ontogenetic changes in host quality correlate with seasonal changes in the ability of herbivores to tolerate or detoxify host tissue (Salminen and Lempa 2002; Haukioja 2003; but see Lahtinen et al. 2006). In the case of plant phenolics, seasonal patterns in the composition of individual phenolics appear to be well correlated with the activation of biosynthetic pathways (Hatano et al. 1986; Salminen et al. 2001). Hybridization may alter the biogenetic control of phytochemical pathways and resource allocation, and changes in the seasonal variation of

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plant chemistry may then have subsequent effects on patterns of herbivory across hybrid taxa.

Typically, summary measures are used by ecologists studying phenolics (e.g., total phenolics, total proanthocyanidins, gallotannins). While there are certainly valid reasons for selecting these types of analyses, individual phenolics rarely have the same biological activity (Feldman et al. 1999), and they exhibit complex patterns of bioactivity and ecological function corresponding to differences in molecular structure (Clausen et al. 1990; Ayres et al. 1997; Barbehenn et al. 2006*a*, 2006*b*). Structural differences determine rates of radical scavenging (Hagerman et al. 1998), protein precipitation (Scalbert 1991), and metal chelation (Mila et al. 1996) as well as levels of toxicity (Nishizawa et al. 1990; Ayres et al. 1997) and prooxidant activity in consumers (Barbehenn et al. 2005). The use of summary measures is also problematic when quantifying seasonal variation in phenolics because concentrations of individual phenolics do not vary in unison across plants or within plants across season (Salminen et al. 2001, 2004; Haukioja 2003). Further, genetic correlations of summary measures of phenolics in hybrid zones may be spurious (e.g., Whitham et al. 2003) because the genetic and enzymatic control of phenolic biosynthesis is complex and not uniform (Niemetz et al. 2001; Dixon et al. 2005). Moreover, methods for the measurement of summary phenolics are poor approximations of the sum of the concentration of individual phenolics and exhibit significant analytical artifacts (Appel et al. 2001; Salminen et al. 2004).

The effects of hybridization on plant defense metabolism cannot be fully understood without considering variation in the relative concentrations of individual compounds. The relative concentrations of plant phenolics may be important in determining the defense capabilities of different phytochemical phenotypes (Rasmussen and Einhellig 1977; Castellanos and Espinosa-Garcia 1997; Barbehenn et al. 2001, 2006*a*, 2006*b*) but have historically received far less attention than absolute concentrations in plant defense theory (but see McKey 1979). The potential for synergistic and antagonistic effects between compounds in mixture (Langenheim 1994; Barbehenn et al. 2006*a*, 2006*b*) emphasizes the need for a more sufficient quantitative measurement of the complex variation in phytochemical phenotypes. A more complete assessment of the effects of hybridization on plant defenses may be obtained through the use of metabolic profiling and subsequent analysis of the absolute and relative differences in phytochemical phenotypes.

Phenolics are the most common group of putative defense compounds produced by oaks and include numerous simple phenolics, hydrolyzable tannins, proanthocyanidins, and flavonoid glycosides (Salminen et al. 2004; Yarnes et al. 2006). While crude fractions of oak phenolics have long been used to study the role of oak phenolics in herbivore ecology, only recently have ecological aspects of individual phenolics in oaks been considered (Tikkanen and Julkunen-Titto 2003; Salminen et al. 2004). The *Quercus gambelii* Nutt. × *Quercus grisea* Liebm. (Fagaceae) oak hybrid complex has been well studied with respect to genetic structure and gene flow (Howard et al. 1997; Williams et al. 2001) as well as associated communities of herbivores and endophytic fungi (Aguilar and Boecklen 1992; Preszler et al. 1996). A previous study of total phenolics in the *Q. gambelii* × *Q. grisea* complex suggests that seasonal variation in phenolics may differ between hybrids, parental species, and their backcrosses (Bihr 2001). In this study, we report the effects of hybridization on seasonal variation in the absolute and relative concentrations of individual phenolics, total proanthocyanidins, and nitrogen within the *Q. gambelii* × *Q. grisea* complex, including the parental species, *Q. gambelii* × *Q. grisea* hybrids, and backcrosses. We discuss patterns of expression between hybrid classes and seasonal variation in the phytochemical phenotype of hybrid oak taxonomic categories.

Material and Methods

Study System

Quercus gambelii, Rocky Mountain white oak, hybridizes with several oak species of the subgenus *Quercus* sect. *Quercus*, resulting in a number of multispecies hybrid complexes distributed throughout the southwestern United States (Tucker 1961). Hybrid progeny within these complexes are often colloquially referred to as "*Quercus undulata*." In the San Mateo Mountains of central New Mexico, a two-species hybrid complex exists between *Q. gambelii* and *Quercus grisea* (Preszler and Boecklen 1994). The genetic identities of trees within the Monica Canyon hybrid complex have been previously described using randomly amplified polymorphic DNA markers (Howard et al. 1997). Through the use of these 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, and (5) *Q. gambelii*.

Leaf Collection

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) and 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. Dried oak leaves were then ground to a fine powder using a ball mill (Wig-L-Bug, Reflex Analytical, Ridgewood, NJ) and pooled within trees. After grinding, phenolics were extracted three times from leaf tissue (ca. 20 mg dry weight) with 70% aqueous acetone + 0.1% ascorbic acid (added to prevent oxidation of phenolics). This extraction solvent was found to provide better recovery than a wide range of aqueous solvents (methanol: 50% MeOH, 70% MeOH, 85% MeOH; acetone: 50% Me_2CO, 70% Me_2CO, 85% Me_2CO; 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, and the supernatants were centrifuged (10 min at 5000 *g*), filtered through a 0.45-μm polytetrafluoroethylene filter, and kept frozen at −20°C until analysis with high-performance liquid chromatography–diode array detection–electrospray ionization–mass spectrometry (HPLC-DAD-ESI-MS).

Table 1

MANOVA of Absolute Concentrations of Phenolics and Nitrogen across Hybrid Classes in the *Quercus gambelii* × *Quercus grisea* Complex Partitioned by Sampling Date

Statistic	Value	*F*	df	*P*	Discriminant function analysis	Eigenvalue	Cumulative variation (%)
June 9:							
Wilks's λ	.012	2.634	80, 101	<.001	DF1	4.026	42.8
Pillai trace	2.526	2.399	80, 112	<.001	DF2	3.22	76.9
Hotelling-Lawley trace	9.417	2.766	80, 94	<.001	DF3	1.662	85
July 1:							
Wilks's λ	.031	1.564	80, 89	.02	DF1	3.769	57.5
Pillai trace	2.16	1.467	80, 100	.035	DF2	1.537	81
Hotelling-Lawley trace	6.552	1.679	80, 82	.01	DF3	.713	91.9
August 10:							
Wilks's λ	.053	1.290	80, 93	.118	DF1	2.752	54
Pillai trace	1.932	1.214	80, 104	.176	DF2	1.356	81
Hotelling-Lawley trace	5.058	1.359	80, 86	.081	DF3	.66	94
September 15:							
Wilks's λ	.046	1.430	80, 97	.046	DF1	3.624	63.4
Pillai trace	1.96	1.297	80, 108	.104	DF2	.985	80.7
Hotelling-Lawley trace	5.712	1.607	80, 90	.015	DF3	.791	94.5

Note. Analyses were performed on the $\log_e + 1$ concentration size variables.

Phytochemical Analysis

Individual hydrolyzable tannins, flavonoid glycosides, and simple phenolics were quantified (mg/g dry weight) following the methods outlined by Salminen et al. (1999). The water-soluble phenolic compounds of oak leaves were analyzed under negative electrospray ionization with HPLC-DAD-ESI-MS (2795 Separations module with 2996 DAD, Waters, Milford, MA; Micromass ZMD 2000) at 280, 315, and 349 nm. Two solvents were used: solvent A, 0.1% formic acid (HCOOH) in H_2O, and solvent 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 mm × 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/h. The DAD was operated at 280, 315, and 349 nm.

Individual compounds were identified on the basis of ultraviolet and mass spectra as well as retention times. Simple phenolics, hydrolyzable and complex tannins, and flavonoid glycosides were quantified using chlorogenic acid, penta-galloylglucose, and kaempferol as external standards. 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 by 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.

Foliar nitrogen content (% dry mass) was measured via elemental analysis as described by Yarnes and Boecklen (2005). Total proanthocyanidins were measured using the butanol-HCl assay as outlined by Ossipova et al. (2001), using a Hach DR/4000U UV-Vis spectrophotometer (Ames, IA). Measurements were quantified using a standard curve prepared using quebracho tannin (Tannin, Peabody, MA). The use of this summary measure of proanthocyanidins was necessary because of extensive chromatographic interference between proanthocyanidin polymers and the abundant ellagitannins in these oaks in normal-phase HPLC (Yarnes et al. 2006).

Table 2

Univariate Repeated-Measures ANOVA Tests of Absolute Concentrations for Each Compound

	Class		Date		Date × class	
Compound	Value	*P*	Value	*P*	Value	*P*
1	1.08	.375	21.78	<.001	1.26	.249
2	.47	.757	92.78	<.001	1.53	.122
3	.88	.481	415.2	<.001	.95	.502
4	5.63	<.001	9.46	<.001	1.09	.376
5	3.54	.013	250.31	<.001	1.52	.124
6	5.16	.002	80.38	<.001	1.36	.192
7	1.18	.332	13.42	<.001	1.95	.034
8	.87	.49	42.39	<.001	1.39	.178
9	3	.028	23.09	<.001	2.31	.01
10	.59	.669	127.92	<.001	.91	.541
11	2.64	.046	112.53	<.001	5.19	<.001
12	4.18	.005	6.66	<.001	1.43	.159
13	1.04	.397	7.84	<.001	.96	.488
14	.41	.801	5.64	.001	1.35	.198
15	1.25	.302	44.27	<.001	1.01	.445
16	2.2	.084	9.15	<.001	1.81	.053
17	1.67	.173	7.02	<.001	1.39	.177
18	.61	.655	2.24	.087	1.99	.03
PA	3.03	.026	175.41	<.001	.96	.494
N	2.28	.075	451.01	<.001	1.5	−.132

Note. Analyses were performed on the $\log_e + 1$ concentration size variables. See "Identification of Individual Compounds" for key to compound numbers.

Table 3

MANOVA of Relative Concentrations of Individual Phenolics across Hybrid Classes in the *Quercus gambelii* × *Quercus grisea* Complex Partitioned by Sampling Date

Statistic	Value	F	df	P	Discriminant function analysis	Eigenvalue	Cumulative variation (%)
June 9:							
Wilks's λ	.014	2.718	76, 104	<.001	DF1	3.934	44.7
Pillai trace	2.485	2.503	76, 116	<.001	DF2	2.757	76
Hotelling-Lawley trace	8.807	2.839	76, 98	<.001	DF3	1.606	94.2
July 1:							
Wilks's λ	.034	1.672	76, 92	.009	DF1	3.765	58.5
Pillai trace	2.122	1.546	76, 102	.02	DF2	1.52	82.1
Hotelling-Lawley trace	6.436	1.821	76, 86	.004	DF3	.644	92.1
August 10:							
Wilks's λ	.057	1.360	76, 96	.076	DF1	2.742	55.7
Pillai trace	1.883	1.263	76, 108	.131	DF2	1.345	83.1
Hotelling-Lawley trace	4.919	1.456	76, 90	.044	DF3	.54	94.1
September 15:							
Wilks's λ	.05	1.516	76, 100	.025	DF1	3.402	62.4
Pillai trace	1.937	1.383	76, 112	.059	DF2	.982	80.4
Hotelling-Lawley trace	5.454	1.686	76, 94	.008	DF3	.785	94.3

Note. Analyses were performed on the $\log_e + 1$ concentration $- \log_e + 1$ geometric mean shape variables.

Statistical Analysis

We used size and shape analysis (Mosimann 1970; Boecklen and Price 1989; Boecklen et al. 1991) to compare oak hybrids on the basis of absolute and relative concentrations of foliar phenolic compounds and nitrogen. 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 1 less than that for the matrix of size variables, we excluded the variable $\log_e + 1$(flavonoid glycoside 18) $- \log_e + 1$(geometric mean) from the analysis. This compound was chosen for exclusion because it was the most invariant across hybrid taxa, was well correlated with total proanthocyanidins ($r = 0.67$), and did not contribute heavily to the discriminant functions for size.

For each date, we compared oak phytochemical phenotypes using a one-factor MANOVA with class (oak taxonomic category) as the main effect and trees as replicates. We used this design for the 20 $\log_e + 1$-transformed concentrations and for the 19 shape variables. We then partitioned the MANOVA by phytochemicals and compared classes using a one-factor repeated-measures ANOVA. We did this largely as a descriptive device to determine which variables contributed most to the MANOVA results. We used P values from the ANOVAs to measure the strength of the contribution and did not consider individual ANOVAs to be independent tests. For compounds found to be significantly different across hybrid-zone taxa through ANOVA, we used the Tukey-Kramer HSD post hoc test to determine patterns of expression within hybrid classes. Our terminology follows that of Orians (2000), where patterns of expression include dominance (equal to one parental species), overexpression (greater than either parental species), underexpression (less than either parental species), additive (intermediate of parental species), and equality (equal to both parental species).

We then compared absolute and relative concentrations of phenolic compounds and nitrogen 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 phytochemicals and also provided information about how oak taxonomic categories differed. We analyzed the correlation coefficients between the absolute and relative concentrations of phytochemicals and the canonical scores of trees to help interpret the weight of contribution

Table 4

Univariate Repeated-Measures ANOVA Tests of Relative Concentrations for Each Compound

	Class		Date		Date × class	
Compound	Value	P	Value	P	Value	P
1	.84	.509	22.03	<.001	1.74	.065
2	.95	.442	98.21	<.001	.67	.773
3	.96	.441	371.17	<.001	.7	.752
4	4.44	.004	31.87	<.001	.68	.768
5	6.83	<.001	150.54	<.001	.53	.894
6	4.68	.003	76.74	<.001	1.13	.343
7	1.17	.335	40.67	<.001	.57	.86
8	.47	.760	11.66	<.001	1.18	.307
9	3.29	.019	49.22	<.001	2.24	.013
10	.68	.606	90.86	<.001	.74	.706
11	5.27	.001	414.78	<.001	5.42	<.001
12	4.37	.004	23.39	<.001	.5	.908
13	.36	.833	13.82	<.001	.49	.915
14	1.8	.144	27.48	<.001	1.11	.355
15	3.45	.015	8.58	<.001	.87	.578
16	4.84	.002	83.78	<.001	1.15	.330
17	2.49	.056	48.55	<.001	1.33	.207
PA	2.19	.085	136.74	<.001	1.6	.099
N	1.37	.261	43.1	<.001	1.91	.039

Note. Analyses were performed on the $\log_e + 1$ concentration $- \log_e + 1$ geometric mean shape variables. See "Identification of Individual Compounds" for key to compound numbers.

Table 5

Tukey's HSD Post Hoc Tests of the $\log_e + 1$-Transformed Absolute and Relative Concentrations of Individual Phenolics, Proanthocyanidins (PA), and Nitrogen (N) across Hybrid Classes

Compound	*Quercus grisea*	*Q. grisea* backcross	*Quercus gambelii* × *Q. grisea*	*Q. gambelii* backcross	*Q. gambelii*
Absolute concentration:					
4	$1.75 \pm .06^{A}$	$1.75 \pm .06^{A}$	$1.85 \pm .06^{A}$	$2.02 \pm .06^{B}$	$2.05 \pm .06^{B}$
5	$.56 \pm .05^{ACD}$	$.72 \pm .05^{B}$	$.62 \pm .05^{ABC}$	$.49 \pm .05^{ACD}$	$.44 \pm .05^{AC}$
6	$1.92 \pm .08^{A}$	$2.19 \pm .08^{BC}$	$2.03 \pm .08^{AB}$	$2.30 \pm .08^{C}$	$2.40 \pm .08^{C}$
9	$1.85 \pm .07^{A}$	$1.99 \pm .07^{A}$	$1.90 \pm .07^{A}$	$1.96 \pm .07^{A}$	$2.19 \pm .07^{B}$
11	$.70 \pm .05^{AC}$	$.60 \pm .05^{A}$	$.55 \pm .05^{B}$	$.59 \pm .05^{AB}$	$.74 \pm .05^{C}$
12	$.73 \pm .06^{A}$	$.71 \pm .06^{A}$	$.70 \pm .06^{A}$	$.96 \pm .06^{B}$	$.92 \pm .06^{B}$
16*	$.57 \pm .05^{A}$	$.61 \pm .05^{A}$	$.43 \pm .05^{B}$	$.54 \pm .05^{AB}$	$.48 \pm .05^{AB}$
PA	$3.36 \pm .12^{A}$	$3.71 \pm .12^{B}$	$3.58 \pm .12^{AB}$	$3.69 \pm .12^{C}$	$3.92 \pm .12^{C}$
N*	$1.21 \pm .01^{AB}$	$1.19 \pm .01^{A}$	$1.23 \pm .01^{B}$	$1.24 \pm .01^{B}$	$1.24 \pm .01^{B}$
Relative concentration:					
4	$.59 \pm .05^{AB}$	$.53 \pm .05^{A}$	$.69 \pm .05^{BC}$	$.78 \pm .05^{C}$	$.79 \pm .05^{C}$
5	$-.60 \pm .05^{A}$	$-.51 \pm .05^{A}$	$-.54 \pm .05^{A}$	$-.75 \pm .05^{B}$	$-.82 \pm .05^{B}$
6	$.75 \pm .07^{A}$	$.95 \pm .07^{B}$	$.87 \pm .07^{B}$	$1.05 \pm .07^{BC}$	$1.13 \pm .07^{C}$
9	$.68 \pm .05^{A}$	$.75 \pm .05^{A}$	$.74 \pm .05^{A}$	$.71 \pm .05^{A}$	$.93 \pm .05^{B}$
11	$-.47 \pm .04^{A}$	$-.65 \pm .04^{B}$	$-.60 \pm .04^{BC}$	$-.66 \pm .04^{B}$	$-.52 \pm .04^{AC}$
12	$-.44 \pm .04^{AB}$	$-.52 \pm .04^{A}$	$-.45 \pm .04^{AC}$	$-.28 \pm .04^{D}$	$-.34 \pm .04^{BCD}$
15	$.61 \pm .05^{AB}$	$.70 \pm .05^{B}$	$.54 \pm .05^{A}$	$.43 \pm .05^{C}$	$.51 \pm .05^{AC}$
16	$-.60 \pm .03^{A}$	$-.62 \pm .03^{AB}$	$-.73 \pm .03^{C}$	$-.70 \pm .03^{BC}$	$-.78 \pm .03^{C}$
17	$-.88 \pm .03^{AB}$	$-.81 \pm .03^{A}$	$-.84 \pm .03^{AB}$	$-.91 \pm .03^{BC}$	$-.95 \pm .03^{C}$
PA*	$2.19 \pm .11^{A}$	$2.48 \pm .11^{B}$	$2.42 \pm .11^{AB}$	$2.44 \pm .11^{AB}$	$2.66 \pm .11^{B}$

Note. Data are presented as least square mean ± 1 SE. All taxa effects are significant at $P < 0.05$, except as noted by asterisks. Values with different superscript letters are significantly different from each other. See "Identification of Individual Compounds" for key to compound numbers.

* $P < 0.10$.

of individual phytochemicals the classification of hybrid-zone taxa (table A1 in the online edition of the *International Journal of Plant Sciences*). We also used the ratio of the eigenvalues to determine the relative contributions of size (absolute concentration) and shape (relative concentration) variables to the classification of oak hybrid phytochemistry. All analyses were performed in SYSTAT 10.2 (SPSS, Chicago).

Results

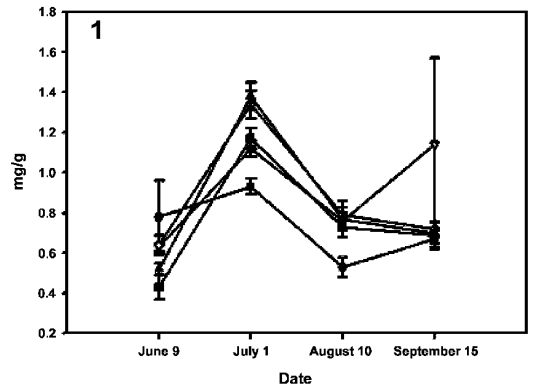
Identification of Individual Compounds

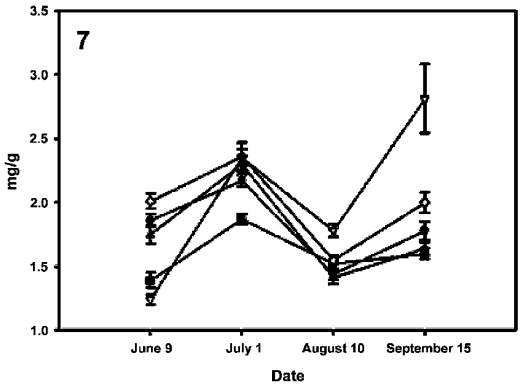
We identified 18 individual phenolic compounds in oaks of the *Quercus gambelii* × *Quercus grisea* hybrid zone in Monica Canyon using negative-ion HPLC-DAD-ESI-MS. Compounds were identified on the basis of their retention times (R_t), ultraviolet spectra, and mass spectral characteristics (Yarnes et al. 2006). One phenolic acid was identified as coumaroylquinic acid (1). Ten phenolics were identified as ellagitannins: vescavaloninic acid (2), castavaloninic acid (3), vescalagin (4), mongolinin A (5), pedunculagin (6), castalagin (7), acutissimin A (8), cocciferin D_2 (9), and two compounds (10, 11) that remained classified as ellagitannins because their chemical structures could not be fully determined. Seven flavonoid glycosides were classified as quercetin glycosides (12, 13), kaempferol glycosides (14, 15), or simply flavonoid glycosides (16, 17, 18).

Absolute and Relative Concentrations of Phenolics and Nitrogen in Hybrid-Zone Taxa

The *Q. gambelii* × *Q. grisea* oak hybrids and parental species differed in absolute and relative amounts of phenolics and nitrogen. For June 9, July 1, and September 15, both absolute and relative concentrations of phytochemicals were significantly different across hybrid taxa (tables 3–5). However, absolute concentrations across hybrid taxa were not significantly different on August 10 (tables 1, 2), and relative concentrations were only marginally significant (tables 3, 4).

Repeated-measures ANOVA of the absolute concentrations of individual phenolics, total proanthocyanidins, and total nitrogen found seven phytochemicals that contributed to the significant effect of hybrid taxa in the MANOVA (tables 1, 2), including vescalagin (4), mongolinin A (5), pedunculagin (6), cocciferin D_2 (9), ellagitannin (11), quercetin glycoside (12), and total proanthocyanidins (PA). Additionally, both nitrogen and flavonoid glycoside (16) were found to be marginally significant ($P < 0.10$). Patterns in the expression of absolute concentrations varied both between compounds and between hybrid classes within compounds (table 5). Cocciferin D_2 (9) concentrations were highest in *Q. gambelii*, and ellagitannin (11) was highest in parental groups. Vescalagin (4) was found in higher concentrations in *Q. gambelii* and its backcrosses, as were pedunculagin (6), quercetin glycoside (12), and proanthocyanidins (PA; table 5). Nitrogen content was lower in *Q. grisea* and its backcrosses relative to other taxonomic groups,


1
mg/g
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
June 9
July 1
August 10
September 15
Date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mg/g
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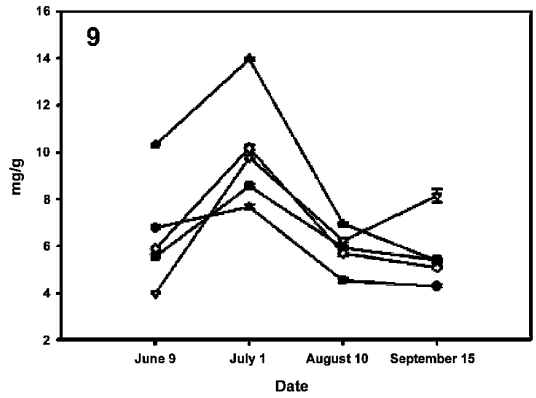
3.0
2.5
2.0
1.5
1.0
June 9
July 1
August 10
September 15
Date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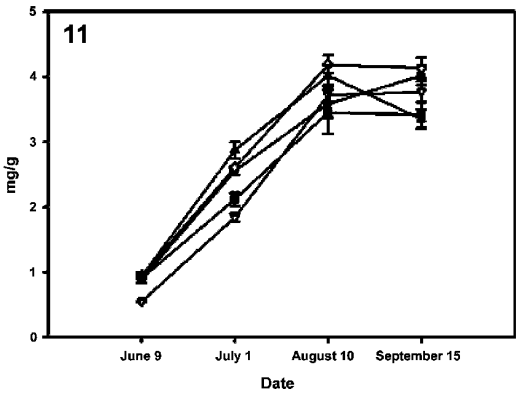
mg/g
16
14
12
10
8
6
4
2
June 9
July 1
August 10
September 15
Date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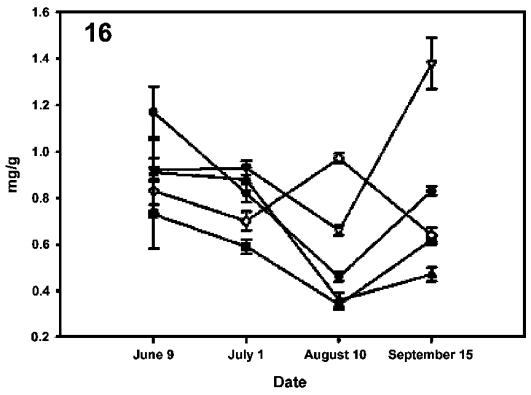
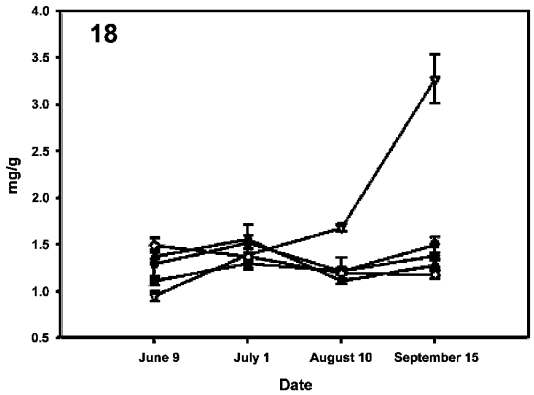
mg/g
5
4
3
2
1
0
June 9
July 1
August 10
September 15
Date


16
mg/g
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
June 9
July 1
August 10
September 15
Date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mg/g
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3.5
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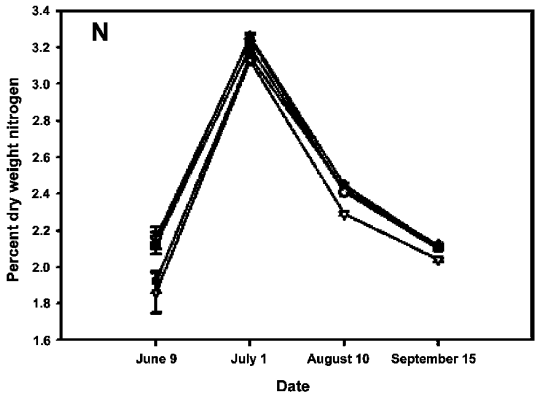
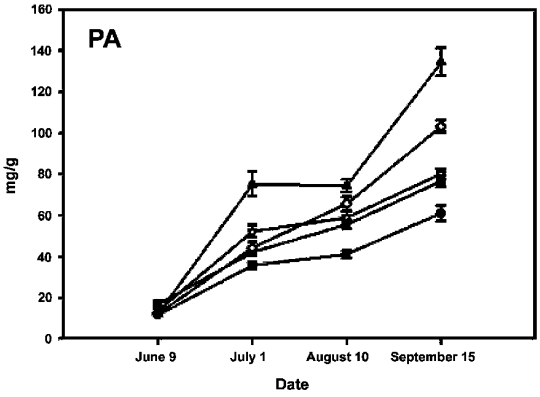
2.5
2.0
1.5
1.0
0.5
June 9
July 1
August 10
September 15
Date


N
Percent dry weight nitrogen
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
June 9
July 1
August 10
September 15
Date


PA
mg/g
160
140
120
100
80
60
40
20
0
June 9
July 1
August 10
September 15
Date

and flavonoid glycoside (16) was lowest in *Q. gambelii* × *Q. grisea* hybrids. When combined across compounds and hybrid combinations, absolute concentrations were differentially expressed across hybrid classes in a dominant fashion 14 times, overexpressed five times, underexpressed two times, and expressed in an additive manner three times.

Repeated-measures ANOVA of relative concentrations of phytochemicals found variation between taxonomic categories similar to the absolute concentrations (tables 3, 4). When combined across compounds and hybrid combinations, relative concentrations were differentially expressed across hybrid classes in a dominant fashion 10 times, overexpressed five times, underexpressed five times, and expressed in an additive manner four times.

Seasonal Variation in Phenolics and Nitrogen

Differences in absolute concentrations of phytochemicals between hybrid-zone taxa also changed over the growing season; seasonal variation the absolute concentration of five compounds, castalagin (7), cocciferin D_2 (9), ellagitannin (11), and two flavonoid glycosides (16, 18), exhibited a significant class-by-time interaction (fig. 1; tables 1, 2). In compound 7, *Q. gambelii* × *Q. grisea* hybrids and *Q. grisea* backcrosses were lower in absolute concentrations early in the year on June 9, while *Q. grisea* backcrosses classes were higher than other taxa on September 15 (fig. 1). In cocciferin D_2 (9), *Q. gambelii* had the highest concentrations on both on June 9 and July 1, and all categories declined through August and September except *Q. grisea* backcrosses. Both parental species had higher absolute concentrations of ellagitannin (11) on June 9, while all taxa varied in a similar fashion across the remainder of the year. *Quercus grisea* had substantially lower production of flavonoid glycoside 16. Late season (August 10, September 15) absolute concentrations of 18 were significantly higher in *Q. grisea* backcrosses, despite its having the lowest amounts among all taxa on June 9. Notably, *Q. grisea* backcrosses had the highest absolute concentrations of 13 of 18 phenolics (1, 2, 5–9, 12–14, 16–18) on September 15 (fig. 1).

Size and Shape Analysis: June 9

Variation in the absolute concentrations of phytochemicals among taxa within the *Q. gambelii* × *Q. grisea* hybrid zone on June 9 produced four significant discriminant functions. The first two discriminant functions accounted for ca. 77% of the variation among hybrid-zone taxa (tables 1, 2). The first axis accounted for 43% of the variation and was largely described by decreasing absolute concentrations of mongolinin A (5), ellagitannin (11), and flavonoid glycoside (16) as well as increasing concentrations of quercetin glycoside (12) and foliar nitrogen. The first discriminant axis primarily separated *Q. gambelii* backcrosses from the remaining taxa within the hybrid zone (fig. 2).

Variation in the relative concentrations of phenolics and nitrogen among taxa within the *Q. gambelii* × *Q. grisea* hybrid zone on June 9 also produced four significant discriminant functions. The first two discriminant functions accounted for 76% of the variation among hybrid-zone taxa (tables 3, 4). The first axis accounted for about 45% of the variation and was primarily described by decreasing relative concentrations of mongolinin A (5) and increasing relative concentrations of foliar nitrogen. The first discriminant axis separated *Q. gambelii*, *Q. gambelii* × *Q. grisea*, and *Q. gambelii* backcrosses from the remaining 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 phenolics and nitrogen on June 9 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 ca. $8.30\%/8.91\% = 93.1\%$ of the total variation in phenolics between hybrid-zone taxa was due to variation in the relative concentrations of phenolics and nitrogen.

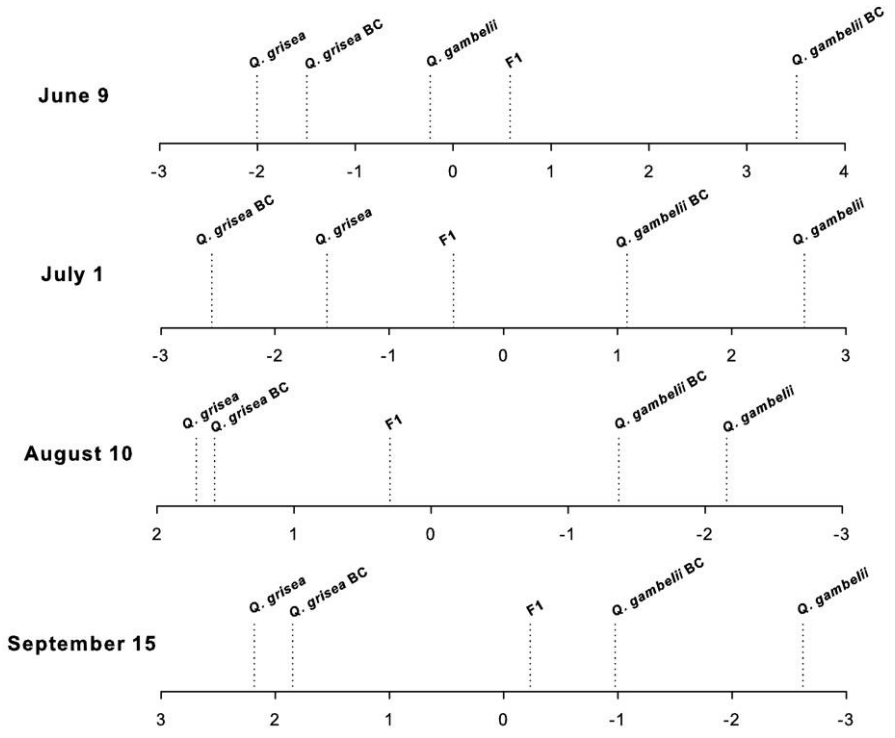
Size and Shape Analysis: July 1

Variation in the absolute concentrations of phytochemicals among taxa within the *Q. gambelii* × *Q. grisea* hybrid zone on July 1 produced four significant discriminant functions. The first two discriminant functions accounted for about 81% of the variation among hybrid-zone taxa (tables 1, 2). The first axis accounted for 58% of the variation and was chiefly described by increasing absolute concentrations of vescalagin (4), pedunculagin (6), and quercetin glycoside (12) and decreasing absolute concentrations of kaempferol glycoside (15). The first discriminant axis primarily separated *Q. gambelii* and *Q. gambelii* backcrosses from the remaining taxa within the hybrid zone (fig. 2).

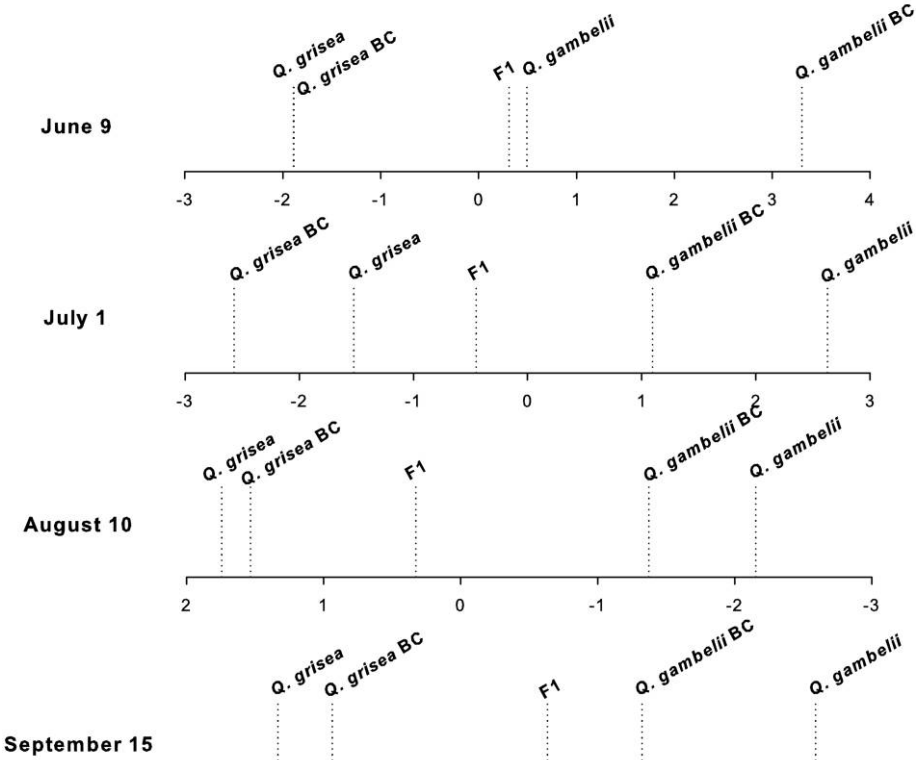
Four significant discriminant functions described variation in relative concentrations of phenolics and nitrogen among taxa within the *Q. gambelii* × *Q. grisea* hybrid zone on July 1. The first two discriminant functions accounted for ca. 82% of the variation among hybrid-zone taxa (tables 3, 4). The first axis accounted for 59% of the variation and largely described increasing relative concentrations of vescalagin (4), pedunculagin (6), quercetin glycoside (12) and nitrogen and decreasing concentrations of mongolinin A (5) and kaempferol glycoside (15). The first discriminant axis separated *Q. gambelii* and *Q. gambelii* backcrosses from the remaining 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 phenolics and nitrogen on July 9 could be attributed to variation in the $\log_e + 1$ relative concentrations. The ratio of the sum of the eigenvalues from discriminant analyses on the absolute and relative concentrations indicates that ca. $5.93\%/6.02\% =$

Fig. 1 Seasonal variation in the production of phenolics and nitrogen within the *Quercus gambelii* × *Quercus grisea* hybrid complex. Compounds shown are those observed to significantly vary in absolute and/or relative concentrations across taxonomic categories and sampling dates. Solid circles: *Q. grisea*; open triangles: *Q. grisea* backcross; solid squares: *Q. gambelii* × *Q. grisea*; open diamonds: *Q. gambelii* backcross; solid triangles: *Q. gambelii*. See "Identification of Individual Compounds" for key to compound numbers.



Absolute Concentrations



Relative Concentrations

98.5% of the total variation in phenolics between hybrid-zone taxa was due to variation in the relative concentrations of phenolics and nitrogen.

Size and Shape Analysis: August 10

Variation in the absolute concentrations of phenolics and nitrogen among *Q. gambelii* × *Q. grisea* hybrid-zone taxa on August 10 produced four significant discriminant functions. The first two discriminant functions accounted for ca. 81% of the variation among hybrid-zone taxa (tables 1, 2). The first axis accounted for 54% of the variation and chiefly described increasing absolute concentrations of castevaloninic acid (3), mongolinin A (5), and castalagin (7) and decreasing absolute concentrations of vescalagin (4), pedunculagin (6), quercetin glycoside (12), and total proanthocyanidins (PA). The first discriminant axis separated *Q. grisea* and *Q. grisea* backcrosses from other taxa within the hybrid zone (fig. 2).

Variation among hybrid-zone taxa in relative concentrations of phenolics and nitrogen on August 10 produced four significant discriminant functions. The first two discriminant functions accounted for ca. 83% of the variation among hybrid-zone taxa (tables 3, 4). The first axis accounted for 56% of the variation and represented increasing relative concentrations of castevaloninic acid (3) and castalagin (7) and decreasing relative concentrations of pedunculagin (6) and quercetin glycoside (12). The first discriminant axis primarily 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 phenolics and nitrogen on August 10 could be attributed to variation in the $\log_e + 1$ relative concentrations. The ratio of the sum of the eigenvalues from discriminant analyses on the absolute and relative concentrations indicates that ca. $4.63\%/4.77\% = 97\%$ of the total variation in phenolics between hybrid-zone taxa on August 10 was due to variation in the relative concentrations of phenolics and nitrogen.

Size and Shape Analysis: September 15

Variation in the absolute concentrations of phenolics and nitrogen among taxa within the hybrid zone on September 15 produced four significant discriminant functions. The first two discriminant functions accounted for ca. 81% of the variation among hybrid-zone taxa (tables 1, 2). The first axis accounted for 64% of the variation and described increasing absolute concentrations of mongolinin A (5) and flavonoid glycosides 16 and 17 and decreasing concentrations of vescavaloninic acid (2) and vescalagin (4). The first discriminant axis separated *Q. grisea* and *Q. grisea* backcrosses from other hybrid-zone taxa (fig. 2).

Variation between hybrid-zone taxa in relative concentrations of phenolics and nitrogen on September 15 produced four significant discriminant functions. The first two discriminant functions accounted for 80% of the variation among hybrid-zone taxa (tables 3, 4). The first axis accounted for ca. 62% of the variation and corresponded to increasing relative concentrations of castalagin (7) and flavonoid glycosides 16 and 17 and decreasing concentrations of vescavaloninic acid (2), vescalagin (4), and total proanthocyanidins (PA). The first discriminant axis primarily separated *Q. grisea* and *Q. grisea* 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 phenolics and nitrogen on September 15 could be attributed to variation in the $\log_e + 1$ relative concentrations. The ratio of the sum of the eigenvalues from discriminant analyses on the absolute and relative concentrations indicates that ca. $5.17\%/5.4\% = 95.7\%$ of the total variation in phenolics between hybrid-zone taxa on September 15 was due to variation in the relative concentrations of phenolics and nitrogen.

Discussion

Hybridization affects the seasonal production of both phenylpropanoids (flavonoid glycosides and proanthocyanidins) and ellagitannins across oak taxonomic categories in the *Quercus gambelii* × *Quercus grisea* hybrid zone. Variation in patterns of expression was highly variable between compound types and individual compounds, between hybrid-zone taxonomic categories within compounds, and between sampling dates. Despite the complexity of biosynthetic variation within the *Q. gambelii* × *Q. grisea* hybrid zone, general trends in seasonality were observed. Ellagitannins either peaked in July and August or steadily accumulated throughout the summer, while flavonoid glycosides were more uniformly produced throughout the summer. Proanthocyanidins steadily increased in concentration, peaking sharply in September and effectively doubling over August concentrations. Nitrogen peaked in July and steadily decreased through September. When phenolics and nitrogen were analyzed as a mixture, different compounds contributed to the classification of hybrid-zone oaks at different times during the growing season, reflecting changes in phenolic biosynthesis. Notably, most of the variation (range: 93%–99% across sampling dates) in phenolic composition between oak taxonomic categories was attributable to differences in the relative concentrations of individual phenolics. This emphasizes the importance of analyzing phenolics as a mixture and the potential reduction of empirical content when only variation in absolute concentrations is considered. More broadly, fine-scale spatiotemporal analysis of plant defense metabolites may facilitate a more comprehensive, metabolic approach toward resolving the role of plant defenses on herbivore populations (Haukioja 2003).

While additive patterns of quantitative expression (hybrid intermediacy) in defense traits are the most commonly reported pattern in the literature (Orians 2000), hybrid equality was the most prevalent pattern observed for phytochemicals in the *Q. gambelii* × *Q. grisea* complex. This may be attributed to the

Fig. 2 Classification of oak phytochemical phenotypes based on the absolute and relative concentrations of individual phenolics, proanthocyanidins, and nitrogen. Oak taxonomic categories are ordered according to the canonical score of each category along the first discriminant axis from discriminant function analysis. BC = backcross.

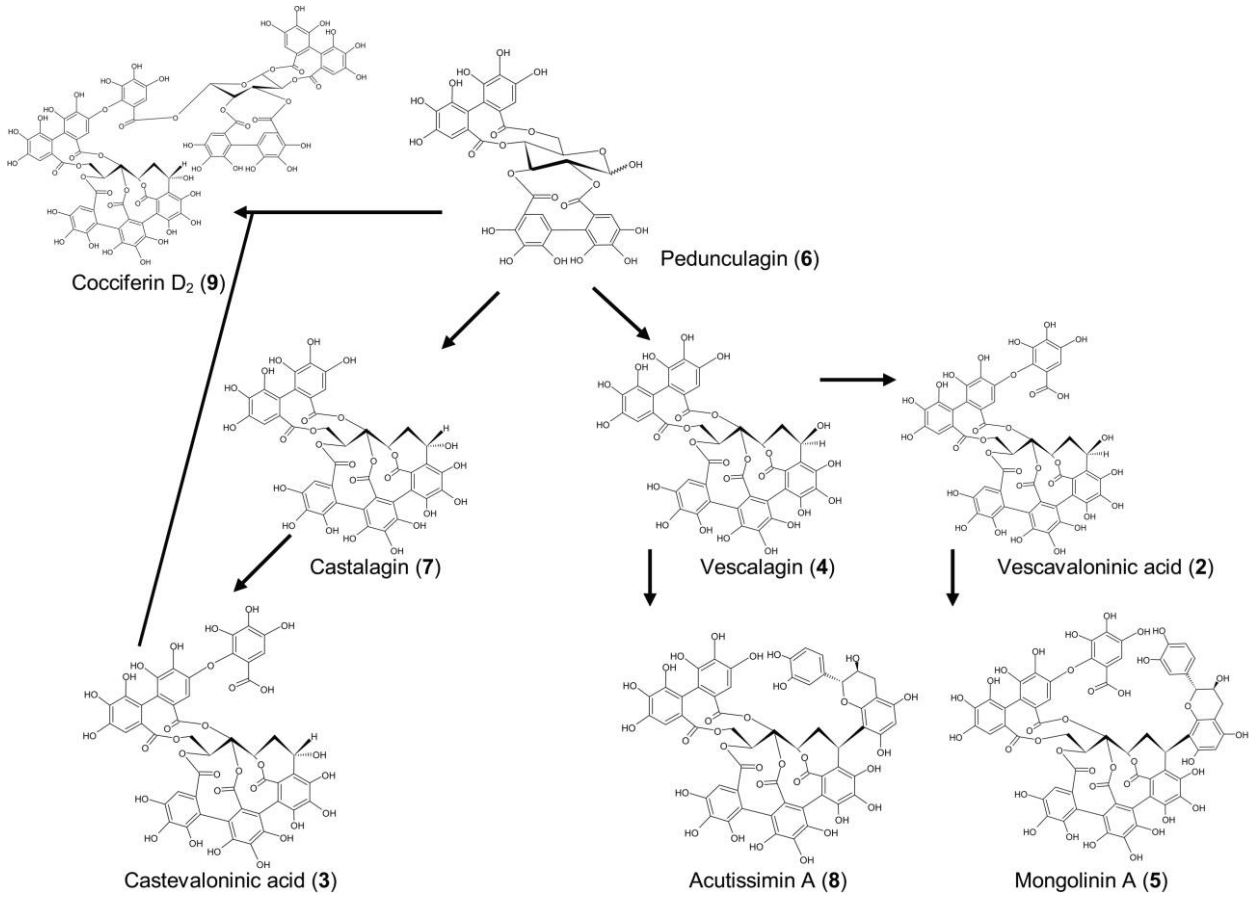


Fig. 3 Proposed biosynthetic pathway of ellagitannins in *Quercus gambelii* × *Quercus grisea*. Modified from Yarnes et al. (2006).

genetic homogenization of *Q. gambelii* and *Q. grisea* within the hybrid zone as a result of extensive introgression (Howard et al. 1997) and/or reduced reproduction between *Q. gambelii* × *Q. grisea* hybrids. Therefore, if phenolics determine abundances of herbivores or microbes within hybrid zones, the hybridization-based facilitation of host switching in consumers may be restricted to contact zones. When concentrations of individual phenolics did vary across hybrid-zone taxa, the pattern of expression often depended on the class of hybrid; *Q. gambelii* × *Q. grisea* hybrids nearly always differed in their patterns of expression from backcrosses to *Q. grisea* or *Q. gambelii* (table 5). Nonequal patterns of expression within the Monica Canyon hybrid zone were most often dominant. In crosses that included *Q. gambelii*, dominant expression in *Q. gambelii* was observed in a 2 : 1 ratio over all other nonequal patterns of expression. In contrast, a pattern of dominant expression could be attributed to *Q. grisea* in only a 1 : 1 ratio. This may be due to maternal effects of inheritance within the *Q. gambelii* × *Q. grisea* hybrid zone. Within these hybrid zones, environmental effects on conspecific pollen viability favor heterospecific fruit set in *Q. gambelii* (Williams et al. 2001). Maternal effects may modify patterns of expression in quantitative chemical defenses if genetic expression is controlled by maternally inherited organelles, such as chloroplasts. This may be likely for ellagitannins because foliar hydrolyzable tannins are concentrated in the mesophyll (Grundhöfer et al. 2001) and may serve a key role in photosystem protection (Close et al. 2003). Strong maternal effects on secondary metabolism have also been reported elsewhere (Spring and Schilling 1989; Buschmann and Spring 1995). Backcrosses generally exhibited greater absolute concentrations than parental species as the year progressed, especially *Q. grisea* backcrosses, which exhibited the lowest production in nine of 10 ellagitannins on June 9 and the highest concentration in seven of 10 ellagitannins on September 15 (table A2 in the online edition of the *International Journal of Plant Sciences*; fig. 1). This suggests a relatively high level of developmental instability in the biosynthesis of ellagitannins in *Q. grisea* backcrosses. The frequency of backcrossing and the relative instability of plant defense metabolism in these hybrid classes may play a role in maintaining host specificity of consumers.

Analysis of oak phytochemical phenotypes within the Monica Canyon hybrid zone indicates that the individual compounds that contribute strongly to the classification of hybrid-zone taxa varied throughout the year (fig. 2; tables 1–4, A1). Classification of oaks based on phytochemistry varied drastically between June and July but afterward consistently intergraded from *Q. grisea* to *Q. gambelii*. Further, the composition of phytochemical phenotypes in the *Q. gambelii* × *Q. grisea* hybrid zone from July to September suggests basic differences between "*Q. grisea*-type" and "*Q. gambelii*-type" oaks in the progression of ellagitannin biosynthesis through two isomeric interme-

diates, vescalagin and castalagin (fig. 3). This may indicate significant differences in the defense capabilities within the hybrid zone because vescalagin is the substrate for the synthesis of a greater diversity and abundance of ellagitannins than of castalagin. At high pH, Barbehenn et al. (2006*a*, 2006*b*) found that, similar to observations in insect digestive tracts, ellagitannins formed much higher concentrations of semiquinone radicals—active oxidative defenses—than of proanthocyanidins and that overall concentration of semiquinones was related to compound structure. However, in experimental mixtures containing both ellagitannins and proanthocyanidins, proanthocyanidins reduced the overall concentrations of ellagitannin-based oxidative defense (Barbehenn et al. 2006*b*). Endophagous insects (leaf miners and gall formers) have been collected from the Monica Canyon hybrid zone for more than 15 yr, and the most common species are additively distributed, with highest densities on *Q. gambelii*. The additive distribution of herbivore abundance may be related to these differences in the relative production of ellagitannins and proanthocyanidins, a gradient of oxidative defenses, produced within the *Q. gambelii* × *Q. grisea* hybrid zone.

Seasonal variation in plant defenses and quality, as observed in *Q. gambelii* × *Q. grisea*, may result in mismatched phenologies between host chemistry and herbivore development in hybrid zones. Ontogenetic variation in the quality of foliage is central to determining patterns of herbivore performance and mortality (Feeny 1970; Mopper and Simberloff 1995; Tikkanen and Julkunen-Titto 2003; Salminen et al. 2004), and phenological effects of host quality on herbivore populations have been attributed to the disruption of host quality and defenses across the growing season (Salminen and Lempa 2002; Haukioja 2003; Tikkanen and Julkunen-Titto 2003; Lahtinen et al. 2006).

The seasonality of phenolics and nitrogen in *Q. gambelii* × *Q. grisea* may also be due in part to the inheritance of leaf senescence and leaf longevity. *Quercus grisea* is typically subevergreen and drought deciduous, while *Q. gambelii* is annually deciduous. Older leaves typically exhibit increased toughness, lower protein content (Lusk 2001), and greater amounts of proanthocyanidins (Salminen et al. 2004). Leaf longevity may be related to the gradient of foliar nitrogen and of mongolinin A and acutissimin A (both consist of an ellagitannin moiety and a proanthocyanidin monomer) during early to midsummer within the *Q. gambelii* × *Q. grisea* hybrid zone.

Hybridization strongly affects the seasonal metabolic variation in phytochemical phenotypes of *Q. gambelii* × *Q. grisea* hybrid-zone taxa. Hybrid oaks vary in the production of phenolics and nitrogen between taxa and across seasons, affecting temporal and taxonomic patterns in host quality and defense. In a hybrid zone with extensive introgression, phytochemical phenotypes exhibit marked differences in the relative concentrations of individual phenolics, total proanthocyanidins, and nitrogen, while absolute concentrations are relatively homogeneous across oak taxa. The analysis of defense metabolites as dynamic, natural mixtures of interacting individual compounds is essential to the understanding of hybridization effects on plant defense. Studies of the defense chemistry of hybrid zones must account for ontogenetic variation in plant defenses. Further study is still required to determine the influence of maternal effects, leaf life span, and the biogenetic control of seasonal variation in phenolic biosynthesis and resource allocation in hybrid zones.

Acknowledgments

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