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Heritability, covariation and natural selection on 24 traits of common evening primrose (*Oenothera biennis*) from a field experiment

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Abstract

This study explored genetic variation and co-variation in multiple functional plant traits. Our goal was to characterize selection, heritabilities and genetic correlations among different types of traits to gain insight into the evolutionary ecology of plant populations and their interactions with insect herbivores. In a field experiment, we detected significant heritable variation for each of 24 traits of *Oenothera biennis* and extensive genetic covariance among traits. Traits with diverse functions formed several distinct groups that exhibited positive genetic covariation with each other. Genetic variation in life-history traits and secondary chemistry together explained a large proportion of variation in herbivory (r^2 = 0.73). At the same time, selection acted on lifetime biomass, life-history traits and two secondary compounds of *O. biennis*, explaining over 95% of the variation in relative fitness among genotypes. The combination of genetic covariances and directional selection acting on multiple traits suggests that adaptive evolution of particular traits is constrained, and that correlated evolution of groups of traits will occur, which is expected to drive the evolution of increased herbivore susceptibility. As a whole, our study indicates that an examination of genetic variation and covariation among many different types of traits can provide greater insight into the evolutionary ecology of plant populations and plant–herbivore interactions.

Introduction

An important goal of evolutionary ecology is to understand how functional trait variation influences ecological interactions and adaptation to various environments. For example, ecophysiological traits relating to photosynthesis and plant structure are key adaptations to different abiotic environments (Ackerly, 2004; Hemsley & Poole, 2004). Similarly, variation in life-history traits, such as lifespan, phenology and biomass, has large impacts on the fitness and success of organisms under different biotic and abiotic conditions (Roff, 1992; Stearns, 1992). Finally, many studies of plant–insect interactions have shown that secondary metabolites are adaptations that provide resistance against herbivory (Dethier, 1941; Berenbaum *et al.*, 1986; Karban & Baldwin, 1997; Agrawal, 2005). Although suites of ecophysiological, life history, resistance and morphological traits have been studied in isolation, surprisingly few studies have integrated these classes of traits into a comprehensive evolutionary ecological framework. Such an approach could provide greater insight into the ecological and evolutionary significance of genetic variation.

The genetic variation and covariation of traits has several important consequences for studying the evolutionary ecology of plants. First, traits must be heritable for natural selection to result in evolutionary change within populations. Second, genetic covariance among traits, as shaped by past ecological and evolutionary processes, may constrain future adaptive evolution. A number of studies have now considered the heritability

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of and covariation among traits and how these may affect their joint evolution (Lynch & Walsh, 1998; Conner & Hartl, 2004). However, typically traits related to only one particular function are examined, such as covariation among physiological (Caruso *et al.*, 2005) or floral traits (Conner *et al.*, 2003). A recent review convincingly argued that more studies are needed that measure traits with disparate function to understand how traits genetically covary, influence important ecological interactions (e.g. herbivory) and potentially constrain future adaptive evolution (Geber & Griffen, 2003).

Two related approaches have been used to understand the evolutionary ecology of functional traits. One recent comparative approach involves studying how functionally related traits may covary to form suites of traits or 'syndromes'. This approach has been applied to such disparate topics as the coexistence of species, community assembly, pollination ecology (Grime, 1977; Chapin *et al.*, 1993; Westoby *et al.*, 2002; Fenster *et al.*, 2004) and, more recently, resistance against herbivores (Kursar & Coley, 2003; Agrawal & Fishbein, 2006). Another approach, typically employed at the intraspecific level, is quantitative genetics. Here, measures of variation and covariation among multiple traits are used to estimate a phenotypic (P matrix) or genetic (G matrix) variance–covariance matrix (Lande, 1979; Falconer & Mackay, 1996), which provides a statistical framework to predict multivariate evolution of traits in response to selection (Lande & Arnold, 1983; Rausher, 1992). Because syndromes must originate via past selection on populations, it is necessary to examine selection and associations among traits to understand the evolutionary forces that give rise to such syndromes.

Here, we used a field experiment to examine 24 traits from the native plant, common evening primrose (*Oenothera biennis*). We measured traits that influence ecophysiology, life-history, resistance to herbivores and morphology. Specifically, we asked the following questions: (1) what is the heritability, and 2) multivariate genetic covariation of plant traits associated with diverse functions? 3) Do single traits or multivariate suites of traits predict resistance to herbivory? 4) Is there evidence for natural selection on individual and/or multivariate suites of traits?

Materials and methods

Experimental system

This study used the herbaceous plant common evening primrose (*O. biennis* L., Onagraceae), which is native to open habitats of eastern North America (Dietrich *et al.*, 1997). Plants form a rosette before bolting and flowering, and *O. biennis* populations frequently genetically vary in life-history strategy, reproducing in their first (annual) or second (biennial) year of growth (Johnson, 2007). Reproduction is fatal, so that plants flower once and then die. *Oenothera biennis* is dominated by a diverse class of phenolic secondary metabolites (Zinsmeister & Bartl, 1971; Howard & Mabry, 1972) and plants are frequently damaged by herbivores that reduce plant fitness (Johnson & Agrawal, 2005). *Oenothera biennis* is also functionally asexual, a consequence of its permanent translocation heterozygote (PTH) genetic system, which occurs in ca. 57 species from three plant families (Cleland, 1972; Holsinger & Ellstrand, 1984). The life-history and genetic system of *O. biennis* make it possible to estimate total male and female lifetime fitness of individual plants and genotypes.

As we discuss below, the functional asexuality of *O. biennis* is predicted to influence the evolution of plant traits. Because all genes in the genome are effectively linked, the evolutionary consequences of selection on one trait are dependent on the strength and direction of selection on other traits (Otto & Lenormand, 2002). As such, data on the genetic (co)variance and selection on multiple traits can help to identify: (1) traits that are likely to reach an optimum, (2) traits on which interference selection constrains their evolution and 3) the nature of correlated evolution.

Plant collections and growth

We obtained 39 genotypes of *O. biennis* from old fields and roadside populations by collecting ripe fruits from single plants separated by 0.5 km or more (Tompkins County, NY, USA, average distance between populations 12 km; maximum 30 km). Delimiting populations of functionally asexual organisms is inherently difficult because individuals do not interbreed, but they may nonetheless interact. In this study, we follow previous convention of viewing the genotypes studied as a random sample from a single large population with multiple subpopulations (Johnson, 2007). Because of *O. biennis*' PTH genetic system, seeds from each collection were genetically identical, and six of the nine microsatellite markers developed for this study were sufficient to distinguish 36 of the 39 genotypes (Larson *et al.*, 2008).

In February 2006, we germinated seeds on moistened filter paper in sealed Petri dishes exposed to direct sunlight. Seedlings were transplanted to 500-mL plastic pots filled with potting soil (Metro-Mix; Sun Gro Horticulture, Bellevue, WA, USA) and grown in a glasshouse with supplemental light at Cornell University. Plants were provided *ad libitum* water and fertilized weekly with a dilute solution of 21 : 5 : 20 N : P : K. After 10 weeks of growth, we transferred plants to shaded cages outside for 10 days and then transplanted them into the ground of an abandoned agricultural field. The site was chosen because it lacked naturally occurring *O. biennis* but had rocky soil characteristic of *O. biennis* habitat.

We created 10 complete experimental blocks, each one having one to two randomized representatives from each

of the 39 genotypes (total $N = 400$, 9–11 replicate plants per genotype). Plants were separated by 1 m among rows and columns and experimental blocks were arranged into columns and rows separated by 5 m. Plants were irrigated upon planting, but not for the rest of the season. We sprayed five of the blocks (half the plants) with esfenvalerate insecticide (Asana XL; DuPont Agricultural Products, Wilmington, DE, USA) every 2–3 weeks to reduce insect herbivory. To understand whether the insecticide could confound our results by directly influencing the performance of plants, we conducted a 6-week growth assay prior to our experiment in which plants were grown in the absence of herbivores and treated three times with esfenvalerate or a water control; we did not detect any effects of the treatment on shoot ($F_{1,40} = 0.18$, $P = 0.28$) or root ($F_{1,40} = 0.26$, $P = 0.61$) biomass. In the field, our insecticide treatment reduced the abundance of herbivores ($F_{1,352} = 54.31$, $P < 0.001$). This reduction had no significant effect on lifetime fruit production ($F_{1,38} = 0.24$, $P = 0.63$) or any life-history trait (effect of insecticide: $P > 0.30$ for all traits; see the Traits measured section), and there were no significant genotype-by-insecticide interactions ($P > 0.10$). Therefore, we combined the life-history data collected from insecticide and control plots when calculating the genotype breeding values of fitness and life-history traits. To estimate constitutive trait values, we only measured physiology, chemistry and morphology from sprayed plots ($N = 200$ plants), whereas herbivory was only measured from unsprayed plots ($N = 200$ plants).

Traits measured

We measured four types of traits: ecophysiological, life history, herbivore resistance and morphological. Ecophysiological traits were defined based on their role in the primary function and metabolism of plants (e.g. C : N ratio and leaf water content). Life-history traits were those most closely related to reproduction, mortality and growth (e.g. flowering time and lifetime biomass). Herbivore resistance traits included concentrations of secondary compounds and direct measures of herbivory. Morphology refers to physical plant traits, of which we only measured trichome density. In late July and early August, we measured traits from all plants in the experiment. Foliar herbivory was almost exclusively due to the exotic Japanese Beetle (*Popillia japonica* Scarabidae) and was estimated following population outbreak as the proportion of leaves with leaf damage (most plants had well over 30 leaves). This introduced beetle is also a frequent pest of fruit crops in the rose family. In Ithaca, *P. japonica* infests plants in June and July, and it has been the dominant leaf-chewing herbivore on most local *O. biennis* populations in recent years (M.T.J. Johnson and A.A. Agrawal, personal observation), and common since at least the late 1970s (Kinsman, 1982). Plants were also point censused for beetles on the day that leaf herbivory was measured; despite low counts there was a genetic correlation between our measure of plant-level herbivory and beetle abundance ($N = 39$, $r = 0.659$, $P < 0.001$).

Two young, fully expanded leaves were taken from every plant for assessment of phenolics (see below) and the ratio of leaf carbon to nitrogen (C : N). Leaves were kept in a cooler on ice, freeze dried in the laboratory and ground to a fine powder. C : N ratio was assessed in an elemental combustion system (Cornell University Stable Isotope Laboratory).

To measure leaf water content, specific leaf area (SLA) and trichome density, we took a single leaf disc (28 mm^2) from the tip of the youngest fully expanded leaf on each plant and placed each disk in a sealed plastic tube in a cooler with ice. We weighed each disc to the nearest microgram on the day of collection (fresh mass) and after 24 h of drying at 40 °C (dry mass). We calculated water content as the ratio of water mass (fresh–dry mass) to the fresh mass of the leaf disc. We counted trichomes on both sides of each leaf disc under a dissection microscope and divided this number by disc area to estimate trichome density per cm^2. We assessed SLA of the disc as leaf area per unit dry mass.

Life-history measures were taken throughout the growing seasons. Plants exhibited one of two life-history strategies: they either remained as a rosette (score 0) or bolted into a flowering stalk (score 1) during summer 2006. We measured the number of days from germination (February 1) until bolting (bolting date) and also until the first flowers (flowering date) opened. Surveys in spring 2007 revealed that all plants that remained as rosettes in 2006 died during winter due to mammalian herbivory, pathogens and other unknown causes. After fruits had finished ripening in November 2006, we harvested all plants, dried the tissue in forced air-drying ovens and weighed the total dry mass. We also counted the number of fruits on each plant, which provided a measure of total male and female fitness.

Plant chemistry

Leaf sample extraction

We characterized and quantified phenolic composition of *O. biennis* leaves using 20 mg of leaf powder from each sample, extracted in 500 μL of acetone/water (7/3, v/v, containing 0.1% ascorbic acid, w/v) for 1 h with a Vortex-Genie 2T mixer (Scientific Industries, Bohemia, NY, USA). After centrifugation (16 000 ***g***, 10 min; Eppendorf centrifuge 5402; Eppendorf AG, Hamburg, Germany), we stored the supernatant in a separate vial and repeated the extraction on the original tissue three times with fresh solvent (total of four extractions). Acetone was removed from the pooled extracts using an Eppendorf concentrator 5301 (Eppendorf AG) and the sample was freeze dried. Prior to analysis, we dissolved

the sample in 1400 μL of water and filtered it through a 0.45-μm PTFE filter.

HPLC-DAD analysis

We analysed phenolics from each sample using high-performance liquid chromatography with a diode array detector (HPLC-DAD). The LaChrom HPLC system (Merck-Hitachi, Tokyo, Japan) consisted of a pump L-7100, a diode array detector L-7455, a programmable autosampler L-7200 and an interface D-7000. Individual phenolics were separated using the Merck Chromolith Performance RP-18e (100 × 4.6 mm i.d.) column with CH_3CN (A) and 0.05 M H_3PO_4 (B) as eluents. After an extensive comparison of solvent and flow rate gradients, we found the following to be most effective. Solvent gradient: 0–2 min, 2% A (isocratic); 2–16 min, 2–30% A in B (linear gradient); 16–18 min, 30–80% A in B (linear gradient); 18–21 min, 80% A (isocratic); 21–23 min, 80–2% A in B (linear gradient); 23–30 min, 2% A (isocratic). Flow-rate gradient: 0–16 min, 1.5 mL min^{-1} (isocratic); 16–18 min, 1.5–2.0 mL min^{-1} (linear gradient); 18–28 min, 2.0 mL min^{-1} (isocratic); 28–28.5 min, 2.0–1.5 mL min^{-1} (linear gradient); 28.5–30 min, 1.5 mL min^{-1} (isocratic). We used four acquisition wavelengths (200, 280, 315 and 349 nm) and UV spectra were recorded for each peak between 195 and 600 nm. For quantitative analyses, 10 μL of the *O. biennis* extract was injected into the Merck Chromolith Performance column where hydrolysable tannins (ellagitannins) were quantified in pentagalloyl glucose equivalents (280 nm), flavonoid glycosides in quercetin equivalents (349 nm) and caffeoyl tartaric acid in caffeoyl quinic acid equivalents (315 nm).

Characterization of Oenothera biennis phenolics

Oenothera biennis phenolics separated by HPLC-DAD were first characterized on the basis of their UV spectra. More specific characterization was achieved by HPLC-ESI-MS in the negative ion mode using a Perkin-Elmer Sciex API 365 triple quadrupole mass spectrometer (Sciex, Toronto, Canada). The HPLC system consisted of two Perkin-Elmer Series 200 micro pumps (Perkin-Elmer, Norwalk, CT, USA) connected to a Series 200 autosampler (Perkin-Elmer) and a 785A UV/VIS detector (Perkin-Elmer). ESI-MS conditions were the same as those described in a previous paper (Salminen *et al.*, 1999). Chromatographic conditions were as described above for HPLC-DAD, but the 0.05 M H_3PO_4 was replaced with 0.4% HCOOH. After UV detection at 280 nm, 20% of the eluate was split off and introduced into the ESI-MS system.

Statistical analyses

Genetic variance, heritability and coefficients of variation

We used restricted maximum likelihood (REML) in Proc Mixed of SAS (SAS Institute, Cary, NC, USA) to estimate the variance explained by plant genotype for each trait. The statistical model included plant genotype and spatial block as random effects, where the significance of genotype was tested using a log-likelihood ratio test (Littell *et al.*, 1996). Because of *O. biennis*' functional asexuality, we calculated broad-sense heritability for each trait as $H^2 = V_g/V_T$ (Lynch & Walsh, 1998), where V_g is the total genetic variance (additive and nonadditive) and V_T is the total phenotypic variance (genetic and environmental) in the trait. The coefficient of genetic variation was calculated as $V_g^{0.5}/\mu_i$, where μ is the mean for trait i. All analyses were performed on untransformed data as recommended by Houle (1992).

Genetic associations among traits

Genetic covariation among traits was characterized using Pearson correlation coefficients and genetic covariances. Pearson correlations were determined for all pairwise combinations of traits using the best linear unbiased predictors (BLUPs) (similar to mean values) of genotype breeding values ($N = 39$ genotypes per correlation). BLUPs are more accurate than family mean values because they are less biased by environmental effects and more robust to unbalanced replication than are family mean values (Shaw *et al.*, 1995; Littell *et al.*, 1996). The genetic covariance among traits was calculated according to the equation: $cov_g = r_g(G_{11}G_{22})^{0.5}$, where r_g is the genetic Pearson correlation coefficient between two traits, and G_{ii} is the genetic variance of each trait from REML. In the case of life-history strategy (rosette vs. flowering), we used generalized linear mixed models in Proc Glimix to estimate the genetic variance and the binomial equation's estimate of variance for total phenotypic variance. The statistical significance of genetic covariances was assessed as the P-value from the t-statistic of r_g (Lynch & Walsh, 1998, p. 641). To assess the role of multiple tests in producing spurious significant effects, we assessed whether the frequency of significant correlations deviated from the random expectation using the binomial expansion test (Zar, 1996).

To determine how covarying traits were related to one another, we performed hierarchical cluster analysis. This was carried out by first calculating a square matrix of genetic Pearson correlations coefficients among all traits, followed by implementing Ward's minimum variance method (Ward, 1963) in SYSTAT (Vers. 9; Systat Software, Chicago, IL, USA) to define linkages among traits and groups of traits (Wilkinson, 1999). We objectively defined a 'cluster' when all traits were linked by < 2 sums of squares variance (i.e. the measure of distance) (Wilkinson, 1999).

Genetic variation in plant traits that predict herbivory

We used two methods to identify how genetic variation in plant traits affected herbivory by *P. japonica*. First, we performed forward stepwise multiple regression in which we regressed the BLUPs for herbivory against the BLUPs of all plant traits, using type II (partial) error in Proc Reg

of SAS. Stepwise regression was used instead of a fully parameterized model because of the limited statistical power associated with the latter. The model was built by allowing a variable to enter the equation if the linear regression coefficient had a partial P-value < 0.10, whereas we excluded variables that had a partial P-value > 0.10. Once the best linear coefficients model was found, we used forward stepwise regression again to explore whether any of the traits already in the model exhibited significant quadratic effects on herbivory. A significant quadratic effect of a plant trait on herbivory indicates that there are nonlinear effects of a trait on resistance to herbivores, which could be caused by intermediate levels of a trait conferring the highest or lowest resistance to a herbivore.

Because of the extensive covariation among traits, we also used principal components analysis (PCA) to reduce the dimensionality of trait variation into a smaller number of variables. We performed PCA in SYSTAT using the Varimax rotation method and Pearson correlations as the distance measure among genotypes. We retained all principal components (PC) with an eigenvalue > 1 (Legendre & Legendre, 1998), which resulted in nine PCs. We then used the PCs in a forward stepwise regression to determine which components best predicted herbivory.

Natural selection on plant traits

We estimated selection on plant traits using conventional covariance measures of selection differentials (Price, 1970), as well as multivariate genotypic selection analyses (Lande & Arnold, 1983; Rausher, 1992). The selection differential on each trait was measured as the covariance between relative fitness of genotypes and normally standardized trait variation among genotypes.

We then used forward stepwise regression to regress the BLUPs of relative fitness against the BLUPs of all traits including herbivory. This process was started by only including linear coefficients in the model, as these coefficients estimate the strength of directional selection (β). We then included all quadratic coefficients for the traits in the best linear coefficients model to estimate the strength of curvilinear selection, and multiplied these quadratic coefficients by two to estimate γ, the strength of quadratic selection (Lande & Arnold, 1983; Stinchcombe *et al.*, 2008). We visually inspected the partial regression plots of relative fitness vs. trait variation, and used the equation for computing maximum/minimum values (i.e. $-\beta/\gamma$) to determine whether there was stabilizing or disruptive selection on traits (Mitchell-Olds & Shaw, 1987). We inferred that stabilizing selection acted on a trait when the quadratic coefficient was negative and exhibited a maximum value within the range of data, and disruptive selection when the quadratic coefficient was positive and showed a minimum value within the range of data. Finally, PCA was used again, here including herbivory, to reduce the dimensionality of the data; nine PCs had eigenvalues > 1. As before, we used stepwise multiple regression to determine how relative fitness related to the PCs.

Results

Characterization of phenolics

Eleven HPLC peaks were uniformly distributed across all plants, which included six ellagitannins, four flavonoids and caffeoyl tartaric acid (Fig. 1a,b). Other peaks were present in only some plants and/or were poorly chromatographically separated (e.g. peaks at retention times 8.5–11.0 min). These latter peaks were characterized as either ellagitannins or flavonoid glycosides based

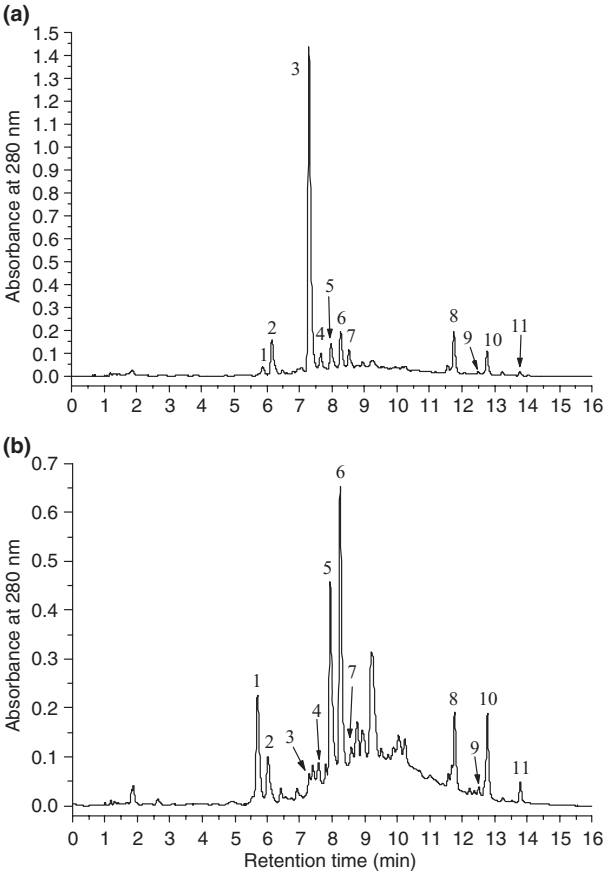

Fig. 1 Characteristic HPLC traces of phenolics found in *Oenothera biennis* leaves. (a) Chromatogram from a plant sample with a high concentration of oenothein B and a low concentration of oenothein A, and (b) a sample with a low concentration of oenothein B and a high concentration of oenothein A. The numbered compounds were quantified individually: (1) ellagitannin, (2) caffeoyl tartaric acid, (3) oenothein B, (4) ellagitannin, (5, 6) isomers of oenothein A, (7) ellagitannin, (8) quercetin glucuronide, (9) flavonoid glycoside, (10) kaempferol glucuronide and (11) flavonoid glycoside.

Table 1 Mean, range, genetic variance (V_g), broad-sense heritability (H^2) and coefficient of genetic variance (CV) for 24 *Oenothera biennis* traits and lifetime fitness from a field experiment.

Trait	Mean	Range	V_g	H^2	CV (%)
Herbivory					
Proportion of leaves damaged	0.06	0–0.88	0.004***	0.19	97.9
Foliar traits					
C : N	15.90	11.2–24.8	2.97***	0.40	10.8
SLA ($mm^2\ mg^{-1}$)	17.62	10.9–28.0	2.21***	0.21	8.4
Water content (%)	72.74	55.5–84.9	3.15**	0.18	2.4
Trichome density (trichomes per cm^2)	343.18	4–989	10 832***	0.43	30.3
Phenolics (mg g^{-1} dry tissue)					
Total phenolics	189.08	97.7–314.6	1148.91***	0.63	17.9
Caffeoyl tartaric acid (**2**)	1.43	0.5–3.4	0.24***	0.77	34.5
Ellagitannins					
Ellagitannin (**1**)	3.66	0.7–20.5	6.88***	0.73	71.7
Oenothein B (**3**)	52.36	0.6–101.4	531.81***	0.78	44.0
Ellagitannin (**4**)	2.91	1.3–6.3	0.33***	0.41	19.6
Oenothein A (**5** and **6**)	13.51	1.8–96.62	232.27***	0.91	112.8
Ellagitannin (**7**)	3.25	1.2–5.5	0.44***	0.51	20.3
Other ellagitannins	20.11	6.3–55.0	72.50***	0.86	42.3
Total ellagitannins	95.81	38.7–158.3	222.63***	0.40	15.6
Flavonoids					
Quercetin glucuronide (**8**)	2.05	0.6–5.2	0.41***	0.61	31.0
Flavonoid glycoside (**9**)	0.17	0–0.4	0.004***	0.71	39.0
Kaempferol glucuronide (**10**)	2.19	0.6–5.2	0.68***	0.73	37.5
Flavonoid glycoside (**11**)	0.17	0–0.57	0.009***	0.69	54.5
Other flavonoids	0.23	0–0.5	0.005***	0.61	30.3
Total flavonoids	4.82	2.0–9.9	1.05***	0.50	21.3
Life-history and phenology					
LH strategy (rosette-0/flower-1)	0.56	0 or 1	5.22***	0.05 (0.52)†	408.0
Bolting date (days since Feb 1)	168.27	146–246	147.72***	0.46	7.2
Flowering date (days since Feb 1)	199.40	139–246	111.78***	0.38	5.3
Biomass (g)	48.43	0–402.3	2293.40***	0.54	98.9
Fitness					
Fruits	115.45	0–800	10 597***	0.38	89.2

There were 5–11 replicate plants from each of 39 plant genotypes. Numbers in bold in parentheses denote peaks from HPLC (see Fig. 1). $^{**}P < 0.01$, $^{***}P < 0.001$.
†Heritability for life-history strategy was estimated by dividing the genotypic variance from generalized linear mixed models by the total phenotypic variance according to the binomial equation: total number of plants × frequency of annual plants × frequency of rosette plants. The value in parentheses shows the heritability from REML where the 0/1 life-history variation was treated as a continuous response.

on their UV spectra and included into the subgroups 'other ellagitannins' and 'other flavonoid glycosides'. Detailed descriptions of the chemical profiling by HPLC-DAD and HPLC-ESI-MS will be presented in a forthcoming article (Salminen *et al.*, unpublished results).

Genetic variation in plant traits

Our study first addressed whether there was heritable genetic variation for the diverse suite of functional traits measured from *O. biennis*, and we found significant genetic variance for all traits (Table 1). Clonal heritabilities ranged from 0.05 to 0.92 (mean 0.57) and the coefficient of genetic variance ranged between 2.4 and 408 (mean 50.0). The amount of herbivory on plants, measured as the proportion of leaves damaged, exhibited low heritability ($H^2 = 0.19$) compared with other traits. Traits associated with the physiology of leaves (sla, C : N ratio, % water) exhibited heritability values (mean 0.26) that were 28% lower than those for life-history traits (bolting and flowering date, life-history strategy and plant biomass) (mean 0.36) (Table 1). The concentrations of secondary compounds exhibited the highest heritability values (mean 0.68). Lifetime fruit

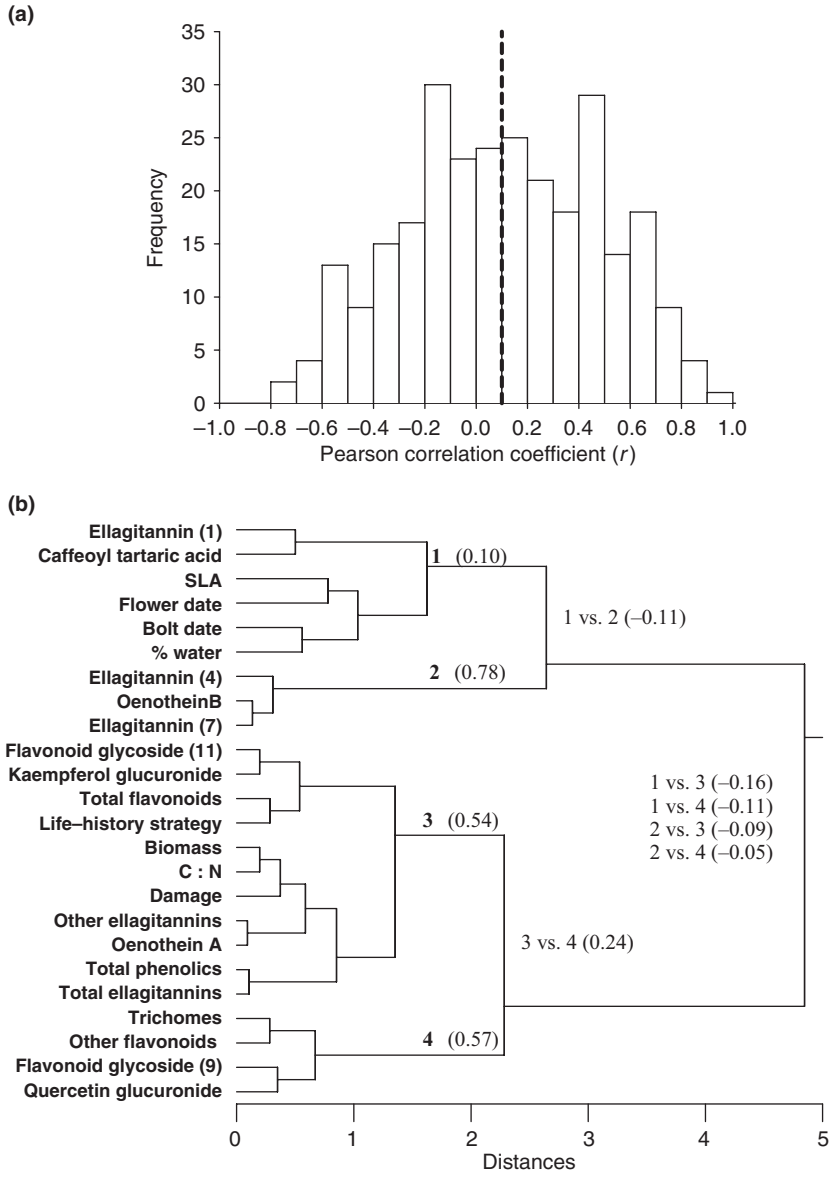

Fig. 2 The distribution of genetic correlations and associations among functional traits in *Oenothera biennis*. (a) The frequency distribution of pairwise Pearson genetic correlations among all traits ($N = 276$ pairwise correlations), where the mean r-value is shown by the dashed vertical line. (b) Associations among traits according to hierarchical cluster analysis using Ward's linkage. We identified four clusters of traits separated by sums-of-squares distance > 2; average pairwise correlations within and between clusters are shown in parentheses.

production was also heritable (Table 1), indicating genetic variance for fitness.

Covariation among plant traits

Our second question sought to understand whether plant traits genetically covaried with one another, and we found that the 276 pairwise tests among the 24 traits revealed extensive covariation among traits, measured as either genetic Pearson correlation coefficients or genetic covariances (Supporting Information Table S1). The high frequency of significant pairwise associations (128 of 276 significant tests at the 0.05 level) was unlikely to have been due to chance (Binomial expansion test: $P = 4.2 \times 10^{-89}$). The average correlation between traits was positive (mean $r_{genotype} = 0.11$), although the distribution of correlation coefficients was approximately normal (Fig. 2a), with many statistically significant positive (88 tests with $P < 0.05$) and negative (40 tests with $P < 0.05$) pairwise correlations and covariances (Supporting Information Table S1). Many of the negative correlations occurred between physiological traits and secondary compounds, or among different types of secondary compounds, principally ellagitannins (Supporting Information Table S1).

We used hierarchical cluster analysis on pairwise genetic correlations to better understand the relationship among traits (Fig. 2b). Cluster analysis identified four groups of covarying traits in which traits positively covaried within these groups. Covariation among groups

Table 2 Plant traits that explain variation in herbivory by *Popillia japonica*.

Trait	Parameter	*P*-value	r^2 (partial)
LH (annual/biennial)	0.012 ± 0.006	0.06	0.10
Ellagitannin (**1**)	0.007 ± 0.003	0.06	0.10
Other ellagitannins	**0.001 ± 0.0003**	0.001	0.29
Quercetin glucuronide (**8**)	**−0.011 ± 0.004**	0.01	0.19
Other flavonoids	**0.173 ± 0.039**	< 0.001	0.38
Principal components			
PC1	**0.013 ± 0.006**	0.03	0.12
PC2	**−0.04 ± 0.008**	< 0.001	0.42

Stepwise regression identified one life-history (LH) trait (annual/biennial) and four phenolic traits that explained 73% of variation in the proportion of leaves damaged by an outbreaking exotic herbivore. No significant quadratic effects were detected. A multivariate distillation of these traits using principal components analysis explained 45% of the variation. The first principal component (PC1) contrasted concentrations of kaempferol glucuronide, flavonoid glycoside (**11**), total ellagitannins and total phenolics (positive loadings) with quercetin glucuronide and a flavonoid glycoside (**9**) (negative loadings). PC2 provided a contrast between bolting date, % water and oenothein B, and two ellagitannins (**4**,**7**) (all with positive loadings) with biomass, C : N, oenothein A, an ellagitannin (**1**), oenothein A, other ellagitannins and total phenolics (negative loadings). The regression coefficient (parameter), *P*-value and partial r^2 values are shown for each trait and PC; the sum of partial r^2 is > 1 because some variables were collinear. Numbers in bold in parentheses denote peaks from HPLC (see Fig. 1). Significant parameters indicated in bold.

was typified by significantly negative or nonsignificant correlations (Fig. 2b). Groups did not clearly separate according to the types of traits associated with them (i.e. ecophysiological, life history, resistance or morphology). The largest groups (1 and 3) contained all main trait types, and smaller groups of traits either contained only secondary compounds (group 2) or a mix of trichome density (a morphological trait) and secondary compounds (group 4).

Herbivory

Our third research objective was to determine whether individual plant traits or multivariate suites of traits predicted resistance to herbivory. Five traits explained 73% of the total variation in herbivory by outbreaking *P. japonica* beetles – the dominant herbivore on plants (overall model: $F_{5,33} = 17.66$, $P < 0.001$, $r^2 = 0.73$; Table 2, Fig. 3a). The most important traits among these were several phenolic compounds. Specifically, quercetin glucuronide (a flavonoid glycoside) was negatively associated with herbivory (Fig. 3b), whereas the cumulative concentrations of several minor flavonoids ('other flavonoids', Fig. 3c) and several ellagitannins ('other ETs', Fig. 3d) were positively associated with herbivory (Fig. 3d). Life-history strategy (rosettes vs. flowering plants) and an ellagitannin (peak 1) were also positively related to herbivory, but the effects of these compounds were only marginally significant ($P = 0.06$, Table 2). The variance–covariance matrix revealed no trade-offs among these traits, whereas several traits positively covaried with one another (Supporting Information Table S1).

A multivariate distillation of all 24 traits identified two PCs that explained 45% of the variation in herbivory ($F_{2,36} = 14.5$, $P < 0.001$, $r^2 = 0.45$). Each significant PC summarized variation in multiple traits (see Table 2).

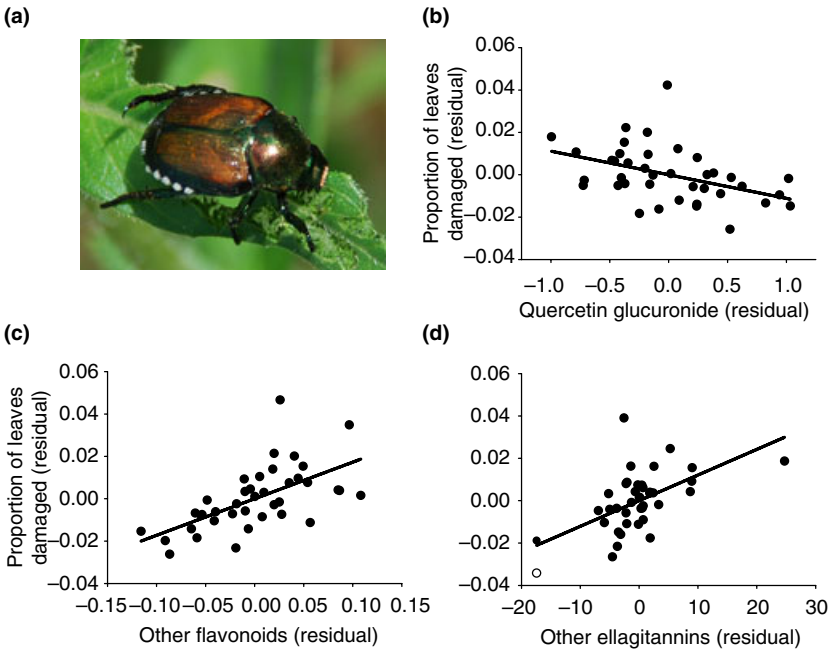

Fig. 3 Plant traits that explained variation in herbivory by (a) *Popillia japonica*, the major herbivore observed on plants (photo credit: A. Agrawal). Multiple regression using the genotype BLUPs revealed that genetic variation in concentrations of: (b) quercetin glucuronide, (c) 'other flavonoids' and (d) 'other ellagitannins' explained the most variation in herbivory (see Table 1). Genetic variation in life-history strategy and an ellagitannin (peak 1; see Fig. 1) also explained variation in herbivory, but these traits explained less variation. All figures show residual herbivory vs. residual trait variation, where variation in other traits has been partialled out.

Natural selection on plant traits

Finally, we asked whether there was selection on plant traits and we found that natural selection acted on multiple life-history and chemical traits of *O. biennis*. Significant selection was detected on 17 of the 24 traits, as measured by selection differentials (Supporting Information Table S2). Conventional multivariate genotypic selection analyses (Rausher, 1992) revealed that selection acted on just four plant traits. There was positive directional selection for an increase in plant biomass (Fig. 4a), flowering during the first year (Fig. 4b) and greater concentrations of the ellagitannin oenothein A (Fig. 4c, Table 3). We also detected directional selection for decreases in the concentration of quercetin glucuronide (Fig. 4d), a compound associated with decreased *P. japonica* herbivory. We found quadratic selection on biomass, which reached a maximum within the range of data at 1.55 standardized biomass units, suggesting the presence of stabilizing selection (Fig. 4a). There was also quadratic selection on quercetin glucuronide, where weak disruptive selection acted with a fitness minimum at 0.89 standardized units (Fig. 4d).

Somewhat counterintuitively, damage by herbivores was positively associated with plant fitness (Supporting Information Table S1), suggesting that this relationship is indirectly mediated by one or more plant traits that jointly influenced both herbivory and plant fitness. Consistent with this idea, quercetin glucuronide and life-history strategy were subject to positive directional selection and also associated with increased herbivory. When we re-examined the relationship between relative fitness and herbivory after accounting for variation in fitness explained by traits under selection (see Table 3), the significant relationship between herbivory and

Table 3 Genotypic selection gradients describing directional (β), nonlinear (γ) selection and selection differentials (S) on *Oenothera biennis* traits.

Trait	β	*P*	γ	*P*	*S*
Biomass	**0.58 ± 0.11**	< 0.001	**−0.37 ± 0.12**	0.003	0.92***
LH strategy	**0.47 ± 0.08**	< 0.001	0.24 ± 0.14	0.09	0.84***
Oenothein A (**5** and **6**)	**0.18 ± 0.07**	0.01	0.05 ± 0.06	0.41	0.62***
Quercetin glucuronide (**8**)	**−0.13 ± 0.05**	0.02	**0.15 ± 0.07**	0.03	0.20
Principal components					
PC1	**0.44 ± 0.21**	0.04			
PC2	**−2.05 ± 0.25**	< 0.001			
PC3	**1.23 ± 0.28**	< 0.001			
PC5	**0.79 ± 0.37**	0.04			

PC1 contrasted total phenolics, total ellagitannins, kaempferol glucuronide and a flavonoid glycoside (**11**) (all with positive loadings) with quercetin glucuronide and a flavonoid glycoside (**9**) (all negative loadings). PC2 contrasted the positive loadings of bolting date, % water, oenothein B and an ellagitannin (**4**) with the negative loadings of herbivory, plant biomass, C : N, oenothein A, other ellagitannins and total phenolics. PC3 contrasted the positive loadings of life-history strategy, trichome density, oenothein B, other flavonoids with the negatively loaded caffeoyl tartaric acid. PC5 contrasted LH strategy (positive loading) with bolting date (negative loading). Numbers in bold in parentheses correspond to HPLC peaks (see Fig. 1). ***$P < 0.001$.

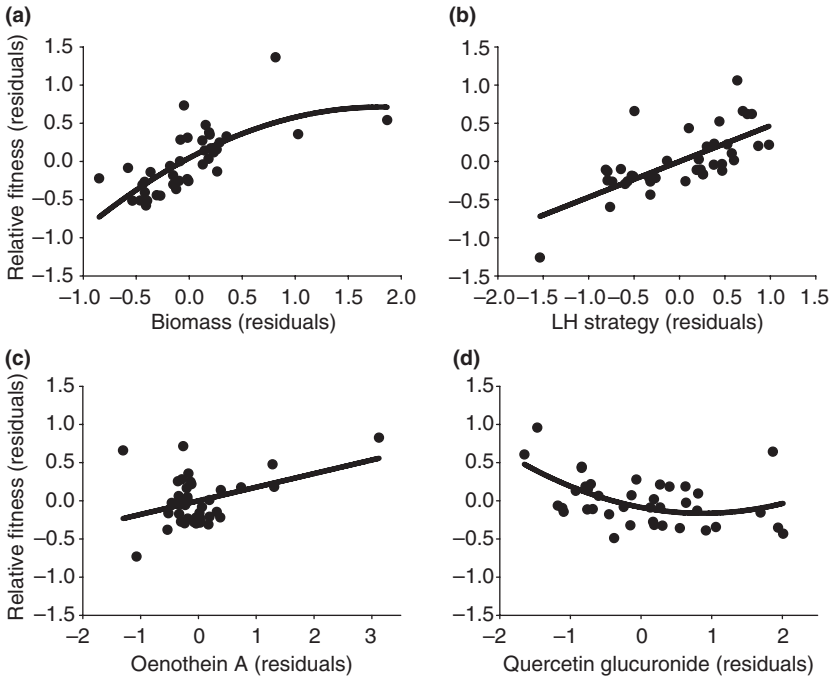

Fig. 4 Directional and quadratic selection on *Oenothera biennis*. Multiple regression using the genotype BLUPs revealed four plant traits that explained 97% of the variation in relative fitness among plant genotypes. (a) Strong directional selection acted to increase plant biomass in plant genotypes with low biomass, but stabilizing selection acted at high biomass values. Directional selection also acted to increase: (b) the initiation of flowering from the rosette stage and (c) the concentrations of an ellagitannin (peak 1). (d) Directional selection acted to decrease concentration of quercetin glucuronide in genotypes with low concentrations of this compound, and we detected significant disruptive selection with a fitness minimum at moderately high concentrations. All figures show residual fitness vs. residual trait variation, where variation in other plant traits was partialled out.

relative plant fitness disappeared ($F_{1,31} = 0.11$, $P = 0.91$). We can infer from this result that herbivory had no direct effect on *O. biennis* fitness. However, that is not to say that resistance to *P. japonica* cannot evolve due to correlated selection.

Discussion

Our study shows the importance of examining both selection on disparate traits, and the genetic variance and covariance among those traits, in order to understand the evolutionary ecology of plant populations and their interactions with herbivores. Our focus on a wide diversity of functional traits enabled us to account for a large amount of the variation in herbivory (> 70%), with both life-history traits and secondary chemistry influencing resistance to an abundant insect herbivore. Although secondary compounds can deter herbivores (Berenbaum *et al.*, 1986; Mauricio, 1998; Agrawal, 2005), a comprehensive characterization of phenolic plant chemistry revealed both positive and negative relationships between plant secondary compounds and herbivory. Natural selection acted on multiple plant traits, which accounted for > 95% of the variation in relative fitness among plant genotypes. Because of the nature of this selection, the extensive genetic covariance among traits and the functionally asexual genetic system of *O. biennis*, we predict that there are strong constraints on adaptive evolution in *O. biennis.* Therefore, individual traits will not respond independently to natural selection and we predict that several maladaptive traits (e.g. susceptibility to herbivores) might be maintained or increased in populations.

Heritability of plant traits

The patterns we observed in heritability estimates from different classes of functional traits were remarkably similar to those patterns reported from a recent review (Geber & Griffen, 2003). Geber & Griffen (2003) found that among 1214 heritability estimates reported in 74 studies, mean heritability of chemical traits was more than two times greater than the mean heritability of morphological, phenological or life-history traits. We also found that the heritability of secondary chemistry was approximately two times greater than other types of traits, and CV values were similarly high (Table 1). Although this pattern could be due to differences in environmental sensitivities (plasticity) among trait types, we believe it is more likely that this pattern reflects greater balancing selection on chemical traits due to the highly variable nature of selection by herbivores, which varies in both space and time (Thompson, 2005). Such variable selection can maintain genetic variation due to genotype-by-environment interactions (Hedrick, 1986) and via frequency-dependent selection (Dybdahl & Lively, 1998). For life-history traits, regardless of whether we treated biomass as a morphological trait (most common in the animal literature) or a life-history trait (most common in the plant literature), we did not find strong evidence for the prediction that life-history traits exhibit the lowest heritabilities due to strong selection that erodes genetic variance (Fisher, 1930; Mousseau & Roff, 1987). Rather, as found by Geber & Griffen (2003), physiological traits exhibited the lowest heritability values (Table 1).

Genetic covariation, evolutionary constraints and correlated evolution

The functional asexuality of *O. biennis* leads to near complete linkage of the genome, which is predicted to have dramatic effects on a population's evolution (Holsinger & Ellstrand, 1984). Although trait evolution in asexual populations is still a function of the strength of selection and the nature of genetic variances and covariances among traits, these traits cannot evolve independently in finite populations, which results in extensive interference selection among loci (Hill & Robertson, 1966). Essentially, the genotype with the highest relative fitness in asexual populations is favoured over all others, leading to the fixation of adaptive and nonadaptive traits (Barton & Turelli, 1989). A corollary of such evolutionary dynamics is that natural selection is expected to drive extensive correlated evolution among traits. Exceptions to this occur in very large populations where mutational variance is sufficient to enable all traits to reach an optimum (Crow & Kimura, 1965), and in small populations where genetic drift leads to greater stochasticity in evolutionary outcomes. The latter may be particularly important in *O. biennis* as its populations are frequently small (Johnson *et al.*, 2009).

Consistent with the prediction that the genetic system of *O. biennis* constrains adaptive evolution and leads to substantial correlated evolution, many traits exhibited significant selection differentials, yet relatively few traits had significant selection gradients. Therefore, many traits indirectly influenced, or were at least associated with traits controlling fitness (*sensu* Geber & Griffen, 2003). Based on our genotypic selection analyses, the traits under strong directional selection (biomass, life-history strategy, oenothein A; see Table 3) all exhibited positive genetic covariances (Supporting Information Table S1). Therefore, selection should effectively increase the values of these traits, provided that populations are sufficiently large to prevent strong genetic drift (Hartl & Clark, 1997). Selection also favoured decreased concentrations of quercetin glucuronide, which exhibited significant positive genetic covariance with plant biomass and nonsignificant positive covariances with life-history strategy and oenothein A. Therefore, evolution for reductions in quercetin glucuronide will probably be constrained by the comparatively strong selection on other plant traits.

Selection on life-history and chemical trait variation is also predicted to drive correlated evolution on plant traits, which may impact interactions with herbivores. For example, the positive covariances between herbivory and two traits under positive directional selection (annual reproduction and oenothein A) are predicted to lead to the evolution of increased susceptibility to *P. japonica*. In fact, several of the traits positively associated with herbivory (Table 2) also positively covaried with biomass (Supporting Information Table S1), which further suggests that *O. biennis* populations may evolve increased susceptibility to *P. japonica*. Although there was no evidence for direct selection by *P. japonica*, herbivores can have negative fitness consequences on *O. biennis* (Johnson & Agrawal, 2005), and we predict that selection by herbivores will probably increase if populations evolve greater susceptibility. As discussed above, these conclusions from the multivariate breeder's equation are most accurate in predicting the outcome of evolution over a single generation (Lande, 1979; Lande & Arnold, 1983).

The extensive positive and negative genetic covariances among secondary compounds allow us to make inferences about the types of genes that cause genetic variation in the production of phenolics. Two related biosynthetic pathways are responsible for the production of phenolics: flavonoid biosynthesis is produced via a combination of acetate-malonate and shikimate (from phenylalanine) pathways, whereas ellagitannin biosynthesis is produced via the shikimate pathway (from gallic acid) (Winkel-Shirley, 2001; Salminen *et al.*, 2004). Therefore, flavonoids and ellagitannins compete for a common precursor, dehydroshikimic acid (Ossipov *et al.*, 2003). The nature of variances and covariances among these chemical compounds sheds light on whether there might be polymorphisms in genes that: (a) influence the total amount or rate at which substrates move down these pathways (i.e. flux), vs. (b) polymorphisms that affect the relative amounts of substrates that move down alternative biosynthetic paths to produce the final compounds. If polymorphisms only influence flux, then we should observe variation in the total concentrations of flavonoids and positive genetic correlations among them (Riska, 1986). Consistent with this interpretation, the total concentrations of flavonoids varied fivefold among genotypes and flavonoid compounds exhibited only significant positive genetic correlations and nonsignificant correlations, suggesting that there is at least genetic variation in genes that control total flux in the flavonoid pathway.

Alternatively, when two or more enzymes compete for a limiting substrate at branching points within a pathway, genetic variation in the competing enzymes' concentrations, or substrate affinities/activities, will cause a greater frequency of negative correlations (Riska, 1986). Consistent with this expectation, we found negative genetic correlations among multiple ellagitannins. We also observed positive correlations among some ellagitannins as well as variation in the total concentration of ellagitannins, suggesting that there are polymorphisms in structural or regulatory genes that control branching points within the ellagitannin pathway, as well as polymorphisms that control total flux.

A wholistic approach to the evolutionary ecology of plants and plant–herbivore interactions

Ever since Fraenkel's (1959) classic paper suggesting that herbivore defence is the 'raison d'etre' of plant secondary compounds, many studies investigating the evolution of resistance have been biased towards testing for the defensive function of plant chemicals and conspicuous physical defences (e.g. thorns and latex). Recent studies, however, indicate that a greater variety of traits may play a role in reducing herbivory, including phenology (Pilson, 2000; Kursar & Coley, 2003), physiological traits (Agrawal, 2004; Johnson, 2008) and even third trophic-level predators and parasitoids attracted by plant volatiles (Thaler, 1999). These studies suggest that a wholistic approach to the study of resistance may help explain a greater proportion of the variation in resistance.

Our results show that plant secondary chemistry played a dominant role in affecting resistance to herbivores on *O. biennis*, yet measurement of multiple types of traits was still beneficial as traits other than plant secondary chemistry (e.g. life-history strategy) also accounted for variation in resistance (Table 2). The effects of secondary chemistry were complex, as different chemicals had either negative or positive effects on herbivory (Table 2) and often correlated with other types of traits (Fig. 2b). At least one secondary compound negatively affected the amount of herbivory by *P. japonica*, whereas others had positive effects. Perhaps surprisingly, variation in relatively minor components of flavonoids (see 'Other flavonoids', Table 2) explained a large proportion of the variation in herbivory. These results support recent conjecture that measures of total concentrations of secondary chemicals of a particular type (e.g. total phenolics) may be a poor indicator of resistance (Salminen *et al.*, 2004).

Our results also provide evidence for intraspecific suites of traits associated with resistance that have the potential to evolve as adaptive syndromes of plant defence against herbivores (Kursar & Coley, 2003; Agrawal & Fishbein, 2006). Specifically, we found that suites of traits covaried with one another to explain variation in herbivory (see PCA results, Table 2; also Fig. 2). Whether the covariance underlying such traits is stable through space and time, allowing for the evolution of clearly defined defensive syndromes, is not yet known and is an avenue for future research (Steppan *et al.*, 2002).

Our approach illustrates that natural selection acts on multiple heritable plant traits, and perhaps suites of covarying traits (Table 3). As might be expected, directional selection was the strongest on life-history traits, but moderately strong selection also acted on specific secondary compounds. Overall, we show that a broad trait-based approach can lead to a better understanding of the evolutionary ecology of species interactions and the processes and constraints that influence adaptive evolution.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Matrix of variances, covariances and genetic correlations among functional plant traits.

Table S2 Selection differentials on each of 24 plant traits. Selection differentials were measured according to Price (1970), by calculating the covariance between the BLUPs of relative fitness and the normally standardized genotypic breeding values (BLUPs). Numbers in bold in parentheses refer to HPLC peaks (Fig. 1).

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