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Ecological patterns and genetic analysis of post-dispersal seed predation in sunflower (*Helianthus annuus*) crop-wild hybrids

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Abstract

Crop-wild hybridization has been documented in many cultivated species, but the ecological and genetic factors that influence the likelihood or rate that cultivar alleles will introgress into wild populations are poorly understood. Seed predation is one factor that could mitigate the spread of otherwise advantageous cultivar alleles into the wild by reducing seedling recruitment of crop-like individuals in hybrid populations. Seed predation has previously been linked to several seed characters that differ between cultivated and wild sunflower, such as seed size and oil content. In this study, seed morphological and nutritional characters were measured in a segregating population of sunflower crop-wild hybrids and wild and cultivated lines. Seed predation rates among lines were then assessed in the field. The relationship between seed predation and seed characters was investigated and quantitative trait loci (QTL) were mapped for all traits. There was no effect of seed type (hybrid vs. parents) on seed predation, although a trend toward more early predation of wild seeds was observed. Within the hybrids, seed predators preferred seeds that contained more oil and energy but were lower in fibre. The relationship between seed predation and oil content was supported by co-localized QTL for these traits on one linkage group. These results suggest that oil content may be a more important determinant of seed predation than seed size and provide molecular genetic evidence for this relationship. The cultivar allele was also found to increase predation at all QTL, indicating that post-dispersal seed predation may mitigate the spread of cultivar alleles into wild populations.

Keywords: herbivory, hybridization, introgression, quantitative trait locus mapping, selection

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Introduction

Consequences of the introgression of crop alleles into wild relatives range from a shift in the genetic composition of natural populations to range expansion or the evolution of weedy or invasive species morphs (Ellstrand *et al.* 1999; Ellstrand 2003). Although much of the attention surrounding crop-wild hybridization has focused on genetically-modified (GM) cultivars (Colwell *et al.* 1985; Goodman & Newell 1985; Ellstrand & Hoffman 1990; Chapman & Burke 2006), the transfer of non-transgenic cultivar alleles to natural populations poses similar risks (De Wet & Harlan 1975; Ellstrand 2003) and can provide a useful experimental model for studying the behaviour of crop alleles in the wild. Gene flow between crop plants and their wild relatives has been documented in the majority of cultivated species (Ellstrand 2003), but the ecological and genetic factors that influence the likelihood of gene escape and the rate at which a cultivar allele will spread into natural populations are not well characterized.

The likelihood that a cultivar allele will spread through a natural population is largely determined by its selective advantage over wild alleles (Rieseberg &

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Burke 2001). Several studies have identified non-transgenic 'domestication' phenotypes, such as accelerated flowering, rapid growth and large inflorescences, which appear to enhance the fitness of crop-wild hybrids under natural conditions (Cummings *et al.* 1999; Snow *et al.* 2003; Campbell & Snow 2007; Mercer *et al.* 2007; Baack *et al.* 2008; Dechaine *et al.* 2009). Despite the apparent fitness benefits, there is little evidence for long-term persistence of cultivar-like phenotypes in many wild populations (Baack *et al.* 2008; but see Whitton *et al.* 1997). One likely explanation is that the selective advantage of crop-like traits is at least partially mitigated by the presence of herbivores in the wild. For example, a recent study found that selection favoured early flowering in sunflower crop-wild hybrids only if the effects of pre-dispersal herbivory were accounted for in the selection analysis, suggesting that cultivar-like flowering would only be advantageous in the absence of herbivores (Dechaine *et al.* 2009). In the presence of herbivores, selection favoured wild-like later flowering, possibly because plants that delayed flowering escaped the most severe herbivory (Cummings *et al.* 1999; Pilson 2000; Dechaine *et al.* 2009). These results demonstrate the importance of characterizing patterns of selection on cultivar alleles under a variety of natural conditions.

Post-dispersal seed herbivory may also mitigate the selective advantage of cultivar-like phenotypes in crop-wild hybrid populations. Large seeds are often predicted to be advantageous because they are more likely than small seeds to germinate and produce seedlings (Westoby *et al.* 1992; Baskin & Baskin 1998) and cultivar lines of several species produce larger seeds than related wild individuals (De Wet & Harlan 1975; Alexander *et al.* 2001). In the absence of granivores (i.e. seed predators), selection in hybrid populations would be expected to favour larger crop-like seeds; but the advantage to large seeds may be reduced or eliminated if they are also subject to greater predation (Westoby *et al.* 1992; Alexander *et al.* 2001; Gomez 2004). Both invertebrate and mammalian granivores have been shown to consume large seeds before smaller seeds (Janzen 1969; Abramsky 1983; Price 1983). Previously reported predators of sunflower seeds in the wild include small rodents such as rats, voles and mice (Alexander *et al.* 2001), ground feeding birds such as quail (Michael & Beckwith 1955) and larger vertebrates such as foxes (Sargeant *et al.* 1986). In a study of sunflower crop-wild hybrids, post-dispersal predation was approximately 50% higher for hybrid than wild seeds and the difference was attributed to the larger size of hybrid seeds (Alexander *et al.* 2001). Consequently, differential seed predation would be expected to reduce the number of hybrid sunflower individuals that establish in the wild.

Although the aforementioned study provided strong evidence that granivores preferred hybrid over wild sunflower seeds (Alexander *et al.* 2001), seed size was not the only possible cause of differential predation between plant types. Rather, seed nutritional characters that differ between cultivated and wild plants could also influence granivore preference. For example, rodents have been shown to prefer seeds with higher levels of protein and soluble carbohydrates (Kelrick *et al.* 1986; Jenkins 1988; Henderson 1990; Lewis *et al.* 2001). Granivore preference has also been frequently linked to the amount of available energy (caloric content) in the seeds, with higher calorie seeds being preferred (Janzen 1971; Kelrick *et al.* 1986; Kerley & Erasmus 1991; Ivan & Swihart 2000; Briones-Salas *et al.* 2006). In oilseed crops, such as canola or sunflower, percent oil content is higher in cultivated seeds than in wild individuals of related species. Cultivated sunflower seeds may contain up to 1.4 times the oil content of wild seeds (Seiler 1983). Since increasing the oil content also increases the caloric content of sunflower seeds, it is likely that oil content would also influence post-dispersal seed predation in sunflower crop-wild hybrids. Moreover, observed relationships between seed size and seed predation may actually reflect a granivore preference for higher energy content, as seed mass and caloric content are often correlated (Janzen 1969; Kelrick *et al.* 1986). Consequently, larger hybrid seeds may not always be subject to increased predation if energy levels are increased in smaller seeds, for example if larger seeds have a higher fibre-to-fat ratio (Kelrick *et al.* 1986).

The genetic basis of susceptibility to post-dispersal seed predation and its relationship to other seed characters is largely unknown. To predict the spread of cultivar alleles into wild populations, it is necessary to characterize the genetic architecture of adaptive phenotypes. For example, pleiotropy of a single locus or physical linkage between loci that confer susceptibility to herbivores and otherwise advantageous crop alleles may constrain the spread of adaptive cultivar alleles into the wild (Dechaine *et al.* 2009). Quantitative trait locus (QTL) mapping provides a method to examine the genetic basis of complex phenotypic traits on a per-locus basis. QTL for oil content and composition have been previously identified in sunflower crop-wild hybrids (Burke *et al.* 2005), but QTL have not been mapped for other seed nutritional characters in crop-wild hybrids or, to our knowledge, for post-dispersal seed predation in any system.

Here we investigate the ecological patterns and genetic architecture of post-dispersal seed predation and its relationship to seed characters in a segregating population of sunflower (*Helianthus annuus* L.) crop-wild hybrids. Sunflower is an interesting model for crop-wild hybridization for several reasons. Wild sunflower occurs throughout the range of sunflower cultivation in North America and crop and wild sunflowers flower coincidently and readily hybridize in the field (Burke *et al.* 2002a). Cultivar alleles have been detected in wild sunflower populations at distances up to 1000 m from the nearest sunflower crop field (Arias & Rieseberg 1994) and can persist for at least five generations (Whitton *et al.* 1997). Cultivated and wild sunflower differ for several seed characters that potentially contribute to variation in seed predation between plant types. There has been strong directional selection for increased achene (hereafter referred to as seed) size over the history of cultivated sunflower (Burke *et al.* 2002b) and more recent selection for increased oil content (Putt 1997; Burke *et al.* 2005). Consequently, cultivated sunflower seeds can be up to seven times greater in mass, five times larger in area and contain up to 40% more oil than wild seeds (Burke *et al.* 2002b, 2005). Seed colour also differs between types, in that wild seeds (at our experimental site) are of a mottled brown colour and cultivated seeds have a striped or black appearance. Up to 100% seed loss to predation has been observed in feeding experiments with wild sunflower (Briones-Salas *et al.* 2006); therefore, post-dispersal seed predation is likely to impact seedling recruitment in natural sunflower populations.

In this study, we examine ecological patterns of phenotypic variation in seed predation and related seed characters in wild, cultivated and crop-wild hybrid sunflowers. We then investigate the genetic basis of these traits in hybrid plants by: (i) quantifying the genetic component of variation in seed predation; (ii) characterizing the relationships between seed predation and seed morphological and nutritional characters; and (iii) elucidating the QTL-architecture of these phenotypes.

Materials and methods

Plant material

We used seeds of F_8 recombinant inbred lines (RILs) generated from a cross between an elite oilseed cultivar line and wild sunflower [cmsHA89 (PI 650572) × ANN1238] (Burke *et al.* 2002b). Development of the RILs has been previously described (Baack *et al.* 2008). Briefly, a single selfed F_1 individual was selected from the initial cross and the resulting F_2 generation was self-pollinated and advanced by single seed descent, ultimately resulting in 184 F_8 RILs from which the linkage map was produced. The F_2 generation was field grown in isolation from other *H. annuus* in Mexico and the F_3, F_4–F_6, and F_7–F_8 generations were greenhouse-grown at the University of Indiana (Bloomington, IN, USA), Oregon State University (Corvallis, OR, USA) and the University of Georgia (Athens, GA, USA), respectively.

Experimental design

Seed predation. Seed predation trials were conducted at the University of Nebraska's Cedar Point Biological Station, Keith County, NE. The ANN1238 parent was collected from a wild population located in the same county; therefore, the wild alleles may be locally adapted to the study site. This site is within the range of wild sunflower and areas of sunflower cultivation and is representative of many of the natural areas where wild and cultivated sunflower come into contact (Burke *et al.* 2002a). Wild *H. annuus* occur within the study area (Pilson 2000; J. Burger, pers. obs.). The experimental sites consisted of a sandhills mixed-grass prairie matrix of fairly homogeneous vegetation (~50/50% mix) dominated by Side-oats Grama (*Bouteloua curtipendula* Michx.) and Little Bluestem (*Schizachyrium scoparium* Michx.) (J. Burger, pers. obs.). Vegetation height ranged from 10 cm–1 m. Although we did not trap or systematically monitor our sites for granivores, teeth marks and chewing patterns on recovered seed hulls, as well as scat found in the area, indicated that rodent predation dominated (J. Burger, pers. obs.). Several rodent species have been trapped in nearby Arthur County, NE (also primarily sandhills mixed-grass prairie), including the plains pocket mouse (*Perognathus flavescens*), hispid pocket mouse (*P. hispidus*), Ord's kangaroo rat (*Dipodomys ordii*), deer mouse (*Peromyscus maniculatus*), northern grasshopper mouse (*Onychomys leucogaster*), western harvest mouse (*Reithrodontomys megalotis*) and prairie vole (*Microtus ochrogaster*) (Lemen & Freeman 1986).

Post-dispersal seed predation was compared across both parental lines and the 85 RILs with enough available seed to use in the experiment. Seed from each line was randomly assigned to one of four quadrants on each of 27 numbered, 930 cm^2 mottled beige ceramic tiles (America™ design #3927). Twenty seeds of a line were attached to a 64 cm^2 circular area within a quadrant using Elmers® rubber cement. Each tile had seeds from four different lines (one line in each quadrant) and each line (including the crop and wild parental lines) was replicated only once per trial (see below). Tiles were placed on the ground and dusted with native soil upon installation to minimize effects of the tile surface and the adhesive on seed predation. Tiles were placed at ~1 m intervals within a ~80 m^2 area.

The experiment was replicated four times (trials), and lines were re-randomized among tile quadrants for each trial. Trials were installed every 2 days, on 11, 13, 15 and 17 September 2007 and therefore overlapped partially in time. Each successive trial was positioned at least 10 m away from the previous trial. Trials were conducted in the fall, because wild sunflower seed heads naturally shatter and scatter seed at this time of the year, making seeds available to ground-feeding predators.

In each trial, seed predation was scored for each line as the number of seeds removed every 2 days for a total of 6 days. The proportion of seeds removed since day 0 (i.e. seeds removed out of 20 seeds) at each time point (day 2, day 4 and day 6) was calculated. Measures of seed predation are cumulative; for example, predation at day 6 includes predation at days 2 and 4. Although these measures are partially redundant, multiple time points were included in order to separate the effects of early predation (proportion of seeds removed by day 2) from total predation over the experiment (proportion of seeds removed by day 6). In addition, correlations with other seed characters and the QTL mapping results differed among seed predation measures (see below), indicating that all time points are important to understanding the genetic basis of seed predation in the field.

Seed characters. Oil content was measured in 115 RILs and the two parental lines following the procedures described in Burke *et al.* (2005). Briefly, seed samples 0.5–1.0 g in mass were phenotyped for percent oil content using pulsed nuclear magnetic resonance (Bruker MQ20 Minispec NMR Analyser, The Woodlands, TX, USA). Five additional seed nutritional characters were analysed for the parents and the 85 RILs that had sufficient sample sizes to produce enough tissue (up to 10 g) for analysis by DairyOne (Ithaca, NY, USA) using standard Association of Analytical Communities (AOAC) methods (http://www.eoma.aoac.org). Crude protein was determined using the Kjeldahl method (AOAC 984.13), which measures percent protein content in the sample including true protein and non-protein nitrogen. The percent neutral detergent fibre (NDF) was measured using the ANKOM A200 Filter Bag Technique (Van Soest *et al.* 1991). NDF (hereafter referred to as fibre) measures the cell wall and structural carbohydrates in the cell; these components likely provide little or no energy benefit to most herbivores. The percent ash content was determined by weighing the samples, incinerating them at 600 °C for 2 h (AOAC 842.05) and weighing the remaining matter. These measures were used to calculate soluble carbohydrates and available energy (kcal) in the seeds. Carbohydrate % was calculated by subtracting the sum of the percent oil, crude protein, NDF, ash and moisture from 100%. Due to sampling error, carbohydrate estimates were sometimes less than 0; these samples were assumed to have no measurable carbohydrates. The available energy (kcal) in 1 kg of sample was calculated by converting percent oil, protein and calculated carbohydrates to g/kg and summing the standard estimates of 4 kcal/g of protein or carbohydrates and 9 kcal/g of oil (Maynard 1944).

Four seed morphological characters were also phenotyped in all available RILs (148) and the parental lines. Ten seeds of each line were weighed on a standard balance and scanned with a flatbed scanner (HP Scanjet 3500c, Hewlett-Packard, Palo-Alto, CA, USA). Scanned images were modified to eliminate shadows and placed onto a black background in Adobe Photoshop (v.9, San Jose, CA, USA). These images were subsequently analysed in Tomato Analyzer (Brewer *et al.* 2006) for seed area, seed shape (maximum length/maximum width) and seed luminosity. Luminosity represents the darkness (0) to lightness (100) of colour (Darrigues *et al.* 2008) and functions here as a surrogate for seed colour. Default settings were used for all measurements. Seed character values were averaged over the 10 seeds per line for subsequent analyses.

Statistical analyses

Tests of significance among the parental and hybrid lines. Analysis of variance (ANOVA) (PROC GLM) and contrast statements were used to test the fixed effect of seed type (wild, cultivar, and hybrid) on seed predation at day 2, day 4 and day 6. Seed predation residuals violated the assumption of normality, so a Kruskal–Wallis non-parametric test was also used to test the effects of seed type on predation. Rates of seed predation among seed types over time (days) were also examined using a repeated-measures ANOVA in which seed type was a fixed effect and day was the repeated measure.

Descriptive statistics within the hybrid RILs. A restricted maximum likelihood (REML) approach (SAS 2001; PROC MIXED) was used to partition total phenotypic variance for seed predation at day 2, day 4 and day 6 into sources attributable to the random factors: trial, tile, RIL (genotypic variance, V_G) and residual error (V_R). Broad-sense heritability (H^2) was calculated using the formula V_G/V_T, in which V_T is the total model variance. The REML procedure was also used to estimate phenotypic means and best linear unbiased predictors (BLUPs) of each RIL for seed predation measures to use in further analyses.

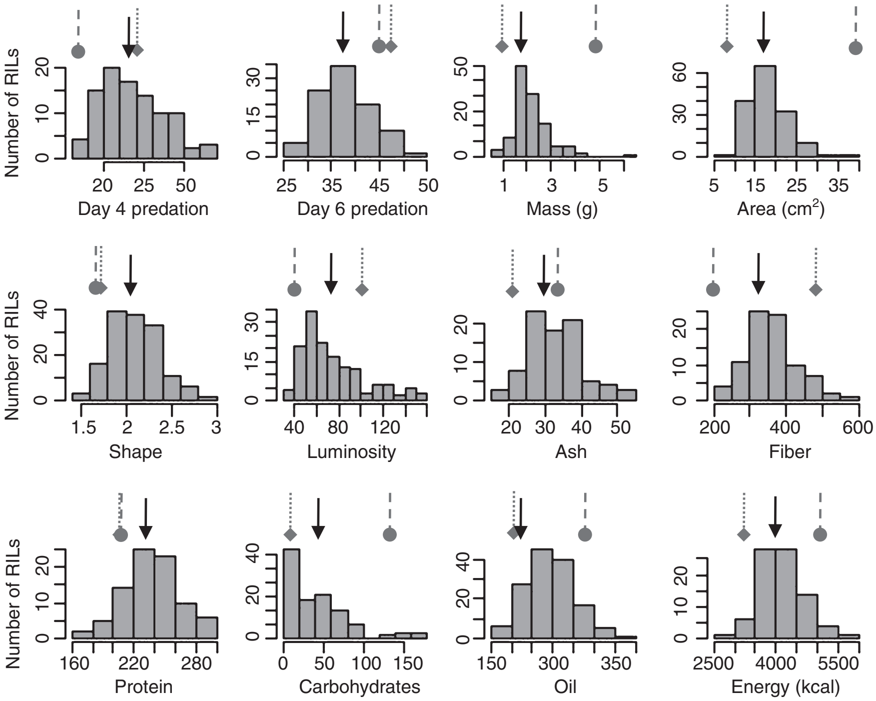

Fig. 1 Histograms for seed predation and seed characters in the recombinant inbred lines (RILs). Seed predation indicates the % of seeds removed after 4 and 6 days in the field; day 2 predation was too low to produce an informative plot. Nutritional characters are in g/kg unless otherwise specified. Shape is a proportion (seed length/seed width). Luminosity is a relative value based on the darkness (0) to lightness (100) of colour. Means are denoted for the hybrid (RILs), cultivar (cmsHA89) and wild (ANN1238) lines using a solid arrow, dashed circle and dotted diamond, respectively. Arrows represent a single sample for the seed nutritional characters in the cultivar and wild lines instead of a mean.

The relationships among seed predation and seed characters were explored using correlational analysis and stepwise regression within the RILs. Pearson correlation coefficients were generated among BLUPs for seed predation measures and RIL means for seed characters and significant correlations were determined using *t*-tests (SAS 2001; PROC CORR) and adjusted for multiple comparisons using Bonferroni correction. No significant correlations were observed for predation at day 2 and this day was excluded from further analyses. We identified the best predictors of seed predation at days 4 and 6 using stepwise regression. This procedure employs a modified forward-selection approach in which variables require a minimum significance level of 0.1 for entry into and to be retained in the regression model (SAS 2001; PROC REG, SELECTION = STEPWISE, SLE & SLS = 0.10). Stepwise regression analyses were based on the 72 RILs with data for all 12 phenotypic measurements.

Quantitative trait locus mapping. Quantitative trait loci (QTL) for seed predation BLUPs and seed character RIL means were mapped using an existing linkage map (Dechaine *et al.* 2009) and the composite interval mapping (CIM) procedure in Windows QTL Cartographer (Wang *et al.* 2007). Up to 10 background control markers were selected for each trait using forward-selection, backward-elimination stepwise regression and a walk speed of 2.0 cM. All CIM analyses used a 5 cM scan window. Permutation tests of 1000 permutations per trait were used to obtain significance thresholds. The additive effect of the cultivar allele (cmsHA89) and the % of phenotypic variance explained (PVE) for each QTL were also estimated in QTL Cartographer. Additive effects were standardized using the standard deviation of the trait (α/SD).

Results

Phenotypic variation in seed predation among parental lines and hybrids

Seed predation over the first 2 days was highest in the wild parent (mean ± SD = 11.3 ± 19.3% removed), lowest in the cultivated parent (1.3 ± 2.5%) and intermediate in the hybrids (RILs) (6.2 ± 11.6%). Predation remained lowest for the cultivated parent through day 4 (12.5% vs. 24–25%), but by day 6, predation was generally equivalent across seed types (38–47%) (Fig. 1). Predation was highly variable and sample sizes for the parental lines were low (one replicate of each line per experimental trial); therefore, no differences among seed types were statistically significant using ANOVA or Kruskal–Wallis tests ($P = 0.4$–0.8 depending on the trait and test).

Genetic analysis within the hybrids (RILs)

Genetic variation in seed predation. The majority of seed characters were normally distributed, the RIL mean was

Table 1 Summary statistics for seed predation (proportion of seeds removed) measures at 3 time points in a sample of 85 sunflower crop-wild hybrid recombinant inbred lines (RILs). Means, standard deviations (SD), and variance components were generated using restricted maximum likelihood (REML) analysis

Predation	Mean % (SD)	V_G	V_R	V_T	H^2
Day 2	6.2 (11.6)	0.000	0.012	0.014	0.000
Day 4	23.9 (28.9)	0.006*	0.054	0.088	0.063
Day 6	38.1 (34.3)	0.008*	0.060	0.128	0.061

V_G: among-RIL variance component, significance indicates genetic variation among RILs from REML analysis, *$P < 0.05$; V_R: residual variance component; V_T: total model variance; H^2: broad-sense heritability (V_G/V_T).

generally intermediate to the two parental genotypes and transgressive individuals were observed for all phenotypic measures [Table S1 (Supporting information); Fig. 1]. Among-line variance (V_G) was not significant for seed predation at day 2 (Table 1), suggesting that seed predation at this time point was too low and variable among RILs to detect an effect of genotype. RILs exhibited significant V_G for predation at days 4 and 6, indicating the presence of genetic variation for these traits. Despite significant RIL effects, broad-sense heritability estimates (H^2) were low, 0.06 for each measure of seed predation. Consistent with low heritabilities, residual variance (V_R) was substantially larger than among-line variance for seed predation at all time points.

Relationships between predation and seed characters. Predation at day 2 was not significantly correlated ($P < 0.05$) with any seed characters (Table 2). Although no correlations between predation at days 4 or 6 and seed characters were significant after Bonferroni correction, several interesting trends emerged. Day 4 and day 6 predation were positively related to percent oil content and energy, in that granivores preferred seeds with more oil and available energy. Seed predation was also negatively correlated with percent fibre content, suggesting that high fibre seeds were less preferred. Only the correlation between seed predation and energy was supported by the results of the stepwise regression analyses, in which energy was one of two significant

Table 2. Among-trait correlations for seed predation best linear unbiased predictors (BLUPs) and RIL means for seed characters. Pearson correlation coefficients (upper) and *P*-values (lower) of t-tests are shown. Negative values are in parentheses. Correlations significant at *P* <0.05 are highlighted in bold. A black border indicates correlations that are significant after Bonferroni correction (*P* <0.0008). Grey fill denotes a significant relationship between seed predation and seed characters using correlational analysis or stepwise regression (*). Shape was calculated from seed length (l) and width (w) measures

	Day 4 Predation	Day 6 Predation	Mass	Area	Shape	Luminosity	Ash	Fibre	Protein	Carbohydrates	Oil	Energy
Day 2	**0.39**	**0.33**	(0.02)	(0.06)	(0.07)	(0.03)	(0.19)	(0.09)	(0.20)	(0.01)	0.12	0.22
Predation	**0.001**	**0.007**	0.865	0.605	0.594	0.785	0.124	0.453	0.076	0.951	0.276	0.078
Day 4		**0.76**	0.15*	0.07	(0.07)	(0.07)	(0.15)	**(0.24)**	(0.10)	0.08	**0.31**	**0.34***
Predation		**<0.0001**	0.157	0.486	0.540	0.501	0.186	**0.032**	0.368	0.513	**0.005**	**0.003**
Day 6			0.04	(0.07)	0.10	(0.04)	(0.17)	**(0.25)**	(0.08)	0.08	**0.24**	**0.31***
Predation			0.723	0.486	0.362	0.713	0.139	**0.027**	0.491	0.463	**0.025**	**0.007**
Mass				**0.79**	**(0.29)**	(0.14)	0.03	0.05	**(0.28)**	**0.38**	(0.13)	(0.19)
				<0.0001	**0.0004**	0.098	0.807	0.661	**0.010**	**0.001**	0.124	0.084
Area					**(0.39)**	0.06	(0.17)	**0.23**	**(0.37)**	0.21	(0.16)	**(0.26)**
					<0.0001	0.473	0.122	**0.041**	**0.001**	0.063	0.057	**0.020**
Shape (l/w)						(0.04)	0.00	(0.12)	0.13	(0.02)	0.15	0.14
						0.668	0.972	0.285	0.233	0.883	0.081	0.228
Luminosity							**(0.39)**	**0.33**	**(0.28)**	(0.05)	(0.13)	(0.21)
							0.0003	**0.002**	**0.011**	0.648	0.128	0.063
Ash								**(0.30)**	**0.56**	0.06	(0.04)	0.10
								0.006	**<0.0001**	0.596	0.731	0.359
Fibre									**(0.24)**	**(0.42)**	**(0.63)**	**(0.85)**
									0.023	**<0.0001**	**<0.0001**	**<0.0001**
Protein										(0.16)	(0.05)	0.12
										0.160	0.124	0.303
Carbohydrates											(0.25)	0.02
											0.731	0.856
Oil												**0.94**
												<0.0001

Table 3 Quantitative trait locus (QTL) mapping results. Columns 2, 3, and 4 indicate the linkage group (LG), left flanking marker, and 1-LOD support limits in cM for each QTL, respectively. Columns 5 lists the standardized additive effect (α/standard deviation) of the cultivar allele (cmsHA89); parentheses indicate negative additive effects. Columns 6 lists the percent variance explained (PVE) by each QTL. Column 7 indicates if the QTL co-localized (C) or mapped to the same LG as a QTL for the same or a similar trait in a previous study

Trait	LG	Left marker	1-LOD (cM)	α (cultivar)	PVE	Prior
Day 4 Predation	8	ORS70	(30.81–48.71)	0.17	11.56	NA
Day 6 Predation	3	ORS488	(32.41–48.31)	0.32	8.81	NA
	8	ORS70	(34.81–45.91)	0.54	24.46	
Mass	2	ORS925	(0.01–8.01)	0.31	9.21	C[1]
	9	CYC5B	(51.61–55.61)	0.32	9.90	C[1]
	10	ORS595	(12.41–24.21)	0.41	13.15	LG[1], C[2]
Area	2	ORS925	(0.01–8.01)	0.35	11.36	C[2]*
	9	CYC5B	(10.41–18.01)	0.41	15.99	LG[1]**
	10	HT347	(47.91–58.11)	0.45	14.73	C[1]*
	12	ORS810	(29.61–43.21)	0.31	8.06	
	16	ORS750	(53.61–55.61)	0.25	5.70	
Shape (length/width)	1	ORS543	(0.01–17.31)	0.28	7.27	
	5	ORS505	(8.71–31.11)	0.31	8.93	LG[1]*, C[2]*
Luminosity	5	ORS1120	(61.01–67.01)	(0.33)	10.29	NA
	11	J22O06	(29.51–37.91)	0.28	7.18	
	16	HT208	(92.21–110.31)	(0.50)	22.34	
Ash	6	HT135	(4.81–34.01)	(0.06)	16.39	NA
	16	HT52	(78.21–108.31)	0.05	16.80	
Fibre	9	C02104	(6.11–18.71)	(0.45)	19.58	NA
Protein	4	ORS366	(8.51–18.01)	0.49	14.57	NA
Oil	3	HT441	(19.01–36.41)	0.27	7.11	
	4	ORS963	(0.01–4.01)	0.41	13.72	LG[3]
	7	ORS331	(2.01–13.71)	(0.30)	8.30	
Energy	4	ORS963	(0.01–6.51)	0.33	8.03	
	9	C02104	(8.11–16.11)	0.50	21.31	
	15	ORS420	(20.61–33.61)	0.39	9.80	
	15	ORS687	(57.51–66.61)	0.32	8.08	

[1]Burke *et al.* (2002); [2]Baack *et al.* (2008); [3]Burke *et al.* (2005); *length; **width.

predictor variables (energy parameter estimate = 0.00003, $P < 0.001$) for predation at day 4 and the only significant predictor (parameter estimate = 0.00003, $P < 0.02$) of predation at day 6. Predation at day 4 was also positively associated with seed mass using stepwise regression (parameter estimate = 0.111, $P < 0.05$). The stepwise regression results indicated that energy and seed mass explained approximately 12% and 5%, respectively, of the variation in predation at day 4 (model $R^2 = 0.174$) and energy explained 7.5% of the variation in predation at day 6.

We observed interesting correlations among a number of the seed characters (Table 2). Energy was highly positively correlated with oil content and negatively correlated with fibre, in that seeds that were more energy rich contained more oil but less fibre. Fibre was also negatively related to protein, carbohydrate, oil and ash contents. Seed size measures were negatively correlated with protein content (seed mass and area) and available energy (seed area), but positively correlated with carbohydrate content (seed mass), indicating that larger seeds were less protein- and energy-rich but contained more carbohydrates. Seed size and shape traits were negatively related, as larger seeds had a lower length-to-width ratio. Seeds that were lighter in colour (higher luminosity value) were higher in fibre but lower in ash and protein.

Quantitative trait locus mapping. No QTL were detected for seed predation at day 2. Three QTL were detected for seed predation at days 4 and 6, each explaining 8.8–24.5% of the phenotypic variance for these traits (Table 3; Fig. 2). In all cases, the cultivar allele conferred an increase in seed predation. One QTL for each seed predation measure mapped to the bottom of LG 8. No additional QTL for any trait mapped to this LG. A second QTL for seed predation at day 6 was detected on LG 3. This QTL co-localized (overlapping 1-LOD support limits) with a QTL for percent oil content, which is consistent with the positive relationship between these traits. Despite the observed relationships between predation measures and seed mass (day 4 only), fibre content and available energy, no QTL for these seed characters mapped to the same genomic regions as seed predation QTL.

In all, 24 QTL were detected affecting nine seed characters. We mapped one to five QTL for each trait and individual QTL explained 5.7–22.3% of the phenotypic variance for a trait. Both positive- and negative-effect QTL were detected for luminosity, percent ash content

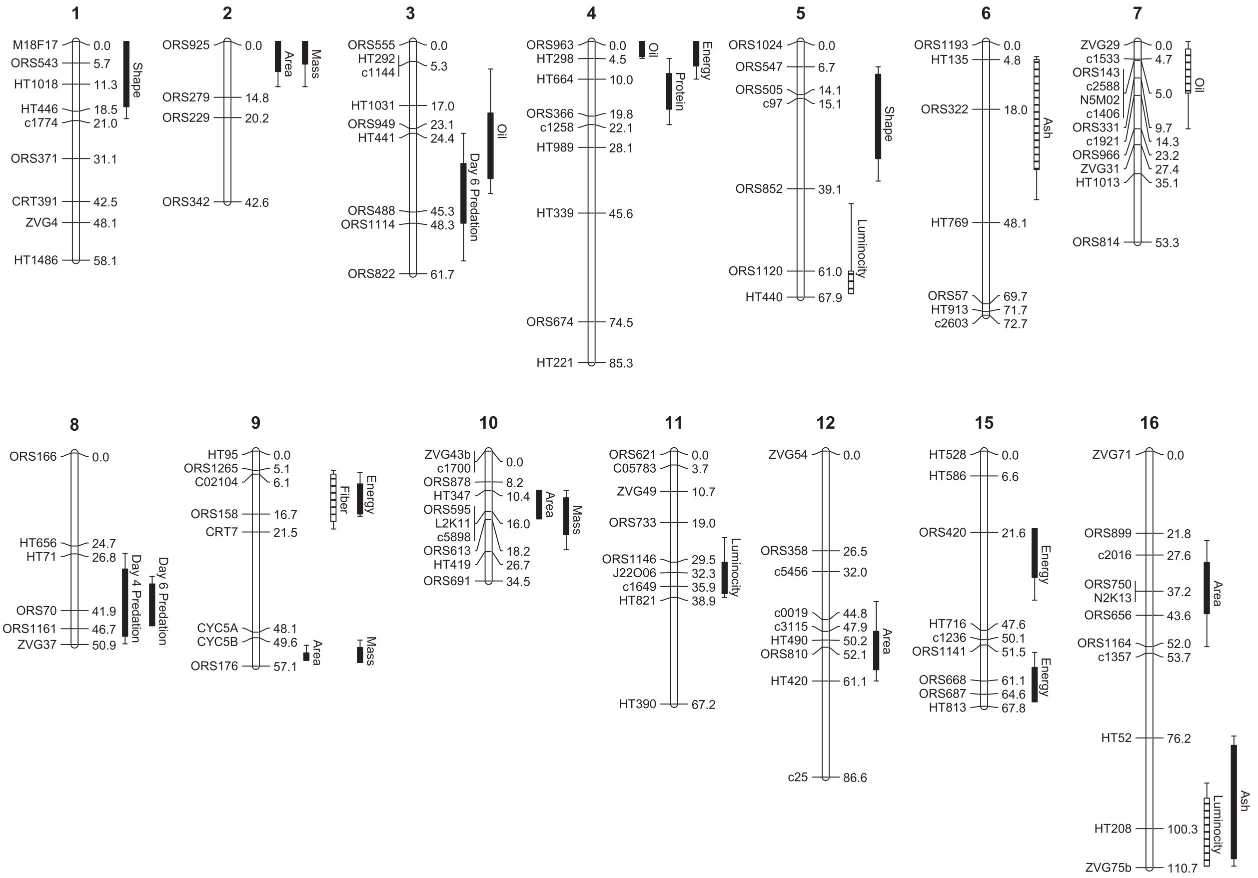

Fig. 2 Quantitative trait locus (QTL) mapping results. QTL for seed predation and seed characters were mapped in RILs of sunflower crop-wild hybrids using composite interval mapping (CIM) in QTL-Cartographer. Bars and tails indicate 1-LOD and 2-LOD support limits, respectively. Additive effects of the cultivar allele (cmsHA89) are displayed using solid (positive) and open (negative) fill.

and percent oil content. The cultivar allele increased the trait value for all remaining QTL, except the one QTL detected for fibre content. No QTL were detected for carbohydrate content. QTL results were generally consistent with correlations among seed characters (Tables 2 and 3; Fig. 2). Positive effect QTL for energy and oil content co-localized at the top of LG 4 (Fig. 2), which is consistent with the positive correlation between these traits. The negative relationship between fibre and energy was supported by co-localization between a negative effect QTL for fibre and positive effect QTL for energy on LG 9. QTL of opposite effects for luminosity and ash content co-localized on the bottom of LG 16, which was consistent with the negative relationship between these traits. QTL for seed area and seed mass co-localized at three positions, on LGs 2, 9 and 10.

Eight of thirteen QTL for seed size characters or oil content co-localized with (six QTL) or mapped to proximal regions of the same LG (two QTL) as QTL for comparable traits in prior studies using lines derived from the same parental cross (Burke *et al.* 2002b, 2005; Baack *et al.* 2008). All three of the seed mass QTL detected in this study were concordant with prior studies (Burke *et al.* 2002b; Baack *et al.* 2008). Although QTL for seed length and width were not directly mapped in this study, two QTL for seed area and one QTL for seed shape co-localized with previously detected seed length QTL (Burke *et al.* 2002b; Baack *et al.* 2008). A third QTL for area mapped to the same LG as a QTL for seed width in a prior study (Burke *et al.* 2002b). One QTL for percent oil content was on the same linkage group as a QTL for the same trait detected in the F_3 generation of these RILs (Burke *et al.* 2005). Additive effects were always in the same direction for similar QTL detected in multiple studies.

Discussion

Phenotypic variation in seed predation among parental lines and hybrids

Patterns of seed predation suggested that granivores initially preferred wild seeds over hybrid or cultivar seeds, but this relationship deteriorated over the duration of the experiment. Granivores may have initially detected the wild seeds, because these seeds were the most similar to the wild sunflower seeds that naturally occur in the study area. After locating the seed patches, granivores may have fed more indiscriminately or slightly preferred cultivar or hybrid seeds, resulting in similar predation estimates among seed types at the termination of the experiment. Unlike a previous study also in sunflower crop-wild hybrids (Alexander *et al.* 2001), we did not detect a clear granivore preference for hybrid seeds at any time point. There are several possible explanations for this inconsistency between studies. First, the two studies were conducted over different time periods (6 vs. 10 days) (Alexander *et al.* 2001). If granivores preferentially fed on hybrid or cultivar seeds after 6 days it would not have been detected in our study. Consistent with this observation, Alexander *et al.* (2001) observed 60–70% seed predation of hybrid seeds after 10 days, whereas seed predation only reached ~50% at the termination of our study, although some lines had been nearly completely consumed at this time. On the other hand, Alexander *et al.* (2001) did not document seed predation before day 10 and consequently, differences in initial seed preferences would not have been detected in that study.

Another possibility is that the inconsistencies between the studies resulted from temporal shifts in seed predation; our study was conducted at a different time of the year than in Alexander *et al.* (2001) (fall vs. spring). For example, in the Midwestern United States, rodents remove the most seeds from the late fall to early spring, whereas invertebrates cause the most damage in the late spring to fall (Whelan *et al.* 1991; Harrison *et al.* 2003). Inconsistencies may have also resulted from genetic or experimental differences between our study and that of Alexander *et al.* (2001). For example, different cultivar lines were used in each study and there may be genetic variation for susceptibility to seed predators across cultivars. Finally, sample sizes of the parental lines were small compared to the RILs and consequently, seed predation never differed significantly among seed types. A comparison of both studies suggests that seed predation is likely to vary temporally, environmentally and across hybrid genotypes. Therefore, long-term studies of multiple hybrid genotypes over different environments will be necessary in order to better predict the effects of seed predation on seedling recruitment in crop-wild hybrid populations.

Genetic analysis within the hybrids (RILs)

Seed predation. Within hybrid lines, granivores generally preferred seeds that were higher in oil content and available energy, as well as lower in fibre. Several previous studies have also detected a positive relationship between seed predation and energy content both within (Lewis *et al.* 2001) and among species (Jensen 1985; 1988; Henderson 1990; Kerley & Erasmus 1991), although seeds with a low fat content are favoured by some granivores for digestibility (Kelrick *et al.* 1986). These results are consistent with optimal foraging theory, which posits that prey choice should maximize net energy intake (Pulliam 1974; Charnov 1976). One caveat is that we performed the nutritional analyses on the entire achene (hull and kernel) but most granivores likely consume only the kernel (Briones & Sanchez-Cordero 1999). This may be important if seed characters differ greatly between the consumed and uneaten portions of the seed. For example, the relationship between carbohydrates and seed predation in bitterbrush seeds (*Purshia tridentata*) is significant only when the effect of the seed hull is included, because the majority of carbohydrates are contained in that part of the seed (Jenkins 1988). In our study, the correlation between fibre and seed predation may be attributable to the fact that sunflower seeds that contain a higher % of oil typically have tighter, thinner and therefore, less fibrous shells (Salunkhe *et al.* 1992). Consistent with this observation, oil content and fibre were strongly negatively correlated in the RILs and QTL of opposite effect were detected for these traits at the top of LG 9. The observed relationships between seed predation and oil content or energy are unlikely to be affected by our measurement of the entire seed, because the majority of oil is contained in the kernel (Fedeli *et al.* 1972; Salunkhe *et al.* 1992).

Fibre, oil content and available energy were all highly correlated, making it challenging to determine which of these traits directly or indirectly attract granivores. For example, in one study, mice generally chose seeds with a high dietary value (an energy measure), but several species were also preferred that had a lower than average dietary value but were high in lipid content, suggesting a preference for high lipid seeds independent of overall energy levels (Briones-Salas *et al.* 2006). In our study, only the relationship between seed predation and oil content was supported by the QTL analysis, in that negative effect QTL for these traits co-localized on LG 3. A large effect QTL (PVE = 23%) for oil content has been previously detected in this region of LG 3 in sunflower cultivars, although no candidate genes have been identified (Ebrahimi *et al.* 2008). Rodents find seeds by olfaction (Howard *et al.* 1968; Vander Wall 1998), which may explain how they are able to detect differences in oil content among seeds. These results provide the first evidence of a molecular genetic mechanism for the observed relationships between granivore preference and seed characters and suggest that vulnerability to seed predation may be associated with genes regulating oil content in sunflower. It is, however, important to recognize that QTL studies can only detect variation between the two parental individuals and more work should be done to better characterize the relationship between predation and seed characters at the molecular genetic level.

In addition to the QTL on LG 3, we detected a large effect QTL for seed predation at days 4 and 6 on LG 8; no other QTL were detected on this LG. The three previous studies to have mapped QTL in lines derived from the same parental cross as in this study found QTL for seed width, flowering date (Burke *et al.* 2002b; Dechaine *et al.* 2009) and plant height (Baack *et al.* 2008) in the same region of LG 8 as the seed predation QTL. A study of a cross between ANN1238 and the primitive sunflower domesticate, Hopi, detected QTL for seed mass and area in the this region of LG 8 and the wild allele led to a decrease in seed size (Wills 2008). The seed size QTL were of relatively small effect (<10%) in previous studies (Burke *et al.* 2002b; Wills 2008) and may have been beyond the power of our study to detect. In all cases, the cultivar allele led to an increase in seed predation, suggesting that granivore preference for cultivar-like seeds may mitigate the escape of cultivar alleles into wild populations.

We also detected limited evidence for a positive relationship between seed predation and seed mass, although this relationship was only observed using stepwise regression and then solely at day 4. Alexander *et al.* (2001) found no relationship between seed predation and seed size within sunflower crop-wild hybrids, suggesting that only the smallest wild seeds escaped predation and that all hybrid seeds were large enough to attract seed predators. These results suggest that seed size may be a less important factor in granivore preference than oil content or available energy within sunflower crop-wild hybrids. We also may have failed to detect stronger correlations between seed size and seed predation because preferences differ among types of granivores. For example, body size influences seed preference even within rodent taxa, in that smaller rodents select smaller seeds and vice versa (Munoz & Bonal 2008). Invertebrates may also consume seeds, although at least one study suggests that arthropods have little impact on *H. annuus* seed predation compared to rodent predation (Briones-Salas *et al.* 2006). It is also possible that we found little variation in predation on hybrid sunflower seeds, because hybrid seeds are generally palatable to granivores. In a study of 12 species, *H. annuus* seeds were the most energy-rich, contained the least secondary compounds and were the most palatable to spiny-pocket mice (Briones & Sanchez-Cordero 1999).

Seed characters. Correlations among seed morphological and nutritional traits were generally consistent with the QTL analysis. For example, the strong positive correlation between oil content and energy was supported by positive effect QTL for these traits on the top of LG 4. Oil content QTL have been previously detected in this region (Burke *et al.* 2005). It is surprising that more QTL for energy did not co-localize with oil content QTL, as the majority of available energy in sunflower seeds comes from oil (Salunkhe *et al.* 1992). This may be the result of a lack of power in our study compared to previous work that detected QTL for oil content or fatty acid composition in all genomic regions in which we detected energy QTL (Ebrahimi *et al.* 2008). These results suggest that energy and oil content are tightly linked at the phenotypic and genetic levels in sunflower seeds; consequently, the effects of oil content and energy on granivore preference cannot be separated.

Several QTL for seed size or oil content have been detected in previous studies of sunflower. For example, QTL for seed mass and area co-localized to the region of LG 10 that contains the *B* (branching) locus, which has also been shown to affect seed size and oil traits in previous studies (Bert *et al.* 2003; Tang *et al.* 2006; Wills 2008). The genomic regions flanking *B* have been suggested to contain pleiotropic or multiple linked QTL with effects on several traits that were selected during sunflower domestication, including reduced branching, earlier flowering, larger seeds and a higher oil content (Mestries *et al.* 1998; Bert *et al.* 2003; Tang *et al.* 2006). A few QTL were detected on the same LG as in previous studies but at different positions (Burke *et al.* 2002b, 2005; Baack *et al.* 2008). QTL expression for oil traits has been shown to be sensitive to experimental conditions (Ebrahimi *et al.* 2008). Our study was conducted in a different location than previous work and used a different set of markers to create the linkage map; both of these factors may contribute to inconsistencies in QTL results among studies.

Conclusions

In this study, we examined the ecological patterns and genetic basis of granivore preference on wild, cultivar and crop-wild hybrid sunflower seeds. We found little evidence for increased predation for hybrid seeds; if anything, predation was more severe for wild seeds, especially at early time points. On the other hand, removal of hybrid and cultivar seeds increased at later time points and may have surpassed predation on wild seeds if the experiment had been continued, as has been previously reported (Alexander *et al.* 2001). Within hybrid lines, we detected a granivore preference for high oil/high energy seeds. At the genetic level, we detected three QTL for which the cultivar allele led to an increase in seed predation within the hybrids. One of these QTL co-localized with a QTL for oil content, providing a genetic basis for the observed positive relationship between this trait and seed predation. Overall, these results suggest that granivores may prefer seeds they recognize, such as wild seeds, but preferentially remove unknown seeds with high oil or energy content. Therefore, granivores would be expected to reduce the number of crop-like individuals in hybrid populations and consequently, mitigate the spread of cultivar alleles in the wild.

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Supporting information

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

Table S1 Means and SD of seed predation and seed characters in the wild, crop and hybrid recombinant inbred lines (RILs)