

Beyond pollinators: evolution of floral architecture with environment across the wild sunflowers (*Helianthus*, Asteraceae)

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Background – There is a growing appreciation that the evolution of floral traits is shaped by selection not only from pollinators, but also factors like abiotic stress, florivory, and disease. Many such pressures impose counter-selection for smaller, less attractive flowers. In self-incompatible taxa, floral investment in attraction is required to ensure cross-pollination, setting up a potential energetic trade-off with investment in fecundity through pollen and seeds.

Methods – This study assesses ecological and evolutionary causes of variation in floral morphology across the diverse genus *Helianthus* (Asteraceae), focusing on floral size, colour, water content, and relative investment in attractive but sterile ray florets versus non-showy but fertile disc florets.

Key results – All floral traits were found to be highly evolutionarily labile, and the trade-off in relative investment between ray and disc florets was found to evolve independently of floral size. Both floral size and disc water content were strongly correlated with source site climate and soil characteristics, with larger heads and higher water content repeatedly evolving in more fertile and drier habitats consistent with aspects of the resource-cost and enemy-escape hypotheses of floral trait evolution, respectively. The evolution of disc colour and relative ray-disc investment was not explained by life history, flowering period, or source site environmental characteristics, suggesting that the evolution of these traits may instead be driven by other selective pressures, including perhaps pollinators.

Conclusions – Together the results of this study suggest that the macroevolution of sunflower floral architecture is likely driven by selective pressures from multiple biotic and abiotic factors, with habitat environmental conditions influencing some but not all aspects of floral morphology.

Key words – Climate, colour, disc, environment, flower, *Helianthus*, morphology, ray, sunflower, soil fertility.

INTRODUCTION

The flower is a key evolutionary innovation, one that has led directly to the numerical dominance of angiosperm species among land plants (Regal 1977, Specht & Bartlett 2009). The staggering floral diversity exhibited by angiosperms has long been of interest to botanists and evolutionary biologists, and long attributed to selection by and coevolution with animal pollinators (Sprengel 1793, Darwin 1862, Knuth & Müller 1908, Stebbins 1970). Despite its central role in angiosperm reproduction, pollination is not the only process that shapes floral trait evolution. Many other aspects of the biotic and abiotic environment have been demonstrated to drive variation in flower size and shape, as have whole-plant life history and phenology (Bazzaz et al. 1987, Galen 1999, Teixido et al. 2016). In many cases such forces impose contrasting se-

lective pressures to the typical selection for larger, showier flowers that often arises through competition for pollinator attraction (Bell 1985, Anton et al. 2013). The production of flowers incurs direct energetic costs in the form of carbon, water, and nutrient resources used for construction, as well as ongoing costs for maintenance of what are typically non-photosynthetic structures. Accordingly, the ‘resource-cost hypothesis’ suggests that smaller, less conspicuous flowers should be favoured in low-resource habitats by virtue of reducing physiological stress from water and nutrient limitation (Galen 1999, Teixido et al. 2016). Indeed, in many systems smaller-flowered species and populations occur in hotter, drier, less fertile environments than those with larger flowers (e.g. Galen 2000, Herrera 2005, Teixido & Valladares 2013). Along with selection imposed by the abiotic environment, interactions with natural enemies such as flo-

rivores and pathogens may also shape floral trait evolution. Traits that make flowers more visible and attractive to pollinators have been shown to have the same effect on florivores in multiple systems (Brody 1992, Galen 1999, Oguro & Sakai 2015), and increased visitation by both beneficial and malevolent floral visitors may increase the risk of vectoring plant pathogens (McArt et al. 2014). In line with this, the ‘enemy-escape hypothesis’ posits that selection for larger, more attractive flowers by pollinators should be counteracted by selection against such traits by florivores and pathogens in environments where either are abundant (Galen 1999, Teixido et al. 2016). In addition to environmental factors, plant life history and phenology influence reproductive traits, as annual species typically have far higher reproductive allocation than their perennial congeners and short flowering periods may place developmental constraints on floral size and shape (Bazzaz et al. 1987, Galen 1999). Together, floral traits likely evolve under selection from combinations of many non-pollinator biotic and abiotic pressures. This may contribute to the high floral diversity observed within many groups, especially those that lack strong pollinator differentiation or specialist mutualisms and for which the causes of widespread floral diversity are therefore not obvious.

The Asteraceae is one of the two largest families of flowering plants, with approximately 23 000 species (Barkley et al. 2006). One of the uniting features of this family is its characteristic composite head structure, called a capitulum, where up to hundreds of individual florets are consolidated in inflorescences that mimic the structure of single flowers (Barkley et al. 2006). In the radiate heads characteristic of much of the family, two types of florets are present – zygomorphic ray florets and actinomorphic disc florets (Gillies et al. 2002, Barkley et al. 2006). Each ray floret has a fused corolla that functions as a single petal, and each disc floret contains anthers, a stigma, and an ovule capable of producing a single seed (Gillies et al. 2002, Barkley et al. 2006). In many genera of Asteraceae (such as *Helianthus*) the ray florets are completely sterile, and serve only to increase attractiveness to pollinators (Gillies et al. 2002, Schilling 2006). As such, increased energetic investment in ray florets may increase attractiveness to pollinators but does not increase maximum potential pollen and seed output like investment in disc florets. This sets up the potential for an evolutionary trade-off between investment in attractive ray florets to ensure successful pollination and the maximization of pollen and seed production through investment in disc florets. Ray florets have been found to be both vital for pollinator attraction and energetically expensive to the detriment of fecundity – separate experimental manipulations of *Achillea ptarmica* show that ray removal heavily reduces pollinator visitation but also significantly improves fruit set (Andersson 1991, 1999). This trade-off between attraction and fecundity is likely to be especially strong among self-incompatible species, given the obligate need for cross-pollination for disc-based fecundity to be realized.

Helianthus L. is an extremely diverse genus of Asteraceae, with both annuals and perennials occupying a wide range of habitats, including forests, deserts, wetlands, prairies, beach dunes, and rock outcrops (Heiser et al. 1969, Mason & Donovan 2015). Of the approximately 50 members of

the genus (Schilling 2006, Timme et al. 2007), all but one wild species are completely sporophytically self-incompatible (the annual *H. agrestis* Pollard), and thus almost all wild sunflowers rely exclusively on cross-pollination in order to reproduce, a service provided primarily by generalist bees (Heiser et al. 1969). With respect to floral diversity, composite heads (hereafter ‘flowers’ for brevity) vary dramatically in size, with wide variation in morphology and colour (fig. 1). In particular, some species of *Helianthus* appear to invest relatively more floral biomass in pollinator attraction (e.g. *H. porteri* (A.Gray) Pruski, *H. niveus* subsp. *tephrodes* (A.Gray) Heiser), as evidenced by the production of larger and more numerous showy (but sterile) petal-bearing ray florets, which are produced at the expense of allocation to fertile disc florets. This contrasts with other species that appear to invest relatively more in the production of pollen and seeds (e.g. *H. radula* (Pursh) Torr. & A.Gray, *H. debilis* subsp. *tardiflorus* Heiser), as evidenced by fewer and smaller (or absent) petals along with larger and more numerous fertile disc florets. This genus thus serves as an ideal system in which to examine the role of environmental variation, plant life history, and flowering phenology in the evolution of floral trait variation. Here we assess the power of the resource-cost and enemy-escape hypotheses to explain the evolution of floral size and disc-ray investment in light of habitat, climate, and soil fertility.

MATERIALS AND METHODS

Study system

To assess floral trait evolution across the wild sunflowers, 27 wild diploid non-hybrid *Helianthus* species were selected for study, as well as the sole diploid member of the sister genus *Phoebanthus* S.F.Blake to serve as an outgroup. Diploid non-hybrids were selected in order to employ phylogenetically explicit analyses with the most recent phylogeny of *Helianthus* (Stephens et al. 2015). The species selected represent four-fifths of the diploid non-hybrid members of the genus, and a majority of the approximately 50 *Helianthus* species (Heiser et al. 1969, Timme et al. 2007). Multiple populations (two to four) were included from across the geographic range of each species in order to capture natural intraspecific variation and improve the assessment of macroevolutionary patterns. Seed from each population was collected in the wild or obtained through the USDA Germplasm Resources Information Network (electronic appendix 1). This system has been used to assess the evolution of leaf economics traits (Mason & Donovan 2015), root physiology (Bowsher et al. 2016), as well as leaf defences and secondary metabolites (Mason et al. 2016).

Plant growth

A common garden approach was used to minimize environmentally-induced trait variation, with plants grown under uniform high-resource greenhouse conditions. To accommodate the study of so many populations, the 28 species were split into two common gardens grown in the summers of 2012 and 2013 (CG-1 and CG-2, respectively) at the University of Georgia Plant Biology greenhouses. To minimize

differences between the two common gardens, experimental timing was identical between years and environmental conditions were kept as similar as possible (pots, soil mixture, fertilizer, irrigation regime, greenhouse temperature controls, etc.). To evaluate and correct for any other potential uncontrollable differences in conditions between the two common gardens, all populations of three species were repeated in both years to serve as phytometers. These three species (*H. annuus* L., *H. radula*, and *H. silphioides* Nutt.) reflect

major aspects of variation in life history, growth form, and overall morphology across the genus.

Given the utility of their size and scope, these common gardens were also used for the study of several other classes of physiological traits, including leaf economics (Mason & Donovan 2015) and leaf defences and secondary metabolites (Mason et al. 2016), and experimental conditions are described in detail in Mason & Donovan (2015). In brief, seeds were germinated in petri dishes and transplanted into



Figure 1 – A selection of floral diversity across the genus *Helianthus*. Note the variation in disc colour, ray number and shape, and overall size (though images are not to scale). Top row: *H. annuus* L., *H. petiolaris* Nutt. subsp. *petiolaris*, and *H. niveus* subsp. *tephrodes* (A.Gray) Heiser. Middle row: *H. debilis* subsp. *tardiflorus* Heiser, *H. mollis* Lam., and *H. giganteus* L. Bottom row: *H. porteri* (A.Gray) Pruski, *H. radula* (Pursh) Torr. & A.Gray, and the outgroup *Phoebanthus tenuifolius* S.F.Blake (photographs: Chase Mason).

6-liter pots with a 3:1 mixture of sand and calcined clay in late May. To account for spatial variation in the greenhouse, the eight replicate plants per population were arranged in a randomized complete block design. To ensure high water availability, pots received daily drip irrigation to field capacity. To ensure high nutrient availability, 20 g of nine-month slow-release fertilizer with micronutrients (Osmocote Plus 15-9-12, Scotts, Marysville, OH, USA) was mixed into the soil, and pots received an initial liquid fertilization with supplemental Ca, Fe, and Mg to promote seedling establishment. Plants received ambient photoperiod throughout the growing season, ranging from a maximum daylength of 14.4 hours in mid-June, to a minimum of just under 10 hours in December. Greenhouse temperatures were set to 27 °C during the day and 18 °C at night (though summer high temperatures varied somewhat above the daytime set point due to limitations of the evaporative cooling system).

Floral trait assessment

Floral trait data collection was standardized by ontogenetic stage. Plants were checked three times per week for the onset of flowering (anthesis), and two of the first few flowers to

open were harvested on the day they fully opened for the assessment of floral traits. The morphological traits of disc diameter, ray length, ray width, and ray number were recorded (fig. 2), at which point both rays and disc florets were separated from the receptacle. The fresh mass of each of these three floral parts was recorded, at which point all tissue was dried at 60 °C for at least three days and weighed for dry mass.

Trait values for the two flowers assessed on each plant were averaged to generate trait values for each individual replicate plant (electronic appendix 1). Not every plant flowered, or was able to be sampled successfully on the day flowers opened. This resulted in a mean of 5.93 ± 2.01 (s.d.) plants sampled per population. To test for any consistent differences between the two common gardens, trait values obtained for all replicates of the three phytometer species were assessed by ANOVA (including year, population, year \times population, and block effects). No traits were found to be significantly different between years in more than one species, so no corrections were performed between years. For each assessed trait, population least-squares means were calculated using ANOVA in order to account for the randomized complete block design (electronic appendix 1). For the nine

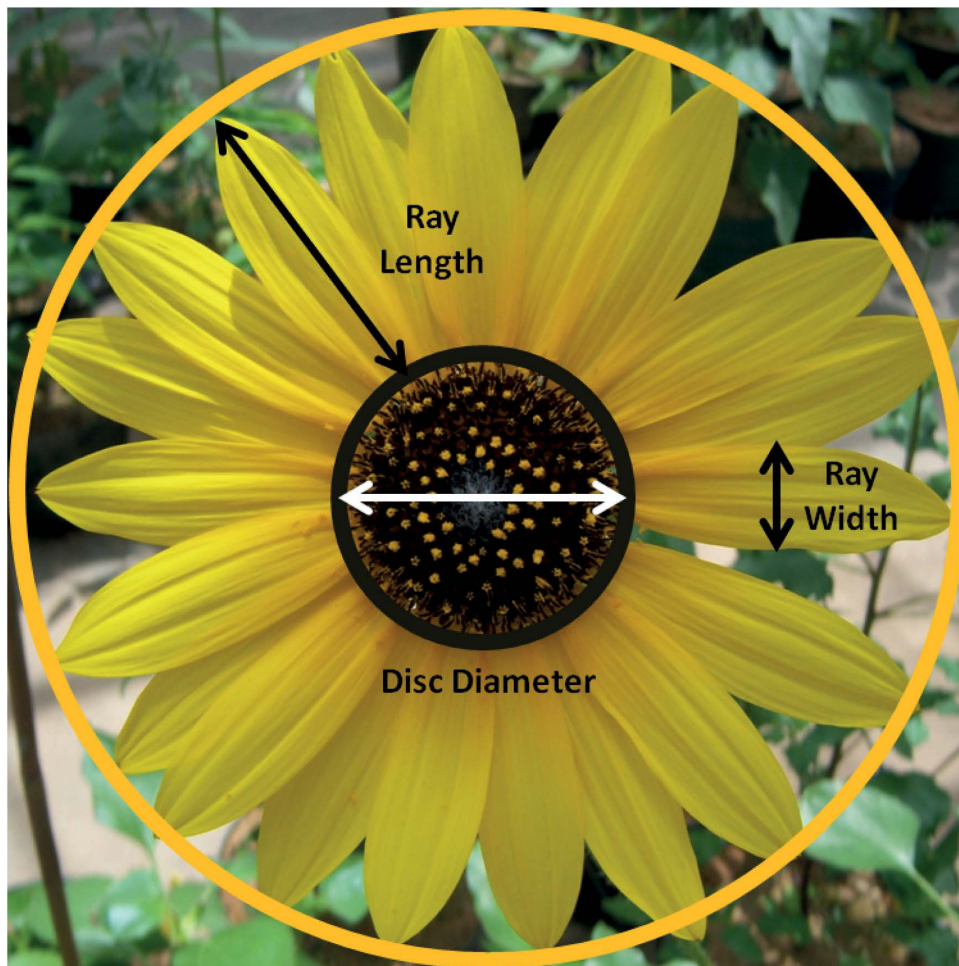


Figure 2 – Diagram of assessed floral morphological traits. Disc area (inner circle) was calculated from the disc diameter, while total flower area (outer circle) was calculated from the disc diameter and ray length. Ray area was calculated as the difference between the total flower area and disc area.

populations serving as phytometers, population least-squares means for CG-1 and CG-2 were averaged.

From population least-squares means of the ten assessed traits, a variety of additional traits were calculated. Floral fresh and dry masses were used to calculate total flower fresh mass, total flower dry mass, ray water content, disc water content, receptacle water content, total flower water content, ray dry mass fraction, disc dry mass fraction, receptacle dry mass fraction, and dry mass investment ratio (DMIR) as the ratio of ray dry mass to disc dry mass. Floral morphological traits were used to calculate flower diameter, disc and flower circumference, disc area, ray area, total flower area, ray density, ray area fraction, disc area fraction, and area investment ratio (AIR) as the ratio of ray area to disc area. This full dataset of population least-squares means for all traits is presented in electronic appendix 1. Given that many of these traits are mathematically linked and convey different aspects of the same phenotypic variation, we focus on nine of these continuous floral traits for subsequent phylogenetic analyses: total flower dry mass, total flower area, disc water content, ray water content, ray length, ray width, ray number, DMIR, and AIR.

Two categorical traits were assessed at the population or species level. First, disc colour was scored as either dark or light based on the presence or absence of abundant anthocyanin pigments in the disc florets. This trait was always invariant within populations, and invariant within species with the single exception of *H. floridanus* A.Gray ex Chapm. (likely the result of introgression with the sister species *H. angustifolius* L.; Heiser et al. 1969). Second, data on flowering period in the wild was obtained for each species from the literature (Heiser et al. 1969, Rogers et al. 1982, Ellis et al. 2006) as well as personal field observations and regional herbarium records.

Environmental data collection

For each population site, climate and soil characteristics were obtained as reported previously in Mason & Donovan (2015). In short, climate data was obtained using the WorldClim database (Hijmans et al. 2005) and the CGIAR Global Aridity and PET database (Zomer et al. 2008), yielding altitude, mean annual temperature (MAT), mean diurnal temperature range, temperature seasonality, mean annual precipitation (MAP), precipitation seasonality, potential evapotranspiration (PET), and aridity index (the ratio of MAP to PET, indicating temperature-adjusted water availability). At each site, five soil cores were taken to a depth of ~20 cm at locations spread evenly throughout the population within the root zone of clumps of plants, dried at 60 °C and homogenized prior to analysis of soil characteristics. Soil nitrogen content, carbon content, and C:N ratio were assessed simultaneously with Micro-Dumas combustion (NA1500, Carlo Erba Strumentazione, Milan, Italy) at the University of Georgia Analytical Chemistry Laboratory. Soil was also submitted to A&L Eastern Laboratories (Richmond, VA, USA) for standard bulk soil analysis, yielding organic matter content, cation exchange capacity (CEC), pH, available phosphorus, and exchangeable potassium, calcium, and magnesium. All soil

metrics were averaged across the five soil cores and resulting population site means were used for subsequent analyses.

Phylogenetic comparative analyses

To assess trait evolution and phylogenetic signal we used the most recent and well-resolved phylogeny of the diploid backbone of the genus *Helianthus*, based on 170 nuclear genes (Stephens et al. 2015). Non-sampled taxa were pruned from the phylogeny, and residual phylogenetic signal was jointly estimated for the dataset of nine continuous floral traits using Pagel's λ (Pagel 1999) in the R package *Rphylopars* (Goolsby et al. 2017). Phylogenetic signal was significant ($\lambda = 1.0$, $p < 0.0001$, $df = 1$), demonstrating the need for analyses that explicitly include phylogeny (Revell 2010). Phylogenetic mixed models on population means were used to assess macroevolutionary correlations among continuous floral traits and between floral traits and environmental characteristics (Housworth et al. 2004, Felsenstein 2008). These were implemented using the *varCompPhyloip* function in the R package *ape* (Paradis et al. 2004) to call the contrast program in Phyloip ver. 3.68 (Felsenstein 2004).

Species means for each trait were then calculated from population means and used for maximum likelihood ancestral state reconstruction using the *fastAnc* function in the R package *phytools* (Revell 2012). The categorical trait of disc colour was reconstructed using stochastic character mapping (Bollback 2006, Huelsenbeck et al. 2003) using 1000 simulations with the *make.simmap* function in *phytools* (Revell 2012). *Helianthus floridanus* was coded as 2/3 light and 1/3 dark given the difference in disc colour among populations. Species means were also used in phylogenetic ANOVA (Garland et al. 1993) to test for associations between continuous floral traits and life history, continuous floral traits and disc colour, as well as associations between disc colour and environmental characteristics. These were implemented using the *phylANOVA* function in the R package *phytools* (Revell 2012). Additionally, species means were used to assess macroevolutionary correlations between floral traits and the species-level trait of flowering period using phylogenetically independent contrasts, implemented with the *pic* function in the R package *ape* (Paradis et al. 2004).

RESULTS

Rapid independent evolution of flower size and allocation

Most continuous floral traits appear to be highly evolutionarily labile, evolving phenotypic extremes independently many times across the genus (fig. 3). Such lability may perhaps be a consequence of the modular nature of the composite head (Gillies et al. 2002). With respect to overall flower size, total flower dry mass and total flower area are of course strongly positively correlated (table 1). Larger flowers have longer, wider, and more numerous rays, as well as higher disc water content (table 1). DMIR and AIR are strongly positively correlated, indicating that these two metrics capture mass and area measures of the same phenotypic variation in allocation between rays and discs. Interestingly, DMIR is uncorrelated with total flower dry mass, and AIR is uncorrelated with total

Table 1 – Macroevolutionary correlations among floral traits as assessed by phylogenetic mixed model.

R² and directionality of significant correlations (p < 0.05) are presented. Abbreviations: WC, water content; DMIR, dry mass investment ratio; AIR, area investment ratio.

	Flower Area	Disc WC	Ray WC	Ray Length	Ray Width	Ray Number	DMIR	AIR
Dry Mass	(+)0.65	–	–	(+)0.19	(+)0.18	–	–	(–)0.23
Flower Area		(+)0.21	–	(+)0.76	(+)0.48	(+)0.46	–	–
Disc WC			(+)0.80	(+)0.16	–	–	(–)0.26	–
Ray WC				–	–	(+)0.32	–	–
Ray Length					(+)0.55	(+)0.61	(+)0.22	–
Ray Width						(+)0.24	(+)0.29	–
Ray Number							–	–
DMIR								(+)0.45

flower area (table 1). This indicates the allocation between rays and discs is largely independent of flower size across species, as can be seen from a trait-space perspective (fig. 4; electronic appendix 2). DMIR is also negatively correlated with disc water content, indicating that species with higher relative mass investment in discs also have higher disc water content. Light disc colour was found to be the most likely ancestral state, with multiple transitions to dark pigmentation and several reversions (fig. 3). Disc colour was not found to be significantly associated with any of the nine continuous floral traits, and thus appears to evolve independently of floral architecture.

Evolution of floral traits with life history and flowering period

Unexpectedly, there are virtually no significant associations between floral traits and life history or flowering period. The one exception is ray width, which is significantly larger in annuals relative to perennials (F = 13.34, p = 0.037), with a magnitude of about 0.36 cm. Overall, these results suggest that flower size and morphology evolve largely independently of species’ life history and the length of time during which flowers are deployed.

Evolution of floral traits across climate and soil gradients

Unlike life history or flowering period, metrics of climate and soil characteristics are evolutionarily correlated with multiple floral traits (table 2). Metrics related to water availability (MAP, precipitation seasonality, aridity index) are strongly correlated with many aspects of total flower size, and larger flowers have evolved repeatedly in drier and more seasonal environments (table 2). Similarly, many metrics of soil fertility are strongly correlated with descriptors of flower size, with larger flowers evolving repeatedly in environments with higher cation exchange capacity and higher levels of mineral nutrients like phosphorus, potassium, magnesium, and calcium (table 2). Interestingly, other metrics of soil fertility, such as organic matter, carbon content, nitrogen content, and C:N ratio are uncorrelated with descriptors of flower size (table 2).

Disc water content mirrors the patterns seen for flower size, with higher disc water content in drier environments and those with higher soil fertility as defined by mineral nutrients (table 2). Disc water content is also positively correlated with altitude (table 2). Ray water content is positively correlated with soil fertility as predicted by calcium and cation exchange capacity, as well as soil carbon content, but is uncorrelated with environmental metrics of water availability (table 2). Additionally, ray water content is the only trait to be correlated with a temperature metric, here negatively with diurnal range (table 2).

In stark contrast to flower size, relative allocation between ray and disc is largely uncorrelated with climate and soil metrics. The only significant relationship is a positive correlation between DMIR and soil organic matter (table 2). Disc colour was not associated with any climate or soil metrics. This suggests that variation in both disc colour and ray-disc investment evolves largely independently of the abiotic environment.

DISCUSSION

Potential drivers of flower size and water content evolution in wild sunflowers

The resource-cost hypothesis predicts that larger and more conspicuous flowers should be selected against in resource-poor environments (Galen 1999). The finding here that smaller flowers have evolved repeatedly across *Helianthus* in response to shifts onto lower fertility soils is consistent with this hypothesis given the higher general nutrient requirements of reproductive structures relative to vegetative biomass (Bazzaz et al. 1987, Galen 1999, Oguro & Sakai 2014). Across *Helianthus*, flower size metrics are most strongly associated with soil phosphorus, a macronutrient which is most abundant in floral structures and tightly related to flower production (Abrahamson & Caswell 1982, Oguro & Sakai 2014), as well as overall soil cation exchange capacity. This response is similar to the plastic response observed in many systems, where increased nutrient availability has been shown to increase flower size (Muñoz et al. 2005, Burkle & Irwin 2009). Conversely, the finding that larger flow-

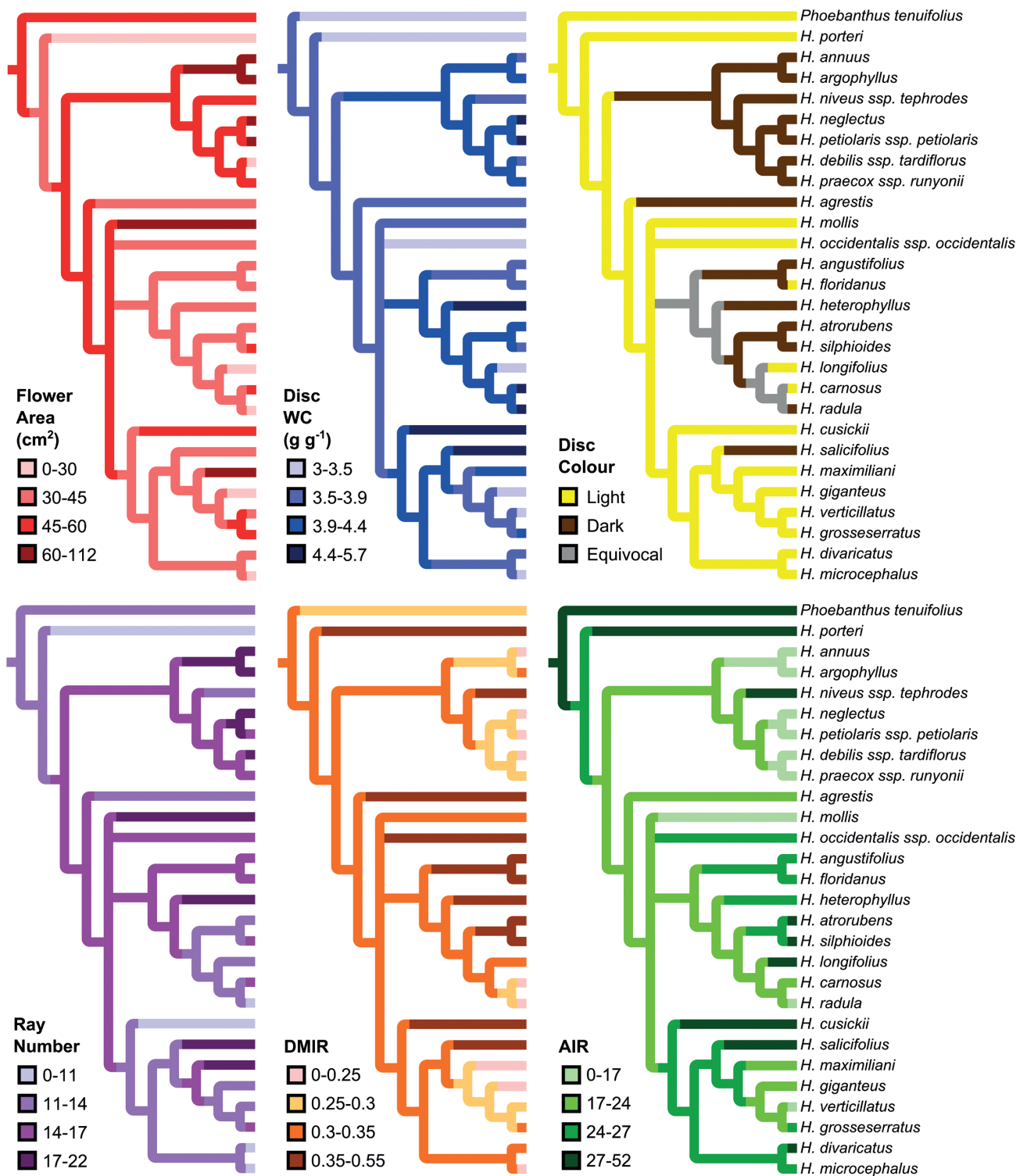


Figure 3 – Ancestral state reconstruction of selected floral traits. For continuous traits – total flower area, total water content, ray number, dry mass investment ratio (DMIR), and area investment ratio (AIR) – reconstructions were performed with maximum likelihood using the *fastAnc* function in the R package *phytools* (Revell 2012). For the categorical trait of disc colour, reconstructions were performed with stochastic character mapping (Bollback 2006, Huelsenbeck et al. 2003) using 1 000 simulations with the *make.simmap* function in *phytools* (Revell 2012). For display, nodes with probabilities > 60 % for a given state are labelled as either light or dark, and other nodes labelled as equivocal. Note that all species are invariant among populations for disc colour with the exception of *H. floridanus* (in which one population had dark discs). See main text for more information.

ers have evolved repeatedly in response to shifts into drier and more seasonal environments is not what would be expected under the resource-cost hypothesis. Floral structures are known to be a significant source of water loss in plants, to the point of reducing plant water status and impacting photosynthetic rate (Whiley et al. 1988, Galen et al. 1999, Teixido & Valladares 2014). From a resource-use perspective it would thus be expected that adaptation to more arid environments would involve a reduction in flower size (Galen 1999, Galen et al. 1999, Teixido et al. 2016). Indeed, the observed pattern in *Helianthus* is also opposite of the plastic response typical of plants exposed to reduced water availability or increased temperatures, which is to reduce overall flower or display size (Lambrecht & Dawson 2007, Vaid et al. 2014). This inconsistency warrants consideration. Teixido et al. (2016) found that large flowered species native to arid environments typically possess other adaptations to mitigate their transpirational costs, for instance opening only noctur-

nally or possessing floral longevities of less than single day. Neither of these explanations holds for *Helianthus*, however, as flowers of all species remain open during the day and typically last for multiple days, with larger flowers actually lasting longer as disc florets open sequentially from the outer to inner whorls.

Two biotic forces may instead explain the evolution of larger flowers in drier environments. First, it is possible that selection by pollinators drives the evolution of flower size. For instance, drier environments may have fewer pollinators, strongly favouring larger display sizes in self-incompatible species. It has been demonstrated that an increase in the display size of artificial inflorescences typically makes flowers more attractive to bumblebees (Ishii et al. 2008), that the removal of display area reduces visitation (Bell 1985), and that display size tends to increase the number of flowers visited per pollinator (Ohashi & Yahara 2001). Additionally, among

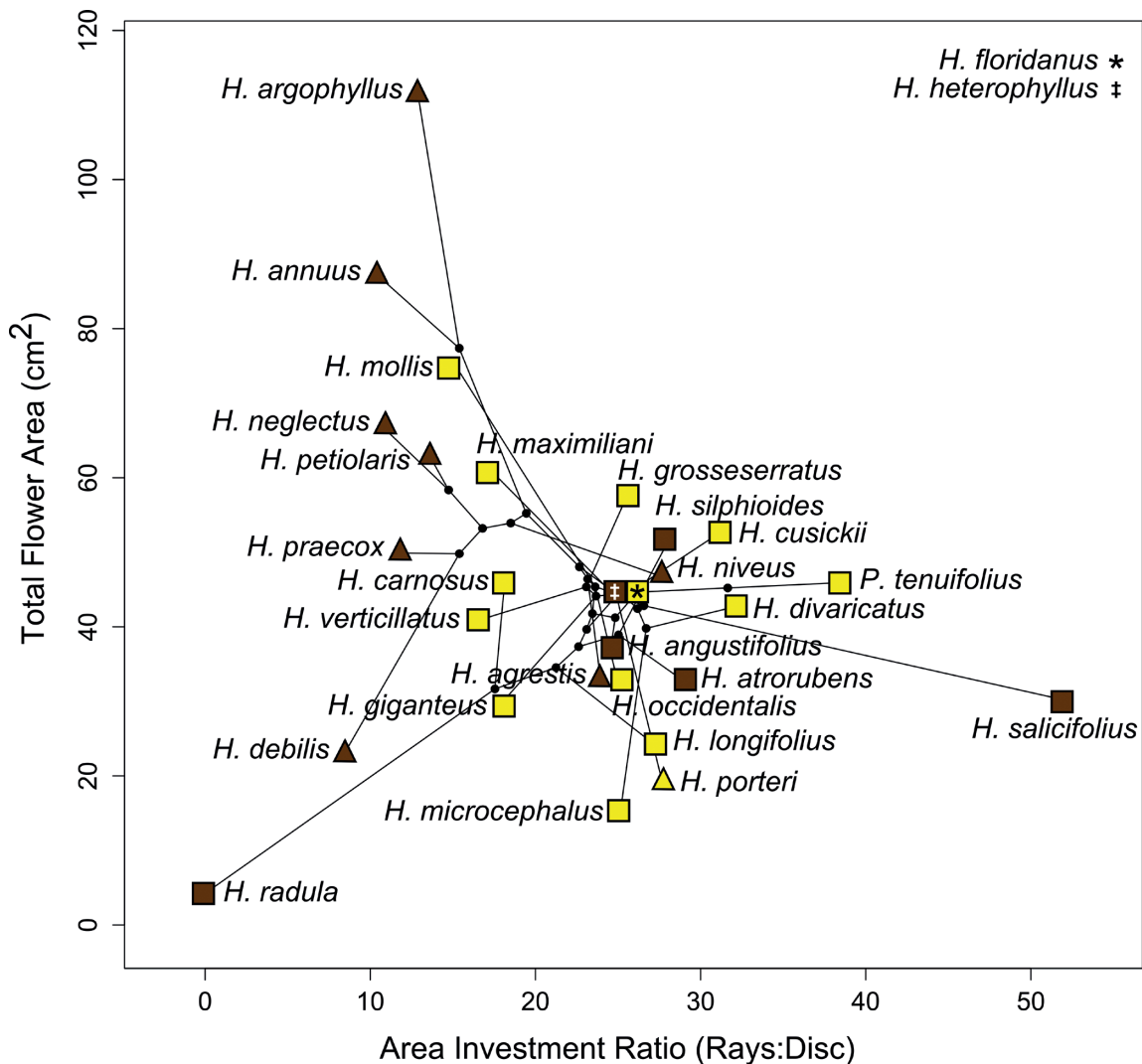


Figure 4 – Diversification of *Helianthus* in floral trait space – total flower area and area investment ratio (AIR), plotted using the *phylo morphospace* function in *phytools* (Revell 2012). Species are coded by disc colour (light or dark) and life history (triangles for annuals, squares for perennials). Species are connected by phylogenetic relationships, with reconstructed ancestral states of internal nodes plotted in trait space. See electronic appendix 2 for the same analysis on a mass basis.

Table 2 – Macroevolutionary correlations between floral traits and environmental characteristics as assessed by phylogenetic mixed model.

R² and directionality of significant correlations ($p < 0.05$) are presented. Abbreviations: WC, water content; DMIR, dry mass investment ratio; AIR, area investment ratio; MAT, mean annual temperature; MAP, mean annual precipitation; PET, potential evapotranspiration; OM, organic matter; CEC, cation exchange capacity.

	Dry Mass	Flower Area	Disc WC	Ray WC	Ray Length	Ray Width	Ray Number	DMIR	AIR
Altitude	–	–	(+)0.41	–	–	–	–	–	–
MAT	–	–	–	–	–	–	–	–	–
Temp. Seasonality	–	–	–	–	–	–	–	–	–
Diurnal Range	–	–	–	(-)0.15	–	–	–	–	–
MAP	(-)0.17	(-)0.24	(-)0.40	–	(-)0.18	(-)0.19	–	–	–
Precip. Seasonality	(+)0.21	(+)0.28	(+)0.32	–	(+)0.16	–	(+)0.22	–	–
Aridity Index	(-)0.18	(-)0.23	(-)0.37	–	(-)0.15	(-)0.14	–	–	–
PET	–	–	–	–	–	–	–	–	–
Soil C	–	–	–	(+)0.20	–	–	–	–	–
Soil N	–	–	–	–	–	–	–	–	–
Soil C:N	–	–	–	–	–	–	–	–	–
Soil OM	–	–	–	–	–	–	–	(+)0.14	–
Soil P	(+)0.31	(+)0.27	–	–	(+)0.17	(+)0.40	–	–	–
Soil K	(+)0.18	–	(+)0.58	–	–	–	–	–	–
Soil Mg	–	–	(+)0.23	–	–	–	–	–	–
Soil Ca	–	(+)0.19	(+)0.34	(+)0.33	–	–	(+)0.29	–	–
Soil pH	(+)0.17	(+)0.22	(+)0.27	–	–	(+)0.21	–	–	–
Soil CEC	(+)0.14	(+)0.20	(+)0.41	(+)0.28	–	–	(+)0.26	–	–

the diversity of habitats occupied by sunflowers, drier sites tend to have less dense vegetation, and thus less obstruction of visual signalling over distance. This may favour the evolution of larger display sizes that would not be favoured in densely vegetated habitats. Second, it is possible that pressure from florivory and/or disease selects for smaller flowers in mesic habitats. Wetter environments tend to support denser vegetation, along with more abundant herbivores and higher ambient pathogen load (Coe et al. 1976, Burdon et al. 1989, Fitt et al. 1989, Bradley et al. 2003, Garibaldi et al. 2011), such that arid habitats may provide a relative refuge from natural enemies (Springer 2009). Larger flowers are known to suffer higher rates of both florivory and disease (McArt et al. 2014, Oguro & Sakai 2015), and thus smaller flowers may have evolved in wetter environments as predicted by the enemy-escape hypothesis (Galen 1999). This may also apply to the evolution of lower disc water content in wetter environments, as low water content has been shown to reduce florivory in members of the Asteraceae (Oguro & Sakai 2015).

In most genera, annual species have higher reproductive biomass allocation in each growing season compared to their perennial congeners (Primack 1979, Bazzaz et al. 1987, Reekie & Bazzaz 2011). Sunflowers have been suggested to

demonstrate this pattern (Gaines et al. 1974, Bazzaz et al. 1987). However, we here observe no differences in flower size or ray-disc investment between annual and perennial species, which suggests that annuals must evolve their higher reproductive output by increasing the number of flowers produced, rather than modification of flower size or structure. While in this study we did not assess flower number given the indeterminate flowering of many *Helianthus* species under greenhouse conditions, trade-offs between flower size and number are known to be absent in many genera (Burd 1999, Sargent et al. 2007). Additional work controlling the length of the flowering period is needed to determine whether *Helianthus* also lacks a flower size-number trade-off.

Do pollinators drive the evolution of colour and relative ray-disc investment?

Across the wild sunflowers, ray-disc investment and disc colour are unrelated to virtually all of the abiotic environmental characteristics quantified in this study. While we included a wide variety of metrics related to temperature, water availability, and soil fertility, the scale at which these metrics were obtained may somewhat limit their interpretation. Climate averages were obtained at a resolution of approximately one square kilometre, and these estimates may be potentially dis-

connected from local temperature and water availability at the plant level due to microclimate and topology. Soil samples were also collected at a single point in time, and nutrient availability may vary temporally at sites. Despite this, variation within sites should be a relatively small proportion of the large environmental variation observed among species' habitats across North America.

An obvious alternative hypothesis to evolution in response to abiotic pressures is evolution in response to pollinators, and this biotic factor is of course the classic driver of floral evolution (Darwin 1862, Stebbins 1970, Fenster et al. 2004). In self-incompatible species, selection imposed by even generalist (non-specialist) pollinators can be extremely strong, enough to explain variation in flower size and shape (Galen 1996, 1999). While *Helianthus* are pollinated primarily by generalist bees (with occasional visits by dipterans and lepidopterans), the abundance and composition of pollinators almost certainly varies substantially among the diverse habitats occupied by each species across North America. Cultivated sunflower, for instance, is known to be pollinated by at least 48 wild bee species where it is grown across the United States, including multiple members each of *Bombus*, *Ceratina*, *Halictus*, *Lasioglossum*, *Melissodes*, *Megachile*, *Osmia*, and several other genera of bees (Parker 1981, Posey et al. 1986, Greenleaf & Kremen 2006). Further work is needed to determine whether pollinators are responsible for variation in relative ray-disc investment or disc colour among wild sunflowers, though this will be difficult given that local pollinator assemblages likely vary heavily not only among species but also among populations within the many widely-distributed members of the genus.

Determining the exact drivers of floral trait evolution is a complex endeavour, especially in a generalist-pollinated clade. While we find reasonable support for the role of aridity and soil fertility in the evolution of floral size and water content, we find no support for abiotic factors in the evolution of disc colour or relative ray-disc investment. Given the rapid diversification of ray-disc investment and the multiple transitions between light and dark disc colouration across *Helianthus*, it seems unlikely that these traits have evolved by drift alone, though it is unclear what selective pressures might be at work. Studies performed at the microevolutionary level may provide insights into mechanisms operating genus-wide and contributing to the interspecific diversity in these traits. Examining population differentiation in floral traits across specific biotic and abiotic gradients may allow for better parsing of focal pressures relative to species-level analyses, where multiple environmental factors co-vary simultaneously. This approach has been used successfully for uncovering adaptive differentiation for flowering time across a latitudinal cline in two widespread sunflower species (Kawakami et al. 2011, Blackman et al. 2011), and could easily be adapted to testing hypotheses across specific biotic and abiotic gradients.

SUPPLEMENTARY DATA

Supplementary data are available at *Plant Ecology and Evolution*, Supplementary Data Site (<http://www.ingentaconnect.com/content/botbel/plecevo/supp-data>) and consist of the following: (1) source information, trait data and environmental

metrics used in this study (Excel spreadsheet); and (2) phylogenetic space of total flower dry mass and dry mass investment ratio (pdf). These data are also deposited in the Dryad data repository (<https://doi.org/10.5061/dryad.8m0q0>).

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