

Biological and environmental drivers of flower-leaf sequence variation in the American Plums (*Prunus*, sect. *Prunocerasus*).

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Introduction

Woody perennials have a unique ability among plants to seasonally begin reproduction prior to vegetative growth. This flowering-first phenological sequence, known as hysteranthly, proteranthly or precocious flowering, is particularly common in temperate deciduous forests around the globe (Rathcke & Lacey, 1985). A number of studies suggest that this flower-leaf sequence (FLSs) is under selection, and that hysteranthly has functional significance, but the importance of variation in FLSs for maintaining fitness (Gougherty & Gougherty, 2018; Buonaiuto *et al.*, 2021; Guo *et al.*, 2014) may vary across functional types and evolutionary clades within the temperate forest biome. With mounting evidence that anthropogenic climate change is driving shifts in flower-leaf sequences (Ma *et al.*, 2020), expanding our understanding of the adaptive benefit of hysteranthly is vital to forecasting the demography and performance of forest communities in an era of global climate change.

The most common, and well-tested explanation for the evolution of hysteranthly in temperate forests is that it is adaptive for wind-pollination, as leafless canopies increase wind speeds for pollen transport and reduce the likelihood of pollen interception by vegetation (Whitehead, 1969; Niklas, 1985). However, this explanation does not address the widespread prevalence of hysteranthly in biotically-pollinated taxa found in temperate regions. This number is not trivial; a recent analysis found that approximately 20% of the hysteranthly species in the moist, Eastern Temperate Forests of North America are biotically pollinated (Buonaiuto *et al.*, 2021).

Several alternative hypotheses to the wind pollination hypothesis have been put forward to explain the advantage of hysteranthly in biotically-pollinated species, but they have not been widely evaluated in the literature. Below, we briefly review these hypotheses and their predictions, and then test these predictions using the American plums (*Prunus* subsp. *prunus* sect. *prunocerasus*), a widespread clade with high variability in flower-leaf sequences as a representative case-study. Our treatment here both clarifies the hypothesized function of flower-leaf sequence variation in biotically-pollinated taxa, and offers insights into how shifting flower-leaf sequences may impact species demography and distributions as climate continues to change.

Hypotheses of Hysteranthous flowering in biotically pollinated taxa

Water limitation hypothesis: In the dry-deciduous tropics of South and Central America, hysteranthly is common (Rathcke & Lacey, 1985; Franklin, 2016), and is regarded as an important adaptation to alleviate water stress by partitioning the hydraulic demand of flowers and leaves across the season in this environment

(Gougherty & Gougherty, 2018; Franklin, 2016; Borchert, 1983; Reich & Borchert, 1984). By contrast, temperate forests are rarely water-limited in the early season during which flowering and leafing occur (Polgar & Primack, 2011), but there is still considerable variation in water availability in space and time within temperate regions of the globe. Under this hypothesis, the function of hysteranthous flowering in these regions parallels that in the dry tropics—partitioning hydraulic demand across the season to allow hysteranthous species to tolerate increased aridity. If this is the case, we would expect to find hysteranthous taxa in locations that are, on average, drier than their non-hysteranthous relatives.

Insect-visibility hypothesis: Hysteranthous flowers are visually conspicuous in the landscape. Thus, as in wind-pollinated taxa, hysteranthous in biotically pollinated taxa may be an adaptation for pollination efficiency as flowering-first species are easier for insects pollinators to locate (Janzen, 1967). This hypothesis predicts that hysteranthous should be associated with smaller floral displays, because flower are not obscured by leaves, they are easier to see, and there is weaker selection for increasing floral display size.

Fruit maturaturion hypothesis: There are several aspects of reproductive development that suggest hysteranthous is a by-product for early flowering, driven by development constraints. Hysteranthous may be common in large fruited species that require lots of time to mature their fruits, or in small, early fruiting species that have evolved dispersal syndromes (wind dispersal, non-dormant seeds) that require dispersal early in the season (Primack, 1987). In either case, we should expect fruit size to associate with hysteranthous.

Alternative to these functional hypotheses is the assertion that hysteranthous flowering is simply a by-product of selection for early flowering. Species that flower before their leaves inherently flower early in the season. Spring flower phenology is less constrained by prior phenological events than leaf phenology (Savage, 2019), which could allow selection to drive flowering into the early season, producing the hysteranthous phenological sequence. Here, there is no specific adaptive advantage to hysteranthous; selection is not operating on the relative timing of flower and leaf emergence, but rather the absolute flowering time alone. If none of the predictions of the previously stated hypotheses are supported, this is the most likely null hypothesis.

A significant challenge for robust testing of hysteranthous hypotheses is that most characterizations of flower-leaf phenological sequences are based on expert-opinion verbal descriptions (e.g. “flowers before leaves” or “flower before/with leaves”), which make comparisons across taxa, time and space difficult and sensitive to observer bias (see; Buonaiuto *et al.*, 2021). This problem can be overcome by adopting standardized quantitative measures of plant phenology for observational studies and applying them to historic data records. Herbarium records are an excellent source of data that can be leveraged for quantitative phenological measurements (Willis *et al.*, 2017), but have not been used widely to investigate variability of flower-leaf sequences variation among and within species.

The American plums offer potential for a high resolution investigation of drivers of hysteranthous flowering in taxa that are not easily explained by the dominant wind-pollination hypothesis. The 16 species that make up the section are distributed across the temperate zone of North America and, like the genus *Prunus* at large, are all insect-pollinated, yet show pronounced inter-specific variation in flower-leaf sequences. Species in this section are well represented in herbaria records (Fig. 1a), making them a tractable group to measure and assess variation in flower-leaf sequences.

In this study we ask:

Do the observed associations between flower-leaf sequence variation and morphological and environmental traits match predicted associations of the hysteranthous hypotheses?

First, we used herbaria records to quantify both within- and across- species level variation in flower-leaf

sequences of the American plums, (subspecies *Prunus*, sect. *prunocerasus*). Then we combined environmental attributes, biological traits and phylogenetic data in statistical models to interrogate the functional hypotheses for hysteranthous flowering described above. Finally, we compared our findings in this clade to patterns observed in larger genus *Prunus* to better understand how phenology-trait associations vary over taxonomic scales.

Methods

0.1 Quantifying flower-leaf sequence variation

We obtained digital herbarium specimens for all members of the section *Prunocerasus* from the Consortium of Midwest Herbaria (CMH) Database (?). To quantify flower-leaf sequence variation within and across species we randomly sampled 200 specimens for each species and scored the phenological development of flowers and leaves using a modified BBCH scale for woody plants (Finn *et al.*, 2007). In total, we evaluated the phenology of 2521 specimens, but only specimens with visible flowers were included in this analysis (n=1009). We reconstructed the phylogenetic relationships among species in this group based on the tree topology in Shaw & Small (2004). We inferred branch lengths following the method of Grafen & Hamilton (1989) in which node heights are estimated in proportion to number of subtending taxa using the R package “ape” (E. Paradis and K. Schliep, 2019).

To quantify FLS variation, we fit an ordinal, hierarchical, Bayesian, phylogenetic mixed model (de Villemereuil P. Nakagawa, 2014) to assess the likelihood an individual would be at any given vegetative BBCH phase while flowering. Our model predicted leaf stage (Y, ordinal with up to j categories) as a function of species and additional phylogenetic effects. Because hysteranthous co-varies with flowering time (i.e., flowering first species will generally flower earlier than other species, on average) we included day of observation as an additional predictor. The model is written below:

$$\text{logit}(P(Y \leq j)) = \alpha_{[j]phylo[i]} + \alpha_{[j]sp[i]} + \beta_{dayofyear[sp[i]]} * X_1 + \epsilon_i$$

$$\epsilon_i \sim N(0, \sigma_y^2)$$

where Y is the ordinal outcome (leaf stage) and j is the number of categories (1,2,...6). $P(Y \leq j)$ is the probability of Y less than or equal to a category $j = 1...j - 1$. $\alpha_{[j]}$ describes an intercept for each category [1,2,...6], while slope $\beta_{dayofyear[sp[i]]}$ is constant across categories.

The influence of the phylogeny α_{phylo} was modeled as follows:

$$\alpha_{sp} \sim N(\mu_\alpha, COR[\sigma_{phylo}^2])$$

The α for species effects independent of the phylogeny was modeled as follows:

$$\alpha_{sp} \sim N(\mu_\alpha, \sigma_{species}^2)$$

We used our model to predict the likelihood each species would be observed at a given vegetative BBCH stage during flowering at the 0%, 25% 50% and 75% quantiles of their flowering period. We then developed a flower-leaf sequence index, by assigning a numerical score to each species per seasonal quantile, and summing over

the full flowering season. In each seasonal quantile, species received a “1” if more than 50% of their probability distribution occurred at the two earliest stages of vegetative phenology—BBCH 0 (“bud development”) and BBCH 09 (“bud break”)—and a “0” if not. We summed these values across the season, generating an index from 0 (never hysteranthous) to 4 (hysteranthous through late season (Q75)), where 1= hysteranthous at start of season, 2= hysteranthous through early season (Q25) and 3 = hysteranthous through mid season (Q50). We also used two alternative indexing schemes (>25% of the probability distribution occurred at BBCH 0 and >40% of the probability distribution occurred at BBCH 0 and BBCH 09) to make sure our result were robust across multiple cutoffs.

0.2 Evaluating hysteranthly hypotheses

To test the predictions of the hypotheses we obtained data on petal length and fruit diameter directly from herbarium specimens. To assess aridity tolerance, we computed the average Palmer Modified Drought Index score from 1900-2017 for every *Prunocerasus* specimen in the database (n=2305) from the North America Drought Atlas (Cook & Krusic, 2004). For any specimens that lacked accurate geo-location information, we extracted PDSI values at the county centroid of the herbaria specimen.

For our morphological measurements, we sampled an additional 321 specimens and measured the petal length of up to 10 randomly selected petals per specimen (n=2757) using ImageJ image processing software. We also used ImageJ to measure the diameter of fruits on an additional 316 specimens, measuring up to 5 fruit per specimen (n=224).

We then used Bayesian phylogenetic mixed models to test the relationship between flower-leaf sequence index scores and each of the variables. In these models, we modeled species and phylogeny as above.

The model structure is written below:

$$y_i = \alpha_{ind/sp[i]} + \alpha_{phylo[i]} + \beta_{hyst.index} * X_{hyst.index} + \epsilon_i$$

$$\epsilon_i \sim N(0, \sigma_y^2)$$

where Y is observed trait values (PDSI, petal length or fruit diameter), and the slope $\beta_{hyst.index}$ describes the relationship between extended hysteranthly (higher hysteranthly index value) and the trait of interest.

$\alpha_{ind/sp[i]}$ and $\alpha_{phylo[i]}$ describes the species and phylogenetic effects respectively. We also ran each model using our two alternative F

Hysteranthly in the larger genus *Prunus*

To better understand how the patterns we identified in our in-depth study of the *Prunocerasus* clade scaled across coarser taxonomic resolution we also evaluated the relationship between hysteranthous flowering and hypothesis-related traits in all of the *Prunus* species that are native to, or established in North America. For this analysis, we obtained categorical descriptions of flower-leaf sequences and mean estimates of fruit diameter and number of flowers/inflorescence as a proxy for floral investment from the Flora of North America (?) for 32 species in the genus. We extracted PDSI values for all herbaria observation of those species in the Consortium of Midwest Herbaria database (n=23,272) as described above.

To account for the influence of evolutionary relationships among species, we reconstructed the phylogenetic relationships in the genus based on the tree topology in Chin *et al.* (2014). As above, we computed branch lengths with the R package “ape” (E. Paradis and K. Schliep, 2019).

We standardized the units of all predictors to make their effect size estimates for the following model structure directly comparable to each other:

$$\text{logit}(P(Y \leq j)) = \beta_{[j]phylo[i]} + \beta_{pdsi[sp[i]]} * X_1 + \beta_{fruitdiameter} * X_2 + \beta_{floralinvestment} * X_3 + \epsilon_i$$

$$\epsilon_i \sim N(0, \sigma_y^2)$$

where Y is the ordinal outcome of flower-leaf sequence category (“flowers before leaves”, “flowers before/with leaves”, “flowers with leaves” and “flowers after leaves”) and j is the number of categories (1,2,...4). $P(Y \leq j)$ is the probability of Y less than or equal to a category $j = 1, \dots, j - 1$. We modeled the influence of the phylogeny (α_{phylo}) as above.

0.3 Model runs

We fit all models in the R package “brms” (Bürkner, 2018) using weakly informative priors, and ran the model on four chains. For the “Quantifying flower-leaf sequence variation” and “Evaluating hysteryanth hypotheses” we ran the models with a warm-up of 3000, and 3500 iterations, and 4000, and 4500 sampling iterations respectively, for a total of 4000 sampling iterations across all chains. For the “Hysteryanth in the larger genus *Prunus*” model, we used a warm up of 6,000 iterations and 8,000 sampling iterations for a total of 8,000 sampling iterations to maximize the effective sampling size. Model fits was assessed with Rhats <1.01, high effective sample sizes and no divergent transitions.

Results

Quantifying flower leaf sequences in the American plums

We found substantial inter-specific differences in flower-leaf sequences within the American plums (Fig. 2, S1). Flower-leaf sequence patterns were strongly dependent on the day of observation, with observations later in the the flowering season of each species decreasing the likelihood of finding flowers open during early vegetative BBCH phases ($\beta_{day} 0.03$, $CI_{50} [0.02, 0.03]$). Based on our flower leaf sequence index, two species (*P. umbellata*, *P. mexicana*) were likely to be hysteryanthous regardless of the time of observation and three species (*P. rivularis*, *P. subcordata*, and *P. texana*) were always most likely to flower after with leaves present (Fig. 1b). All other species displayed intermediate phenotypes, with five species mostly likely to hysteryanthous at the start of the season (*P. alleghaniensis*, *P. americana*, *P. hortulana*, *P. munsoniana* and *P. nigra*), one species through early season (*P. gracilis*) and two species through mid season (*P. angustifolia*, *P. maritima*) (Fig 1b).

Associations between hysteryanth and environmental and morphological traits

In the American plums clade, aridity (lower average PDSI) was associated with higher flower-leaf sequence index scores (β : -0.03, $CI_{50} [-0.05, 0.02]$, Fig. 3a.), suggesting that species that displayed hysteryanthous flowering later into their flowering season are found in dryer locations.

Shorter petal and smaller fruit diameters were also associated with higher flower-leaf sequence index scores (β : -.21, $CI_{50} [-0.38 -0.04]$, β : -1.40, $CI_{50} [-1.97 -0.82]$ respectively, Fig. 3b.,c.). This suggests that smaller fruits

and flowers are associated with increased hysteranthly.

At the genus level, there was a positive association between increasing PDSI and inflorescence size and increasing overlap between flowers and leaves (i.e., decreasing hysteranthly, β : 2.50, CI_{50} [1.17, 3.371] and β 6.41, CI_{50} [3.86, 8.05] respectively, Fig. 4a), suggesting that hysteranthly is associated with drier locations and smaller floral displays (Fig. 4b). Hysteranthly was associated with larger fruits (β : -1.24, CI_{50} [-1.95,-0.21], 4b)] though there was high uncertainty around these estimates in our model.

Discussion

Our study provided foundational insights into the evolution of flower-leaf sequences in biotically pollinated plants. Our findings that hysteranthous flowering has potential to be linked to both aridity tolerance and pollination success through the predictions of the water limitation and insect visibility hypotheses increases the urgency for advancing our understanding phenological sequences as human-caused global change continues to disrupt pollinator services and impact environmental variability.

Hysteranthly hypotheses

Using North American *Prunus* species as a case study, our analyses support that flower-leaf sequences are under selection by biological and environmental drivers, and that variation in these patterns across species may reflect adaptive tradeoffs. We found that hysteranthous flowering is associated with smaller floral displays and increased aridity in both the American plum clade of the genus *Prunus*, and more broadly the members of the full genus that are the native or established in North America. While we did not find support for the fruit maturation hypotheses, the relationships between hysteranthly and aridity, and hysteranthly and floral display size support the predictions of the water limitation hypothesis and the insect visibility hypothesis, respectively.

The support for both the water limitation hypothesis and insect visibility hypothesis highlights that these hypotheses are not mutually exclusive, and could be related. Selection on floral size represents a classic evolutionary tradeoff where larger floral displays may generally be more effective for attracting pollinators but demand more resources, including water, to maintain turgor and reproductive function than smaller ones (Galen *et al.*, 1999; Lambrecht & Dawson, 2007). With this trade-off, reproductive displays are often small in harsher environments (), and hysteranthly could represent a compensatory mechanism that both reduces hydraulic demand while increasing pollination efficiency in these environments.

Studies that have compared the transpiration rates among flowers and leaves provide insights to the potential importance of this seasonal partitioning for maintaining water status. Measurements of water movement (transpiration rates, sap flow, hydraulic conductivity) to flowers range from 20%-60% of that of leaves under comparable conditions (Whiley *et al.*, 1988; Roddy & Dawson, 2012; Liu *et al.*, 2017; McMann *et al.*, 2022). This level of additional hydraulic demand can drive loss of stomatal conductance and decrease photosynthetic rates (Galen *et al.*, 1999).

Yet the flower-leaf sequences of even the hysteranthous species in our study were markedly different from patterns of hysteranthly in the dry-tropics where the water limitation hypothesis developed. While flowering can precede leafout by as much several weeks for species in our focal clade, the process of fruit development, which is also water intensive, occurs when leaves are present. By contrast, in the dry tropics hysteranthous flowering is initiated at the time of leaf drop (Borchert, 1983; Franklin, 2016). This makes it that the full

reproductive cycle occurs in the leafless period. The comparatively small window of leafless reproductive development in our temperate clade, may in part, explain why the association we observed between hysteroanthy and aridity in our study was relatively weak with high residual variance. Our result suggest that hysteroanthy may allow temperate species to occupy marginally drier environments than non-hysteroanthous species, but may not facilitate species' persistence under extreme aridity, like the conditions encountered by hysteroanthous species of the dry tropics. Instead, the aridity tolerance conferred though hysteroanthy could serve as mechanism for niche partitioning between the closely related and morphological similarity species in our study, enhancing their ability to coexist with highly overlapping ranges.

Inter-and intra-specific variation in flower-leaf sequences

In our study, we developed a novel approach to assessing flower-leaf sequences that scales from quantitative, individual-level observations to species levels characterizations based on empirical likelihood estimates. With this approach, we were able to for the first time quantitatively assess intermediate cases of hysteroanthy (ones that are typically described as “flowers before/with leaves”). Previous studies of hysteroanthous flowering have either excluded these cases from their analyses (e.g.; Gougherty & Gougherty, 2018) or binned them with the well defined cases (e.g.; Buonaiuto *et al.*, 2021). We found that eight of the thirteen American plum species expressed this intermediate flower-leaf sequence, but by estimating the likelihood of hysteroanthy across the growing season with Bayesian methods, our approach identified substantial differences in FLSs among them (Fig. 2, Fig. S1), which allowed us to robustly assess the trait associations related to the hysteroanthy hypotheses addressed above.

Our quantitative analysis of the American plums clade revealed that flower-leaf sequences—often described as a species-level trait—are highly variable within species (Fig. 2, Fig. S1). For all members of the clade, the day of phenological observation was a strong predictor of the likelihood that flowers would be visible before the emergence of leaves. In many cases, there was high likelihood that individuals of a species may be observed at different vegetative stages during flowering (Fig. 3, S1). The intra-specific variability we detected in our study furthers a growing call to adopt an individual, observational approach to the study of flower-leaf sequences by quantifying flower-leaf sequences at the individual level and modeling these patterns at coarser taxonomic scales, rather than treating them as immutable categorical patterns at the species level (Buonaiuto *et al.*, 2021).

Additionally, by related these individual, quantitative observations as ordinal response categories with our hysteroanthy index, we were able to consider our results in context with existing categorical, species-level characterizations based on expert opinion. The coherence of inference between our individual based observational approach for the American plum clade and the top-down, categorical data we analyses larger genus *Prunus* is an encouraging demonstration that the categorical, expert opinion-based data can still offer useful insights the the drivers of hysteroanthous flowering when higher-resolution data is not available. Our modeled flower-leaf sequences patterns of the American plums also qualitatively agreed with previous characterizations of the the species-level variation in this group (Shaw & Small, 2004), indicating that the biological patterns we observed are relatively robust to these methodological choices.

Future directions

In this study, we focused on a well-studied, and economically important clade of morphologically similar species, that allowed us to control for unmeasured biological variation on our traits of interest. This case-study critically provides a road map to evaluate the role of hysteroanthy more broadly in temperate biotically

pollinated plant taxa (groups with high interspecific flower-leaf sequence include *Magnolia*, *Rhododendron*, *Acer* and *Cornus*).

To advance our collective understanding of the adaptive significance of flower-leaf sequences, the research community should complement the observational approach we employed in this study, with novel experiments to demonstrate benefits to hysteranthous flowering. To test the water-limitation hypothesis, researchers could plant sister-taxa with contrasting flower-leaf sequences in common environments across a gradient of aridity, and evaluate their performance. To test the insect visibility hypothesis, researchers should also consider hysteranthy—and phenology in general—in the more general framework of tradeoffs in pollination biology. The trade off between phenology and pollination investment should not only consider flower size, but also the number of flowers, nectar and pollen reward investment, volatiles between related hysteranthous and non-hysteranthous taxa. For a simple experiment to test this hypothesis, researchers could place hysteranthous and non-hysteranthous individuals in a controlled environment, and systemically release pollinators to observe their preference, search times and foraging behavior.

With a better mechanistic understanding of the relationship between flower-leaf sequences and ecological performance in hand, researchers could then use experiments to assess how differences in floral and leaf physiological responses to temperature variation may alter the adaptive benefits of flower-leaf sequences with climate change. The measurement and modeling approaches we developed in our observational study can be readily implemented to analyze data from these experimental settings, representing an important opportunity to unite observations of broad ecological patterns with targeted mechanistic explanations in order to better understand both the evolutionary past and ecological future of flower-leaf sequences in temperate woody plants.

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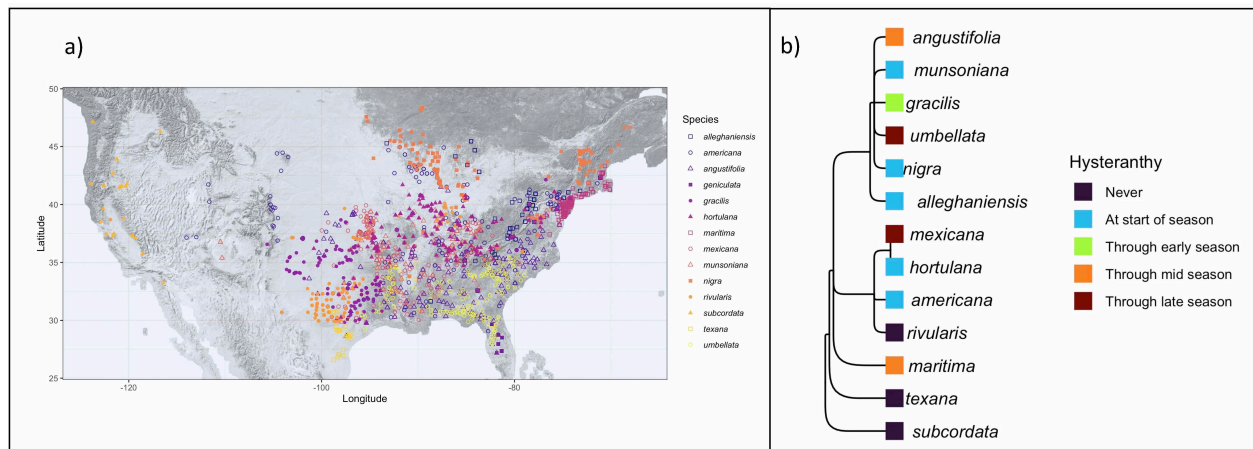


Figure 1: Geographic distribution and taxonomic relationships among the focal clade *Prunocerasus*. a) Maps the localities of all the herbaria records used in this study. b) Depicts phylogenetic relationships among the American plums and the duration of their flowering period they are hysteresis. These categorizations are based on ordinal phylogenetics mixed models. Tree topology is from Shaw & Small (2004)

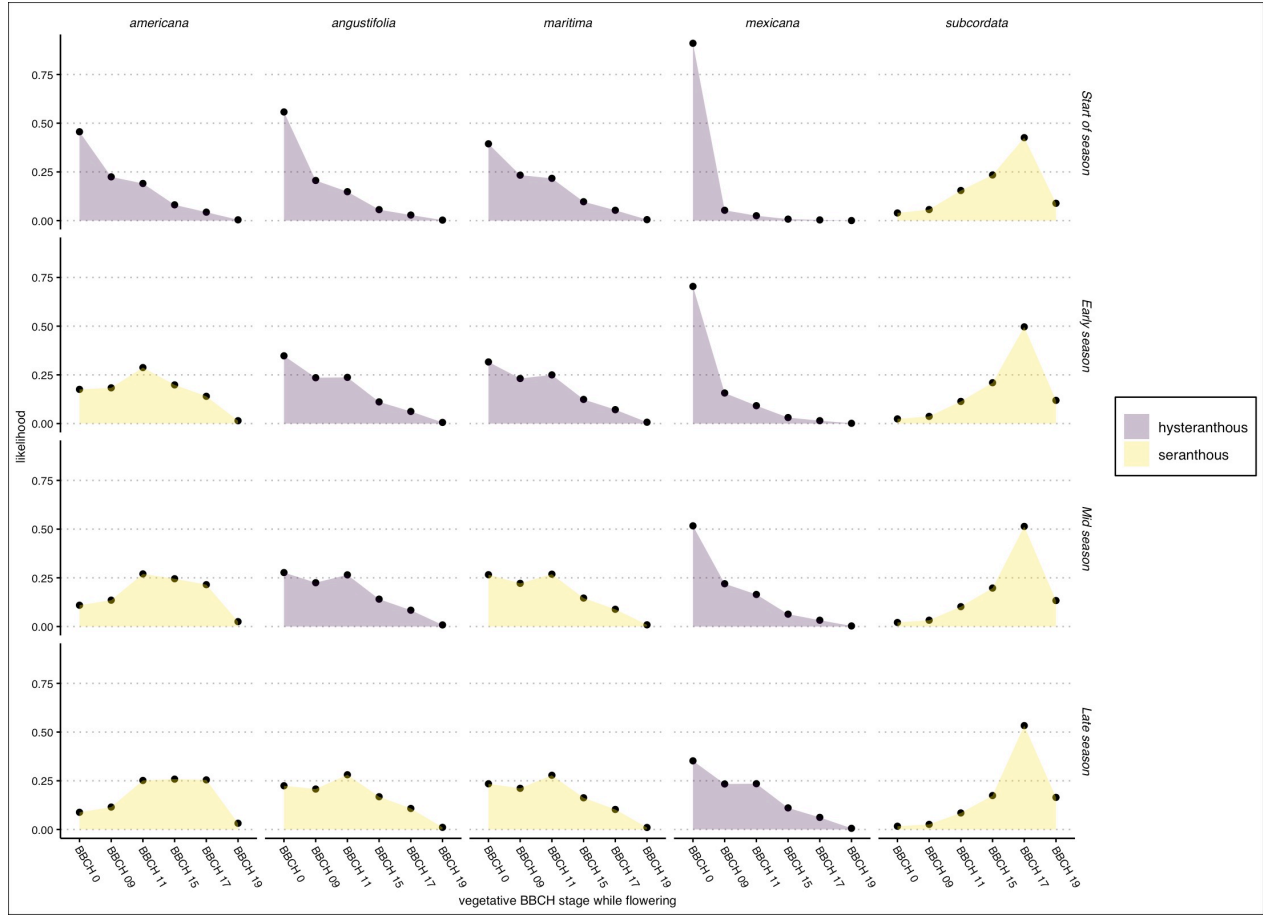


Figure 2: Predicted likelihood that a species would be in flower during each vegetative BBCH phase for five example species in the American plums. Points are the mean likelihood and bar the 95% uncertainty intervals. Species were classified as hysteranthous if greater than 50% probability flowering occurred in BBCH 0 and BBCH 09 (colors) for each part of the flowering season. See Fig. S1 for all species and alternative hysteranthous classification schemes.

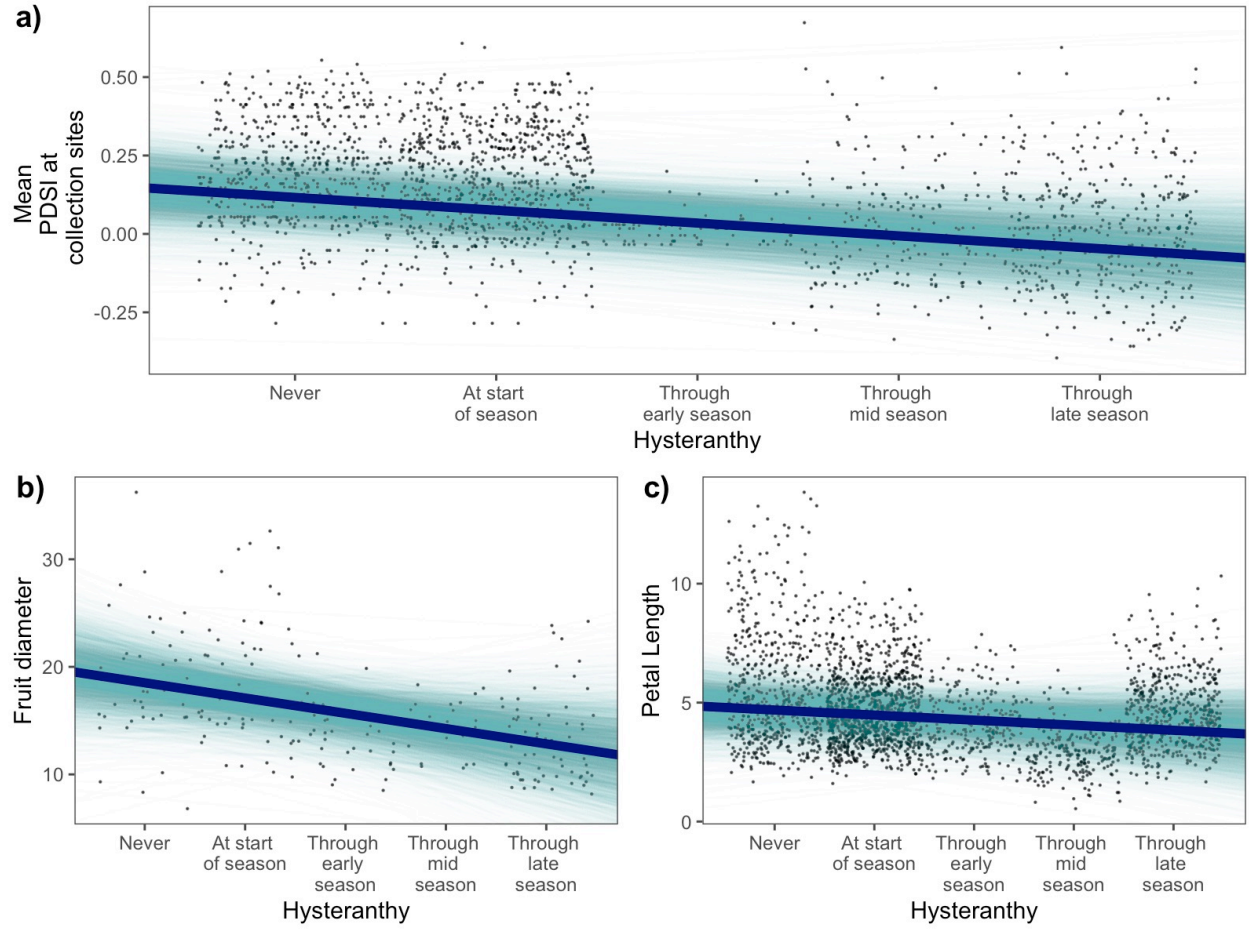


Figure 3: Relationships between the duration of hysteresis across the flowering period and environmental and biological traits based on Bayesian phylogenetic mixed models. a) b) and c) depict the relationships between the duration of hysteresis and mean PDSI, fruit diameter, and petal length respectively. Solid lines indicate the mean posterior estimate and shaded areas X draws from the posterior distribution as a display of uncertainty.

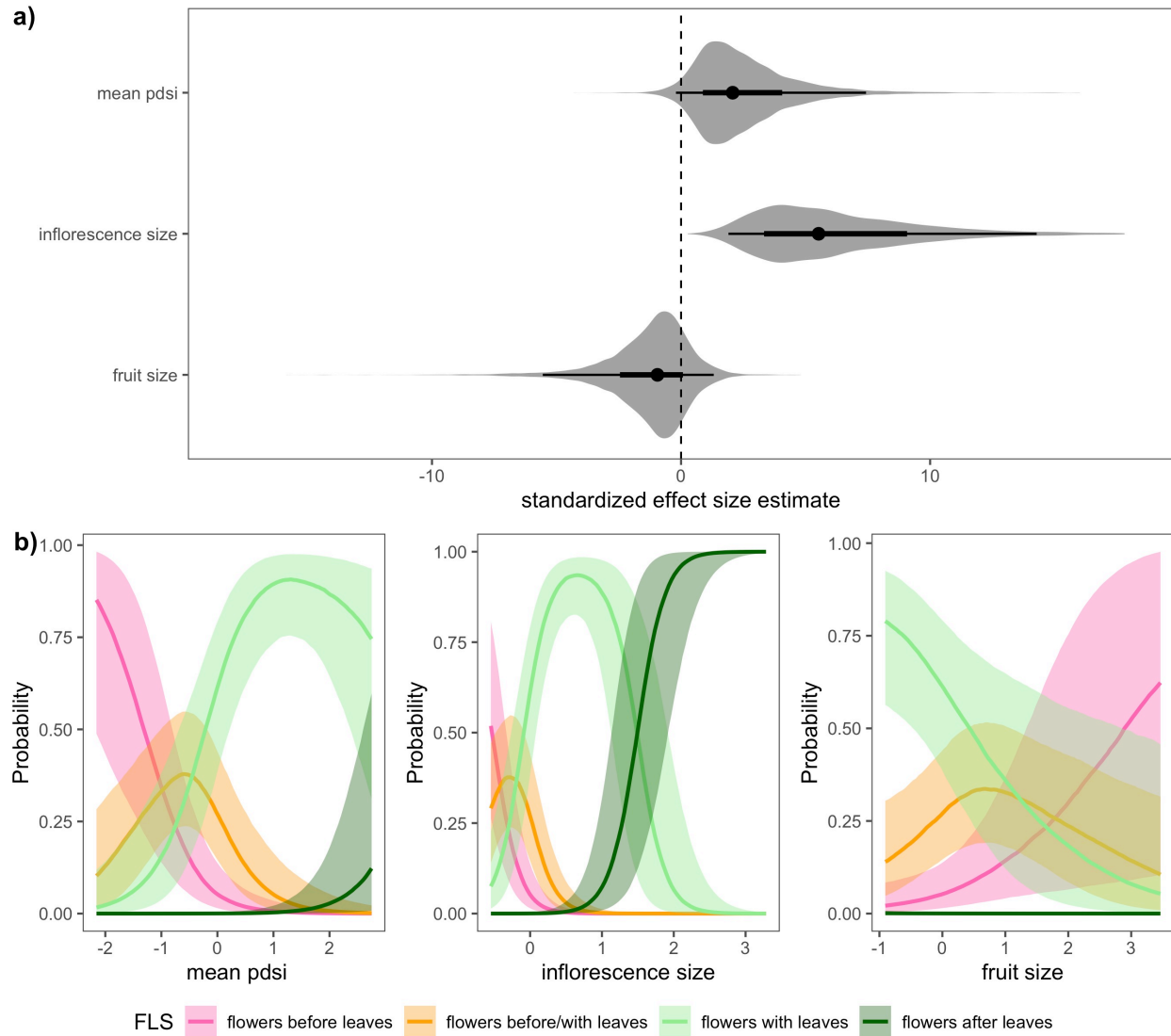


Figure 4: Relationships between the likelihood of of hysteranthly and environmental and biological traits in the genus *Prunus* based on Bayesian phylogenetic mixed models. Panel a) shows the estimated effect size of each predictor with negative values indicating an increased likelihood of hysteranthly. Points indicate the mean posterior estimate for each predictor, and thick and thin bars the 50% and 97.5% uncertainty intervals respectively. We also show the full posterior distribution as an additional measure of uncertainty, Panel b), c) and d) show the marginal effect of mean PDSI, inflorescence size and fruit size respectively, on the likelihood that of each FLS category. Solid lines indicate the mean likelihood and shaded areas the 50% uncertainty intervals.