Title: Stomatal responses of herbaceous plants to climate change since the 1800s: A case study using herbarium specimens

Authors: Nia Richardson[1,2,3], Léa Vilna-Santos[4], Axel Cortes[5], Mia K. Gaughan[3,4], Adam B. Smith[3], Matthew W. Austin[3]*

[1]Howard University, Washington, D.C., USA
[2]University of Michigan, School for Environment and Sustainability, Ann Arbor, Michigan, USA
[3]Missouri Botanical Garden, Saint Louis, Missouri, USA
[4]Washington University in Saint Louis, Saint Louis, Missouri, USA
[5]Collegiate School of Medicine and Bioscience, St. Louis, Missouri, USA

* Corresponding author:
Missouri Botanical Garden
4344 Shaw Blvd.
St. Louis, MO 63110
maustin@mobot.org

Author ORCiDs:
Nia Richardson: N/A
Léa Vilna-Santos: N/A
Axel Cortes: N/A
Mia K. Gaughan: 0009-0008-6175-9615
Adam B. Smith: 0000-0002-6420-1659
Matthew W. Austin: 0000-0002-1231-9081

Abstract

- **Background and Aims:** As climate change accelerates, plants are expected to respond in myriad ways, such as shifting phenology or altering the density or size of stomata. Nonetheless, most studies to date have focused on just a single facet at a time.
- **Methods:** Here, we used herbarium specimens to examine stomatal responses to climate change in two perennial herbaceous species that have not shifted flowering time across the past century, *Oenothera macrocarpa* (Onagraceae) and *Viola sororia* (Violaceae). These trends were evaluated across time and in relation to three major climate drivers: temperature, precipitation, and atmospheric CO_2 concentrations.
- **Key Results:** Stomatal lengths in *O. macrocarpa* became smaller, significantly changing by -0.345 μm per decade (95% confidence intervals: -0.505 to -0.184), amounting to a ~17.5% reduction in size from the 1880s to the present. Otherwise, stomatal densities and lengths did not change significantly over time and were unrelated to temperature, precipitation, and atmospheric CO_2, except for a marginally significant decline in *O. macrocarpa* stomatal length with CO_2.
- **Conclusions:** Previous research on stomatal trends obtained from herbarium specimens across time have largely focused on trees, while the stomatal response of herbaceous plants to climate change has been relatively understudied. These findings demonstrate that species lacking a phenological response to climate change may exhibit other types of response, such as decreased stomatal size. Accordingly, this study underscores the importance of simultaneously investigating the response of multiple morphological and physiological facets for a more holistic understanding of how climate change influences species.

Keywords: atmospheric carbon enrichment, biological collections, climate change, forb, herbaceous plant, herbarium specimens, leaf morphology, natural history collections, *Oenothera macrocarpa*, phenology, stomata, *Viola sororia*

Introduction

Since the pre-industrial era, atmospheric CO_2 levels have risen from ~290 ppm to more than 430 ppm, leading to cascading responses among Earth's biota (IPCC 2022; NOAA n.d). Plants in particular have responded in multiple ways, including changes in physiology, morphology, phenology, and distribution (Miller-Rushing et al. 2009; Rubenstein et al. 2023; Austin et al. 2024). Most research to date has focused on characterizing changes in single types of response (e.g., flowering phenology, morphology, or abundance), ignoring the potential for concomitant changes across multiple facets (counterexample: Zhang et al. 2026). Examination of multiple facets provides a more holistic understanding of potential plant responses. For example, changes in multiple physiological facets could enhance, retard, or cancel one another in their combined effect on fitness (Doak & Morris 2010; DeMarche et al 2017; Coblentz et al. 2024). In an era of rapid environmental change and concurrent biodiversity loss, it is critical to examine multiple facets of how plants respond to climate.

Changes to the timing of life history events, or phenology, are among the most widely studied plant responses to climate change (Cleland et al. 2007; Ahlstrand et al. 2025). Globally, warming temperatures are generally associated with earlier and extended flowering seasons, although responses vary among species and regions (Chen et al. 2003; Austin et al. 2024; Williamson et al. 2025). While evolutionary relatedness, variation in climatic trends, habitat, and other factors can affect phenological shifts, there is still wide variation in changes of reproductive timing across species (Chen et al. 2003; Bartlett et al. 2023; Austin et al. 2024; Williamson et al. 2025). One possible explanation for this lack of consistency lies in potentially compensatory changes in one facet offsetting the need for changes in another (DeMarche et al 2918; Coblentz et al. 2024). For example, alterations in a species' ability to regulate water exchange with the atmosphere through stomata might offset the effects of higher temperatures, thereby obviating the need to shift phenologically (e.g., Rahman et al. 2021).

Stomata control the exchange of water vapor and CO_2 between the interior of the leaf and the atmosphere, and are thus potentially responsive to changes in CO_2 concentration, temperature, and water availability--three environmental conditions that are largely indicative of climatic changes (Hetherington & Woodward, 2003). Various morphological features have been shown to regulate

overall stomatal conductance at the level of the leaf, such as the density of stomata and their size (Assmann, 1999). As atmospheric CO_2 concentrations rise, a plant's need for high stomatal conductance decreases, favoring lower stomatal densities and size to reduce water loss (Miller-Rushing et al. 2009; Xu et al. 2016). Indeed, reductions in stomatal densities and size have been demonstrated in many taxa (e.g., Penuelas and Matamala 1990), and overall stomatal conductance has been predicted to decline from 10% to 40% in response to a doubling of atmospheric CO_2 (Morison 2001; Medlyn et al. 2001).

Long-term herbarium datasets are invaluable for quantifying changes in plant traits such as shifts in flowering date or stomata over time (Lister & the Climate Change Research Group 2011; Lang et al. 2019). However, few studies simultaneously explore how plant phenology and stomata have responded to climate change, and most stomatal work on herbarium specimens to date has focused on stomatal responses of trees and domesticated plants, with less attention to uncultivated, herbaceous specimens (Lin et al. 2015; Liang et al. 2023). Herbaceous plant specimens are more challenging to work with compared to trees as they tend to have more fragile leaves and are more likely to be damaged by procedures used to study stomata, such as stomatal peels. As a result, stomatal responses of herbaceous species across decadal and centennial time scales are less well-known.

To help fill these gaps, we examined two perennial herbaceous plant species native to Missouri, USA: the Missouri evening primrose (*Oenothera macrocarpa*, Onagraceae) and the common blue violet (*Viola sororia*, Violaceae). *Oenothera macrocarpa* is a self-incompatible, late-spring–summer blooming herb, which occurs on limestone glades and rocky prairies, habitats characterized by low moisture (Kucera & Martin 1957; Moody-Weis and Heywood 2001). *Viola sororia* is a self-compatible spring ephemeral, which typically occurs in moist meadows and woods (Solbrig et al. 1980). A recent study of a floral community in the Missouri Ozark Highlands combined long-term field and herbarium data of these species and found that the flowering time of both species is sensitive to temperature, with flowering occurring earlier in years with warmer springs, though the two species have shifted shifted neither the beginning or ending of their flowering seasons across the past century (Austin et al. 2024) (Fig. 1A-D). Further, neither species' flowering time is sensitive to spring precipitation (Austin et al. 2024) (Fig. 1E-F). Accordingly,

while the flowering phenology of both species appear similarly sensitive to climate, it is unknown whether these species have responded to climate change in other ways, such as by changing stomatal density or size. We predicted that because *O. macrocarpa* resides in drier habitats, compared to *V. sororia*, it would display greater decreases in stomatal densities and size over time. We also hypothesized that stomatal densities and size in *O. macrocarpa* would be more sensitive (i.e., larger absolute slopes) to climatic indices indicative of stress and resources. Namely, we expected stomatal densities and size would decline with greater temperature and atmospheric CO_2 concentration, and increase with greater precipitation.

Materials and Methods

Climate data

Temperature and precipitation data were obtained from parameter-elevation regressions on independent slopes model (PRISM) climate coverages (LT81m version at 30-arcsec, ~800-m spatial resolution at the latitude of Missouri, USA; Daly et al. 2002). These data span 1895 to the present, so could only be paired with specimens collected since their inception.

Globally averaged atmospheric CO_2 concentrations were obtained from the European Environment Agency (2024), which compiled data sources from the US National Oceanic and Atmospheric Administration (no date), to generate a time series spanning 1750 to 2018. Values were reported every 5 years prior to 1978 and annually from 1978 onward. For specimens collected prior to 1978 and not in a year cleanly divisible by 5, we linearly interpolated CO_2 concentration based on the values of the two bracketing years. We used globally-averaged CO_2 concentrations owing to a lack of spatially-explicit CO_2 estimates across the time period of our investigation, and due to the fact that over a year's time, CO_2 mixes fairly well across the globe (IPCC 2022).

To understand how stomatal density and length responded to climatic factors, we used species- and record-specific values. For *O. macrocarpa*, we paired each record with mean temperature and total precipitation from April through November of the year of collection, and for *V. sororia*, April through June. We selected these temporal windows based on the first and last flowering months of each species across our data set. For CO_2, we used the annual value of the year of observation.

Since many specimens were only able to be georeferenced to the level of a county, we used county-wide average values from the PRISM rasters (Park and Davis, 2017).

Stomatal lengths and densities

We obtained herbarium specimens of *O. macrocarpa* and *V. sororia* from the Missouri Botanical Garden Herbarium (MO). Our protocol was designed to minimize the chances of damage to herbaceous leaves, and we describe it in detail to enable others to apply it to their case. For each species, we selected up to three specimens from each decade (29 *O. macrocarpa* specimens dating from 1884-2014 and 38 *V. sororia* specimens dating from 1835-2012), choosing specimens with flat leaves, and a collection year and county of origin listed on the collection label. Using a dental putty dispenser (House Brand High-Performance Dispensing Gun, 1:1 / 2:1 ratio for 50ml, manufacturer code UC-IMDG, from Net32, https://www.net32.com/), we expelled a ~10-mm wide dab of dental putty (Affinis, Single Pack - Regular Set, Light Body: A-Silicon Impression Material, part number 6501, from Net32, https://www.net32.com/) onto the abaxial surface of a leaf on each herbarium sheet. After allowing at least 5 min for the putty to dry, we gently removed each putty dot from the leaf using needle-nosed tweezers. To prepare slides for each specimen, we applied a layer of clear nail polish (CotyBeauty's Sally Hansen Miracle Gel Nail Polish Shiny Top Coat, 101 Miracle Gel 3.0 Shiny Top Coat) on the imprinted side of each putty dot and allowed it to dry for at least 20 min. Using clear (i.e., not frosted) tape, we applied the sticky side of the tape to the polished side of the putty and gently applied pressure to ensure that the dried nail polish would not crack. We then lifted the tape carefully to ensure the dried nail polish was stuck to the tape, mounted the tape on a glass microscope slide, and observed the imprint under a microscope. To obviate issues from skin oils and fingerprints, we wore latex gloves during the preparation procedure.

To measure stomatal density and size, we first created a series of images of four non-overlapping frames per imprint at 20x magnification using a Nikon Coolscope II microscope (Appendix 1, Supplementary Figures S1 and S2). We selected each frame by dividing the imprint into quadrats, and then focused on a region within each quadrant. We moved the frame if there were artifacts from air bubbles, larger veins, or other apparent damage. For each frame, we created a "z-stack" by taking a set of images along a series of focal adjustments (Appendix 2). This allowed the images

to capture different parts of the leaf that may have been out of focus at any one focal setting. We analyzed stomatal counts taken from the z-stacks using ImageJ (Schneider et al. 2012) because we were more confident in identifying stomata from them than when viewing through the CoolScope. We included stomata in our counts if they were wholly visible in the frame or if they abutted the top or left side of the frame. To account for uncertainty due to obstructive leaf morphology and artifacts of slide preparation, we scored each purported stomate by our confidence ("high" or "low" confidence) in it being an actual stomate. Stomatal density was calculated per frame as the number of stomata divided by frame area (145883.472 μm^2). For each frame, we calculated stomatal density twice, once only using "high confidence" stomata and once using both "high and low confidence" stomata.

We measured stomatal length for up to 10 randomly selected "high confidence" stomata per frame using ImageJ. Whenever a frame had fewer than 10 "high confidence" stomata, we measured stomatal length for the total number of "high confidence" stomata in the frame. Here, we define stomatal length as the distance between apices of guard cells (i.e., length of the stomatal pore; Fig. S3).

We examined the relationship between stomatal length and density using a linear mixed-effects model for each species, where mean stomatal length per frame was the response and stomatal density the predictor variable, with specimen as a random effect.

Statistical analysis of stomatal trends

We analyzed changes to stomatal density using a series of linear mixed-effects models. To evaluate changes to stomatal density across years, we used stomatal density as the response variable, collection year as the predictor variable, and specimen as a random effect. To evaluate the sensitivity of stomatal density to climate, we used temperature, precipitation, and atmospheric CO_2 plus their two- and three-way interactions as predictors, and specimen as a random effect. Continuous predictor variables in each model were centered and scaled by their standard deviations prior to analysis. We ran two versions of each model: one that included only "high confidence" stomata and one that included both "high and low confidence" stomata.

We analyzed changes to stomatal size using another set of linear mixed-effects models. To evaluate changes to stomatal size across years, we used stomatal length as the response, collection year as the predictor, and frame nested within each specimen as random effects since we had multiple measurements of stomatal size per frame. To evaluate the sensitivity of stomatal size to climate, we used stomatal length as the response; temperature, precipitation, CO_2, and their two- and three-way interactions as predictors; and frame nested within each specimen as random effects. Again, all continuous variables were centered and scaled before analysis.

All analyses were conducted using R version 4.3.1 (R Core Team 2024). Mixed effects models were implemented using the 'lmer' function from the *lme4* package version 2.0-6 (Bates et al., 2015). All code and data necessary to implement the analysis are found in the online supplement (Appendices 3 & 4).

Results

Analyses based on "high-" and "high and low"-confidence stomata demonstrated qualitatively similar trends, so in the main text we report results for the "high confidence" class. Results for "high and low confidence" stomata are reported in Tables S1 and S2. Of all identified stomata, 97.3% were scored as "high-confidence" for *O. macrocarpa* and 86.3% were scored as "high-confidence" for *V. sororia*.

Stomatal lengths of *O. macrocarpa* significantly decreased across time with a slope of -1.145 μm / standard deviation of year ($P < 0.005$), which translates to a change of -0.345 μm / decade (95% CI: -0.505 to -0.184; Fig. 2H; Table 1). The *O. macrocarpa* specimen with the highest mean stomatal length was collected in 1887, with a mean stomatal length of 21.5 μm, and the lowest mean stomatal length was collected in 2012, with a mean of 13.8 μm. Stomatal lengths of *V. sororia* did not change across time ($P = 0.513$; Fig. 3H; Table 2).

For *O. macrocarpa*, there was a marginally significant negative correlation between stomatal length and CO_2 (slope = -0.961 μm / standard deviation of year, $P = 0.0527$; Fig. 2F; Table 1). For *V. sororia*, the relationship between stomatal length and the interaction between CO_2 and

temperature was also marginally significant (slope = 1.301 μm / standard deviation of year, P = 0.0567; Fig. 3F; Table 2). None of the other relationships between climatic variables -- or their interactions -- and stomatal lengths were significant for either species (all $P > 0.05$; Figs. 2 and 3; Tables 1 and 2).

Stomatal density has not changed since the 1880s for either *O. macrocarpa* or *V. sororia* (*O. macrocarpa*: P = 0.787; *V. sororia*: P = 0.923; Figs. 2G and 3G; Tables 3 and 4). For *O. macrocarpa*, stomatal density and length exhibited a significant negative correlation (slope = -0.021, $P < 0.05$; Fig. S4), indicating that for every 1 stomate/mm² increase in stomatal density, mean stomatal length decreases by approximately 0.021 μm on average, while accounting for differences among specimens. For *V. sororia*, stomatal density and length did not exhibit a significant relationship (slope = -0.010, $P = 0.1$; Fig. S5).

Discussion

Understanding the complex effects of climate change on organisms requires consideration of multiple variables. We examined changes in stomatal density and size for two herbaceous species of the US Midwest, and contrasted these trends with trends in flowering phenology across the same general time period. Across the ~130 yr spanning 1884-2014, stomatal lengths of *O. macrocarpa* significantly decreased at a rate of ~ -0.345 μm per decade, yielding a total decrease of ~17.5% from a mean of ~20.0 μm in the later part of the 19th century to ~16.5 μm in the early 21st century (Fig. 2H). Stomatal length in *O. macrocarpa* also declined with increasing CO_2 concentrations, although the relationship was only marginally significant (Fig. 2F). In contrast, for *V. sororia,* neither stomatal size nor density were related to year or any single environmental variable we tested, though there was a marginally significant interaction effect CO_2 concentration and temperature on stomatal length (Fig. 3). While temperature was identified as a key regulator predicting flowering time of these species, there has not been a significant shift in these species' flowering phenology across the past century (Austin et al. 2024) (Fig. 1). These analyses demonstrate that species experiencing the same macroclimate respond differently to the same environmental variables, and even though we expect warming to induce changes to stomata and phenology, responses can be decoupled within a species. In sum, understanding how changing

environments affect the degree of coupling between multiple morphological and physiological facets is essential to understanding how climate change influences species.

Numerous experimental studies have demonstrated that under higher CO_2 concentrations, plants decrease stomatal density (e.g., Woodward 1987; Xu et al. 2016), a trend that has also been found across time using long-term herbarium data (Woodward 1987; Beerling & Chaloner 1992; Large et al. 2017). A handful of studies have found a similar negative relationship between CO_2 concentration and stomatal size (e.g., Fortier et al. 2026). Our result of *O. macrocarpa* decreasing stomatal length in relation to rising atmospheric CO_2 concentrations is consistent with this prior work. Interestingly, however, our finding that stomatal density did not change across time nor in relation to CO_2 concentration contrasts with these prior studies. Several non-mutually exclusive explanations may account for these contrasting results. One notable difference of our study is that while most prior work focused on woody plants, we investigated stomatal responses of herbaceous species, which generally have different life history strategies and physiological requirements (Illuminati et al. 2024). The lack of stomatal density response found in our study may also be due to interactive environmental factors that we did not account for. For example, in some species, soil water status or local temperature may override the effect of CO_2 on stomata (Xu et al. 2016). It is also possible that the lack of stomatal density trends is due to our regional sampling of herbarium specimens across Missouri, which may spatially obfuscate stomatal responses that have occurred at a local scale.

The variation in *O. macrocarpa* and *V. sororia* stomatal length trends through time could be explained by species-specific characteristics. For example, while gene knock-out studies have demonstrated a genetic component of stomatal responses to climate change (Lang et al. 2024), the degree to which stomatal length can plastically respond to environmental variation may differ between species. If stomatal density is a fixed morphological feature of *O. macrocarpa* while stomatal length is plastic, adjusting stomatal length may be a compensatory adjustment that could allow *O. macrocarpa* to successfully respond to the stress of increased temperature, despite maintaining stomatal density (Franks & Beerling 2009). In general, anatomical compensation is not uncommon across plant species (Stewart et al. 2018, Horiguchi & Tsukaya 2011). However, we did not find stomatal length to be related to year-by-year variation in climate, which suggests

that within these species, there is only nominal plasticity in length across changing environmental conditions. Alternatively, differences in habitat may explain why *O. macrocarpa* decreased stomatal length while *V. sororia* did not. One key difference between these species' habitat requirements is soil moisture availability; *O. macrocarpa* occurs in water limited glades and rocky prairies (Kucera & Martin 1957; Moody-Weis and Heywood 2001), while *V. sororia* occurs in moist meadows and woodlands (Solbrig et al. 1980). Soil water deficit is often associated with reduction in the size of stomatal openings (Xu et al. 2016), which may lead to interactive effects between water availability and CO_2, whereby stomata are more likely to decrease in size in environments with both increased CO_2 and limited soil water content. A follow-up study that tests the degree to which stomatal length and density are plastic in these species, and whether stomatal responses are interactively affected by CO_2 and soil water content, would be able to test these hypotheses.

Besides changing stomatal size or density, one proposed strategy for stomatal optimization is altering the rate of stomatal opening and closing (Katul et al. 2010, Lawson et al. 2010, Lawson & Blatt 2014). Quick stomatal response allows stomata to open rapidly and take advantage of irradiance for photosynthesis, and also allows stomata to close quickly when conditions become unfavorable (Lawson & Blatt 2014). Smaller stomata typically respond faster to environmental cues; thus, the observed decrease in stomatal length may enable *O. macrocarpa* to increase the rate of stomatal opening and closing (Drake et al. 2018).

Life-history and physiological strategies are often shared among species within growth form, which may explain why – with the exception of stomatal length – *O. macrocarpa* and *V. sororia* have generally exhibited similar responses to climate change. Herein, although *O. macrocarpa* and *V. sororia* demonstrated a general phenological sensitivity to temperature (Fig. 1C,D; Austin et al. 2024), their shared maintenance of flowering time and stomatal density may be reflective of the strategies typical of herbaceous, slow-return species that conserve traits and resources (Wright et al. 2004, Reich 2014). However, while both *O. macrocarpa* and *V. sororia* flower in late spring, *V. sororia* generally begins flowering before *O. macrocarpa*. If phenology and stomatal architecture are related, the slightly earlier flowering time of *V. sororia* may offer a potential

explanation of why both stomatal length and density were maintained for *V. sororia*, whereas only stomatal density was maintained for *O. macrocarpa*.

Our work demonstrates that species experiencing the same macroclimate can exhibit species-specific responses to environmental variation, differences which may be related to habitat and phenology . Future work should expand upon these results by including more species along environmental gradients. Likewise, opportunities exist for examining other variables that can be measured in the field and/or from herbarium specimens such as changes in leaf and floral morphology (e.g., size, shape, thickness, leaf mass per unit area), herbivore damage, and genetics (Meineke & Davies 2018; Meineke et al. 2018). Including multiple responses will be key to understanding and forecasting species' vulnerability to global change (Green et al. 2022).

Conclusions

These findings highlight important interspecific variation in functional trait responses to global change that may reflect differences in habitat and physiological strategy. *Oenothera macrocarpa*, adapted to xeric glade (shallow-soiled prairie) environments, reduced stomatal size in response to increasing atmospheric CO_2 concentrations, a pattern that may be consistent with reduced conductance as a water-saving mechanism. *Viola sororia*, in contrast, occupies more mesic habitats and may face reduced selective pressure to alter stomatal morphology. Such divergence underscores how environmental context may mediate the degree and nature of plant physiological adaptation to global change. Continued integration of herbarium-based trait data with climate histories will be essential to forecast how plant community composition, productivity, and water balance will shift under future climate scenarios.

Supplementary Information

Appendix 1: **Table S1**. Regressions on stomatal density of *Oenothera macrocarpa* using "high- and low-confidence" stomata. **Table S2**. Regressions on stomatal density of *Viola sororia* using "high- and low-confidence" stomata. **Figure S1**. Example frame showing high- and low-confidence stomata of *Oenothera macrocarpa.* **Figure S2**. Example frame showing high- and low-confidence stomata of *Viola sororia.* **Figure S3**. Example length measurement of a *O. macrocarpa* stomate. **Figure S4.** Relationship between stomatal density and mean stomatal length for *Oenothera macrocarpa*. **Figure S5.** Relationship between stomatal density and mean stomatal length for *Viola sororia*.

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Tables

Table 1. Regressions on stomatal lengths of *Oenothera macrocarpa* using "high-confidence" stomata. The models included "frame" (portion of the leaf imprint) nested within "specimen" as random effects. Significant and marginally significant *P*-values are given in bold. "r^2m" is marginal R2 and "r^2c" conditional R^2.

	Slope	SE	t-value	*P*-value
Temporal model (r^2m = 0.102, r^2c= 0.401, df = 26.998)				
year	-1.145	0.338	-3.387	**0.002**
Climate model (r^2m = 0.1305022, r^2c= 0.4347003, df = 17.999911)				
precipitation	0.412	0.442	0.931	0.3641
temperature	0.004	0.453	0.009	0.9925
CO_2	-0.961	0.463	-2.074	**0.0527**
temp x precip	0.121	0.408	0.297	0.7698
temp x CO_2	-0.656	0.614	-1.069	0.2991
precip x CO_2	0.738	0.706	1.046	0.3096
temp x precip x CO_2	-0.722	0.635	-1.137	0.2703

Table 2. Regressions on stomatal lengths of *Viola sororia* using "high-confidence" stomata. The models included "frame" (portion of the leaf imprint) nested within "specimen" as random effects. Significant and marginally significant *P*-values are given in bold. "r^2m" is marginal R2 and "r^2c" conditional R^2.

	Slope	SE	t-value	*P*-value
Temporal model (r^2m= 0.005, r^2c= 0.494, df = 38.1815)				
year	-0.232	0.351	-0.661	0.513
Climate model (r^2m= 0.1004759, r^2c= 0.5598602, df = 24)				
precipitation	0.891	0.694	1.283	0.2114
temperature	0.260	0.653	0.398	0.6939
CO_2	0.120	0.624	0.193	0.8487
temp x precip	0.634	0.641	0.989	0.3324
temp x CO_2	1.301	0.652	1.997	**0.0567**
precip x CO_2	0.894	0.816	1.096	0.2836
temp x precip x CO_2	0.732	1.050	0698	0.4919

Table 3. Regressions on stomatal density of *Oenothera macrocarpa* using the "high-confidence" stomata counted using ImageJ. The models included "specimen" as a random effect. "r^2m" is marginal R2 and "r^2c" conditional R^2.

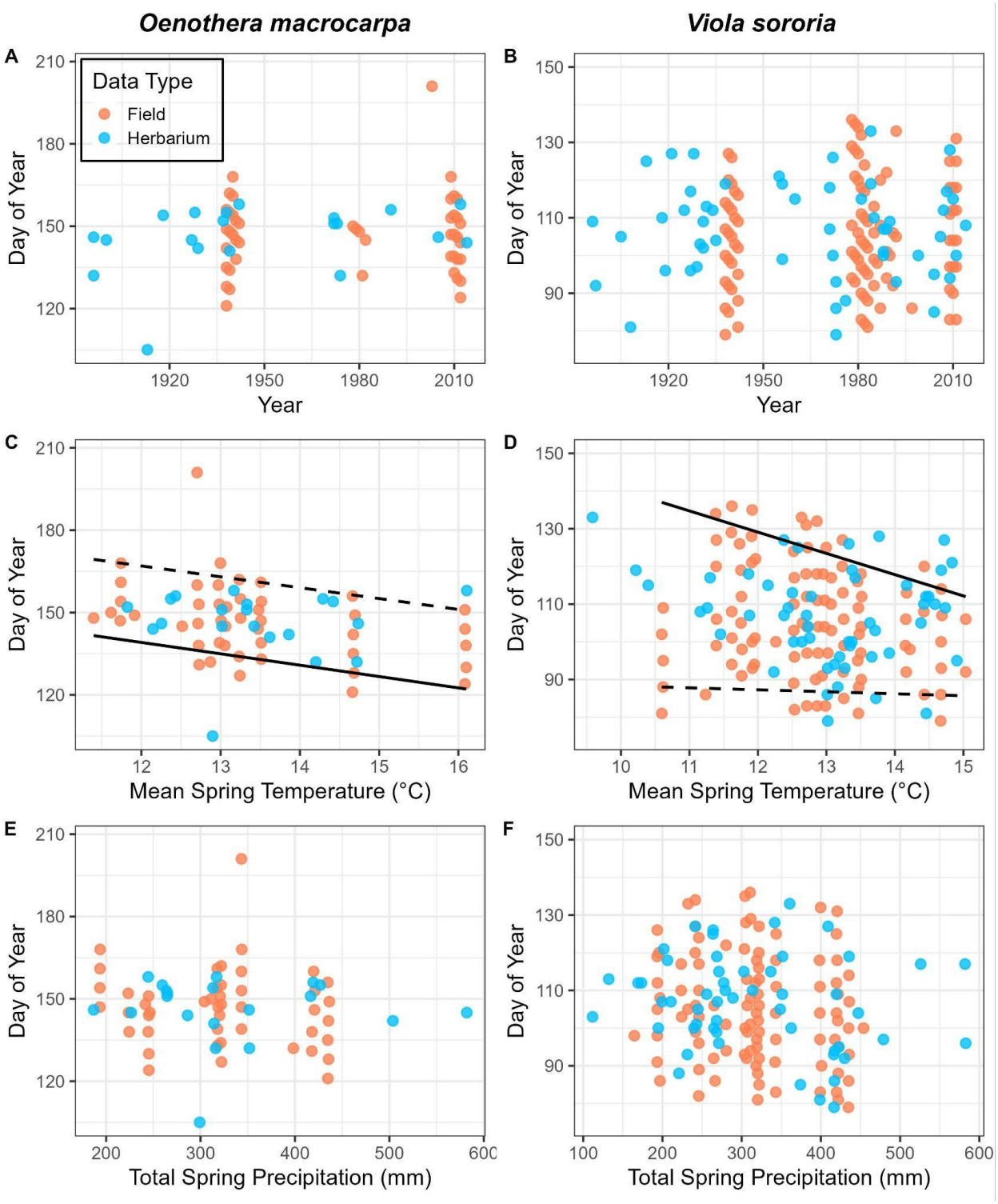
	Slope	SE	t-value	*P*-value
Temporal model (r^2m = 0.002, r^2c= 0.651, df =27.0)				
Year only	-0.026	0.095	-0.273	0.787
Climate model (r^2m = 0.080, r^2c= 0.728, df = 18.0)				
precipitation	-2.159	5.553	-0.389	0.702
temperature	-4.642	5.692	-0.816	0.425
CO_2	-0.763	5.814	-0.131	0.897
temp x precip	-3.198	5.143	-0.622	0.542
temp x CO_2	-2.021	7.738	-0.261	0.797
precip x CO_2	-11.799	8.898	-1.326	0.201
temp x precip x CO_2	5.154	8.038	0.641	0.529

Table 4. Regressions on stomatal density of *Viola sororia* using the "high-confidence" stomata. The models included "specimen" as a random effect. "r^2m" is marginal R2 and "r^2c" conditional R^2.

	Slope	SE	t-value	p-value
Temporal model (r^2m = 0.0002, r^2c= 0.674, df = 37.182195)				
year	-0.009	0.095	-0.098	0.923
Climate model (r^2m = 0.077, r^2c= 0.726, df = 24)				
precipitation	2.393	9.153	0.261	0.796
temperature	4.995	8.608	0.580	0.567
CO_2	-2.767	8.234	-0.336	0.740
temp x precip	3.125	8.472	0.369	0.715
temp x CO_2	-0.008	8.638	-0.001	0.999
precip x CO_2	-8.965	10.844	-0.827	0.416
temp x precip x CO_2	4.172	13.954	0.299	0.767

669 **Figures**

670



671

672 **Figure 1.** Flowering phenology responses to climate change for *O. macrocarpa* and *V. sororia*.

673 Flowering day of year for both species (A & B) has not shifted across the past century; (C & D)

but is sensitive to mean spring temperature; although (E & F) not to total spring precipitation. Data derive from Austin et al. (2024), and consist of field and herbarium flowering observations from the Missouri Ozark Highlands. Regression lines are the 10th and 90th quantile lines for the field data, which respectively represent responses of the beginning and ending of each species' flowering season. Solid and dashed lines represent >90% and >75% probabilities, respectively, of flowering responses, as derived from Bayesian quantile regressions. Regression lines are not shown for relationships with probabilities ≤75%. Mean spring temperature is the mean of mean monthly temperatures from March through May. Total spring precipitation is the sum of total monthly precipitation from March through May. Further details and the data used to create these plots can be found in Austin et al. (2024).

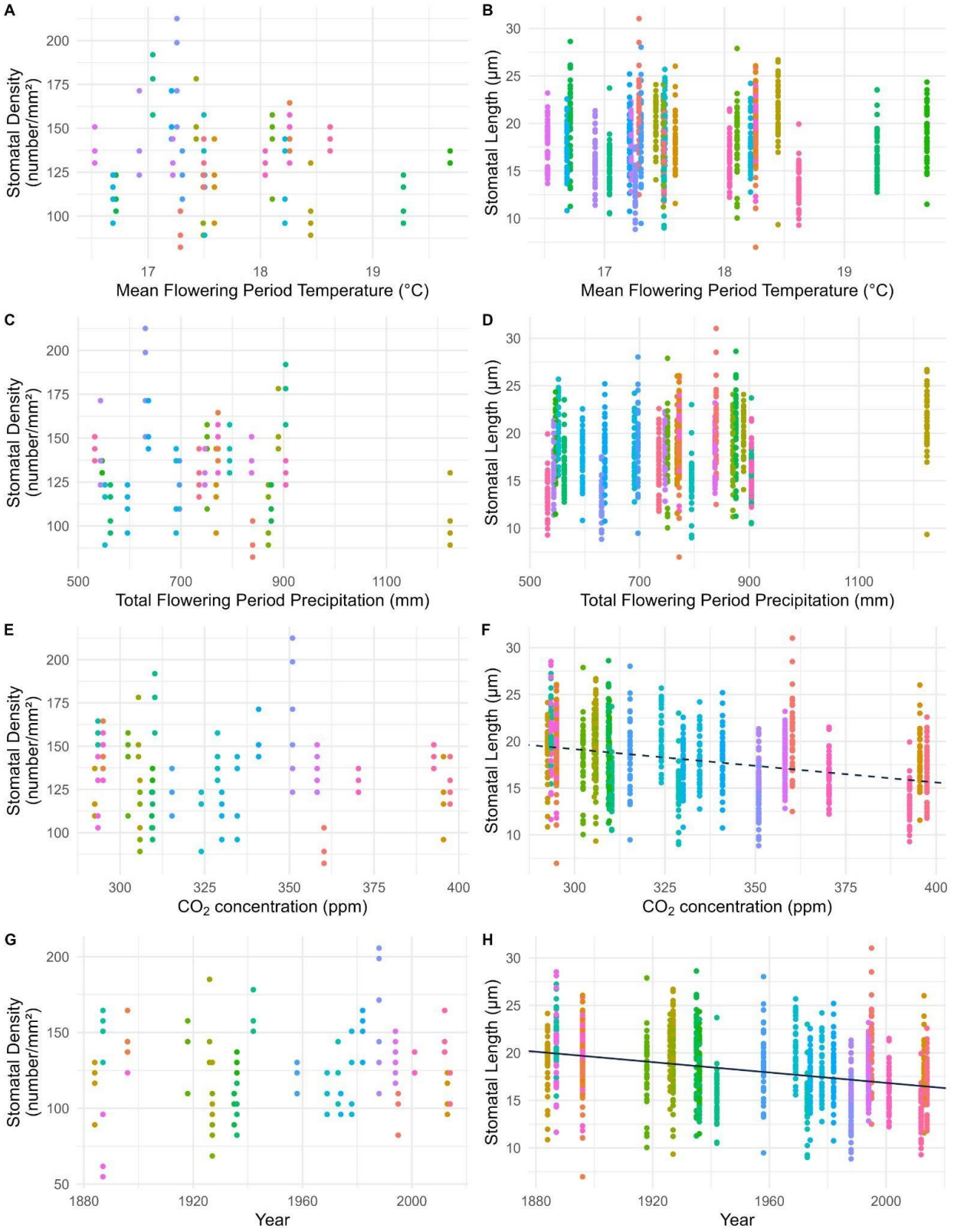


Figure 2. Responses of stomatal density (left column) and length (right column) of *Oenothera macrocarpa* to climatic variables and year of collection. Stomatal length decreased significantly

(P = 0.0218) through time (panel H) and marginally significantly (P = 0.0567) with rising atmospheric CO_2 (panel F). For stomatal density, each dot represents an estimate from a single frame (portion of a leaf imprint), with points of the same color belonging to the same specimen. For stomatal length, each point represents the size of an individual stomate, with points of the same color belonging to the same specimen.

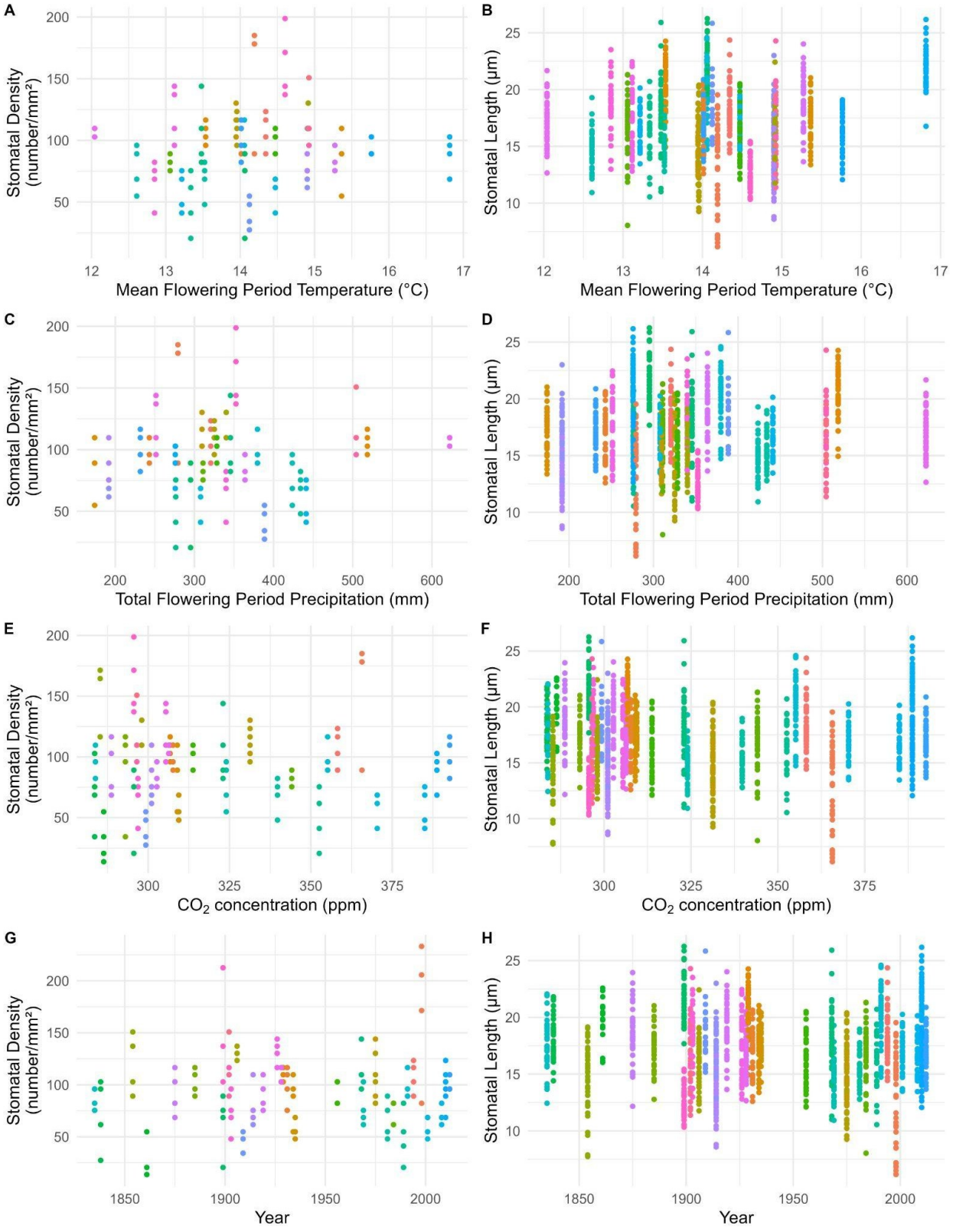


Figure 3. Responses of stomatal density (left column) and length (right column) of *Viola sororia* to climatic variables and year of collection. None of the relationships were significant (all $P >$

0.05). For stomatal density, each dot represents an estimate from a single frame (portion of a leaf imprint), with points of the same color belonging to the same specimen. For stomatal length, each point represents the size of an individual stomate, with points of the same color belonging to the same specimen.

Supplementary Information for

Stomatal responses of herbaceous plants to climate change since the 1800s: A case study using herbarium specimens

Nia Richardson[1,2,3], Léa Vilna-Santos[4], Axel Cortes[5], Mia K. Gaughan[3,4], Adam B. Smith[3], Matthew W. Austin[3]*

[1]Howard University, Washington, D.C., USA
[2]University of Michigan, School for Environment and Sustainability, Ann Arbor, Michigan, USA
[3]Missouri Botanical Garden, Saint Louis, Missouri, USA
[4]Washington University in Saint Louis, Saint Louis, Missouri, USA
[5]Collegiate School of Medicine and Bioscience, St. Louis, Missouri, USA

* Corresponding author:
Missouri Botanical Garden
4344 Shaw Blvd.
St. Louis, MO 63110
maustin@mobot.org

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Table S1. Regressions on stomatal density of *Oenothera macrocarpa* using "high- and low-confidence" stomata counted using ImageJ. The model included "specimen" as a random effect.

	Slope	SE	*t* value	*P* value
Temporal model (r^2 = 0.001, df = 27.0)				
Year only	-0.019	0.089	-0.219	0.828
Climate model (R^2 = 0.08643715 0.710779, df = 18.000)				
precipitation	-1.202	5.207	-0.231	0.820
temperature	-5.179	5.337	-0.979	0.345
CO_2	0.806	5.452	0.148	0.884
temp x precip	-3.563	4.822	-0.739	0.469
temp x $CO2_2$	-1.532	7.255	-0.211	0.835
precip x CO_2	-11.434	8.343	-1.370	0.187
temp x precip x CO_2	5.691	7.536	0.755	0.460

Table S2. Regressions on stomatal density of *Viola sororia* using "high- and low-confidence" stomata. The model included "specimen" as a random effect.

	Slope	SE	*t* value	*P* value
Temporal model (r^2 = 0.006, df = 37.7)				
year	-0.061	0.118	-0.522	0.604
Climate model (R*2* = 0.05754916 0.8162953, df = 24)				
precipitation	-1.270	11.603	-0.109	0.914
temperature	2.012	10.914	0.184	0.855
CO_2	-7.617	10.415	-0.731	0.471
temp x precip	-0.732	10.746	-0.068	0.946
temp x CO_2	-4.871	10.924	-0.446	0.659
precip x CO_2	-13.910	13.698	-1.016	0.319
temp x precip x CO_2	-6.316	17.661	-0.358	0.724

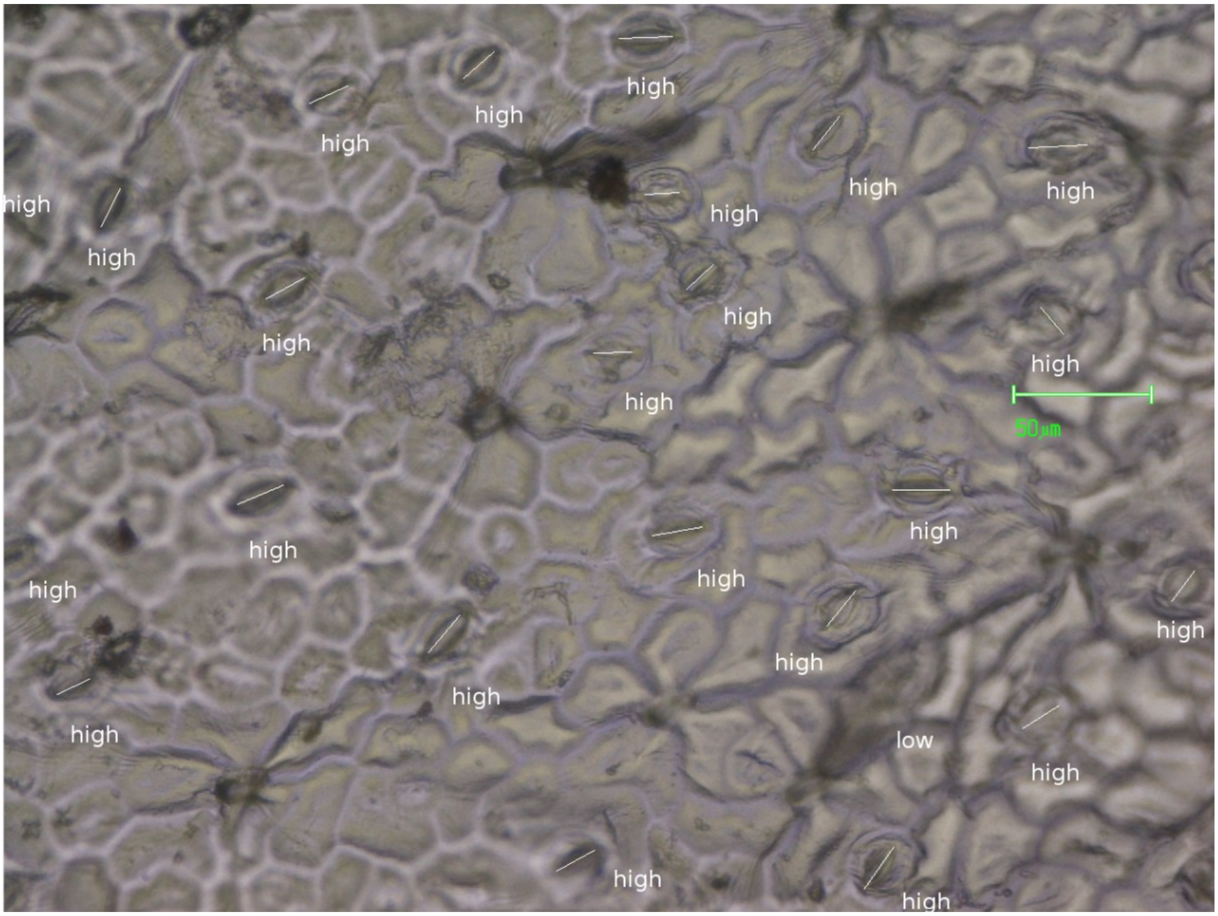

Figure S1. Example frame showing high- and low-confidence stomata of *Oenothera macrocarpa.*

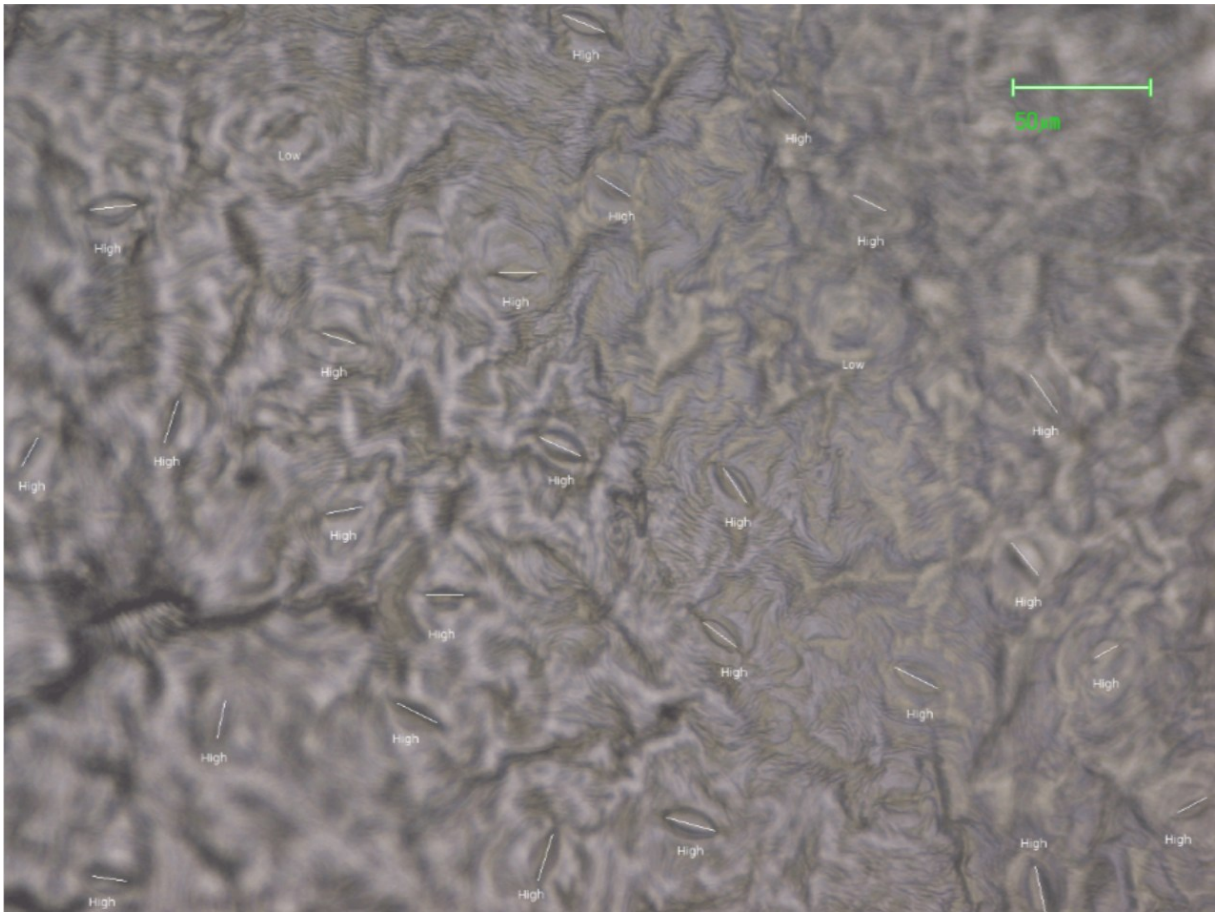

Figure S2. Example frame showing high- and low-confidence stomata of *Viola sororia.*

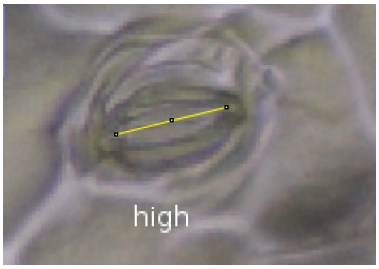

Figure S3. Example length measurement of an *O. macrocarpa* stomate. The length is the distance of the yellow line, which spans the stomatal pore.

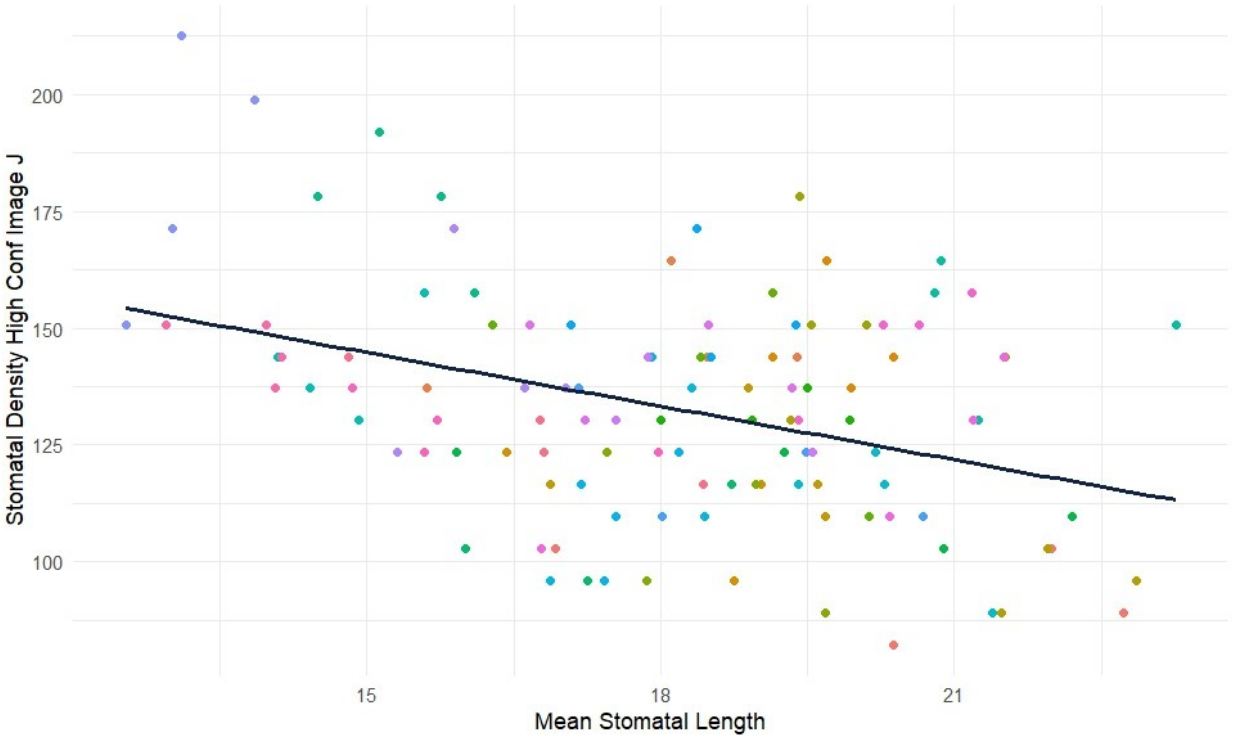

Figure S4. Relationship between stomatal density and mean stomatal length for *Oenothera macrocarpa* (slope = -0.021, $P < 0.05$).

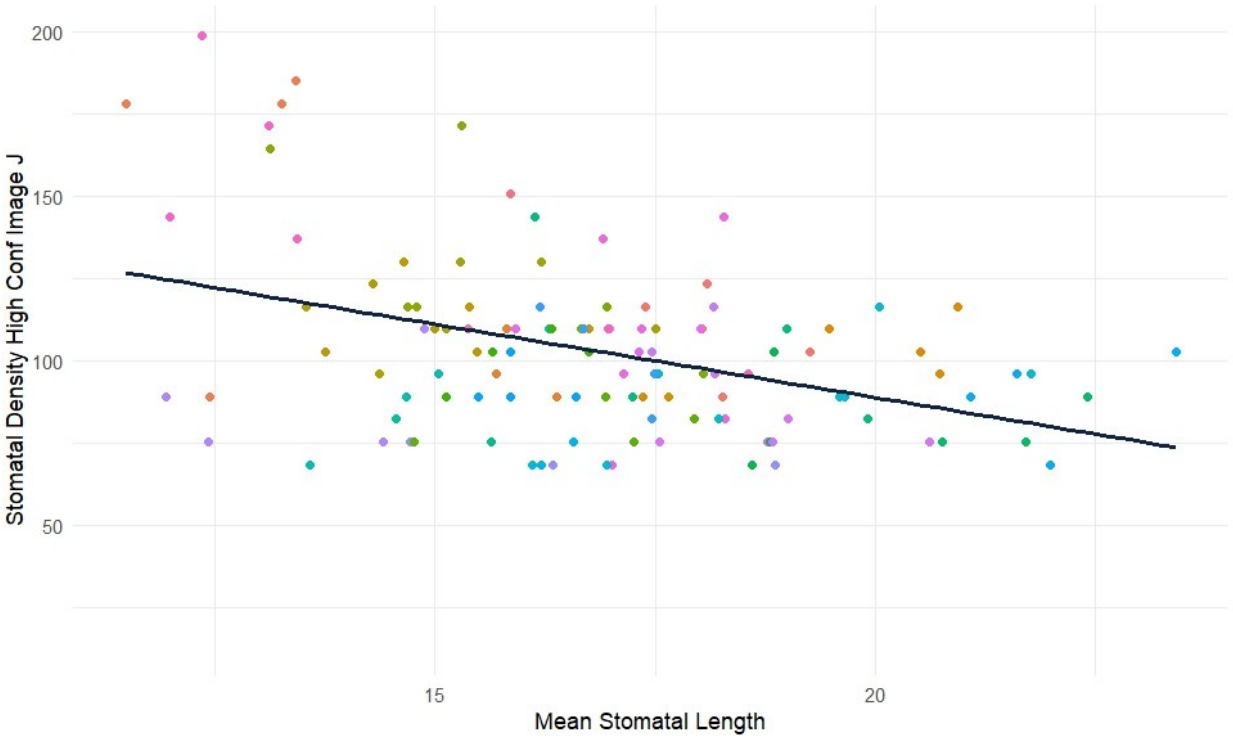

Figure S5. Relationship between stomatal density and mean stomatal length for *Viola sororia* (slope = -0.010, $P = 0.10$).