HERBARIUM SPECIMENS REVEAL A SIGNIFICANTLY EARLIER FLOWERING TREND FOR *CYPRIPEDIUM ACAULE* (ORCHIDACEAE) IN NORTH CAROLINA (U.S.A.)

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ABSTRACT

Cypripedium acaule, the pink lady's slipper, is an orchid native to eastern North America, ranging from central and eastern Canada to the southeastern United States. It is a rather common and striking spring wildflower in North Carolina (NC), extending from the mountains to the coastal plain region. A preliminary survey of herbarium specimens at Duke University (DUKE) suggested that flowering times for *C. acaule* collected in NC in recent years were notably earlier than for those from the last century. Here we set out to investigate this phenological hypothesis by accessing the Southeast Regional Network of Expertise and Collections (SERNEC) portal to extract the metadata from 57 herbaria for 502 herbarium records of *C. acaule* from NC. Of these, only 193 herbarium specimens from 55 counties, spanning the years 1886–2022, had been collected when the plant was in flower. Each of these "time-stamped" records was manually georeferenced to include latitude, longitude, and elevation coordinates, using tools in Google Earth Pro. Because NC varies by over 2037m in elevation, nearly 3° in latitude, and 10° in longitude, the flowering times recorded for *C. acaule* spanned 83 spring days. To control for the effect of location on flowering time, we implemented Hopkins' Bioclimatic Law to normalize flowering times across the state. A linear regression of the 193 normalized flowering dates suggests an overall shift in blooming that has advanced by 21 days since 1886. A mixed-effects regression was performed to determine the relationship of elevation, latitude, average winter temperature, total winter precipitation, and the total number of winter frost days from the year and location of collection (as fixed variables) on the effect of flowering day of year (DOY). The most significant effect on the flowering DOY was from average winter temperature: for each increase in 1° C, the flowering DOY was 3.23 days earlier. If this trend continues, the flowering time of *C. acaule* could become decoupled from the peak activity of its pollinators, increasing the risk of reproductive failure. This study highlights the vital role of herbarium specimens in understanding the effects of climate change on shifts in phenological patterns.

RESUMEN

Cypripedium acaule, el zapatito de dama rosa, es una orquídea nativa del este de Norteamérica, desde el centro y el este de Canadá hasta el sureste de Estados Unidos. Es una flor silvestre primaveral bastante común y llamativa en Carolina del Norte (NC), que se extiende desde las montañas hasta la región de la llanura costera. Un estudio preliminar de los especímenes de herbario de la Universidad de Duke (DUKE) sugirió que las épocas de floración de *C. acaule* recolectadas en Carolina del Norte en los últimos años eran notablemente más tempranas que las del siglo pasado. Aquí nos propusimos investigar esta hipótesis fenológica accediendo al portal Southeast Regional Network of Expertise and Collections (SERNEC) para extraer los metadatos de 57 herbarios para 502 registros de herbario de *C. acaule* de Carolina del Norte. De éstos, sólo 193 especímenes de herbario de 55 condados, que abarcaban los años 1886-2022, habían sido recolectados cuando la planta estaba en flor. Cada uno de estos registros «con sello de tiempo» se georreferenció manualmente para incluir coordenadas de latitud, longitud y elevación, utilizando herramientas de Google Earth Pro. Dado que Carolina del Norte varía en más de 2037 m de altitud, casi 3° en latitud y 10° en longitud, las épocas de floración registradas para *C. acaule* abarcaron 83 días de primavera. Para controlar el efecto de la ubicación en el tiempo de floración, aplicamos la Ley Bioclimática de Hopkins para normalizar los tiempos de floración en todo el estado. Una regresión lineal de las 193 fechas de floración normalizadas sugiere un cambio general en la floración que se ha adelantado 21 días desde 1886. Se realizó una regresión de efectos mixtos para determinar la relación de la elevación, la latitud, la temperatura invernal media, la precipitación invernal total y el número total de días de heladas invernales del año y la ubicación de la recolección (como variables fijas) sobre el efecto del día del año de floración (DOY). El efecto más significativo sobre el DOY de floración procedía de la temperatura media invernal: por cada aumento de 1° C, el DOY de floración se adelantaba 3,23 días. Si esta tendencia se mantiene, la época de floración de *C. acaule* podría desvincularse del pico de actividad de sus polinizadores, aumentando el riesgo de fracaso reproductivo. Este estudio pone de relieve el papel vital de los especímenes de herbario para comprender los efectos del cambio climático en los cambios de los patrones fenológicos.

KEY WORDS: climate change, *Cypripedium acaule*, flowering times, herbarium specimens, linear regression, North Carolina, phenology

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INTRODUCTION

"In North Carolina, as elsewhere, the flowers of spring attract more attention and are of more general interest than those of any other season" (Blomquist & Oosting 1934)

Phenology is the study of the timing of annually recurrent biological occurrences (Ettinger et al. 2022). In plants, it includes the study of changes in the timing of seasonal events, such as budburst, flowering, and fructification (Lieth 1974) and how these events may have been influenced by climate variation through time, as well as by other ecological factors (e.g., elevation). Because these phenomena can be very sensitive to small variations in climate, especially temperature, phenological records can be a useful proxy for investigating the impacts of climate change and global warming (Bolmgren et al. 2012). Herbarium records provide a historical baseline that is being increasingly used to reconstruct a record of phenological patterns going back more than 200 years (Primack et al. 2004; Davis et al. 2015; Jones & Daehler 2018; Meineke et al. 2018; Geissler et al. 2023; Park et al. 2025). Ongoing digitization efforts to mobilize herbarium specimens have made them widely available (Soltis 2017; Hedrick et al. 2020; Park et al. 2023), enabling the efficient extraction of phenological information.

Cypripedium acaule, the pink lady's slipper, is an orchid native to eastern North America, that produces two basal leaves and a distinctive solitary flower that is rosy purple in color (Blomquist & Oosting 1934). It ranges from central and eastern Canada to the southeastern United States and is found in forests and woodlands, often near pines or conifers, and occasionally in bogs or swamps (Gupton & Swope 1986). The conservation status of *C. acaule* is listed as secure (G5) globally (NatureServe 2024), and it is also listed as secure in North Carolina, where it is fairly common in the mountains (up to 1650 m) and parts of the piedmont and extends to the northern and central coastal plain region (Weakley & Southeastern Flora Team 2024). An initial cursory examination of Duke University (DUKE) herbarium specimens of the pink lady's slipper in North Carolina led to our hypothesis that it may be exhibiting earlier flowering times today than it was more than 100 years ago (Fig. 1).

Here, we set out to investigate our phenological hypothesis by accessing the Southeast Regional Network of Expertise and Collections (SERNEC 2024) portal to extract flowering dates and locations from all confirmed herbarium records of *C. acaule* from North Carolina. Our objective was to determine whether *C. acaule* was experiencing significantly earlier flowering dates now compared to the past, and if so, whether it might be related to historical trends in warming in North Carolina.

MATERIALS AND METHODS

Species Occurrence Records.—The SERNEC (2024) portal, including all Symbiota collections, was queried for all records of *Cypripedium acaule* from North Carolina. Complete record data were downloaded for a total of 502 collections from 57 herbaria into an Excel spreadsheet. The metadata from each vetted collection were sorted, as available, into their respective fields, including county, locality, elevation, latitude, longitude, habitat, collector(s), collector number, date of collection, and voucher location. Reproductive status was recorded for each collection either as flowering, fruiting, or none. Specimens that were not imaged, were without flowers, a collection date, or lacked sufficient information necessary to properly georeference them were excluded from further study (309 collections in total). Specimens that had more than one individual were treated as one sample.

Georeferencing Species Occurrence Records.—A total of 193 specimens (Supplementary Appendix 1) had metadata that accurately and consistently conveyed location data such that we were able to georeference latitude, longitude, and elevation coordinates accurately using an approach that combined Google Earth Pro (Google Earth v.7.3.2.5495, 2018) and a topographic map overlay (https://www.earthpoint.us/topomap.aspx).

Map and Figure Generation.—A vector shapefile of North Carolina counties was extracted from the national county map in US Census Bureau's MAF/TIGER geographic database (https://www.census.gov/geographies/mapping-files/time-series/geo/carto-boundary-file.html). Digital elevation model (DEM) mosaics for all 100

Fig. 1. Herbarium specimens as climate indicators. A preliminary glance through DUKE herbarium specimens of *Cypripedium acaule* from North Carolina led to our phenological hypothesis that this spring flowering orchid had experienced earlier time shifts in flowering in the last 150 years.

of North Carolina's counties were obtained at a resolution of 20 ft^2 from the NC Emergency Management GIS Section Spatial Data Download (https://sdd.nc.gov). The georeferenced coordinates were plotted onto a county map of North Carolina and a combined county DEM using QGIS (v.3.34.0; QGIS.org, 2023).

Standardizing and Normalizing Collection Dates.—To compare flowering dates across years (while also accounting for leap years) we calculated "Day-of-the-Year" (DOY) to standardize collection dates from specimens across years (Supplementary Appendix 1; Arietta 2020; Almorox & Martí 2022). We used the Excel formula [= A1 – DATE(YEAR(A1), 1, 1) + 1] to convert dates to DOY from 1900 onward. Four dates prior to 1900 were converted manually using the OMNI Calculator (https://www.omnicalculator.com/everyday-life/day-of-the-year).

Collection data from the 193 specimens were normalized by implementing Hopkins' Bioclimatic Law (HBL), which relates phenological transitions to latitude, longitude, and elevation (Hopkins 1919, 1920). Although not a true "law", it has been shown to be remarkably robust especially in eastern North America (Richardson et al. 2019) and has even been shown to be effective for studying the breeding phenology of

European roe deer (Peláez et al. 2020). Because HBL states that phenological events are shifted by four days for 1° latitude north, 5° longitude west, and 400 ft (122 m) of elevation increase (Richardson et al. 2019), we used the southernmost latitude point 33.85096° N in North Carolina (and its corresponding longitude point 78.540845 °W, and elevation of 0 ft (0 m)) to construct an Excel formula that was used to normalize

[=DAY OF YEAR-(4*(ELEVATION IN FT/400))-(4*(LATITUDE-33.85096))-(4*(((-LONGITUDE)-78.540845)/5))]

the collection DOY across specimens through time and space (each result was rounded to the nearest whole day; Supplementary Appendix 1, column Q).

Linear Regression Analysis.—When exploring phenological trends, a common method used is a linear regression analysis in R with the stats package (R Core Team, 2023), in which change is expressed as the value and direction of slope in days per year and can also be expressed as the difference in days between the beginning and end of the linear trend (Schleip et al. 2008). This method was applied here to analyze 193 phenological time points over a 136-year period in North Carolina. Using R (v.4.3.2) with RStudio (v.2023.12.0 + 369), a linear regression was performed to determine whether there was a relationship between the normalized DOY of flowering and the year of specimen collection. A scatter plot was created (also using R) to visualize the data points and the line-of-best-fit. The R code applied here is shown in Supplementary Appendix 2.

Mixed-Effects Linear Regression.—To test the partial fixed effects on the flowering DOY, a mixed-effects linear regression was performed that included elevation, latitude, average winter temperature, total winter precipitation, and total number of winter frost days at a given site from the year of collection (Supplementary Fig. 1). Longitude was excluded from this analysis because it is highly correlated with elevation in our data set ($R^2 = 0.5436$; $p = 2.2e{-}16$, standard error = 2.605e-04). The mixed-effects linear regression was performed in R with the package lmerTest (Kuznetsova et al. 2017) and with the collection site as the random effects for the model. Average winter temperature, total number of winter frost days, and total winter precipitation for each collection site for the given year of collection was extracted from CRU TS v. 4.08 (Harris et al. 2020) using the R package simpleRCRU (https://github.com/ahmad-alkadri/simpleRCRU/) for each month and averaged for January, February and March for the year of collection. Plots were generated in R using sjPlot (https://strengejacke.github.io/sjPlot/) for the fixed effects coefficient plot, using ggeffects (Lüdecke 2018) and ggplot2 (Wickham 2016) for the predicted value for average winter temperature and the total number of winter frost days, and the R package car (Fox & Weisberg 2019) for the partial regression plots for all fixed effects (Supplementary Appendix 2).

North Carolina Climate Data.—Data for North Carolina average annual and average winter temperatures were downloaded from the NC State Climate Office (products.climate.ncsu.edu/climate/trends) for the years 1895–2020. Using R (v.4.3.2) with RStudio (v.2023.12.0+369), average annual and average winter temperatures were plotted, and a linear regression was performed to determine the amount and significance in change in average temperature over the last century.

RESULTS

Results from this study support our phenological hypothesis that plants of *Cypripedium acaule* in North Carolina flower much earlier now than they did 136 years ago. Georeferenced and normalized data derived from 193 specimen records from 55 herbaria were the basis of our investigation, and their distribution across North Carolina counties is shown on a map in Fig. 2. North Carolina varies by over 2037m in elevation, and nearly 3° in latitude and 10° in longitude. Spring flowering times for *C. acaule* spanned 83 days from the years 1886–2022. Specimen locations were most dense in the Appalachian Mountains region of North Carolina, then scattered through the Piedmont to the Coastal Plain regions.

Figure 3 plots the normalized DOY of flowering (*y*-axis) against the calendar year of collection (*x*-axis) for each of the 193 specimens. A linear regression analysis resulted in a best fit equation of $y = -0.15251x + 402.47225$ ($R^2 = 0.05798$; $p = 0.00074$, standard error = 0.04448) showing a significant trend towards earlier

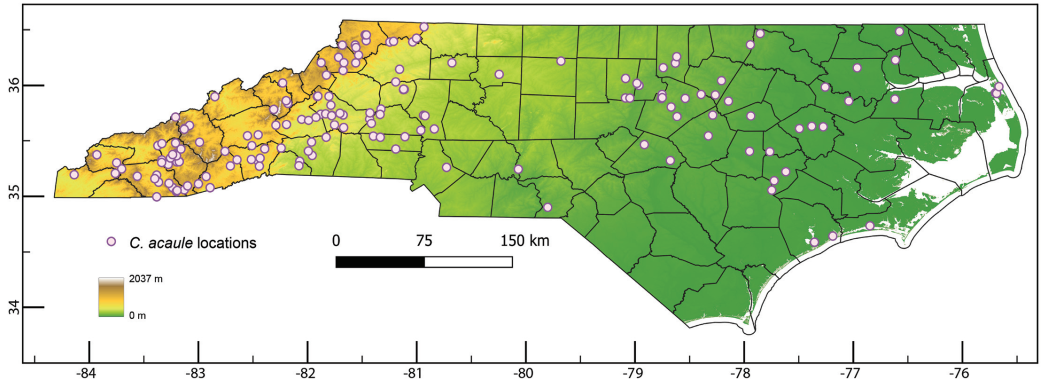

Fig. 2. Map of North Carolina collections of *C. acaule* used in this study overlaid onto a digital elevation model of NC.

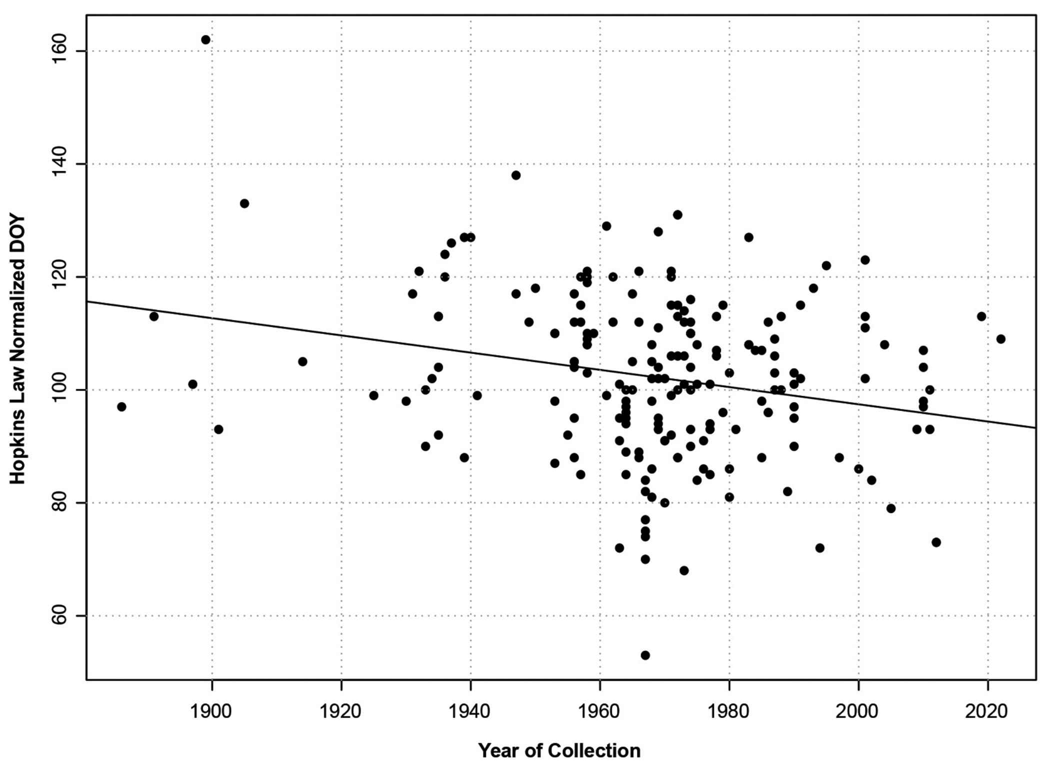

Fig. 3. Scatter plot of normalized flowering day of year (DOY) for herbarium records of *Cypripedium acaule* from NC 1886 to 2022. Line of best fit is shown: $y = -0.15251x + 402.47225$; $R^2 = 0.05305$, $p = 0.00074$. Flowering date is estimated to be 21 days earlier now than it was in 1886.

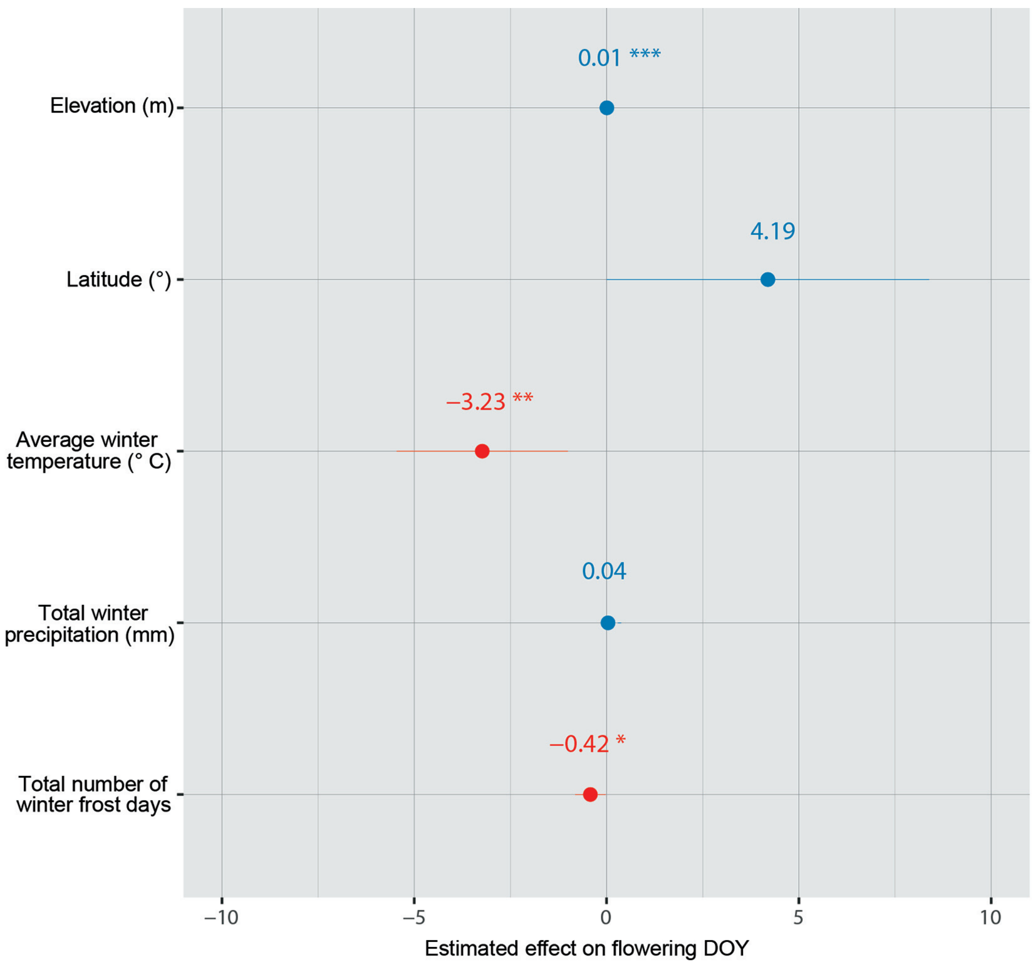

Fig. 4. A fixed effects coefficient plot displaying the estimated effect (negative effects are colored blue and positive effects are colored red) on DOY from the latitude, elevation in meters, average winter temperature in°C, total winter precipitation, and total number of winter frost days in the year of collection and the confidence intervals for each effect. Only elevation, average winter temperature, and total number of winter frost days show a significant effect. The coefficient shown is the amount of expected change on DOY from a given fixed effect. For elevation an increase in one meter has an estimated effect of increasing flowering DOY by 0.01 days. For average winter temperature an increase of one°C results in an estimated decrease in DOY of 3.23. For the effect from the total number of winter frost days, each additional frost-free day results in a decrease in the DOY by 0.42.

flowering. Extrapolating from the regression line, flowering time in 2022 has shifted 21 days earlier since 1886 (Fig. 3).

The results of the mixed-effects linear regression (Fig. 4) also accounted for random effects in the variability and differences across collection sites. A significant effect was found for: elevation (estimated effect on flowering of 0.01 days later per meter increase, standard error = 0.0023, and $p = 0.0009$), average winter temperature (estimated effect on flowering of 3.23 days earlier per °C increase, standard error = 0.39, $p = 0.0048$), and total number of winter frost days (estimated effect on flowering of 0.42 days earlier per frost day, standard error = 0.2, $p = 0.038$). Latitude and total winter precipitation do not have a significant effect on flowering DOY. Figure 5 shows linear regressions of the raw data for flowering DOY for a given elevation (y = 0.0112427 x + 122.5218), average winter temperature (y = –1.74763x + 137.7907), and total number of winter frost days (y

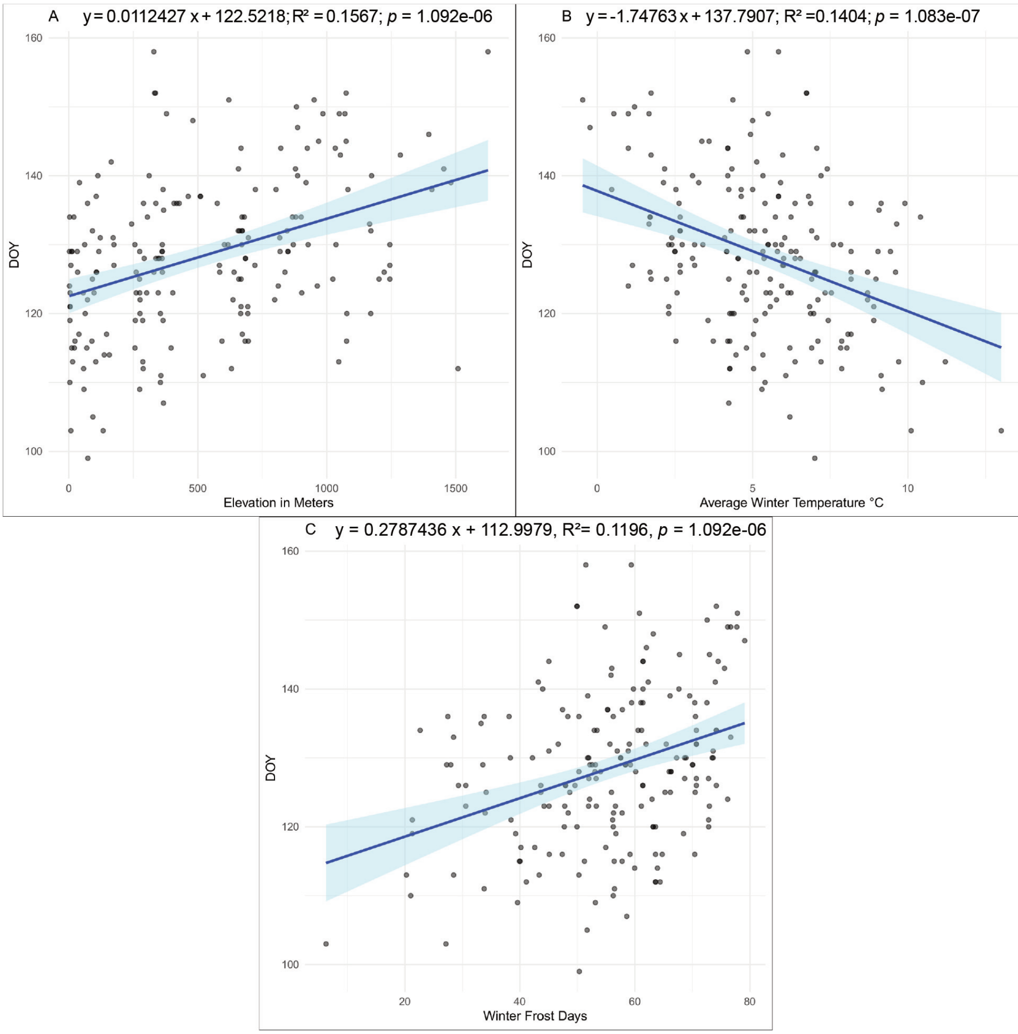

Fig. 5. Plots of linear regressions for the raw data for the most significant of the tested variables in this study on the day of year (DOY) of flowering in *Cypripedium*: elevation A., average winter temperature B., and total number of winter frost days C. The line of best fit for elevation A. shows that the lower the elevation the earlier the flowering DOY, for average winter temperature B. shows that the higher the average winter temperature the earlier the flowering DOY, and for total number of winter frost days C. shows that the fewer winter frost days the earlier the flowering DOY. Shaded bands along lines of best fit indicate 95% confidence intervals. Equations for each line of best fit are reported on each figure panel.

= 0.2787436x + 112.9979). As average winter temperature increases or the total number of winter frost days decreases, flowering DOY is estimated to be earlier as a partial effect of the regression.

Figure 6 plots the average annual (black lines) and average winter (blue lines) temperatures in North Carolina from 1895–2020. During this time period, we show that average annual temperatures have increased by 0.62 °C with a line of best fit of y = 0.005033282x + 5.00972247 (R^2 = 0.09253; p = 0.0005628). On the other hand, the average winter temperatures have increased by 1.17°C with a line of best fit y = 0.009401035x –13.198486651 (R^2 = 0.04848; p = 0.01362). This trend in warming has intensified since 1980.

DISCUSSION

Using herbarium specimens to explore phenological changes through time has become an increasingly valuable tool to inform questions about climate change impacts (Primack et al. 2004; Panchen et al. 2014; Park et al. 2018; Davis & Yost 2020; Gaira et al. 2024). Such long-term baseline phenological data can help to identify whether there have been significant changes in "Day-of-the-Year" (DOY) flowering dates over decades that can then lead to further studies to investigate whether they can be attributed to shifts in certain environmental variables such as temperature and precipitation that are influenced by climate variation.

In our study, we used herbarium specimen data spanning 136 years (collected from SERNEC 2024) to investigate our phenological hypothesis that plants of *Cypripedium acaule* in North Carolina were experiencing significantly earlier flowering dates now, compared to the past (Fig. 1). A linear regression analysis of our data determined there was indeed an important relationship between the normalized DOY and the calendar year of collection, showing a significant trend towards earlier flowering (Fig. 3, $p = 0.00074$). Extrapolating from the regression line, average flowering time in 2022 has shifted 21 days earlier since 1886 (Fig. 3).

The most significant effect on flowering time was from elevation (Figs. 4, 5). When controlling for non-environmental variables, the average winter temperature has the largest and most significant effect on flowering time. This shift is 3.23 days earlier for each °C that the winter is warmer. Changes in the total number of winter frost days are also significant, shifting flowering time 0.42 days earlier for each additional frost-free day (Figs. 4, 5).

In North Carolina, annual average temperatures have increased about 0.05°C per decade since 1895, according to the NC State Climate Office (Kunkel et al. 2020). This increase is even more pronounced (0.1°C per decade) when examined over just the winter months (December–February). Overall, average winter temperatures in North Carolina have increased 1.17°C between 1895–2020 as compared to a 0.62°C increase in average annual temperatures (Fig. 6). The global average annual temperature increase was about 1.18°C during that same period (NOAA National Centers for Environmental Information 2023). Average annual temperatures have been consistently above normal since the 1990s, with the most recent 10 years (2013–2023) being the warmest 10-year period on record. This historical warming trend in North Carolina (Fig. 6) has a striking association with the earlier flowering times we find here for roughly the same time period, even when elevation, location, and total winter precipitation are controlled for. Our results are well in line with several other studies across the northern (Primack et al. 2004; Calinger et al. 2013; Wolkovich et al. 2013; Szabó et al. 2016; Berg et al. 2019) and southern (Song et al. 2020) hemispheres that have also reported a significant correlation between earlier DOY of flowering and warming winters.

Shifts in the timing of life history events might sometimes be a positive way to cope with climate change, but they can be detrimental if changes in one species cause a misalignment in ecological relationships (Miller-Rushing et al. 2010; Visser & Gienapp 2019). When plants flower earlier than they used to, and this happens within a relatively short time span, it can impact other ecological factors at play (Badeck et al. 2004). For example, very little work has been done to investigate whether the timing of the annual activities of various species of the bumblebee *Bombus* sp., which act as primary pollinators for *Cypripedium*, have also shifted in the same direction as the orchid (Koppel & Ker 2022; Suzuki-Ohno et al. 2020). Even slight changes in flowering times can cause issues for pollination timetables. As climate change effects become more prominent, and these orchids continue to adapt to increasingly warm average winter temperatures, this could most likely lead to the disruption of these intimate ecological relationships.

Our study would not have been possible without the continued conservation and preservation of herbarium collections. Herbaria are the product of the collective hard work of generations of botanists. These collections contain snapshots of the life history of plants through time and space. The role of herbaria in contributing data to climate change research studies is vital for understanding the effects of shifting phenological patterns.

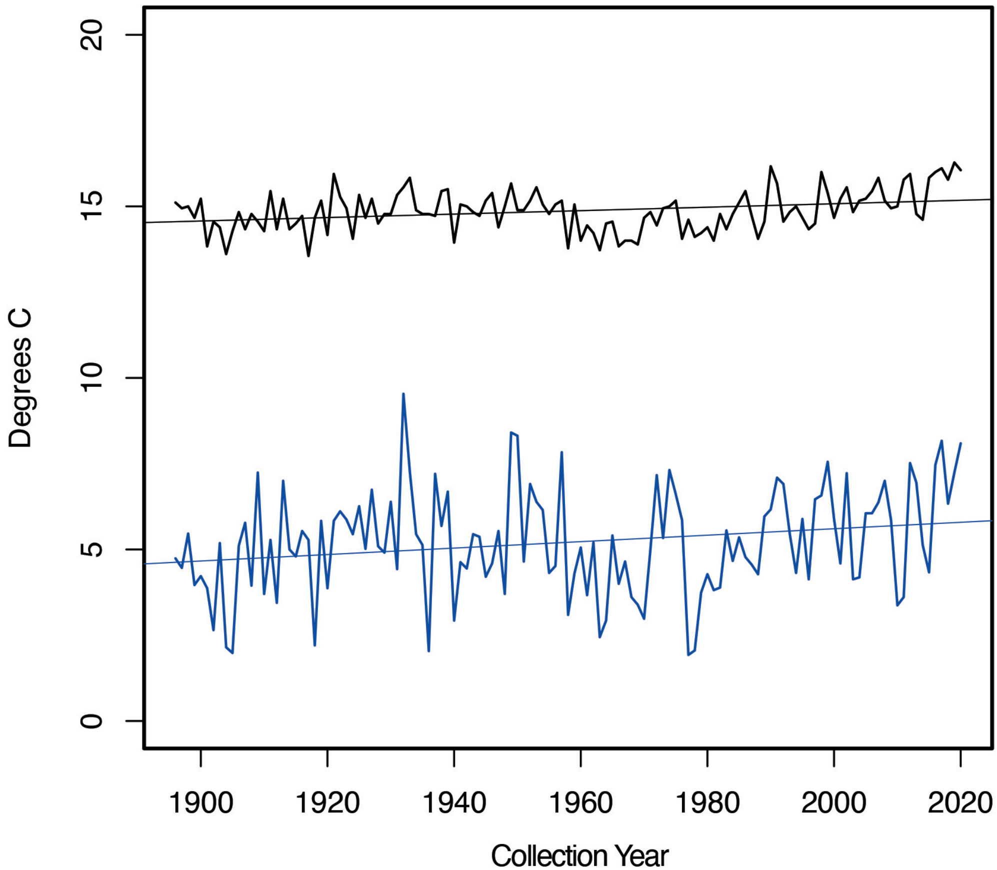

Fig. 6. The annual average temperatures (black; y = 0.005033282x + 5.00972247 ($R^2 = 0.09253$; $p = 0.0005628$) and average winter temperatures (blue; y = 0.009401035x -13.198486651 ($R^2 = 0.04848$; $p = 0.01362$) across North Carolina from 1895–2020. The change in annual average temperature is 0.62°C and 1.17°C for the average winter temperature.

DATA AVAILABILITY

All supplemental files are available at https://github.com/nikolaihay/Supplementary-data-for-Flowering-times-for-Cypripedium-acaule-Orchidaceae-manuscript.

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