



Grass flowering times determined using herbarium specimens for modeling grass pollen under a warming climate

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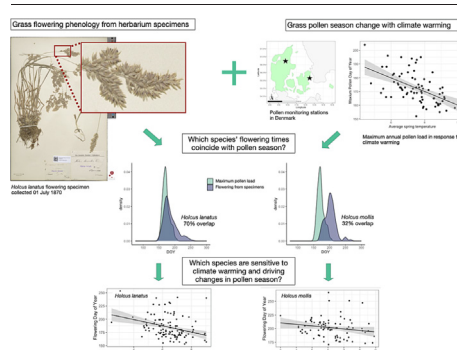
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HIGHLIGHTS

- Herbarium specimens were used to identify flowering times for 12 grass species over long time series
- Coincidence in specimen flowering time with the pollen season and flowering response to climate warming were determined
- Seven grass species overlap with the time of maximum airborne pollen detection
- Four grass species show shifts in flowering time in response to warming temperatures
- Herbarium collections can contribute to forecasting pollen allergy seasons

GRAPHICAL ABSTRACT



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ABSTRACT

The grass family is responsible for most of peoples pollen allergies, and the severity of pollen-based asthma and allergies is expected to increase with global climate change. Identifying grass species through standard pollen monitoring techniques have limitations due to challenges in species-specific pollen identification. As a result, these monitoring methods end up grouping all Poaceae species together, even though there are hundreds of grass species in Europe with flowering times that may vary drastically among species. Given this lack of specificity, it is hard to know which grass species are responsible for causing allergies over the pollen season, and how different species are affected by climate change. To address these issues, we obtained phenological data from thousands of herbarium specimens collected across Denmark spanning 190 years and used pollen monitoring data collected over the last four decades to determine the response of flowering time to climate change for 12 allergenic grass species, and identify which species are likely the biggest contributors to grass pollen loads throughout the pollen season. We find that pollen season duration is lasting longer and starting earlier, and the maximum pollen loads are occurring earlier in response to climate warming. Herbarium specimens provide taxonomic resolution and reveal that many grass species are flowering earlier in response to warmer spring temperatures. Seven out of the 12 species studied in Denmark are identified as major contributors to airborne pollen based on their flowering times, relative abundance and overlap with the time of the year when maximum pollen loads are detected. Four species (*Poa pratensis*, *Dactylis glomerata*, *Festuca rubra*, *Holcus lanatus*) significantly shifted their flowering time in response to warming temperatures and are flagged as of particular concern to allergy sufferers. Using data derived from natural history collections can contribute to the advancement of pollen forecasting for asthma and allergy patients under both current conditions and amidst future global changes.

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1. Introduction

The Poaceae or grass family is one of largest plant families in the world and provides numerous agricultural, ecological, and socioeconomic benefits (Hodkinson, 2018). Grasses are also recognized as among the top allergenic pollen producing plants, due to their copious production of wind-borne pollen (Ziska, 2021). While grass pollen in general is a major public health concern, the contribution of individual grass species to allergies and asthma is relatively under-studied (García de Leon et al., 2015; García-Mozo, 2017; Zhang et al., 2015; Rowney et al., 2021).

Over the last 50 years, there has been a notable rise in asthma and allergies worldwide, which has been attributed in part to factors associated with anthropogenic climate change including expansion of growing seasons and increases in temperature and CO₂ (Ziello et al., 2012; Fernandez-Rodriguez et al., 2016; D'Amato et al., 2020). Changes in the start of the pollen season and increases in its duration and severity have been reported across the Northern Hemisphere (Zhang et al., 2014; Ziska et al., 2019; Anderegg et al., 2021; Gehrig and Clot, 2021; Adams-Groom et al., 2022; Hanson et al., 2022). Under future climate change scenarios (IPCC, 2013; Collins et al., 2013), the severity of pollen-based asthma and allergies is expected to increase due to prolonged flowering periods, extreme weather events and atmospheric changes (Zhang and Steiner, 2022). Some predict that climate change may lead to as much as a doubling in allergy cases (Grewling et al., 2012; Schmidt, 2016). There is an urgent need to study the seasonality of grass flowering given that climate change will likely alter patterns of grass phenology and abundance in coming decades (García-Mozo, 2017).

To inform health professionals and asthma and allergy patients, pollen data is collected daily from monitoring stations across Europe and published by agencies such as Astma-Allergi Danmark (AAD), the Danish Meteorological Institute (DMI), and the European Allergy Network (www.ean-net.org). The airborne pollen data are used to make predictions on the on-set, duration, and severity of the pollen season in a particular region (Ziello et al., 2012; Smith et al., 2014). Allergenic pollen monitoring and forecasting for grass pollen, however, is limited in two ways: 1) by difficulties in identifying the species of grass pollen, and 2) by the sparsity of long-term monitoring datasets. Difficulties in identifying species based on pollen grains from pollen traps and a poor understanding of what species are flowering at specific time points from the grass family make it challenging to assess which grass species pose the largest risk to human health (Fernandez-Rodriguez et al., 2016; García de Leon et al., 2015; Zhang et al., 2015; Ghitarrini et al. 2017). In practice, all types of airborne grass pollen from monitoring stations are typically lumped together, without distinguishing species. Furthermore, airborne pollen monitoring only dates back 40–50 years and is spatially limited (i.e., there are only two monitoring stations in Denmark: Copenhagen and Viborg). This lack of species-specific resolution, lack of longer-term data, and poor spatial coverage hinders our ability to determine historical and region-specific trends in grass pollen in response to climate, and limits accurate modeling and forecasting of allergen-producing grass species under projected climatic change (Zhang and Steiner, 2022).

Phenological data linked with airborne pollen monitoring data allows for the identification of the species whose flowering coincides most closely with the maximum airborne pollen concentrations (Romero-Morte et al., 2018). While a number of field-based phenology studies exist for the grass family in Europe, they are from very localized areas including urban and peri-urban areas in Spain, Italy, and Austria (Frenguelli et al., 2010; León-Ruiz et al., 2011; Tormo et al., 2011; Kmenta et al., 2016; Ghitarrini et al. 2017; Cebrino et al., 2018). These studies are limited to studying flowering phenology over one to three seasons (apart from one study carried out over 13 years, see Cebrino et al., 2016). Such studies are time consuming to conduct and require specialized botanical training to be able to identify species in the field, limiting their potential for contributing to studies of allergy-causing grass pollen. Furthermore, their short-term nature doesn't allow for detecting phenological responses to climate over time and in relation to temperature, as a greater number of years are needed to ensure that inter-annual fluctuations in warm years and cool years are

reflected in the data (Iwanycki Ahlstrand et al., 2022). The use of DNA metabarcoding methods show considerable potential in characterizing individual genera in pollen (Brennan et al., 2019; Hanson et al., 2022) and demonstrate that airborne pollen generally corresponds to local grass species observed in flower and the timing of prescribed allergy medicines (Rowney et al., 2021). However, DNA metabarcoding methods are not always able to distinguish grass species within a genus, and distinguishing between certain key genera such as *Agrostis* and *Alopecurus* has not been possible (Brennan et al., 2019; Rowney et al., 2021). In addition, the high costs associated with this method make it impractical to carry out in large monitoring programs, and because past pollen monitoring samples are not archived, it also cannot be used to investigate historical change over time.

As an alternative approach, herbarium specimens offer enormous potential in global change studies as they provide long-term species records of flowering phenology over extensive spatial scales and past time periods (Lang et al., 2019; Primack and Gallinat, 2017). In particular, herbarium specimens can help to fill a gap in species specific phenology knowledge in the grass family (Munson and Long, 2017). However, it remains unknown how information of the flowering phenology of individual grass species obtained from herbarium specimens corresponds to the airborne pollen record of a region. Furthermore, while the annual mean temperature in Denmark has increased significantly by 0.01 °C per year during the period 1768–2019 (Cappelen et al., 2020; Iwanycki Ahlstrand et al., 2022) it is unknown how this warming influences grass flowering and the grass pollen season. To fill these gaps, we used extensive herbarium collections and long-term airborne pollen monitoring data from Denmark to address the following questions:

- 1) Has the timing of the airborne pollen season and the flowering periods for key grass species changed over time in Denmark, and, if so, what climatic factors are responsible for the annual variation?
- 2) Can herbarium specimens help determine which species are most likely responsible for contributing to the peak grass pollen season in Denmark?
- 3) Can herbarium specimen phenology provide insight into how the pollen season is responding to climate warming?

Our study helps to address the gap in knowledge regarding species-specific contributions to the pollen season, and their response to climate change.

2. Materials and methods

2.1. Data collection

2.1.1. Pollen data

Pollen season monitoring data for the grass family (Poaceae) was collected from 1977 to 2019 by Astma-Allergi Danmark and the Danish Meteorological Institute (Astma Allergi Danmark, 2021). Data were obtained using 7-day volumetric spore samplers of the Hirst design (Hirst, 1952) installed at two pollen monitoring stations (positioned at heights of 15 m above sea level) in Denmark: 1) Copenhagen on the roof of the Danish Meteorological Institute (55°43'N 12°34'E), and 2) Viborg on the roof of Regional Hospital (56°27'N 9°24'E). A total of 9430 daily observations were available for the pollen seasons over these four decades. Daily pollen level data for the Poaceae (daily average number of pollen grains per cubic meter detected in the air over a 24 h period) were summarized following the 5 percentage method to estimate the pollen season, a procedure comparable to other studies (Nilsson and Persson, 1981; Estrella et al., 2006; Zhang et al., 2014; Ghitarrini et al. 2017; Panchen and Johnston, 2018). The following pollen season parameters were calculated: the day of the year (DOY) in each sampling year when 5 % of the total pollen loads were obtained (start DOY [5 %]), the DOY for the highest pollen count obtained each year (maximum pollen DOY), the DOY in each sampling year when 95 % of the total pollen loads were obtained (end DOY), and the duration of the pollen season from start DOY to end DOY (duration [5 %–

95 %)). We acknowledge that applying different pollen season definitions can influence the degree and directionality of trends detected in a pollen season (Gehrig and Clot, 2021; Tasioulis et al., 2022); however, the highest pollen DOY (season peak date) remains comparable across all definitions (Tasioulis et al., 2022) and is therefore included in our comparisons of pollen season and flowering phenology.

2.1.2. Herbarium data

Digitized herbarium specimens (high-resolution digital photos) in the grass family collected in Denmark over a 200-year period were examined at the Natural History Museum of Denmark (Herbarium C). Specimens representing 12 common species from 10 genera in the Poaceae family were selected for further analyses (Table 1) based on the allergenic pollen production previously reported for these genera (Prieto-Baena et al., 2003; León-Ruiz et al., 2011; Brennan et al., 2019). All 12 of these species are considered native to Denmark, and are widespread, perennial grasses (Hartvig, 2015). Where possible, multiple species were selected from a single genus (*Alopecurus*, *Holcus*) to investigate the degree of intraspecific differences in flowering times.

A total of 3854 specimens from Denmark were available for the 12 species, as their labels had complete dates (collection day, month, and year) and location information. For each specimen, we recorded date of collection (hereafter, day of year), collection locality, and the topographical botanic unit (TBU) district number, a landscape classification system that divides Denmark into 57 floristic units (Lawesson and Skov, 2002). All phenological assessments were performed by an experienced botanist trained in grass morphology. Phenophase categories followed a modified protocol of Yost et al. (2018) to ensure for congruence with other published herbarium studies, and these phenological categories were translated to the BBCH scale (Meier, 1997). High resolution digital specimen images which allowed for the magnification of the small grass florets were used for determining the following phenophase categories for each specimen: immature flower (BBCH = 51 to BBCH = 59), early flower (BBCH = 60 to BBCH = 61), peak flower (BBCH = 61 to BBCH = 67), late flower (BBCH = 67 to BBCH = 68), early fruit (BBCH >69), peak fruit (caryopses visible) (BBCH >69), and late fruit (fruiting spikelet dehiscing) (BBCH >93). If anthers from the male flower parts (which bear pollen) or the stigmas from the female flower parts were visible on greater than half of the florets within a grass inflorescence, a phenophase of peak flowering was assigned; however, this wasn't always visible, depending on the traits of a species' florets, and in such cases flowering phenophase was assumed providing specimens resembled others confirmed to be flowering. Flowering specimens

(BBCH = 61 to BBCH = 67) accounted for 77 % of the samples. When we omitted specimens in the fruiting stage, specimens that were not reproductive, specimens without complete collection dates and locality data, and specimen that were extreme outliers (such as flowering in the months January and February), we were left with 2122 flowering specimens collected between 1825 and 2014 (Table 1), which provided the data for this study.

2.2. Climatic variables

Historical monthly weather data for the period from 1768 to 2019 was obtained from the Danish Meteorological Institute (DMI) for five weather stations in Denmark (Cappelen et al., 2020). Average monthly temperatures (degrees Celsius) and monthly precipitation (mm) for March, April, May, and June of the year of flowering were used in the analysis. Average spring temperature and average spring precipitation were calculated by combining the monthly averages for March, April, and May for temperature and precipitation variables, as three-month averages (for temperature) in the season before flowering have been found to be strong predictor variables in other phenology studies (Jones and Daehler, 2018). Each observation in our herbarium and pollen datasets was linked to its closest weather station and matched with the climate variables corresponding to the day of year from the specimen and/or pollen observation. We initially included latitude as a factor in our analysis, but because this was not significant beyond what would be accounted for by local weather conditions, we omitted this variable in later analyses.

2.3. Candidate species linked to pollen production throughout the season

To identify candidate grass species producing pollen coinciding with the pollen season parameters, kernel density plots were created in R to graphically represent distributions and visualize of the overlap in flowering DOY for each grass species and the entire pollen season, the start DOY, and maximum pollen DOY. Kernel density plots take each observation from a data set and a Gaussian bell curve is drawn over it, then all bell curves are summed and a final curve is drawn (Howell, 2010). This approach is better suited than histograms for comparing data, as the distribution of observations in density plots are not affected by the number of bins used. Two herbarium data sets were compared: the full herbarium data (1825–2014) and a reduced herbarium data spanning the same time of the pollen monitoring (1977–2014; note that there were no flowering specimens collected between 2015 and 2019 at Herbarium C). The percentage of overlap between the species-specific flowering periods derived from specimens and

Table 1

Species investigated from herbarium specimens, including number of specimens for each species from the full (1825–2014) and reduced (1977–2014) data sets, and the earliest (Min), mean, and latest (Max) flowering day of year and standard deviation (SD) for each species based on the full data set. Habitat information follows Hartvig (2015).

Species name	No. flowering specimens / total specimens checked FULL DATA	No. flowering specimens / total specimens REDUCED DATA	Min DOY	Max DOY	Mean DOY	SD	Habitat
<i>Agrostis gigantea</i> L.	300 / 475	141 / 197	141	247	203	17.5	rough grasslands
<i>Alopecurus geniculatus</i> L.	130 / 213	3 / 6	123	249	174	25.9	wet areas
<i>Alopecurus pratensis</i> L.	124 / 192	11 / 12	120	310	159	29.0	moist grasslands, meadows
<i>Cynosurus cristatus</i> L.	125 / 156	9 / 10	145	252	181	19.3	moist grasslands
<i>Dactylis glomerata</i> L.	258 / 460	35 / 40	126	242	182	21.9	meadows, pastures, roadsides
<i>Elytrigia repens</i> (L.) Desv.	89 / 581	10 / 14	166	246	200	17.4	meadows, roadsides
<i>Festuca rubra</i> L.	284 / 491	38 / 45	135	229	171	15.7	shaded meadows
<i>Holcus lanatus</i> L.	132 / 179	11 / 12	153	253	185	20.3	meadows, roadsides
<i>Holcus mollis</i> L.	104 / 138	9 / 9	171	266	202	17.1	meadows
<i>Lolium perenne</i> L.	166 / 290	15 / 17	121	296	188	34.8	meadows, pastures, roadsides
<i>Phleum pratense</i> L.	213 / 326	11 / 15	123	247	190	18.3	meadows, roadsides
<i>Poa pratensis</i> L.	198 / 353	18 / 23	127	195	166	13.2	meadows, pastures, roadsides
Total	2122 / 3854	311 / 400					

the pollen season parameters were calculated using the “overlapping” and “lattice” packages in R (Pastore, 2018; Sarkar, 2008). Furthermore, because the relative abundance of a particular species is likely to be a factor in the amount of pollen released to the atmosphere, we used two measures of abundance for each species in Denmark, the percentage of atlas squares covered by each grass (Hartvig, 2015), and the total number of specimens collected for each species (Gotelli et al., 2021).

2.4. Effect of year and climate on airborne pollen and flowering times

2.4.1. Airborne pollen

To determine the change in timing of the pollen season over time (42 years), we conducted simple linear regressions for each of the pollen season parameters start DOY, maximum pollen DOY, end DOY, and duration, as described above, using year as the predictor variable. Similarly, we used simple linear regressions to determine the response of the pollen season parameters to changes in temperature and precipitation, running separate analyses for each pollen season parameter and each of the following predictor variables: average temperatures in March, April, May, June, and July; average spring temperature (average of the monthly means for March, April, and May), average annual temperature, average precipitation in April, May, June, and July; average spring precipitation (average for March, April, and May). As we did not find any significant correlation between precipitation and any of the phenological metrics and precipitation, we exclude it from further analysis. Similarly, we did not find any significant differences between the two pollen stations, so this was not a focus in this study.

2.4.2. Herbarium specimen data

To assess how individual grass species' flowering time has shifted over time and/or has responded to annual differences in temperature and precipitation, we ran simple linear regression analyses for each species using either year, or individual climatic variables as described above for pollen data. As a methodological note, we ran all analyses on the flowering phenophase only, as well as data including all reproductive phenophases. Only slight differences were found between using specimens covering all reproductive phenophases versus just flowering specimens with respect to the effects of spring temperature, indicating that our method would still be valid if it is not possible to distinguish between reproductive phases in

the grass family. However, we did find that including the later phenophases increased the effect of year (data not shown). All analyses were run in R v.4.1.3. A level of $p < 0.05$ was used to define level of significance. Outliers were not removed from our analyses. We acknowledge that herbarium specimens that demonstrate late flowering may represent grasses collected in areas that were subject to disturbance (mowing or grazing); however, because this investigation focuses on identifying candidate species coinciding with the pollen monitoring data, late-flowering observations were retained as they may still contribute to pollen loads.

3. Results

3.1. Candidate species linked to pollen production throughout the season

The grass pollen season in Denmark spans from May until August, with the maximum pollen levels occurring in June (Fig. 1). The number of flowering specimens that were collected from the period matching that of the pollen data (reduced data set) ranged from 3 to 141 (Table 1). At least half of the species had fewer than 15 specimens available for this time period, making the reduced data set not as statistically robust for analyses; however, the patterns observed in the density graphs for the reduced data set were very similar to that of the full data (Table 2, Fig. S1, Supplemental Material). The mean, minimum and maximum flowering DOYs for the reduced data set were 188, 130, and 286, while for the full data set they were 183, 120, and 310. The full data set, which spans 190 years, captures a greater spread of natural phenological and climatic variability.

Mean flowering DOY for each of the grass species spans 44 days (Table 1) with *Alopecurus pratensis* (mean DOY = 159) being the earliest species and *Holcus mollis* (DOY = 202) and *Agrostis gigantea* (DOY = 203) being the latest species. The species with the longest flowering periods (calculated as max flowering DOY – min flowering DOY) are *Alopecurus pratensis* (190 days) and *Lolium perenne* (175 days), while the species with the shortest flowering is *Poa pratensis* (68 days).

Flowering times of all the 12 grass species combined fall completely within the curve of the full grass pollen season, yet species-specific differences between flowering time and duration are evident (Fig. 2). Certain species had a very high percent of overlap in their flowering season with the full duration of the pollen season, with 79 % for *Lolium perenne* (Table 2). The overlap of airborne pollen with flowering of *Alopecurus*

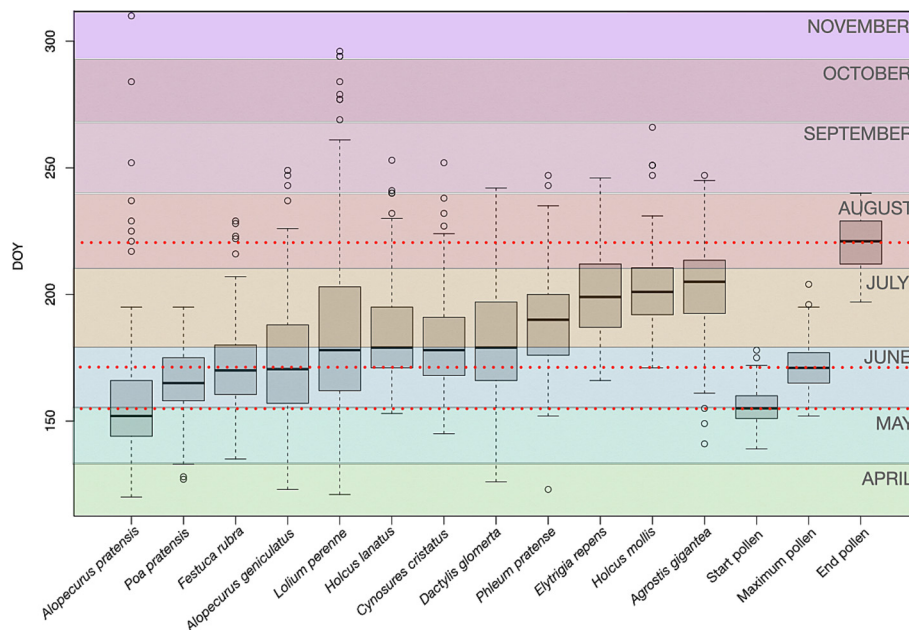


Fig. 1. Boxplot showing the flowering DOY ranges for 12 grass species in Denmark based on 190 years of herbarium phenology data, and the maximum pollen DOY from 42 years of pollen monitoring data. Bold lines within boxes indicate the Mean flowering DOY, and the three dashed red lines indicate (from bottom to top) mean DOY for start, maximum, and end of the pollen season.

Table 2

Percent of overlap in species-specific flowering DOY from the full herbarium dataset (reduced data presented in square brackets “[]”) with the pollen season, start of the pollen season (start DOY), and maximum pollen loads (maximum DOY). Relative species' abundances based on the percent atlas squares occupied by each species (Hartvig, 2015), and the number of specimens in the collections at Herbarium C, Natural History Museum of Denmark. Density plots illustrating the overlap between maximum pollen DOY and flowering of each grass species are presented in Fig. S2 for the full dataset and in Fig. S3 (Supplemental Material), and distribution maps for each species are presented in Fig. S3 (Supplemental Material).

Species	% overlap with pollen season	% overlap with start of season	% overlap with maximum pollen	% of atlas squares covered	Number of specimens
<i>Agrostis gigantea</i>	57 [55]	9 [6]	31 [25]	72	475
<i>Alopecurus geniculatus</i>	70 [32]	49 [25]	63 [80]	96	213
<i>Alopecurus pratensis</i>	49 [61]	65 [59]	42 [44]	85	192
<i>Cynosurus cristatus</i>	60 [75]	32 [38]	73 [68]	66	156
<i>Dactylis glomerata</i>	69 [66]	35 [35]	64 [67]	100	460
<i>Elytrigia repens</i>	57 [59]	10 [14]	37 [43]	100	491
<i>Festuca rubra</i>	52 [52]	48 [52]	81 [79]	100	581
<i>Holcus lanatus</i>	61 [79]	27 [43]	70 [67]	99	179
<i>Holcus mollis</i>	53 [38]	7 [9]	32 [45]	77	138
<i>Lolium perenne</i>	79 [81]	38 [39]	58 [62]	100	290
<i>Phleum pratense</i>	59 [68]	19 [25]	53 [59]	97	326
<i>Poa pratensis</i>	47 [35]	57 [72]	78 [68]	100	353

pratensis (49 %) and *Poa pratensis* (47 %) were much lower. Three species had high overlap with the start of the grass pollen season: *Alopecurus geniculatus*, *Alopecurus pratensis*, and *Poa pratensis*. *Poa pratensis* and *Festuca*

rubra had the highest percent of overlap with the dates of maximum pollen loads (Fig. S2, Supplemental Material [SM]). Other grass species had lower percent of overlaps. Percent overlap between individual species flowering

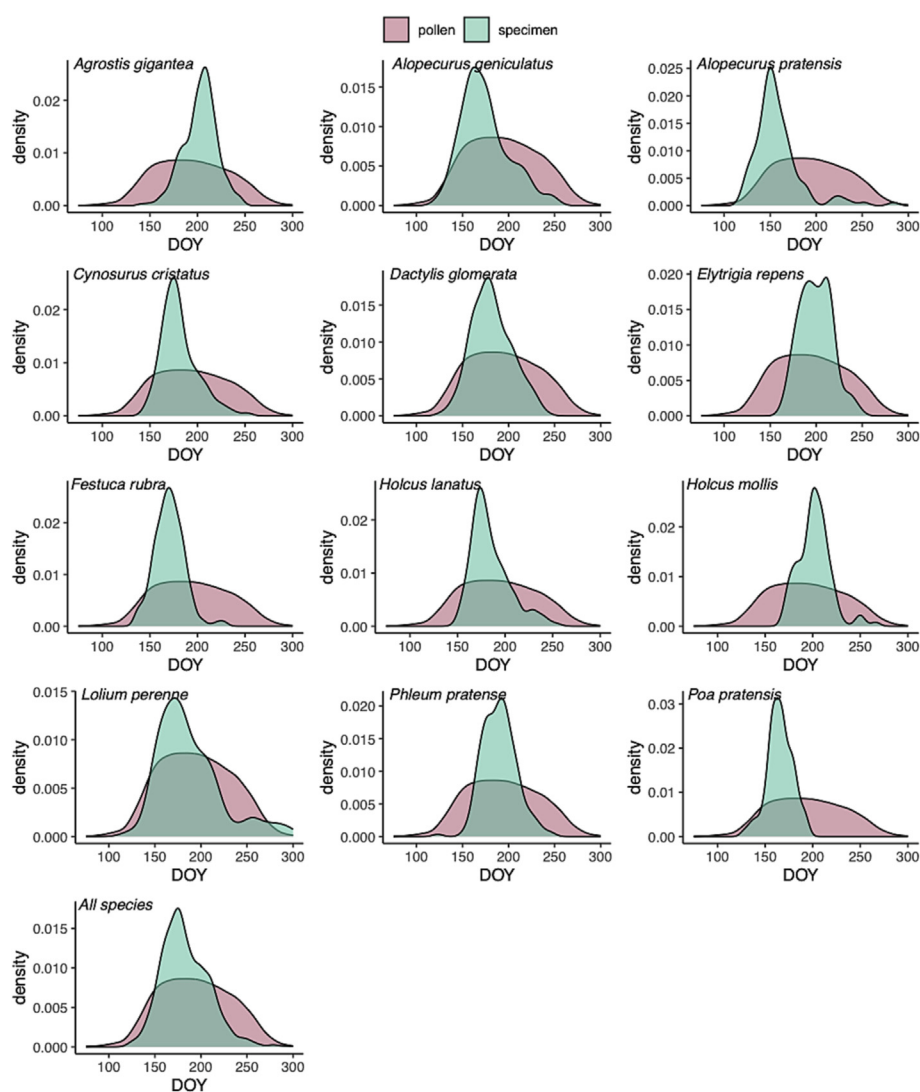


Fig. 2. Density plots comparing the total pollen day of year (DOY) over the entire grass pollen season (shown in pink) calculated from 44 years (1977–2019) of pollen monitoring data, and the flowering DOY derived from herbarium specimens (shown in green) spanning the years 1825–2014 for each of the 12 species examined, and all species combined. Note that the y-axes are somewhat different in each density plot, depending on the height of the species peak.

times from both the full and reduced data sets and the pollen parameters are presented in Table 2. For species with >30 specimens available for the reduced data (*Agrostis gigantea*, *Dactylis glomerata*, and *Festuca rubra*), a very high level of congruence exists in the percent overlap with the full herbarium flowering data and the pollen season parameters.

Nine of the 12 species included in the study (Table 2) occupied 90 % or more of the atlas squares in Denmark based on herbarium specimens, and all species occupied 66 % or more of the atlas squares (Fig. S3, SM). Species varied from 138 to 581 specimens in the sample, but there was not an obvious connection between the number of atlas squares occupied and the number of herbarium species in the museum.

3.2. Effects of year and temperature on grass pollen season

The grass pollen season has advanced significantly over the four decades that airborne pollen has been recorded in Denmark (Fig. 3; Table S1 [SM]). Because there are no significant differences in pollen season parameters between the two airborne pollen monitoring stations (Table S1, Fig. S5 [SM], results are presented for the combined pollen data. The start DOY changed at a rate of 2.5 days earlier per decade ($R^2 = 0.15$, F-stat = 13.61, $p < 0.001$, not shown). The maximum pollen DOY has changed at a similar rate, with maximum pollen levels being recorded 3.0 days earlier per decade ($R^2 = 0.12$, F-stat = 10.68,

$p < 0.01$, not shown). The overall duration of the pollen season increased by 4.4 days per decade ($R^2 = 0.28$, F-stat = 31.74, $p < 0.01$); the duration was 58 days long during the decade 1980–1989 and increased to 71 days in 2010–2019. The end DOY was not found to change significantly over time; that is, the ending of the pollen season has not changed over time (co-efficient = 0.17, $R^2 = 0.04$, F-stat = 3.35, $p = 0.07$, not shown).

Linear regressions between pollen count data and climate variables show that start DOY and maximum pollen DOY are significantly earlier in years with warmer spring weather (Fig. 3). An increase in one degree Celsius in the average spring temperature results in an advance in the start of the pollen season by 4.2 days ($p < 0.001$), and an advance of the maximum pollen DOY of 5.0 days ($p < 0.001$). The highest volume of pollen recorded (grains per m^3) has also increased in relation to temperature, with more pollen in years with warmer springs ($R^2 = 0.28$, coefficient = 3.18, f-stat = 31.91, $p < 0.001$, not shown).

3.3. Effects of year and climate on flowering time

Two species, *Agrostis gigantea* and *Poa pratensis* were found to flower significantly earlier by 1.3 days and 0.9 days, respectively, per decade based on herbarium data ($R^2 = 0.06$, coefficient = 0.13, F-stat = 19.11, $p < 0.001$; see Table S2).

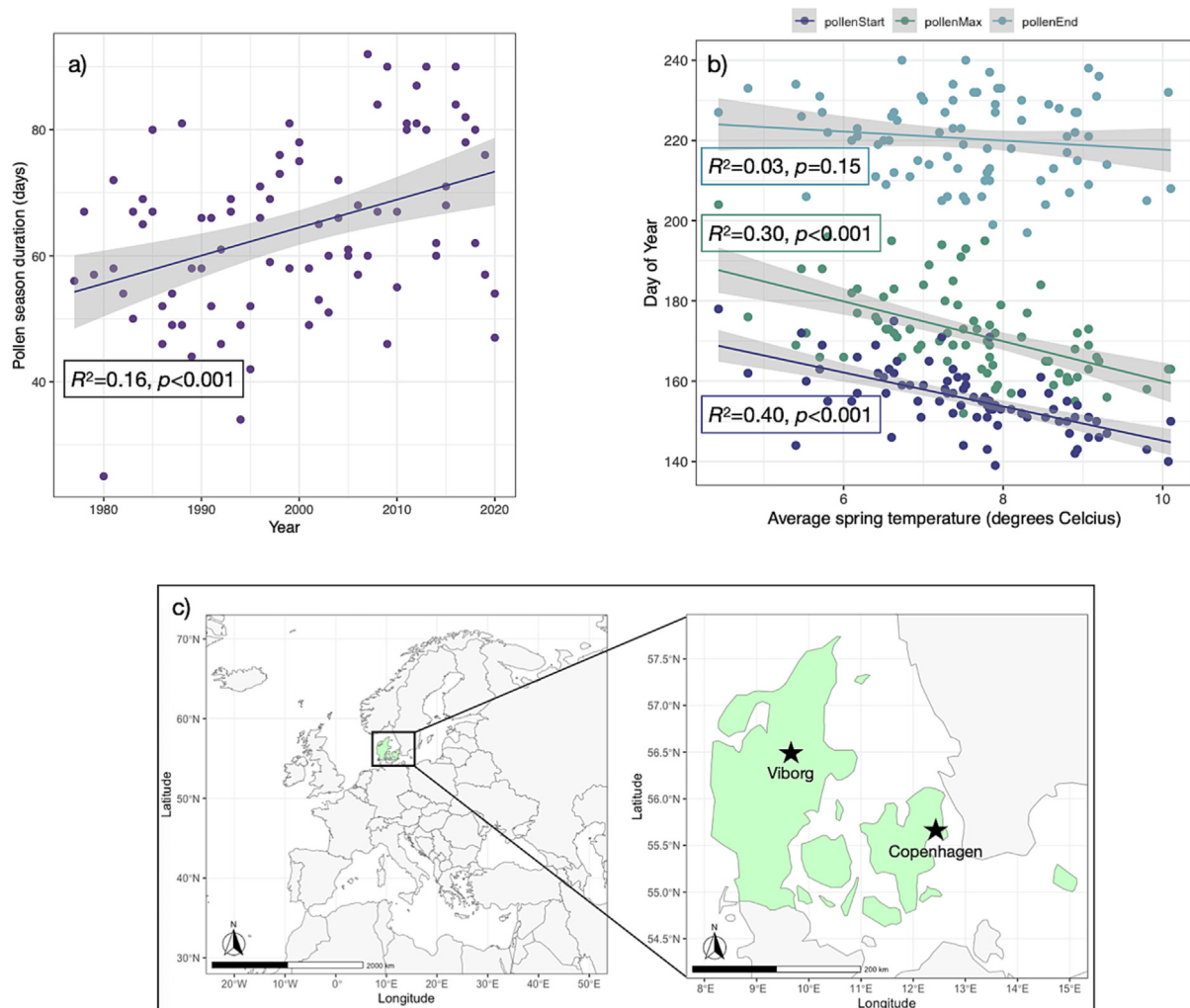


Fig. 3. Regression plots for airborne grass pollen parameters for the period 1977–2019 collected from Denmark a) pollen season duration and year; b) pollen season parameters start, maximum, and end DOY and average spring temperature in the months of March, April, and May in year of collection; c) map of Denmark showing the geographical position of the country within Europe, and marking the two pollen monitoring locations: Copenhagen and Viborg. Output from regression analyses can be found in Table S1 (Supplemental Material).

Nine of the 12 species we investigated were found to flower significantly earlier as temperatures increase in early spring or the month of June (Fig. 4; Table S2, SM), and no species flowered significantly later in response to warming temperature. For species with earlier mean flowering DOYs, average spring temperature or average temperature in May were the best predictors (*Dactylis glomerata*, *Festuca rubra*, *Holcus lanatus*, and *Poa pratensis*); however, for species with later flowering DOYs (*Elytrigia repens* and *Phleum pratense*), it was the month of June (Table S2, SM). Three common meadow / fodder species, *Poa pratensis*, *Dactylis glomerata*, and *Holcus lanatus*, show the strongest relationships (steepest slopes) between flowering time and the temperature in spring; these species were found to flower 4.6 days, 5.6 days, and 5.9 days earlier, respectively, for one-degree Celsius increase in temperature. These rates are similar to the advances seen for the start of the grass pollen season (advancing by 4.2 days) and the maximum pollen day (4.9 days) with respect to increasing spring temperature.

4. Discussion

The grass pollen season in Denmark has changed significantly over time due to warming temperature. Our findings are similar to Gehrig and Clot (2021) who found the grass season to be advancing by seven days in response to a warmer climate over the last 50 years in Basel, Switzerland. Similarly, in the UK, earlier onset of the grass pollen season was found in response to temperature in three out of six monitoring sites, though the pollen season duration was found to be geographically variable (Adams-

Groom et al., 2022). The increasing number of phenology studies in the Poaceae (Frenguelli et al., 2010; León-Ruiz et al., 2011; Tormo et al., 2011; Kmenta et al., 2016; Ghitarrini et al. 2017; Cebrino et al., 2018; Romero-Morte et al., 2018) demonstrate tremendous variability in flowering times and durations among grass species; these flowering differences could confound any grass pollen season trends detected with data in which all Poaceae are grouped together.

Combining herbarium phenology data spanning a 190-year period with four decades of airborne pollen monitoring data allowed us to determine species-specific seasonal patterns for the grass family, thereby enabling us to determine how specific grasses have responded to recent climate change, and, as a result impacted pollen seasons. To our knowledge, this is the first study of its kind using flowering phenology from herbarium specimens to investigate taxon-specific contributions to the airborne pollen season. Producing this much-needed species-specific information at a regional scale over a period of decades using herbarium specimens is a highly efficient way to help address the sparsity of phenology data needed to direct future pollen allergy research and to target management efforts needed to reduce the specific grasses causing pollen allergies (Rowney et al. 2021).

A few studies to-date have attempted to unravel the species-specific grasses that coincide with the pollen season; however, challenges in logistics and costs involved in setting up field-based phenology monitoring and/or DNA barcoding across large geographical areas limit this type of research to localized areas (León-Ruiz et al., 2011; Cebrino et al., 2016; Kmenta et al., 2016; Brennan et al., 2019; Hanson et al., 2022). Our approach demonstrates the valuable potential of herbarium specimens to

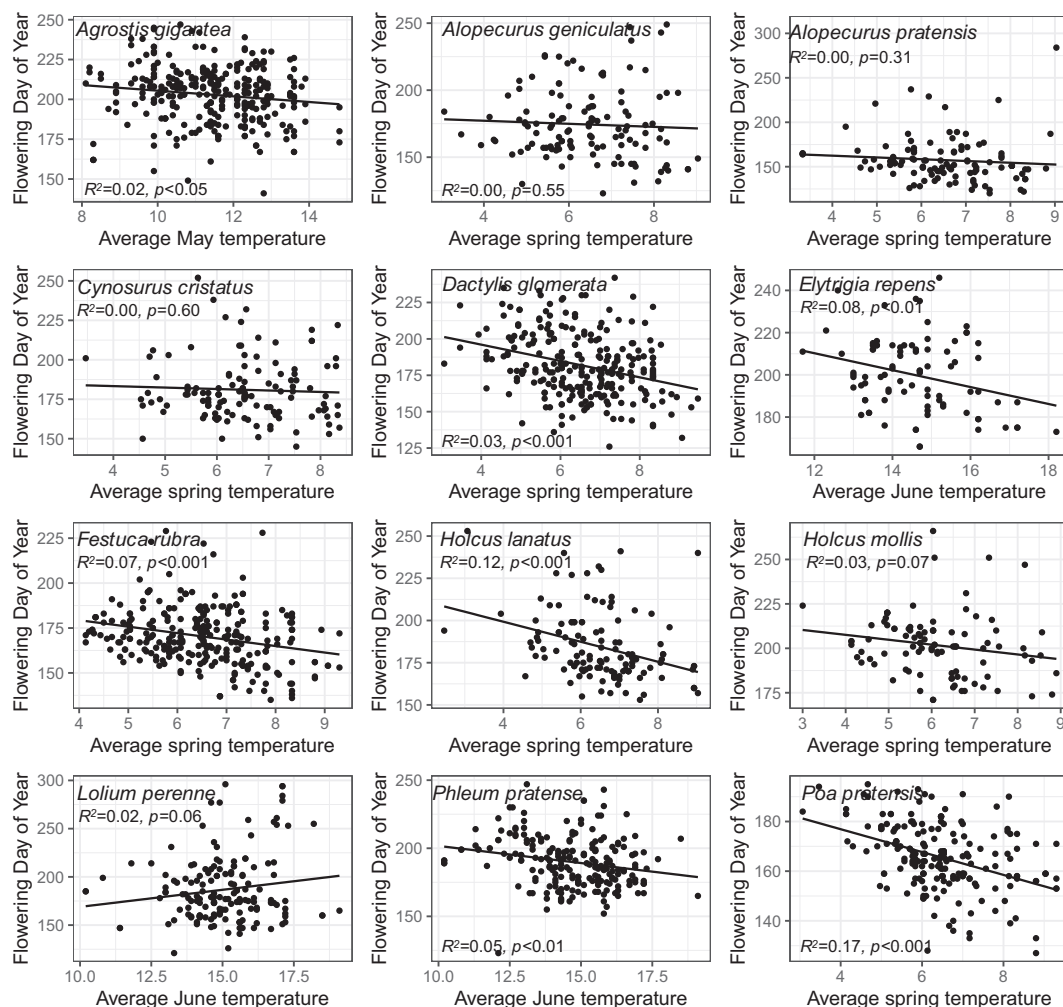


Fig. 4. Simple linear regressions of grass species and the most explanatory climatic variable tested for each of the 12 individual species. Regression lines shown as black solid lines, 95 % confidence intervals shown in grey. Details of the regression analyses are presented in Table S2 (Supplemental Material).

filling in gaps in understanding the phenology of such events, over broad geographic areas (Willis et al., 2017). We demonstrate that phenology data from herbarium specimens can be used to show that the species-specific timing of grass flowering, and therefore pollen production, in Denmark changes temporally throughout the season. We also show species-specific differences in flowering lengths, along with a high degree of overlap in flowering among species. Even though there are regional differences in the distribution of grasses across Europe, there is reasonably good overlap between our findings and those of recent studies using metabarcoding to determine the composition of the pollen season in the UK (Brennan et al., 2019; Hanson et al. 2022), and field-collected phenology data in Austria, Italy, and Spain (Frenguelli et al., 2010; León-Ruiz et al., 2011; Cebrino et al., 2016; Kmenta et al., 2016). For example, the progression of species-specific flowering that we find, as well as the overlap of flowering and peak pollen season, and the length of flowering reported for *Lolium perenne*, are highly consistent with the phenological field study of Kmenta et al. (2016) in Vienna. Our approach demonstrates the usefulness of herbarium phenology data in providing species-level resolution that is better than current DNA metabarcoding methods which are often accurate only to the genus level. Using herbarium specimens, we were also able to confirm that grass species belonging to the same genus flower at different times and for differing lengths, which means that genera-level taxonomic resolution is of limited value to base pollen season predictions on—at least in the case of multi-taxa genera such as *Alopecurus* and *Holcus* (Rowney et al. 2021; Zhang and Steiner, 2022).

Herbarium data are unique in highlighting abundant species that are not driving the start of the pollen season or maximum pollen loads based on their flowering phenologies. For example, *Phleum pratense* is often considered to contribute to pollen allergies in Europe (Garcia-Mozo, 2017) but was a later flowering species and did not coincide with peak pollen counts in this study. *Phleum* was also not found to be one of the main contributors to airborne pollen in the UK based on its proportion of the barcoding reads (Brennan et al., 2019), and similarly not found to be flowering during the pollen peak in Italy (Frenguelli et al., 2010).

Further research is needed to determine if standard airborne pollen monitoring traps are capturing the same species and abundance of pollen grains as those which are triggering allergies (Kmenta et al., 2016; Rowney et al. 2021). While this remains a problem, herbarium phenology data at least can tell us which species have the potential to be causing pollen allergies. Such data can be combined with allergy and asthma patient data as has been done elsewhere in the Europe, and even with metabarcoding data from samples of grass pollen caught in pollen traps. In our current study we were able to compare the start of flowering and the distribution dates of flowering for individual species with the seasonal abundance of grass pollen to gain insights into which species might be causing allergies.

Our study is limited to 12 widely distributed species in the grass family in Denmark (Fig. S3, SM), and we acknowledge that other species may also contribute to the airborne grass pollen abundance. Thus, a wider representation of grass species in an area should be considered for future studies. Herbarium specimens provide historical flowering times that can fill in spatial gaps in the sparse pollen monitoring networks and can greatly improve the spatial and temporal coverage of flowering phenology. However, specimens do have inherent biases (Daru et al., 2018), such as over collection and under collection in certain locations and during certain years and times of the year. Particularly relevant in our data was the sparsity of herbarium records that spanned the same period as the pollen data. The decrease in number of specimens collected during recent decades is a common bias of herbarium collections from well-studied floristic regions of the world. While we limited our study of herbarium specimens to a single herbarium, pooling specimens from multiple herbaria might be a way to reduce sampling bias. This work does demonstrate that using specimens spanning a time period longer than pollen monitoring still produces findings congruent with phenology-based field studies and/or DNA barcoding methods in the UK, Austria, Spain, Italy (León-Ruiz et al., 2011; Cebrino et al., 2016; Kmenta et al., 2016; Ghitarrini et al. 2017; Brennan et al., 2019; Hanson et al., 2022).

4.1. Change in grass flowering in response to climate warming

Phenological shifts in flowering and pollen projection time for plants depends on the temperature response of the individual grass species (Zhang and Steiner, 2022). While pollen from all 12 species in this study overlap with the pollen season, we found that nine of the species shift their flowering earlier in response to warming. The long-term phenology record covering a large geographic area obtained from herbarium specimens (in this present case spanning 190 years) often allows a more accurate estimation of species responses to a changing climate than is possible with short-term studies. We identified four species (*Poa pratensis*, *Festuca rubra*, *Dactylis glomerata*, and *Holcus lanatus*) that are highly sensitive to warming temperatures and are among the most abundant species in the landscape. Prior studies have found that these species also produce the highest amounts of pollen per inflorescence (Prieto-Baena et al., 2003), and therefore are among the grass species that are likely responsible for the earlier and possibly the more severe grass pollen season that is happening in Denmark. Our work not only confirms that flowering phenology and airborne pollen shift in the same direction in response to climate warming (Garcia-Mozo, 2017; Gehrig and Clot, 2021), but the species were also found to shift their flowering times at rates very similar to that for the grass pollen season. This important finding is a strong validation that flowering time of grasses from herbarium specimens can be used to gain insight into the pollen season of grasses.

In this study we used grass herbarium specimens to study flowering times. Specimens can also be used to study range shifts in allergenic species, and how this might be related to the changing distribution of pollen allergies. Additionally, specimens can also be used to examine how plant traits related to pollen production and therefore allergies have changed over time within species, such as the number and size of fertile inflorescences (Prieto-Baena et al., 2003). Herbarium specimens provide a potential, and to-date under-utilized, resource for studying how pollen production may be changing over time, and in relation to carbon dioxide levels, soil nutrient levels, and urbanization.

4.2. Recommendations for management

Species generally considered to be responsible for grass pollen allergies are widespread and abundant in Denmark, and several of these species are even cultivated as fodder for cattle. As a result, eradication is an unrealistic option for reducing their pollen production. Mowing is a possible management option to curb pollen production but may only prolong the grass flowering period and may conflict with other biodiversity conservation and carbon emission goals. Many species will flower again after mowing, which might explain the second smaller “peak” in flowering time for some of our grass species (Fig. 4). We suggest that continued efforts to rewild the Danish landscape (currently the country has the lowest percent natural forest cover in Europe), to increase the area under forest cover and increase species diversity in grass- and pasturelands, would also help to reduce the abundance of these problematic grass species. Additionally, future plant selection or breeding programmes for grass fodder plants could focus on selecting for plants that produce less (allergenic) pollen (Jung et al., 2018).

Land managers could consider converting existing pastures to grass species known to have lower pollen production or perhaps even sterile varieties and cultivars. Another possible management option might involve converting grasslands in densely settled urban areas to forest, where the combination of many introduced species and air pollution makes allergies and asthma worse (Bernard-Verdier et al., 2022; Ziska, 2021). Of course, tree species that do not produce allergenic pollen would have to be selected for such efforts.

4.3. Limitations of herbarium specimens

Determining the phenological stage of grass herbarium specimens is difficult due to the small size of their flowers (Munson and Long, 2017). This is

a task best carried out by plant taxonomists rather than citizen scientists or other volunteers. However, because most reproductive structures on herbarium specimens of grasses are inflorescences with flowers, we found that analyses done with just flowering specimens and analyses done with all reproductive specimens (including the pre-flowering and post-flowering stages) gave virtually the same result. This suggests that for many investigations of the impact of climate change on allergies and asthma, incorporating citizen scientists into time-intensive phenological evaluations of grass herbarium specimens would be a feasible approach, as the specimens have already been identified to species.

In all such studies using herbarium specimens in ecological research, scientists need to be aware of the biases associated with herbarium specimens (Daru et al., 2018). These include biases in spatial collections, season of collection, and pseudo-replication when many samples come from intensive collecting trips. Of special concern to the present study are collector biases, where some collectors might avoid collecting grass specimens while other collectors might deliberately seek out grass specimens. In addition, we found relatively few herbarium specimens collected from the same period as pollen monitoring. However, the phenological patterns uncovered in this study using 190 years of specimen collections are sufficiently robust to be evident even with these potential sources of bias.

While this study compared the flowering time of herbarium specimens of grasses to the time of grass pollen, similar studies should be taken of other plant species, genera, and families known for producing allergenic pollen, including the families Betulaceae (birches) and Fagaceae (oaks and beech), and genera such as *Artemisia* (mugwort, sagebrush) and *Ambrosia* (ragweed).

4.4. Concluding remarks

Herbarium specimens offer a valuable new source of data and a practical means to greatly advance the study of grass pollen allergies in a changing world. In particular, herbarium specimens can be used to investigate how individual grass species contribute to overall pollen patterns, how individual species flowering times are responding to a changing climate, and how these species-specific changes in flowering drive the changes observed overtime in pollen season duration and severity. In this study, we found that results from herbarium specimens and pollen sampling showed similar seasonal patterns and similar responses to temperature. This suggests that in the absence of a field-based phenology monitoring program, data from herbarium specimens can be used to estimate the pollen season and used to predict how the airborne pollen season will shift in response to future climate change.

CRedit authorship contribution statement

NIA planned and designed the research together with RBP. NIA and HME assessed grass phenology, and NIA carried out data analyses. NIA and RBP wrote the manuscript.

Data availability

Images of grass herbarium specimens used in this study will be available on The Global Biodiversity Information Facility (www.gbif.org).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2023.163824>.

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