Article

EFFECT OF CLIMATE, ANTHER MORPHOLOGY AND POLLINATION SYNDROME ON POLLEN AVAILABILITY IN *PENSTEMON*

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Abstract—Traditionally, pollen presentation is thought to be a function of pollinator type and visitation frequency. However, despite the repeated observation that pollen presentation is influenced by flower morphology and abiotic factors, these aspects have been little studied in the wild. Here, we evaluated the effect of climate, anther morphology, and pollination syndrome on anther dehiscence time (the length of time an anther takes to fully dehisce after a flower opens). We recorded anther dehiscence time in twelve species of *Penstemon* including the four major anther types and the two most common pollination syndromes. We also conducted an experiment to measure the effect of humidity and temperature on anther dehiscence. We found that anther morphology was correlated with anther dehiscence time. Anthers with wide openings take the longest time to dehiscence. These results provide some support for the hypothesis that anther dehiscence time has evolved to decrease pollen wastage. Contrary to the assumption that bird-pollinated species have simultaneous pollen presentation, hummingbird-pollinated species had longer anther dehiscence time than most bee-pollinated species. The experiment revealed that high humidity and low temperature increase anther dehiscence time. Our results suggest that pollen presentation is influenced by anther morphology, pollination syndrome, and the physical environment. Optimal pollen presentation presumably maximizes conspecific pollen transfer and reduces pollen thieving.

Keywords—Anther morphology, climate, open-top-chamber, *Penstemon*, pollination syndrome, pollen presentation

INTRODUCTION

Early studies of pollen presentation focused on agriculturally important honeybees (*Apis mellifera*) and aimed to understand honeybee pollen preferences and pollen availability (Synge 1947; Percival 1955; Free 1962). Later, pollen presentation theory helped in the evaluation of alternative pollinators that might be more efficient at pollen transfer to enhance crop production (Thomson & Goodell 2001; Cane & Schiffhauer 2003; Ricketts 2004). Moreover, the later body of work aimed to evaluate evolutionary consequences of different modes of pollen presentation in different pollination syndromes (Thomson et al. 2000; Zhang et al. 2005; Castellanos et al. 2006). As an important component of male fitness, pollen presentation is tied to floral evolution. Animal-pollinated species have evolved a particular flower morphology to attract their important pollinators (Fenster et al. 2004) and, presumably, a particular anther dehiscence time (the length of time an anther takes to fully dehisce after a flower opens) to allow pollen to be available when those effective pollinators are active. This has prompted several studies on the relationship between pollen presentation and pollination syndrome (Martén-Rodríguez et al. 2009; Reynolds et al. 2009). Nevertheless, pollen presentation is still an understudied aspect of plant reproduction; many relevant questions, such as the role of anther morphology on anther dehiscence time and the effect of abiotic factors on pollen presentation, are yet to be explored.

Optimal pollen presentation can be the result of natural selection caused by pollen vectors (Thomson et al. 2000), limited pollen viability (Fernando & Cass 1997; Sarala et al. 1999), male-male competition (Wilson 1995), environmental adaptation, or a combination of factors. From these, the effect of pollen vectors on pollen presentation has been more frequently studied. In particular, Thomson & Thomson (1992) and Thomson et al. (2000) outlined several predictions about pollen presentation when pollinators vary. First, there is a decelerating relationship between the amount of pollen removed and the amount of that pollen that gets deposited, and the curvature of this relationship depends on the behaviour of the pollinator (Thomson et al. 2000). This means that the amount of pollen removed by a pollinator will always be greater than the amount of pollen deposited. Second, the optimal pollen presentation is dependent on pollinator frequency. Finally, high-removal and low-deposition pollinators become functional parasites when more effective pollinators are present. This set of ideas was further extended to bee- and bird-pollination syndromes in *Penstemon* (Thomson et al. 2000; Castellanos et al. 2006). Plants visited by high-removal high-deposition pollinators, like hummingbirds, should have simultaneous pollen presentation, and plants pollinated by bees, which generally are high-removal low-deposition pollinators, should have slow pollen presentation (Thomson et al. 2000). This also means that plants pollinated by hummingbirds should dehisce their anthers faster than bee-pollinated species.

Another aspect of pollen presentation that has received little attention is the effect of climate on anther dehiscence time. Abiotic factors, such as temperature and humidity, are thought to affect pollen presentation, particularly anther dehiscence time. Generally, anther dehiscence is viewed as a desiccatory process in which water is lost via evaporation, causing the anthers to dehisce (Coulter et al. 1911). This view is consistent with several observations that indicate that pollen presentation changes with varying weather conditions (Synge 1947; Percival 1955; Schmid & Alpert 1977; Goodwin 1986; Pearson et al. 1995). Laboratory studies indicate that both humidity and temperature affect anther dehiscence time, but the magnitude of the effects varies between species (Schmid & Alpert 1977). Observational studies have documented a decrease in pollen presentation associated with decreasing temperature (Percival 1955; Synge 1947). Additionally, high humidity increased anther dehiscence time in *Erythronium grandiflorum* Purch (Liliaceae) to the point of complete closure of the anthers when rain droplets landed on the flowers (Thomson & Thomson 1992). This indicates that high humidity is correlated with reduced pollen availability. Finally, Parker (1926) noticed that the weather affected pollen availability. The author observed that relative humidity decreases with temperature and that on warmer days plants open their anthers earlier. Parker (1926) concluded that high temperature coupled with low humidity increases transpiration, which leads to a higher rate of anther desiccation. Despite repeated observations that abiotic factors influence anther dehiscence time, to our knowledge, no study has quantified the effect of temperature and humidity on anther dehiscence time under field conditions.

Anther morphology is an essential component of pollen release and collection and thus an important aspect of pollen presentation. Anther shape can limit the type of animal that can extract pollen and pollinate the flowers. For example, plants with poricidal anthers are mostly pollinated by sonicating bees (Buchmann 1983; Cardinal et al. 2018). However, little is known about how anther morphology aids or limits pollen collection by different pollen vectors. It has been hypothesized, and to some extent observed, that bee-pollinated penstemons have narrower anther openings than bird-pollinated species to restrict bees from harvesting the pollen all at once (Thomson et al. 2000; Castellanos et al. 2006). But it is still unclear whether anther morphology and anther dehiscence time are comparable between species with different pollination syndromes. For example, anthers with a bigger opening could dehisce more slowly than anthers with a smaller opening, which means that, in *Penstemon*, the amount of pollen released between the two anther types could be similar regardless of the pollination syndrome.

In *Penstemon*, anther morphology has been used to taxonomically delimit subgenera within the genus. However, phylogenetic analysis indicated that anther morphology is not conserved within the conventional subgenera (Wolfe et al. 2006), so

the subgenera might be thought of more as functional groups than as reciprocally monophyletic clades. Recently, Freeman (2019) divided the genus into two subgenera: *Dasanthera* and *Penstemon*. However, in the current study, we will follow the earlier taxonomy, as it corresponds to anther morphology. *Penstemon* also presents diverse pollination syndromes that include bee-, wasp-, hummingbird- and fly-pollination (Straw 1956, 1963, Wilson et al. 2004). The diversity in anther morphology and pollination syndrome (Straw 1956, 1963; Wolfe et al. 2006; Katzer et al. 2019) make *Penstemon* an ideal system to study pollen presentation. Other groups of plants that also show great anther diversity and merit similar consideration include Miconieae (Brito et al. 2016), Justicieae (Kiel et al. 2017), and Mimoseae (Hughes 1997).

Our main goal is to evaluate the effect of climate, anther morphology (subgenus membership), and pollination syndrome on anther dehiscence time, using observational and experimental approaches. We predict that anther morphologies with wider openings will have longer dehiscence times than anther morphologies with narrow anther openings, because longer dehiscence time can reduce pollen waste, regardless of pollination syndrome (assuming high visitation frequency). We also expect that, between species with the same anther morphology, anther dehiscence time will be similar. Finally, we predict that high humidity and low temperature increase anther dehiscence time because this will allow pollen to be available when pollinators are active. We test this prediction using species of *Penstemon* representing the four major anther morphologies found in the genus across a large distributional range and pollination syndromes.

MATERIALS AND METHODS

STUDIED SPECIES

We studied 12 species: *P. albidus, P. digitalis, P. eatonii, P. fruticosus, P. glandulosus, P. leonardii* var. *higginsii, P. palmeri, P. rostriflorus, P. speciosus, P. utahensis, P. venustus,* and *P. whippleanus*. These species represent the four main anther morphologies (subgenera) and the two major pollination syndromes (bird and Hymenoptera; Supplementary Data Fig. S1). We sampled seven Hymenoptera-pollinated (*P. albidus, P. digitalis, P. fruticosus, P. glandulosus, P. leonardii* var. *higginsii, P. palmeri, P. speciosus, P. venustus,* and *P. whippleanus*) and three bird-pollinated species (*P. eatonii*, *P. rostriflorus*, and *P. utahensis*). We were not able to sample any bird-pollinated species from the subgenus *Dasanthera*, whereas the Hymenopteran-pollinated *P. (Dasanthera) fruticosus* was sampled. This group has anthers that dehisce from end-to-end and across the connective and are covered in wooly hair (type-A anthers, Fig. 1). We sampled two species, *P, eatonii and P. speciosus*, from subgenus *Habroanthus*, which has anthers that dehisce at the tips only (type-B anther, Fig. 1). Species from the subgenus *Penstemon* with anthers that dehisce from end-to-end and across the connective (type-C anthers, Fig. 1) are *P. albidus, P. digitalis, P. palmeri, P. utahensis,* and *P. whippleanus*. Finally, from the subgenus *Saccanthera,* with anthers that dehisce from a pore-like structure at the connective, forming a sac (type-D anthers, Fig. 1), we sampled *P. glandulosus*, *P. leonardii* var. *higginsii, P. rostriflorus,* and *P. venustus*. See the Supplementary Data, Note S1, for more species descriptions and distributions.

SAMPLING AND ANTHER DEHISCENCE TIME OBSERVATIONS

In the summer of 2018 and 2019, the pollen presentation schedule was estimated in each site by recording the time to full anther dehiscence in 5–10 flowers within each population (Table 1). The results presented here are based on data collected in situ and within 48 hours. Flowers with completely closed anthers were selected and marked, and the time they took to dehisce each anther was estimated based on observing them every 60 minutes until full dehiscence. The four functional anthers were assigned a number relative to their position in the flowers. Anther 1 was the front left, anther 2 was the front right, anther 3 was the back left, and anther 4 was the back right (Supplementary Data Fig. S1). Every hour, anthers were checked and classified as closed, opening (when some pollen was visible, but the anther was not completely open), or open. For all the sites, we recorded the temperature, humidity, and wind speed every hour.

We encountered noteworthy complications during the sampling process. First, anther dehiscence was initially recorded on cut inflorescences that were kept in plastic containers

Figure 1. Panel of anther types. From top to bottom: a picture of a completely dehisced anther, and another picture of the same anther but closed. From left to right, *P. fruticosus* (Anther type A; subgenus *Dasanthera*), *P. speciosus* (Anther type B; subgenus *Habroanthus*), *P. whippleanus* (Anther type C; subgenus *Penstemon*), *P. glandulosus* (Anther type D; subgenus *Habroanthus*). Photo credit: Andrea D. Wolfe.

inside a cooler or a flower vase. In those conditions, anthers took longer to fully dehisce because of the high humidity, which altered the results and impeded the comparison between species; hence, these observations are not included in this report. Second, in several populations of *P. albidus* and *P. whippleanus,* at least one anther in several flowers did not open when we ended observations for the day. These observations were included in the analysis as censored data (see Statistical Analysis section). Finally, in one *P. glandulosus* var. *chelanensis* site, at least one anther in all the flowers had opened by the time we arrived at the site. Hence, we were unable to record anther dehiscence time and thus did not include these observations in the sample.

Experimental design

For the experiment, we chose to sample a *Penstemon digitalis* population that has been planted in Scioto Grove Metro Park, Franklin County, Columbus, Ohio (39°51'30.0"N 83°01'26.2"W). A large number of individuals were available for our experiment. The experiment aimed to test whether high humidity and high temperature affected anther dehiscence time. To increase the temperature and humidity around the experimental plants, an open-top chamber was used. The chamber designed in this study is a modification of widely used open-top chambers (Welshofer et al. 2018). Open-top chambers were built based on Vapaavuori et al. (2012). Specifically, our open-top-chambers were rectangular, approximately: 0.25 L × 0.25 W × 1 H meters (Supplementary Data Fig. S2). Two squares made from PVC poles constituted the top and bottom of the chamber. Four vertical PVC poles located in each corner connected the top part of the chamber to the bottom. The top part of the chamber was smaller to trap humidity. The PVC tubes formed the skeleton to which the polyethylene film (width = 7.87 μm) was attached with transparent tape.

Our experimental setup consisted of the following treatments: control (C), high humidity and high temperature (HH), and low humidity and high temperature (LH). The control plants were not enclosed in chambers. The HH treatments constituted plants that were enclosed inside an open-top chamber. The LH treatments consisted of plants inside a chamber with a metal canister containing 680 grams of silica gel. The canisters had small holes and were placed inside the chambers of the LH treatment close to the ground on top of a rock. All the treatments were carried out simultaneously every day from May 27 to June 2, 2019. Each morning, we marked open flowers whose anthers had not started to dehisce yet. Every day, a new set of plants/flowers was selected. Multiple flowers in a plant were marked by piercing a hole with a 0.05 mm pencil lead

Table 1 List of sampled locations. * Denotes observations made in the same geographic location.

Species/subgenus/site	Longitude	Latitude	Elevation (m)	County	State
Dasanthera					
P. fruticosus site 1	-115.218072	44.232784	1778	Boise	Idaho
P. fruticosus site 2	-116.097343	44.957030	1536	Valley	Idaho
Habroanthus					
P. eatonii site 1*	-113.098024	37.287605	1585	Washington	Utah
P. eatonii site 2	-112.968765	37.646169	2022	Iron	Utah
P. speciosus site 1	-115.0222426	41.0620267	2084	Elko	Nevada
P. speciosus site 2	-119.1995119	44.7349105	1291	Grant	Oregon
P. speciosus site 3	-116.998797	39.462200	2166	Austin	Nevada
P. speciosus site 4	-119.986445	38.693491	2610	Alpine	California
Penstemon					
P. albidus site 1	-104.339695	40.646503	1477	Weld	Colorado
P. albidus site 2	-104.919855	43.635799	1336	Weston	Wyoming
P. albidus site 3	-109.202718	45.444733	1299	Carbon	Montana
P. palmeri site 1*	-113.098024	37.287605	1585	Washington	Utah
P. utahensis site 1	-113.098024	37.287605	1585	Washington	Utah
P. utahensis site 2	-112.068460	37.490490	1807	Kane	Utah
P. whippleanus site 1	-108.105885	39.031747	3193	Mesa	Colorado
P. whippleanus site 2	-106.628496	40.400099	2903	Grand	Colorado
P. whippleanus site 3	-106.324935	38.519098	3195	Chaffee	Colorado
P. whippleanus site 4	-107.713241	37.819575	2889	San Juan	Colorado
Saccanthera					
P. rostriflorus site 1*	-113.098024	37.287605	1585	Washington	Utah
P. leonardii var. *higginsii* site 1*	-113.098024	37.287605	1585	Washington	Utah
P. glandulosus site 1	-116.8964785	44.3886345	882	Washington	Idaho
P. venustus site 1	-117.670475	46.362698	710	Garfield	Washington

through the corollas' leftmost lower lip. This action did not alter the flowers' appearance and did not cause the flowers to wilt within a daily observation period (7 am to 6 pm). Once the treatment plants were marked, a chamber was placed around each plant. We established three chambers per treatment per day for 6 days, and we attempted to randomly scatter the chambers across the sampling site. Temperature and humidity were measured with a Kestrel 5500 hourly during the observation period inside each chamber (treatments) and outside (control).

STATISTICAL ANALYSIS

To compare the anther dehiscence times, we used survival analysis. To estimate the survival curves and the mean anther dehiscence time, we used the Aalen-Johansen estimator as implemented in the R package "survival" and the function "survfit" (Therneau 2021). This method allowed us to include in the analysis anthers that did not experience the event (full dehiscence) by the time observations ended (Therneau & Grambsch 2000). Here, the survival time is the time in minutes it took an anther to fully dehisce from when we first marked the flower, and the mean anther dehiscence time was estimated for each anther individually. An anther was the unit of replication used in analyses, not a flower, a plant, or a species. Nevertheless, it is useful to visually compare species in terms of the difference in time when a flower's last anther (anther 3 or 4) dehisced minus the time when the first anther dehisced (anther 1 or 2), what might be thought of as the 'androecium dehiscence time'.

Survival analyses were conducted where the mean survival times within the following queries were compared: (1) species collected in 2018, (2) species collected in 2019, (3) the four subgenera, and (4) the experimental groups (two treatments

and the control). For these four groups, a second set of survival analyses was conducted to calculate the androecium dehiscence time. For these analyses, we took as input the differences in dehiscence time between anthers 4 and 1. We decided not to combine the data from different years, because the samples collected in 2019 either came from one location or nearby locations, which means that all these species have been exposed to very similar environmental conditions throughout their life span. The comparison between subgenera only included species collected in 2018 that were Hymenoptera-pollinated. Also, pollination syndromes were not compared because bird-pollinated species were under-represented in the data, while Hymenoptera-pollinated species were more thoroughly sampled. In each of the survival analyses, we tested the hypothesis that any two groups (i.e. two species pollinated by birds) being compared had significantly different anther dehiscence times. We used the function "survdiff", which is similar to Tukey's HSD (honestly significant difference) test, to determine which pairs of species (or other groups) were significantly different from each other. The effect size between species and subgenera was estimated using Hedge's g formula and is reported in Supplementary Data tables S1-S3.

For the experiment, we first evaluated the effectiveness of the open-top chamber by comparing the temperature and humidity between treatments with two ANOVA tests, one for each variable. Tukey's HSD test was used to determine pairwise significant differences between treatments. Then, we carried out a survival analysis to calculate the mean anther dehiscence time in a treatment and to compare the mean survival times between groups as described above. The effect size was estimated using Hedge's g formula (Supplementary Data Table S4).

Results

In 2018, we marked 165 flowers and recorded the time in minutes each anther took to dehisce fully. The number of flowers marked were as follows: 18 (*P. albidus*), 22 (*P. eatonii*), 12 (*P. fruticosus*) 1 (*P. glandulosus*), 2 (*P. leonardii* var. *higginsii*), 10 (*P. palmeri*), 34 (*P. rostriflorus*), 22 (*P. speciosus*), 30 (*P. utahensis*), 2 (*P. venustus*), and 12 (*P. whippleanus*). For the experiment, 260 *P. digitalis* flowers were monitored: 117 controls, 66 HH treatments, and 77 LH treatments. Within the chambers (treatments), multiple flowers per plant were selected.

Hymenoptera syndrome

The initial results from the survival analysis of the Hymenoptera-pollinated species showed that androecium dehiscence time, the time it took for all anthers to dehisce, varied between species. In particular, anther 1 took a similar amount of time to fully dehisce in all species ($P > 0.01$), but anthers 2–4 had significantly different anther dehiscence times in at least one pair of species ($P \leq 0.01$). This means that after flowers were marked, these Hymenoptera-pollinated species started their pollen display at similar times. After this initial pollen presentation, pollen became available at different rates. The results of the pairwise significance tests show that species with anthers that dehisce from end-to-end and across the connective took the longest to fully dehisce all their anthers (*P. albidus* and *P. whippleanus*). Consequently, species with anthers with narrower anther openings (*P. glandulosus* and *P. speciosus*) had faster anther dehiscence times (Table 2, Supplementary Data Fig. S3, Table S1). This is consistent with the result from the androecium dehiscence time analysis. This means that, for Hymenoptera-pollinated species, anthers that dehisce from end-to-end and across the connective present their pollen more slowly than species with more restrictive anther types.

Similar to the Hymenoptera-pollinated species analysis, the results of the survival analysis of the different anther morphologies (subgenera) indicated that for anther 1, dehiscence time was not statistically different between the subgenera, which means that pollen display started at a similar time in all subgenera. For anther 2, two pairs of subgenera (*Habroanthus-Dasanthera* and *Habroanthus-Penstemon*) had different dehiscence times ($P < 0.05$). For anthers 3 and 4, the anther dehiscence time estimated for the subgenus *Penstemon* was larger than in *Dasanthera* and *Habroanthus* ($P < 0.05$). In contrast, the androecium dehiscence time between *Habroanthus-Dasanthera* was not statistically different (Table 2). Additionally, the genus *Penstemon* had significantly longer dehiscence time than *Dasanthera* and *Habroanthus*. This supports our

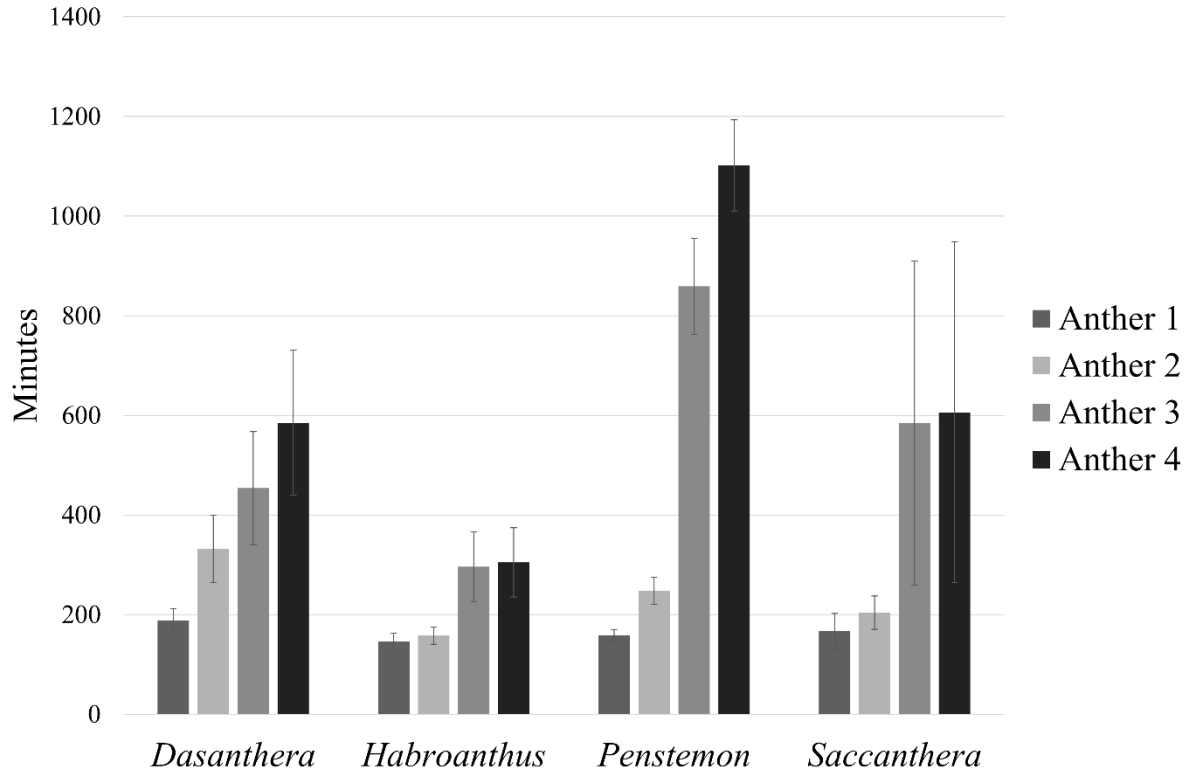

Figure 2. Mean anther dehiscence time per subgenus obtained from the survival analysis. The figure indicates that wider anthers (subgenus *Penstemon*) take longer to fully dehisce. The error bars represent the standard error. The species by subgenera are—subgenus *Dasanthera: P. fruticosus*; subgenus *Habroanthus: P. speciosus*; subgenus *Penstemon: P. albidus*, *P. whippleanus*; subgenus *Saccanthera: P. glandulosus*, *P. venustus*.

hypothesis that species with anthers with wide openings (subgenus *Penstemon*) have longer anther dehiscence times than the other species in the study (Fig. 2; Table S2).

Bird syndrome

The three red-flowered species (*P. eatonii*, *P. rostriflorus*, and *P. utahensis*) showed some important differences in anther dehiscence time. The results showed that for all the anthers, except for anther 2, at least one pair of species was significantly different. This finding contrasts with the results for Hymenoptera-pollinated species where pollen display started at a similar time after flowers were marked. The result from the pairwise comparison indicated that for anther 1, 3, and 4, *P. rostriflorus* had longer anther dehiscence time than *P. utahensis* and *P. eatonii* (Table 2, Supplementary Data Table S3, Fig. S4). *Penstemon eatonii* had longer dehiscence time than *P. utahensis* for anther 3 ($P = 0.09$) and 4 ($P = 0.05$). This means that a bird-pollinated species from the subgenus *Saccanthera* had longer anther dehiscence time than species from the subgenera *Habroanthus* and *Penstemon*. This is the opposite of what we found for Hymenoptera-pollinated species (Table 2, Supplementary Data Table S3, Fig. S4).

Two additional species were sampled in the same area where the red-flowered species occur. One species was *P. palmeri* (subgenus *Penstemon*) and the other species, *P. leonardii* var. *higginsii*, (subgenus *Saccanthera*). The survival analysis indicated that *P. leonardii* var. *higginsii* had slower anther dehiscence time than *P. palmeri* (Table 2) with only anthers 3 and 4 statistically different (anther 1, $P = 0.15$; anther 2, $P = 0.03$; anther 3, $P \leq 0.01$; anther 4, $P \leq 0.01$). Among all the species sampled in Utah (Table 1), the bee-pollinated *P. palmeri* (subgenus *Penstemon*) had the longest anther dehiscence time, followed by *P. rostriflorus* (subgenus *Saccanthera*), and *P. leonardii* var. *higginsii* (*Saccanthera*) had the shortest anther dehiscence time, which is consistent with the result from androecium dehiscence time analysis (Table 2). This means that the bird-pollinated species, *P. rostriflorus,* had slower (more prolonged) pollen presentation than the bee-pollinated species, *P. leonardii* var. *higginsii*.

Table 2. Anther dehisce time calculated with survival analyses using the Aalen-Johansen estimator. *N* indicates the number of flowers. NA means that the median time in minutes could not be calculated due to the low number of observations; rm is the restricted mean calculated in the survival analysis, which is different from the arithmetic mean. The md is the median. Androecium represents the dehisce time calculated for the whole flower. The [b] and [H] superscripts indicate the bird and Hymenoptera syndrome, respectively.

		Anther 1		Anther 2		Anther 3		Anther 4		Androecium	
	N	r-m	md	r-m	md	r-m	md	r-m	md	r-m	md
Species											
P. albidus[H]	18	167	180	259	240	827	498	1018	1401	842	1238
P. fruticosus[H]	12	182	191	228	197	454	248	585	309	251	101
P. glandulosus[H]	1	253	253	253	253	253	253	253	NA	0	0
P. speciosus[H]	22	142	135	158	135	296	205	305	231	156	65
P. venustus[H]	2	124	124	180	180	741	124	782	124	626	658
P. whippleanus[H]	12	145	141	157	141	882	NA	1229	NA	1037	1263
Subgenera											
Dasanthera[H]	12	189	191	332	197	454	248	585	309	251	101
Habroanthus[H]	22	146	135	158	135	296	205	305	231	156	65
Penstemon[H]	30	159	163	248	240	859	958	1102	1401	921	1238
Saccanthera[H]	3	167	124	204	236	585	253	606	124	418	0
Treatments (*P. digitalis*)[H]											
Control	132	230	220	231	220	471	370	478	426	248	124
HH	94	266	252	267	262	692	428	699	428	338	154
LH	83	227	220	232	220	364	272	382	272	155	121
Species 2018											
P. eatonii[b]	25	244	236	307	236	1082	1512	1184	1512	900	1276
P. palmeri[H]	10	414	487	764	487	1410	1512	1512	1512	1098	1025
P. rostriflorus[b]	34	366	399	409	399	1196	1512	1257	1512	893	1025
P. utahensis[b]	51	215	199	455	250	1018	1290	1090	1290	820	1091
P. leonardii var. *higginsii*[H]	3	308	337	308	337	308	337	308	337	0	0

Effect of temperature and humidity on anther dehiscence time

Our open top-chamber design was effective and produced differences in temperature and humidity between treatments (Fig. 3). The Tukey's HSD showed that the HH treatment had significantly higher humidity than the control and the LH treatment ($P < 0.01$). For temperature, we found that the LH treatment and the control had the largest mean difference in temperature (2.06°C, $P < 0.05$). Overall, the open-top chambers were more effective at significantly increasing the humidity than the temperature (Fig. 3). Moreover, the survival analysis revealed that at least one pairwise comparison was significant ($P < 0.001$ for all the anthers; Fig. 4). For anthers 1, 2, and 4, Control-HH and HH-LH were significantly different. Control-LH did not have significantly different dehiscence times for anthers 1, 2, and 4. For anther 3, all the pairwise comparisons were significant. Finally, the androecium dehiscence time was significantly faster in the low humidity treatment but there were no significant differences between the HH treatment and the control (Table 2). The results show that high humidity caused anthers to dehisce more slowly and low humidity caused rapid dehiscence.

Discussion

We examined the role of anther morphology and climate on anther dehiscence time. We found that anther dehiscence time corresponded to anther morphology such that Hymenoptera-pollinated species with anthers that dehisce from

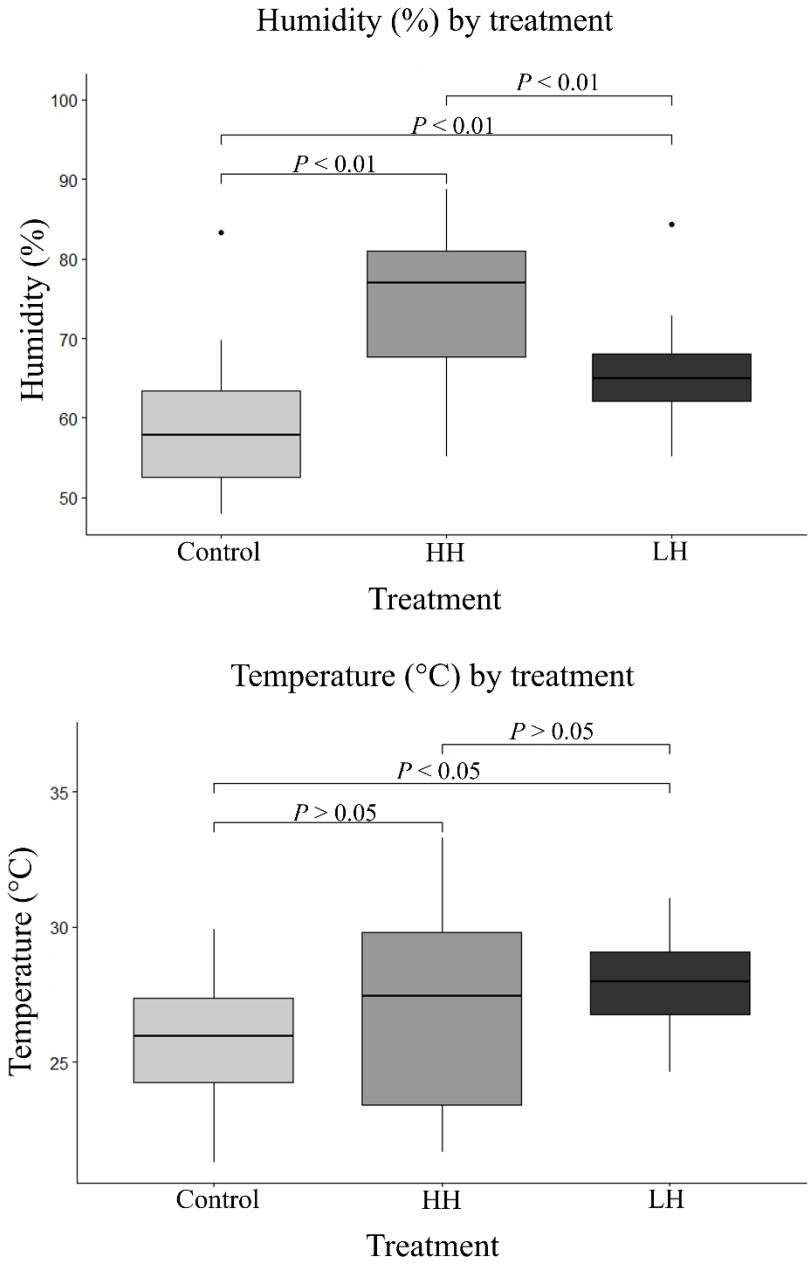

Figure 3. Box plots of humidity (top) and temperature (bottom) recorded per experimental treatment. The graph shows that humidity varied significantly between the treatments while for the temperature only the control and the low humidity treatments were significantly different ($P < 0.05$).

end-to-end and across the connective (anther type C, subgenus *Penstemon*) are the slowest to fully dehisce. The results partially support our expectation that species with the same anther morphology dehisce at similar rates. They also provide some support for the hypothesis that anther dehiscence time has evolved to prevent pollen waste by reducing the pollen available per hour in anthers with wide openings. The experimental approach used here successfully increased the humidity around the plants and revealed that anther dehiscence time increased with humidity. In addition, increases in temperature accelerated anther dehiscence time, but this effect was smaller than the effect of humidity.

Our results indicated that anther morphology has a significant impact on the pollen presentation schedule. It also partially confirmed our expectation that anthers with wide openings (anther type C) take longer to fully dehisce than any other anther type (Table 2). Pollinator type and behaviour have been previously invoked to explain pollen presentation strategies (Harder & Thomson 1989; Thomson et al. 2000; Castellanos et al. 2006). In particular, bees waste about 99% of the

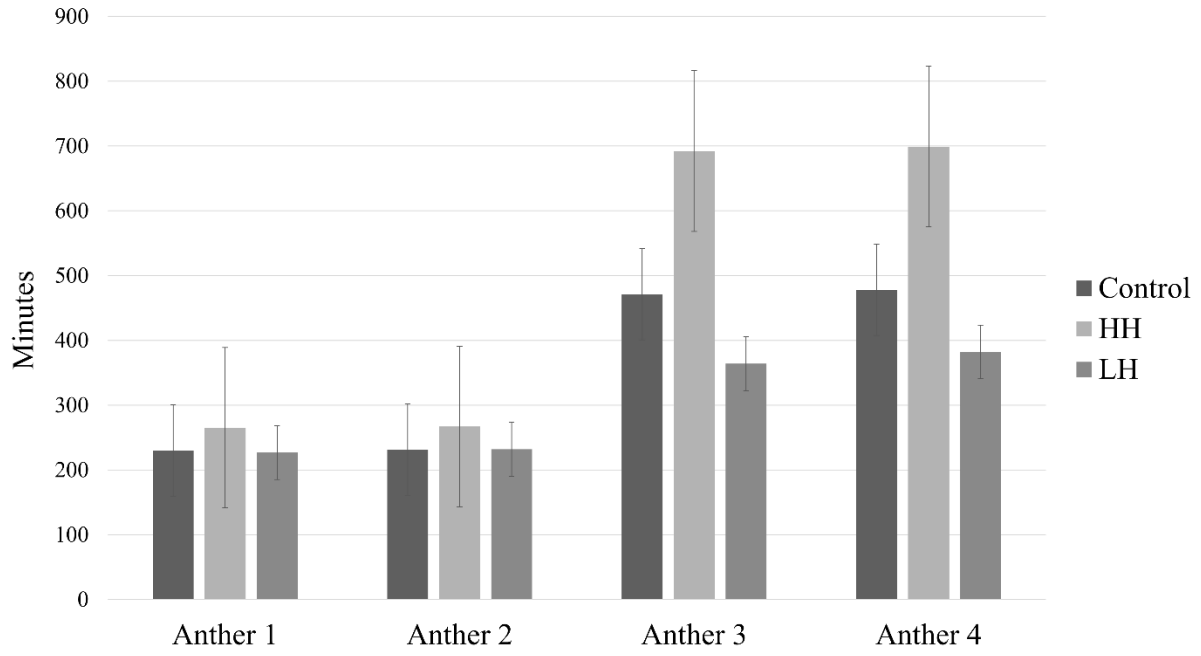

Figure 4. Mean anther dehiscence time per treatment obtained from the survival analysis. The graph shows that high humidity (HH treatment) caused anther to dehisce more slowly. The error bars represent the standard error. Species: *P. digitalis*.

pollen they collect. This is thought to be because of grooming and/or because the Hymenoptera involved place pollen in pollen-carrying structures where it is unlikely to be transferred to a stigma; this can be true even when bees act as legitimate pollinators (Holsinger & Thomson 1994; Thorp 2000). Species pollinated by bees have developed diverse strategies to maximize pollen transfer to the stigma when pollinator frequency varies (Thomson & Thomson 1992; Thomson et al. 2000), when there is strong male-to-male competition (Fernando & Cass 1997; Sarala et al. 1999), and when pollen viability is limited (Wilson 1995). One of these strategies is to present the pollen in doses. By staggering anther dehiscence, plants reduce pollen waste when pollinators are frequent because a small amount of pollen is available to each visitor. However, if pollinators were scarce, staggering would cause an accumulation of pollen in the anther, such that, when a pollinator does visit, a larger amount of pollen is available (Harder & Thomson 1989; Castellanos et al. 2006).

Another potential benefit of staggering pollen availability is to limit the amount of pollen available to pollen thieves. Bees that collect pollen but seldom contact the stigma are called pollen thieves (Inouye 1980). These insects can also be considered pollen parasites when more effective pollinators are present (Thomson & Thomson 1992). By increasing anther dehiscence time, plants not only reduce the amount of pollen each pollinator gets but also how much pollen can be thieved (Parker et al. 2018). Previously, Rodríguez-Peña & Wolfe (2022) established that pollinators and pollen-collecting bees are numerous in populations of *P. albidus* and *P. whippleanus* (anther type C, subgenus *Penstemon*), but pollen-collecting bees were scarce in populations of species with the other three anther types. This means that staggering pollen presentation could have evolved either in response to pollinator type and behaviour, to reduce pollen collection by pollen thieves, or a combination of both.

The Hymenoptera-pollinated species with one of the other three anther morphologies (anther type A, B, and D) in the genus might have other methods to prevent overharvest of the pollen. The other anther morphologies likely have less need for staggering because other aspects of their anther morphology prevent or reduce pollen parasitism. For instance, anther type A has woolly hair covering the anther opening, which indicates that even though pollen is displayed, it is not easily available or visible to all visitors. Rodriguez-Peña and Wolfe (2022) observed that species with anther type A were visited by bees that either vibrated the

anther or chewed off the woolly hair to obtain pollen. This means that the energy reward from extracting pollen from species with anther type A is lower because some energy must be expended removing the wooly hair. Additionally, anther types B and D have small anther openings, and the pollen is not fully visible. Species with anther type B were reported to be pollinated (in large part) by *Pseudomasaris* wasps, which extract pollen by intensely rubbing their heads against the anthers, producing a scratchy sound. Similarly, species with anther type D were sonicated by several species of bees, which generally extract pollen and nectar from those plants (Rodriguez-Peña & Wolfe 2022). This suggests that pollen is not readily available in anther types B and D and that physical force is necessary to collect the pollen. Future work should aim to understand the role of the wooly hair in *Dasanthera* species and whether it reduces the pollen harvest by bees, whether sonication is necessary for anther type D to release their pollen, and what mechanisms allow *Pseudomasaris* wasps to extract pollen from anther type B.

We initially hypothesized that species with the same anther morphology have similar anther dehiscence times. Our results partially support this hypothesis. Species with anther type C dehisce at a similar rate (Table 2) independent from their pollination syndrome; however, species with anther types B and D pollinated by birds dehisce more slowly than their Hymenoptera-pollinated counterparts. Overall, species with anther type C have the longest anther dehiscence time. Among species pollinated by birds, *P. rostriflorus* with anther type D had the longest anther dehiscence time. *Penstemon rostriflorus* has anthers that dehisce only at the connective (anther type D), and its corolla is typical of a bird-pollinated species: red-orange flowers, tubular corolla, and exerted anthers. Pollen presentation in this species happened in two tiers, the front-most set of anthers dehisced in about six hours, while the rear set of anthers dehisced in the next 12 hours. We noticed that flowers only dehisced one set of anthers per day, which usually happened either in the early morning or the afternoon.

Penstemon rostriflorus pollen presentation appears to occur when hummingbirds are most active. However, we did not collect pollination data in Utah. Castellanos et al. (2006) also reported a similar pattern for *P. rostriflorus*. In particular, *P. rostriflorus* released only 5% of its pollen within 4.5 hours of sampling, which is minimal in comparison with other bird-pollinated species (77% and 90%). The other two bird-pollinated species in this study did not display the same pattern. Differences in temperature and precipitation could not explain why only *P. rostriflorus* synchronously opened its anthers with hummingbird activity, while the other bird-pollinated species did not, as the mean temperature and humidity in all sites were nearly identical (Table 1). The anther dehiscence pattern of *P. rostriflorus* was like species well-adapted to bird pollination (Martén-Rodríguez et al. 2009). The other two bird-pollinated species did not show the same pollen presentation pattern, maybe because they are less exclusively adapted to the bird syndrome. *Penstemon eatonii* (anther type B), has diluted nectar and flower morphology typical of the bird-pollination syndrome; however, field observations indicate that this species is effectively pollinated by both bees and birds (Cane & Dunne 2014). Also, *P. utahensis* is considered bird-pollinated (Wilson et al. 2004); however, there is no empirical evidence that supports this assumption. *Penstemon utahensis*, like many bird-pollinated species, has red flowers, but unlike bird-pollinated species, it has a short and tubular corolla with a big mouth opening (Supplementary Data Fig. S1). *Penstemon utahensis* flower morphology resembles *P. albidus* (a bee-pollinated species) more closely than bird-pollinated species.

Thomson et al. (2000) evaluated pollen presentation in sister-species pairs with bee- or bird-pollination syndrome and classified pollination syndrome based on the relative anther opening size. Likewise, Castellanos et al. (2006) ranked 69 *Penstemon* species (from 1 to 69) based on anther opening size to determine whether pollination syndrome was correlated with anther opening. Thomson et al. (2000) and Castellanos et al. (2006) found that species pollinated by birds have bigger anther openings in comparison with their bee-pollinated counterparts. The authors concluded that a larger anther opening allows species to release more pollen and that when birds visit their flower, a larger amount of pollen is deposited on the animal's body in comparison with bee-pollinated species. Our result partially contradicts previous assumptions of pollen release

on bees- versus birds-pollination syndromes. Species from the subgenus *Penstemon* consistently had more prolonged anther dehiscence but species pollinated by bees had similar anther dehiscence time as species pollinated by birds, which contradicts previous hypotheses. Additionally, several bird-pollinated species had significantly larger anther dehiscence time than some bee-pollinated species, which is inconsistent with the expectation that bird-pollinated species should not present staggered pollen presentation. We believe that species well adapted to bird pollination should stagger pollen presentation when (1) pollen viability is low or/and (2) when pollen parasites are common. Further research is needed to answer this question as there is no study on pollen viability on *Penstemon* and there are no records of non-pollinator visitors of *P. rostriflorus*.

The experimental approach applied to *P. digitalis* effectively increased the temperature and the humidity of the plants' surroundings. The high temperature and high humidity treatment had the slowest anther dehiscence time, whereas, in the high temperature and low humidity treatment, anthers dehisce the fastest. Several intrinsic and extrinsic factors could explain these results. Anther dehiscence has been generally viewed as a desiccatory process; flowers lose water after opening due to increasing transpiration, and the loss of water to the environment is thought to cause anthers to dehisce (Coulter et al. 1911). Several species have been shown to employ other mechanisms to dehisce their anthers. Structural changes in the filaments that prevent the transport of liquid to and from the anthers cause localized desiccation and, subsequently, anthers dehisce (Schmid 1976). Another mechanism is the redirection of water from the anthers to the petals (Bonner & Dickinson 1990). Also, Burck (1906) hypothesized that anthers dehisce by losing water to the nectary organs via osmosis, but this claim has found little to no support (Schmid & Alpert 1977).

In *Penstemon*, there have been no studies on the physiology of anther dehiscence. We showed that high environmental humidity retards anther dehiscence, but there was some variation in the response of individual flowers to high humidity. At nearly 90% humidity, dehiscence time was the longest; some flowers did not dehisce any of the anthers, others dehisced a couple of anthers, and some dehisced all the anthers. In a laboratory experiment, *Peritoma arborea* Nutt. (Cleomaceae) only dehisced slightly at high humidity, while *Epilobium canum* (Greene) P. H Raven (Onagraceae) anthers opened fully under the same conditions (Schmid & Alpert 1977). Since in *Penstemon digitalis,* anther dehiscence continued even in high humidity, physiological processes independent of humidity should also control when pollen is released. Similar to *Penstemon digitalis*, anthers of *Lycopersicon esculentum* Mill. (Solanaceae) dehisce by a combination of mechanisms. Liquid is redirected away from the anther to the petals, and a small but relevant amount of liquid is lost to the environment via evapotranspiration (Bonner & Dickinson 1990).

Pollen presentation schedules should reflect adaptation toward successful pollination, and as such, plants should present their pollen when their pollinators are active. Plants pollinated by bees present their pollen at sunrise or during daytime (Reynolds et al. 2009), while plants pollinated by nocturnal animals open their anthers at dusk or as the night progresses (Martén-Rodríguez et al. 2009; Reynolds et al. 2009). Therefore, it is not surprising that environmental variation in temperature and humidity that affects some pollinators also influences anther dehiscence time. Although the available literature is limited, the evidence suggests that the insect-pollinated species *Penstemon digitalis, Erythronium grandiflorum* (Thomson & Thomson 1992), and *Peritoma alborea* (Krupnick et al. 1999) retard anther dehiscence when humidity is high and the temperature is low, which correlates with the period when insects are less active (Heinrich & Esch 1994). On the other hand, *Epilobium canum* pollinated by birds (Murray 1981), appears to dehisce its anthers more independently of the environment, perhaps because birds, unlike insects, can regulate their body temperature (Heinrich & Raven 1972; Heinrich 1977).

Conclusions

Plant reproduction is a complex system that for most angiosperms requires interactions with animals. Key aspects of reproduction establish how these animals collect and transfer pollen, which aids our understanding of the evolution of plant-animal interactions. Here, we probed into

the detailed behaviour of anther dehiscence, the effects of anther morphology, with regard to principal pollinators, as well as temperature and humidity. We observed four species of penstemons co-flowering in one Utah site, and all these species dehisce their anthers at different rates. Our results provide substantial evidence that anther dehisce is a process that depends on abiotic factors and taxon-specific anther morphology. Anthers that dehisce from end-to-end and across the connective have, in general, the longest anther dehiscence time. To our surprise, a hummingbird-pollinated species had a longer anther dehiscence time than many Hymenoptera-pollinated species. Perhaps pollen presentation is also a reflection of the level of specialization to specific pollinators and that for pollinator-generalist plants, the environment plays a bigger role in the timing of pollen presentation. High humidity had a strong retarding effect on anther dehiscence time, while the effect of temperature was less pronounced, likely because our experiment was unable to induce a sufficiently large increase in temperature. Finally, our experimental and statistical approaches can be extended to other plants to better understand the role of anther characters and the environment on pollen presentation schedule.

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AUTHOR CONTRIBUTION

R.A.R.P. designed the project, conducted data collection, performed data analysis, and wrote the manuscript. A.D.W. oversaw the project design, manuscript preparation, and edited the manuscript.

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the author(s).

DATA AVAILABILITY STATEMENT

Data available in the Supporting Information. Also, the raw data and R codes are in Dryad: https://datadryad.org/stash/share/ncceQVsDXbdc9t7_aKfbxRfbYLtSoxmAqb83BQ589rY

APPENDICES

Additional supporting information (four figures, four tables, and a note) may be found in the online version of this article:

Figure S1: Flower morphology of the *Penstemon* species included in this study.

Figure S2: Design and usage of the open-top chambers.

Figure S3: Anther dehiscence time by species collected in 2018.

Figure S4: Anther dehiscence time by species collected in 2019.

Table S1: Hedge's g effect size between pair of species pollinated by Hymenoptera.

Table S2: Hedge's g effect size between pair of subgenera.

Table S3: Hedge's g effect size between pair of species sampled in Utah.

Table S3: Hedge's g effect size between pair of treatments.

Note S1: Studied species additional description details.

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