Trees (2018) 32:549–557
https://doi.org/10.1007/s00468-018-1655-6

ORIGINAL ARTICLE

Down to the wire: late season changes in sex expression in a sexually labile tree species, *Acer pensylvanicum* (Sapindaceae)

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Received: 25 August 2017 / Accepted: 28 December 2017 / Published online: 25 January 2018

Abstract

Key message **In sexually plastic *Acer pensylvanicum*, determination of sex can occur extremely late, within three weeks of spring flowering. Physical damage causing complete vascular tissue severance results in increased female expression.**

Abstract Species with environmental sex determination are rare amongst angiosperms but widely distributed across taxa. The timing of floral development in species that change sex based on environmental cues is unexplored. We investigated the timing of differentiation of sexual organs in buds of *Acer pensylvanicum*, an understory tree in eastern North America with environmental sex determination. We collected branches from individuals at three collection times in the early spring of 2016 and kept them in a warm greenhouse until anthesis. All individuals exhibited complete or partial female inflorescences in the greenhouse in one or more collection. However, none of these same individuals produced only female flowers in the field. Unlike many other woody species that differentiate bud sexual primordia 9–12 months prior to flowering, *A. pensylvanicum* may differentiate the sexual organs in its flower buds as late as three weeks prior to anthesis. In a separate series of branch collections in 2017, we found that the stress response to cutting leads to increased female sex expression in branches, while earlier warm temperatures (e.g., those caused by growing in a protected greenhouse environment) or increased carbohydrate availability does not. Given the labile sex determination system of *A. pensylvanicum*, the ability to delay differentiation of buds into male or female until shortly before spring flowering would allow individual trees to respond to sex-determining damage cues as late as mid-spring. This supports the hypothesis that *A. pensylvanicum* may not exhibit the lag-time characteristic of temperate spring and early-summer flowering woody species and may change sex expression in response to stress.

Keywords *Acer* · Dioecy · Environmental sex determination · Flowering · Phenology · Sex expression

Introduction

Sex expression in perennial plants is usually a fixed characteristic over the lifetime of an individual, however, in some rare cases, sex expression may be labile and change from year to year (Schlessman 1986; Korpelainen 1998). Although environmental sex determination is found within various angiosperm groups, few studies document the time scale on which changes in sex occur in the absence of applied hormones. Elucidating the phenology of floral sex expression, particularly in sexually labile species, provides foundational data for understanding the necessary temporal proximity of cues for triggering sex change. Changes in sex expression can have immediate effects on sex ratios (Charnov and Bull 1989), and longer-term effects on population viability (Sinclair et al. 2012).

Labile sex determination in plants (also known as environmental sex determination or ESD) may be affected

Communicated by R. Guy.

Electronic supplementary material The online version of this article (doi:https://doi.org/10.1007/s00468-018-1655-6) contains supplementary material, which is available to authorized users.

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by many environmental cues (Charnov and Bull 1977; Schlessman 1986; Korpelainen 1998; Bachtrog et al. 2014). These include abiotic factors such as nutrient availability or factors particular to a specific individual such as size, energy reserves, or the experience of physical trauma (Heslop-Harrison 1957; Freeman et al. 1980; Korpelainen 1998). As a general rule, when compared to male plants, female plants grow in more advantageous microhabitats, are in better condition, have more access to resources, and manifest a larger size (Heslop-Harrison 1957; Charnov and Bull 1977; Freeman et al. 1980; Korpelainen 1998). To understand the proximity and magnitude of environmental cues that trigger changes in sex expression, it is crucial to know when reproductive primordia in the flower buds differentiate and complete development. Our study addresses this aspect as it is exhibited in *Acer pensylvanicum* (Sapindaceae) from eastern North America.

Floral development includes the whole process from bud initiation through differentiation of floral organ primordia and eventually anthesis, i.e., flowering (Sedgley and Griffin 1989). Here, we define differentiation as determination of floral primordia identity into their eventual parts such as sepals, petals, stamens, or carpels (Fabbri and Benelli 2000) and final fate. While differentiation occurs for numerous flower parts, we are primarily interested in the development and fate of reproductive structures. In both sexually plastic and sexually fixed species, hormones induce meristematic tissues and trigger differentiation of sex in flower primordia and later development into sexual organs (Bernier 1988). Earlier work on physiology and phenology in northern temperate deciduous trees that flower in spring and summer seasons shows that bud induction (initiation of development) and differentiation (determination of floral primordia identity and subsequent development) often occur 9–12 months prior to flowering (Sedgley 1989; Fabbri and Benelli 2000; Wilkie et al. 2008).

Woody species are known for their lagged responses to environmental cues so that in some situations the effects of disturbances are not manifested for years (Davis 1986; Kozlowski et al. 1991; Hogg 1997; Mohan et al. 2009; Svenning and Sandel 2013). If the result is to be seen in a particular year, a cue must occur prior to the end of a sensitive period. For individuals to respond to sex-determining triggers, stimuli must occur prior to sexual organ differentiation in flower buds. If sexually labile woody plants differentiate flower buds the summer prior to flowering, only environmental triggers prior to the differentiation period in the previous season would be reflected in the next flowering year, leading to response lag times of one to two years for trees with ESD. Conversely, if sexually labile species differentiate the sexual organs of flower buds closer to anthesis, then the lag in response to sex-determining cues will be much reduced.

To address the question of bud development in sexually labile woody perennials, we used the sexually plastic species *A. pensylvanicum* (Sapindaceae) (Hibbs and Fischer 1979). This species, commonly known as striped maple in the United States, is a small understory maple tree found in rocky soils at higher elevations along the Appalachian Mountains (Hibbs et al. 1980). It is a subdioecious species, with most of the individuals bearing only staminate or pistillate inflorescences in a given season. Less than 5% of a population may express both male and female inflorescences on the same tree (Hibbs and Fischer 1979) and, very rarely, male and female flowers in the same inflorescence (de Jong 1976; Blake-Mahmud and Struwe 2016). Male-flowering individuals outnumber female individuals by five to one (Hibbs and Fischer 1979). Environmental cues for sex switching are currently under investigation. Anecdotal evidence attributes the switch in expressed sex to crown closure in canopy trees (Hibbs and Fischer 1979), or to resource status in a related *Acer* species native to eastern Asia (Nanami et al. 2004).

In *A. pensylvanicum*, functionally unisexual flowers are arranged in pendulous, racemose inflorescences. The flowers are yellowish green, with free, linear-lanceolate to obovate calyx lobes and a campanulate corolla up to 5 mm long and 8(− 11) mm wide with obovate corolla lobes. In the small fraction of individuals, flowering monoeciously (i.e., bearing both staminate and pistillate inflorescences), there are occasionally inflorescences that also bear both staminate and pistillate flowers. These bisexual inflorescences are usually found in a transition area between male and female flowering zones. These do not occur on all monoecious trees, nor do they appear to occur on trees without both male and female unisexual inflorescences. Although some male flowers are observed to contain a highly reduced abortive pistil (de Jong 1976), this has been found to be non-receptive, therefore, these flowers are functionally male (Sullivan 1983). While de Jong (1976) reported rare fully developed morphologically perfect flowers on cultivated trees, subsequent observations in the field have failed to find any morphologically or functionally perfect flowers (Hibbs and Fischer 1979; Sullivan 1983; Blake-Mahmud and Struwe 2016).

Over the course of two years, we investigated the following questions in a combined forced flowering greenhouse and field study:

1. What is the timing of sex differentiation in *A. pensylvanicum* buds? Do branches excised from trees consistently express the same sex throughout flower development in a given year?
2. What is the impact of environmental and physiological cues on flowering sex, specifically earlier warm tem-

peratures, damage via cutting, and increased sugar availability?

Materials and methods

To assess the timing of sex determination, we used trees from our study site located in Jenny Jump State Forest (40.913, -74.922 Warren County, New Jersey, USA). Over a period of seven weeks in the late spring of 2016, we collected branches from 35 *A. pensylvanicum* trees of unknown sex, with diameters at breast height (DBH) of 1–3 cm. Sampled trees were in good condition, had not lost branches, and displayed no visible infection, cankers, or other wounds. We collected two branches from each tree every two weeks on March 8th, March 23rd, and April 6th (group one, two, and three, respectively). Based on previous pilot studies conducted in 2015, we had narrowed the window of bud differentiation to less than six weeks prior to blooming (Blake and Struwe 2015). Collected branches were tagged with individual numbers and collection dates and kept in a cooler with ice until they were brought to a research greenhouse in New Brunswick, NJ. In the greenhouse, we recut the stems under water and placed them in a mildly acidic water solution, randomly assigned into one of two replicates. The water solution contained tap water, 1.5 ml of 3% bleach, and 0.32 g of granulated citric acid per liter of solution for a pH of 3.5 (16.7 mM citric acid). Water was kept in translucent brown glass bottles to reduce microbial growth and was not changed during the seven weeks of the experiment. Bottles were kept in a random design (blocked by collection time) away from artificial greenhouse lights. Temperatures in the greenhouse approximated ambient warm spring temperatures of the study site (10–12 °C at night, 20–22 °C during the day). Branches remained under full shade and flowered approximately 14–28 days after collection. Branches that did not develop flowers were excluded from the study. Trees at Jenny Jump State Park began to flower the week of April 26th 2016. We scored the sex of the trees from which we collected branches and counted the number of female and male inflorescences per individual. As the individual trees were relatively small, all inflorescences were visible without binoculars.

To address the question of triggering cues, we used trees located in our other study site at Wawayanda State Park (41.210, -74.464, Passaic and Sussex Counties, New Jersey, USA). In early April of 2017, we collected branches from 40 *A. pensylvanicum* trees of unknown sex, 1.0–5.5 cm DBH. We collected six branches from each tree between April 5th and April 7th, during a time when we would expect to see variation in flowering sex, based on previous data (Blake and Struwe 2015). In 2017, anthesis in the field was slightly later than anticipated, due to cold periods in April. Collected branches were put in one of three treatments: plain water in the field, sugar water in the field, and plain water in the greenhouse. "Plain" water solutions consisted of the same water mixture used in 2016 (i.e., water, 1.5 ml of 3% bleach, and 0.32 g of granulated citric acid per liter of solution for a pH of 3.5). The sugar water solution consisted of water, citric acid, bleach, plus sucrose (40 g of granulated sucrose, 1.5 ml of 3% bleach, and 0.32 g of granulated citric acid per liter water; 117 mM sucrose). Every plastic bottle contained two branches from each tree and was painted black to inhibit microbial growth. Field bottles were covered with parafilm to prevent unintended additions from rainwater or leaf litter and tied securely to the base of the tree from which the branches were taken. Two branches per tree were tagged and kept in a cooler with ice until they were brought to a research greenhouse in New Brunswick, NJ. In the greenhouse, we recut the stems under water and placed them in the plain water solution in black plastic bottles. Temperatures in the greenhouse approximated the ambient warm spring temperatures of the study site (10–12 °C at night, 20–22 °C during the day). Branches remained under full shade and flowered 14–24 days after collection. Branches that did not develop flowers were excluded from the study. Trees at Wawayanda State park began to flower the week of May 1st 2017. We scored the sex of the trees from which we collected branches and counted the number of female and male inflorescences per individual.

We used McNemur's test to test for consistency in sex expression in treatment and control groups as well as consistency in flowering sex expressed in the greenhouse and field. Unlike a Chi-square analysis, McNemur's test may be used for paired categorical data (McCrum-Gardner 2008). McNemur's test statistic with a Yates' continuity correction (Yates 1934) follows a Chi-square distribution and is computed as follows:

$$X^2 = \frac{(|b-c|-1)^2}{b+c}.$$

In this formula, b represents the number of individuals in state one in the control but state two in the treatment and c represents the number of individuals in state one in the treatment but state two in control. Those individuals that are in the same state (in this case, expressed sex) in both control and treatment are not included in the computation of the test statistic (McNemar 1947).

Voucher specimens are deposited in the Chrysler Herbarium (CHRB) at Rutgers University in New Brunswick, NJ, USA.

Results

Timing of flower development—experiments in 2016

Of 35 trees, seven did not flower in the field or had collected branches that produced only leaves and were excluded from the study. Each of the remaining 28 trees had at least one collected branch that bloomed fully or partially female in the greenhouse, while all of the trees bloomed partially or completely male in the field. Collected branches showed only 50% fully male sex expression when collected as late as three weeks prior to anthesis.

Of the 28 study trees, sampled branches from 19 individuals produced flowers following the first collection of branch segments (group 1) in March (Table 1). All 19 flowered female on one or both of the two branches collected in this initial sample; one individual (tree 26) had one branch that bloomed male and one that bloomed female. Of the branches collected as part of the second sampling in March (Group 2), branches from 23 trees (out of 28) produced flowers. From this collection, 74% of those individuals produced only female inflorescences and 17% produced only male flowers. Two individuals flowered differently, with male flowers and female flowers produced on different branches in different replicates. From the third and last collection in early April, 43% of individuals flowered female, 48% male, and 9% of individuals produced inflorescences including both male and female (but no perfect) flowers. Interestingly, the two individuals producing inflorescences with both staminate and pistillate flowers in the greenhouse (trees 17 and 21) were not among the three trees flowering monoeciously in the field. When study trees flowered in the field, 25 of 28 flowered exclusively male, while three trees flowered monoeciously, bearing male inflorescences, female inflorescences, and inflorescences with both male and female flowers.

We did not detect a significant difference between the two replicates in any collection group (group 1: McNemur's $X^2 = 0$, $p > .05$, $df = 1$; group 2: McNemur's $X^2 = 0.5$, $p > .05$, $df = 1$; group 3: McNemur's $X^2 = 0$, $p > .05$, $df = 1$). Because of this, we combined data across replicates (Table 1) to give a more complete picture of floral development. Data divided into replicate groups are available online (see online resource 1).

We found that branches kept in the greenhouse environment began by presenting female inflorescences before trees reached anthesis in the field. Later and closer to their natural flowering time in the field, approximately half-exhibited inflorescences with male reproductive structures (see Table 1). Furthermore, all trees that had early-flowering female inflorescences in the greenhouse flowered male (partly or exclusively) in their natural forest habitats. Of the 28 trees that flowered, nine differentiated sex as late as three weeks before flowering, while 32% of trees differentiated their buds less than three weeks before flowering. The mean time for trees determining sex expression was approximately four and half weeks prior to anthesis (Table 1). Results from McNemur's test showed that collected branches bloomed differently in the greenhouse than they did in the field at each collection (group 1: McNemur's $X^2 = 15.06$, $p < .001$, $df = 1$; group 2: McNemur's $X^2 = 12.07$, $p < .001$, $df = 1$; group 3: McNemur's $X^2 = 7.01$, $p < .01$, $df = 1$).

Cues for flower development—experiments in 2017

In 2017, we collected branches from 40 different trees from a different site to investigate cues for changes in sex expression. Of 240 branches collected, 160 were kept in the field until anthesis. A large proportion of these field bottles were disturbed by wildlife and destroyed. The data resulting from the fraction of branches that remained in bottles until anthesis is presented here. Of the 40 trees, 24 flowered male in the field, eight flowered female in the field, five exhibited both male and female flowers, and three did not flower. While populations were still overwhelmingly male, the increased proportion of female trees in 2017 is consistent with sex ratios across a larger sample of multiple populations. Responses of excised branches are summarized in Table 2.

To examine the effect of earlier warmer temperatures, we made a pair-wise comparison of flowering specimens kept in the greenhouse with surviving branches kept in the plain water in the field ($n = 15$ pairs available for comparison, see Table 2). All of these co-flowering specimens expressed the same sex (female). These results support the conclusion that earlier, warmer temperatures do not affect flowering sex.

To examine the effect of increased carbohydrates, we compared flowering specimens kept in the field in plain water and in sugar water ($n = 14$ comparisons, see Table 2). Of these, branches from 13 individuals flowered completely female in both treatments, one individual flowered female in plain water and monoeciously in sugar water, and one individual flowered female in the plain water and male in the sugar water. This lends no statistically significant support to the theory that increased carbohydrates affect flowering (McNemur's $X^2 = 0$, $p > .05$, $df = 1$).

To examine the effect of the shock administered by cutting, we compared intact trees with flowering specimens kept in the plain water in the field ($n = 17$ comparisons, see Table 2). Nine trees flowered fully or partially female in the field (five female, four monoecious), while all of their branches kept in situ flowered female; eight male-flowering trees had branches in the field that flowered female. This supports the conclusion that removing branches from a tree through cutting had a significant effect on flowering sex of

Table 1 Timing of sex expression of *Acer pensylvanicum*

Individuals	Group 1	Group 2	Group 3	In Field	Differentiation occurred
Tree 1	○	○	○	○ ∗ ●	within 3 weeks prior to flowering (most likely)
Tree 2		○	○	○ ∗ ●	within 3 weeks prior to flowering (most likely)
Tree 3		○	○	○ ∗ ●	within 3 weeks prior to flowering (most likely)
Tree 4			○	●	within 3 weeks prior to flowering
Tree 5	○			●	within 7 weeks prior to flowering
Tree 6	○			●	within 7 weeks prior to flowering
Tree 7	○	○	○	●	within 3 weeks prior to flowering
Tree 8	○	○	○	●	within 3 weeks prior to flowering
Tree 9	○	○	○	●	within 3 weeks prior to flowering
Tree 10	○	○	○	●	within 3 weeks prior to flowering
Tree 11	○	○	○	●	within 3 weeks prior to flowering
Tree 12	○	○	○	●	within 3 weeks prior to flowering
Tree 13	○	○	●	●	3-5 weeks prior to flowering
Tree 14	○	○	●	●	3-5 weeks prior to flowering
Tree 15	○	○	●	●	3-5 weeks prior to flowering
Tree 16		○	●	●	3-5 weeks prior to flowering
Tree 17		○	∗	●	within 3 weeks prior to flowering
Tree 18	○		●	●	3- 7 weeks prior to flowering
Tree 19		○	●	●	3-5 weeks prior to flowering
Tree 20		○	●	●	3-5 weeks prior to flowering
Tree 21		●	∗ ●	●	within 3 weeks prior to flowering
Tree 22	○	●	●	●	5-7 weeks prior to flowering
Tree 23	○	●		●	5-7 weeks prior to flowering
Tree 24	○			●	within 7 weeks prior to flowering
Tree 25		○ ●	●	●	3-5 weeks prior to flowering
Tree 26	○ ●	○ ●	●	●	3-5 weeks prior to flowering
Tree 27	○	○	●	●	3-5 weeks prior to flowering
Tree 28	○	●	●	●	5-7 weeks prior to flowering
Female trees	19	17	10	0	trees differentiating between 5 and 7 weeks before = 7 trees differentiating between 3 and 5 weeks before =9 trees differentiating between 0 and 3 weeks before = 12 Average time to differentiation = 4.6 weeks, based on most conservative time estimate
Male trees	0	4	12	25	
Monoecious trees	1	2	2	3	

Table 1 (continued)

Sex expression data from all branches. Empty circles denote inflorescences with female flowers, filled circles indicate inflorescences with male flowers, asterisks indicate both male and female flowers within a single inflorescence (monoecious), blank boxes represent non-flowering branches. We collected branches in group 1 seven weeks prior to flowering, group 2 at five weeks prior, and group 3 at three weeks prior to anthesis in the field. In group three, Tree 17 had one branch that bloomed with male inflorescences and female inflorescences and another branch with male inflorescences and monoecious inflorescences. In the same group, Tree 21 had one branch with male inflorescences only and another with male and monoecious inflorescences. Trees 25 and 26 had one branch that bloomed female and one branch that bloomed male in collections one and/or two. Trees 4–28 bloomed entirely male in the field. Trees 1–3 had male, female, and monoecious inflorescences in the field. Differentiation was assumed to occur in the window between changes in sex expression. In most cases this is a two week period, but may extend upwards to seven weeks in cases where intermittent branches did not flower in the greenhouse such as tree 5, 6, or 24. In this situation, the most conservative time to differentiation (i.e.—the longest) was used to calculate mean time to sexual differentiation. In trees 1–3, we observed different-sexed inflorescences dispersed throughout the tree and not concentrated on some branches, therefore, differentiation for these individuals most likely occurred within three weeks. However, the small possibility that 4–6 randomly chosen branches were all destined to be female prior to the start of collection cannot be ruled out

Table 2 Flowering in *Acer pensylvanicum* across experimental treatments

Location *Unit* Treatment	A: field *Whole tree*	B: field *Branch* Plain water	C: field *Branch* sugar water	D: greenhouse *Branch* Plain water
Males	24	0	2	0
Females	8	17	18	29
Monoecious	5	0	1	1
Non-reproductive/bottle destroyed	3	23	19	10

Sex was monitored in whole study trees (A) and from branches collected from study trees kept in three different treatments (B-D). Approximately half of branches kept in bottles in the field were destroyed by animals and did not flower. We conducted analyses on individuals that co-flowered in two treatments. To assess the effects of temperature we compared treatment B and D. To assess the effect of sugar we compared B and C. To assess the effect of cutting we compared A and B

the branches (McNemur's $X^2 = 6.125$, $p < .05$, $df = 1$) and was the crucial cue triggering early female flowering of branches collected over a 6-week period in 2016.

Discussion

Our results show that the determination of sex expression in flower buds can occur within three weeks of flowering and is strongly influenced by physical damage in *A. pensylvanicum*. The 2017 findings on sex-determining cues are consistent with the hypothesis that physical damage triggered the female sex expression seen both in cut branches in the field in 2017 and in the greenhouse in 2016. The 2016 experiment indicates the speed at which these cues can be processed by the tree. The late timing of sex determination in *A. pensylvanicum* is in stark contrast to longer timescales found in many other woody species with constant sex expression, and some other species in *Acer*. When combined with the labile sex determination system of this species, the late differentiation of buds suggests that sex expression in *A. pensylvanicum* may be influenced by injury caused by storms and extreme weather events at a speed unusual for woody plants.

Timing of flower development—experiments in 2016

Ideally, studies addressing flower development use histological methods, but sometimes this is not possible. For example, in *A. pensylvanicum*, tough hairs present within the inflorescence buds prevent traditional sectioning via conventional anatomical histology procedures such as paraffin embedding, preventing the investigation of sexual development prior to flowering (Blake-Mahmud, unpubl. data). Furthermore, Sullivan found that flower bud sex could not be determined using dissection techniques (Sullivan 1983). In these types of situations, plant phenology and flower development may be addressed via twig studies in which dormant twigs or small branches are collected and allowed to flower in a greenhouse. In sexually stable species (no ESD), the flowering behavior of cut branches parallels the behavior of trees in the field (Vitasse et al. 2014) and may be used to explore changing plant phenology in response to particular climatic drivers such as earlier warmer temperatures or reduced chilling (Primack et al. 2015). De Jong addressed the response of floral sex and timing to various plant hormones of selected *Acer* species by collecting branches over the winter and forcing them into flower via applied hormones (gibberellins). He associated the changes in flowering in the greenhouse

with corresponding development of floral primordia. Samples that bloomed consistently as one sex were assumed to have differentiated their buds prior to the first collection, while those that changed flowering sex were differentiating their sexual structures during the collection period. While his study used only one to two individuals per species, it provided anecdotal evidence regarding the complexity of flower development in maples and showed that development spans a wide temporal range. In cultivated trees in the Netherlands, *Acer* flower buds are generally initiated in June or July, approximately 8–9 months before flowering (de Jong 1976). For example, in *A. rubrum*, buds begin development as perfect (bisexual) flowers for the first two months, before differentiating into male and female buds at the end of August (de Jong 1976). In *Pistacia vera (Anacardiaceae*, also classified in Sapindales like *Acer*), unisexual buds differentiate reproductive primorida at approximately the same time, roughly 10 months prior to flowering (Hormaza and Polito 1996).

There are some previous observations of late flower differentiation in *Acer* and related species. For example, sexual determination in buds of *A. platanoides* occurs four weeks before blooming (Haas 1933). *Acer platanoides* is monoecious with heterodichogamous flowering (Renner et al. 2007), meaning then every tree bears both male and female flowers and does not change sex expression. Given the occurrence of both staminate and pistillate flowers on every individual in a single flowering year in *A. platanoides*, it is not unexpected that sex could remain flexible until shortly before anthesis as this is a way to adjust maternal investment and energy expenditure in monoecious species (Lloyd 1980). De Jong (1976) found a similar response with a single individual of the sexually labile *A. davidii* subsp. *grosseri*, with sampled branches blooming first female in the presence of added gibberellic acid, then male in a greenhouse before blooming monoeciously in the field. In *Kirkia wilsii* (Kirkiaceae: Sapindales), trees flower in successive unisexual flushes so that a tree is functionally dioecious at any one time but monoecious over a 40-day flowering season (Immelman 1984). Observations of herbarium specimens indicated that flower buds were the opposite sex of the preserved open flowers. The author did not investigate when trees initiated buds nor when buds differentiated sexual primordia (Immelman 1984).

In *A. pensylvanicum*, however, approximately 95% of trees growing in their natural habitats flower with unisexual inflorescences of a single sex in a given year (Blake-Mahmud and Struwe 2016; Hibbs and Fischer 1979). The plasticity of flower sex not induced by addition of artificial hormones so close to blooming time is highly unusual for a functionally dioecious, sexually plastic species, and thus notable in comparison to results observed in other woody species with separate sexes. Future work might employ other methods, such as a scanning electron microscopy, to address the timing of initiation of bud primordia, which cannot be ascertained via branch studies.

In other temperate taxa such as *Malus*, buds for the next year differentiate approximately six weeks after the current year's flowering (Buban and Faust 1982, see Fig. 1). Other trees also begin bud development and differentiation the previous year and well in advance of flowering: *Diospyros kaki* in early June (Yonemori et al. 1993) and *Prunus avium* in July to August (Guimond et al. 1998). In temperate trees with separate unisexual male and female

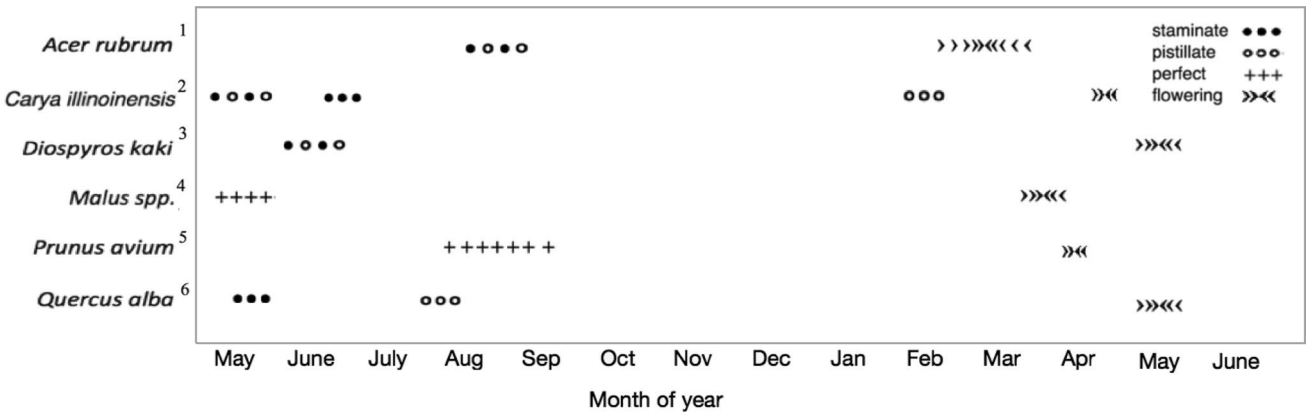

Fig. 1 Differentiation of bud sexual structures in relation to flowering. Most woody species differentiate the primordial reproductive structures within buds the year prior to flowering. In species with separate male and female flowers, staminate buds are often determined first (as in *Quercus alba*) or simultaneously (such as in *Carya illinoinensis*). Differentiation of sexual structures may occur over the course of 2–6 weeks and will vary according to location, population, climate, and current weather. 1—Wageningen, the Netherlands (de Jong 1976), 2—Georgia, USA (Woodroof and Chapman Woodroof 1926), 3—Kyoto, Japan (Yonemori et al. 1993), 4—Northern Hemisphere, flower buds initiated 2–5 weeks following anthesis (Buban and Faust 1982), 5—Prosser, Washington, USA (Guimond et al. 1998), 6—Cambridgeshire, England (Longman and Coutts 1974)

inflorescences, buds will often differentiate at different times, usually with staminate buds preceding the development of pistillate buds (Longman and Coutts 1974). For example, *Quercus alba* differentiates staminate buds in late May through July; pistillate buds develop in August of the previous year (Longman and Coutts 1974; Lavender 1986). In dioecious *Carya illinoinensis*, all female and male buds differentiate in May for the following flowering year, but flowers develop at different times. Following sex differentiation in *C. illinoinensis*, male buds continue to develop that year, while female trees arrest bud development until late winter. Buds complete the development of female reproductive structures during the last week of February (Woodroof and Chapman Woodroof 1926; Wetzstein and Sparks 1986; Wetzstein 1989, See Fig. 1). In *Juglans regia*, male primordia initiate in May the year prior to flowering, while female primordia initiate in August. Both male and female buds are dormant during the winter before completing development in March and flowering in April (Lin, personal communication).

Results from studies of *Carya, Juglans*, and previous work on *A. pensylvanicum* (deJong 1976) might suggest that male flowers develop first due to earlier initiation of stamen primordia compared to gynoecial primordia. This, however, was not the case. Our findings also illustrate that buds maintain a totipotency to express either sex until shortly before flowering. Even though almost all individuals express only one sex in the field, trees maintain the capacity to express both sexes sequentially under the experimental condition of earlier anthesis.

Cues for flower development—experiments in 2017

The influence of branch excision on female sex expression is the first experimental evidence linking damage and expressed sex in *A. pensylvanicum*. Interestingly, other experimental manipulations in which phloem and xylem conductivity were reduced via 50% removal did not result in changes in sex expression significantly different from background rates (Blake-Mahmud, unpub. data). This suggests that a complete (or near complete) severance of conductive tissue is required to change the sex of an individual branch. Whether the severance in conductive tissues results in a lack of root-produced hormones (such as cytokinins), an overabundance of bud-produced hormones (such as auxins), or something else (not associated with a growth regulator) warrants further investigation. Taken with the results from 2016 involving branch collection over a 6-week time period, it appears that while complete severance of conductive tissue triggers femaleness, its effectiveness as a cue for female sex expression in the current year decreases slightly as flowers approach anthesis and does not continue to override male primordia development (as evidenced by the approximately 50% male flowering cut branches three weeks prior to anthesis in the 2016 studies). We are still elucidating the proximate environmental triggers for changes in sex expression in entire individuals, though preliminary unpublished data indicate that diminished plant health (due to extreme physical damage or infection) correlates with changes to female sex expression (Blake-Mahmud and Struwe 2016).

Other studies have indicated that higher nonstructural carbohydrates are present in female flowering individuals in the winter prior to flowering (Blake and Struwe 2017), but we did not find a significant influence of increased carbohydrates (i.e., the sugar added in 2017) on sex expression in our studies. This may mean that sugar does not trigger female sex expression, that the amount of added sugar was below the threshold level needed, or that sugar was not taken up into the developing buds. This may also be due to limits in study design. The number of co-flowering specimens was low due to animal disturbance (i.e., only 14 comparisons). Earlier spring temperatures as simulated in the greenhouse did not affect the sex expression of individual branches. This supports the hypothesis that the earlier onset of spring we have seen in recent decades (Cleland et al. 2007) and the progression of this phenomenon we will expect in the future will affect timing of flowering in *A. pensylvanicum*, but will likely not affect the sex ratios present in natural populations.

Because of the nature of ESD, the late determination of sex may allow *A. pensylvanicum* to remain potentially receptive to sex-determining cues for a longer time period. This may have important ramifications for sex ratios in populations and subsequent fruit set. Depending on the timing and nature of a disturbance, most forest trees exhibit a one to two year lag in their responses to stimuli (Holmes and Likens 2016). However, in the case of *A. pensylvanicum*, the sensitive period for sex determination lasts until April of the flowering year, translating into a time lag potentially shorter than three weeks prior to anthesis. While the impacts of large storms on forests remain complex, the potential lack of lag time in changing sex expression for *A. pensylvanicum* may allow this species to respond reproductively to storm damage at previously unanticipated rates.

Acknowledgements We would like to thank Dr. Jason Grabosky, Dr. Peter Morin, Dr. Greg Anderson, the Research and Horticultural greenhouse staff, Carlos Olivares, Anny Marchioni, and Pepe Bowman for help with this research. Research permits were obtained from the New Jersey Department of Environmental Protection. This work was supported by the Ecology and Evolution Graduate Program, Rutgers University; the Torrey Botanical Society; the Botanical Society of America, and NSF IGERT Grant (NSF-DGE/IGERT 0903675).

Author contributions J. Blake-Mahmud designed and conducted the experiments, collected and analyzed data, and wrote the manuscript as part of a doctoral dissertation. L. Struwe co-led the embedding and sectioning investigation and serves as the doctoral dissertation advisor.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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