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BRIEF COMMUNICATION

UNIFORM FEMALE-BIASED SEX RATIOS IN ALPINE WILLOWS¹

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- *Premise of the study:* The development of biased sex ratios in dioecious plant species has been ascribed to either (1) factors influencing differential adult mortality of male and female plants or (2) factors acting at an early life stage that determine seed sex ratio or seedling survival.
- *Methods:* To discriminate between these two competing hypotheses, we surveyed sex and age of 379 individuals from five species of the genus *Salix* across 11 alpine valleys in the southwest Yukon.
- *Key results:* We observed uniformly female-biased sex ratios of approximately 2:1 across all adult age cohorts and patch sizes of the five willow species. No spatial variation in sex ratio occurred that could be associated with site-specific characteristics such as elevation or aspect.
- *Conclusions:* Our results indicate that the female-biased sex ratios in the alpine willow species investigated in this study are not a consequence of ecological processes acting on established adult plants. The sex ratio is instead determined at an early life stage by a mechanism that remains unknown.

Key words: dioecy; female bias; Salicaceae; *Salix* (willow); sex ratios; spatial sex segregation.

The evolutionary benefits of dioecy, having male and female reproductive organs borne on separate individuals of the same species, are still relatively poorly understood (Barrett, 2010; Barrett et al., 2010). The ratio of male to female plants can vary for dioecious species, with most plant species exhibiting equal or male-biased sex ratios, and female-biased sex ratios occurring less frequently (Lloyd, 1974; Barrett et al., 2010). The evolutionary basis of biased sex ratios has been a topic of interest, as sex ratio could influence reproductive success and play an important role in the evolution of gender differences in plant traits (Ashman and Diefenderfer, 2001). Theory predicts that if production costs and reproductive fitness over time are equal between male and female plants, then natural selection will act to balance a population sex ratio at 1:1; however, many dioecious plants exhibit biased sex ratios, and the direction of this bias cannot always be explained by life history characteristics (Sinclair et al., 2011).

Female-biased sex ratios are often reported for species of the genus *Salix* (Faliński, 1980; Crawford and Balfour, 1983; Elmqvist et al., 1988; Alliende and Harper, 1989; Dawson and Bliss, 1989; Alstrom-Rapaport et al., 1997; Rottenberg, 1998; Predavec and Danell, 2001; Jong and Meijden, 2004; Dudley, 2006; Ueno et al., 2007; Hughes et al., 2009), with a ratio of 2:1 females to males observed in many studies (Crawford and Balfour, 1983; Alliende and Harper, 1989; Dawson and Bliss, 1989; Predavec and Danell, 2001; Jong and Meijden, 2004; Ueno et al., 2007). However, in taxonomically related poplars, male-biased sex ratios have been observed (Yin et al., 2008). An interesting ecological and evolutionary puzzle arises from the observation of female-biased sex ratios in willows. Why might species that are dioecious, both wind- and insect-pollinated, have wind-dispersed seeds, are clonal, long-lived, and often early colonizers of disturbed terrain have female-biased rather than equal or male-biased sex ratios?

The ecological processes promoting biased sex ratios have been the subject of some debate. Two broad hypotheses could explain biased sex ratios: (1) differential adult mortality between male and female plants or (2) the existence of early-acting factors that affect the number of individuals of each sex that establish. If adult mortality is the primary cause of female-biased sex ratios, the female-bias should be greater with older vs. younger individuals. Studies have evoked a variety of explanatory factors influencing differential adult mortality such as herbivory or stress tolerance (Elmqvist et al., 1988; Hughes et al., 2009; Yu and Lu, 2011). In willows, differential adult mortality has been linked to either better competitive abilities of females (Dawson and Bliss, 1989; Rottenberg, 1998), better tolerance to environmental disturbance by females (Hughes et al., 2009), or greater herbivory on male plants (Elmqvist et al., 1988; Predavec and Danell, 2001).

As an alternative to factors influencing adult mortality, biased sex ratios in willows might result from processes occurring

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during pollination, seed development, seed dispersal, or germination (Crawford and Balfour, 1983; Taylor et al., 1999; Jong and Meijden, 2004; Jong et al., 2005; Stehlik and Barrett, 2006; Ueno et al., 2007; Stehlik et al., 2008; Barrett et al., 2010). If sex ratio is determined at an early life stage, then no variation in sex ratio should be observed with age of adult plants. In two planting experiments conducted with the European willow species *Salix viminalis* L. (Alstrom-Rapaport et al., 1997) and *Salix repens* L. (Jong and Meijden, 2004), female-biased sex ratios were found. In each case, seeds from the respective *Salix* species were planted and grown to a reproductive stage to assess sex ratios. In both studies, germination approached 100% for most crosses and postgermination mortality was near 0%; however, the resulting sex ratios were still strongly female-biased, indicating that in these species, the sex ratio was determined at or before seed set. However, these studies lacked a field component to verify results in natural settings.

To investigate female-biased sex ratios, we sampled individuals from five species of alpine willows at their elevational range limits across 11 separate valley drainages within a nearly 100 × 100 km area of the Kluane region, southwest Yukon, Canada. We aged all individuals surveyed, by counting annual growth rings, to test whether sexes differed in mortality. Our data provide a unique test of whether factors influencing adult mortality are contributing to the development of female-biased sex ratios across an alpine tundra landscape. We tested predictions of two hypotheses: (1) If adult mortality influences sex ratios, then sex ratios closer to 1 : 1 will occur in young willows, with higher frequencies of females among older individuals. (2) If sex ratio is determined by a process acting at an early life stage, then sex ratio will not vary with age.

MATERIALS AND METHODS

This study was conducted in alpine tundra in the Kluane region, Yukon Territory, Canada. Eleven separate valleys, 1 to 100 km apart, were included in this survey (Table 1). On opposite aspects, we established a transect parallel to the shrubline, defined as the maximum elevation at which tall willows grow. We identified individuals of each species within 3 m of transect points located at 10-m intervals along the slope until we had sampled eight individuals. If no willows were present at a sample point, we walked 10 m or more until we were within 3 m of the next willow. We repeated this sampling farther down the slope at the elevation where ca. 50% of the ground cover was occupied by shrubs. At two valleys (Gladstones and Observation), we also sampled at a lower elevation where shrub cover was ca. 75%. For each willow surveyed, we identified the species and sex if the individual had visible catkins and recorded the location, elevation, slope, and aspect. We measured the largest diameter of the shrub patch and the maximum height and took a 3–5 cm long section of the

largest stem from the stem base, just above the soil surface, for growth ring analysis.

Ten species were recorded during our survey of 379 individuals. These species vary in flowering phenology, chromosome number, ploidy level, and evidence of hybridization (Table 2). The six most abundant species were *S. pulchra* (diamond-leaf willow), *S. niphoclada* (barren-ground willow), *S. glauca* (gray-leaf willow), *S. richardsonii* (Richardson's willow), *S. barrattiana* (Barratt's willow), and *S. alaxensis* (felt-leaf willow). We were not always able to distinguish between *S. niphoclada* and *S. glauca* during field surveys, and so have combined these individuals into one taxonomic category; however, we believe that most individuals sampled in this group belong to the species *S. niphoclada*. Species identifications were confirmed by George Argus (Emeritus, National Herbarium of Canada).

To estimate the age of willow individuals, we cut thin sections of the shrub stems, mounted the sections on glass slides, and took digital images. Rings were counted and measured with a resolution of 0.0001 mm (WinDendro, Québec, Canada or ImageJ, Research Services Branch, National Institute of Mental Health, Maryland, USA) along four arbitrary radii placed on each shrub section, each at 90° from the other unless radii had to be shifted to avoid growth deformities or rotten wood. We manually cross-dated radii to determine the total number of annual rings per shrub section.

An apparent pattern of biased sex ratios or age-dependent sex ratios could arise due to (1) inaccurate estimates of plant age, (2) repeat sampling of the same clone, and (3) differences in our ability to determine the sex of males and females. However, we were able to address each of these issues in our study, as explained below.

The age of the largest stem of these small-statured individual plants is likely to correlate closely with the date of recruitment. No major disturbances, such as fire, have occurred in recent times, and large herbivores that can consume the entire aboveground biomass of an adult willow plant do not occur here. During our surveys, we did not observe dieback that would suggest that the oldest portion of the willow patches could be dead. In northern alpine systems, decomposition rates are very slow; therefore, any dead portions of individuals would be visible on the landscape for decades. For these reasons, we are confident that our stem ages are accurate estimates of the overall patch age at our sampling sites.

We ensured that the individuals sampled in our survey were genets rather than ramets. Although willows can reproduce through clonal spread (Argus et al., 1999), we restricted our sampling to individuals of the same species growing greater than 10 m apart. Tundra willows growing at elevations between 1500–2000 m in the Kluane Region are found in discrete patches that have likely established from seed over the past century and have not yet grown to cover larger areas, as observed at lower elevations (I. H. Myers-Smith, personal observations). Willows growing in these relatively extreme environments do not tend to spread over more than a few meters and have been shown to be genetically distinct even when growing in close proximity (Douhovnikoff et al., 2010).

We found no evidence that reduced detection of male plants relative to female occurred in our survey. We monitored 20 individuals from three species (*S. pulchra*: 11 individuals, *S. richardsonii*: 5 individuals, *Salix glauca*: 4 individuals) at the Pika Camp site over 5 yr to address whether our detection of male plants was reduced relative to female (I. H. Myers-Smith and D. S. Hik, unpublished data). Reproduction varied between years for the three common species (*S. pulchra*, *S. richardsonii*, *S. glauca*), and male catkin longevity was shorter than female; however, when an individual was reproductive, the catkins of both species were visible on the plant until early August. We did not detect more nonreproductive males vs. females in years when reproduction was low for a given species; in fact, we observed more nonreproductive female individuals than male, though this difference was not significant.

To account for the possibility that more unidentified individuals could be male, we recalculated the sex ratios assuming that all unidentified individuals were male. Using this conservative assumption, we found that one of the six valleys had female-biased sex ratio not significantly different from 2 : 1, four had a sex ratio not different than 1 : 1 and one had a sex ratio of ca. 1 : 2. Since it is highly unlikely that all unidentified individuals were male, we are confident that the majority of sampling transects have a female-biased sex ratio.

All statistical analyses were conducted using R version 2.14.0 (R Development Core Team, Vienna, Austria). We had a nested sampling design, with sampling transect nested within aspect nested within valley. We calculated binomial confidence intervals using the exact method and we used χ^2 tests to test for differences between observed sex ratios and both a 1 : 1 and 2 : 1 female to male sex ratio for each of our study valleys, by species, and for categories of the sampling elevation, shrub patch size (patch volume), and stem age (Fig. 1).

TABLE 1. Locations of the 11 alpine valleys sampled in this study (decimal degrees, datum: WGS 84).

Site	Latitude	Longitude
Gladstones	61.38	–138.21
Five Lakes	61.26	–138.18
Bison	61.21	–138.18
Pika	61.22	–138.28
South	61.22	–138.28
Burwash	61.40	–139.46
Copper	61.24	–139.08
Kluane Plateau	60.96	–138.43
Cairnes	60.90	–138.25
Decoeli	60.83	–137.87
Observation	60.82	–138.73

TABLE 2. Distribution, phenology, chromosome numbers, and ploidy levels reported for the most common tall willow species sampled in this study (Argus et al., 1999); the number of females, males, and unidentified individuals for each of the different species at valleys sampled before August; and the χ^2 and P value for a deviation from a 2♀:1♂ sex ratio.

Willow species	Timing of pollination	Chromosomes	Ploidy levels	N females	N males	N unidentified	χ^2	P
<i>Salix alaxensis</i> (Andersson) Cov. var. <i>alaxensis</i>	before leaves	$2n = 38$	2x	7	5	7	0.4	0.54
<i>Salix barrattiana</i> (Hook.)	before leaves	unknown	unknown	15	5	2	0.6	0.43
<i>Salix glauca</i> L. var. <i>acutifolia</i> (Hook.) C. Schneider	with leaves	$2n = 76, 95,$ and 114	4x, 5x, 6x	45	20	17	0.2	0.66
<i>Salix niphoclada</i> Rydb.	with leaves	$2n = 38$	2x					
<i>Salix pulchra</i> Cham.	before leaves	$2n = 76$	4x	29	12	26	0.3	0.58
<i>Salix richardsonii</i> Hook.	before leaves	$2n = 38$	2x	15	5	17	0.6	0.43

We tested for factors influencing the variation in sex ratios between survey transects using a generalized linear model with binomial errors and valley (Gladstones, Five Lakes, Bison, Kluane, Cairnes, Observation), aspect (north, south, east, or west) and sampling transect (shrubline, 50% shrub zone, or 75% shrub zone) as random effects to account for our nested study design. We tested for differences in sex ratio between sampling elevation, shrub patch size, and stem age. The shrub patch size measurement was log-transformed.

The proportion of unidentified individuals differed among valleys and sampling transects. Shrubs that were smaller were less likely to be reproductive, and valleys sampled at the end of the growing season had a higher proportion of individuals with unidentified sex. Patch size and date of sampling were significant predictors of the proportion of individuals with unidentified sex (generalized linear model, $N = 42$ sampling transects, age: $z = 0.44$, $P = 0.66$; patch size: $z = -6.07$, $P < 0.01$; date of sampling: $z = 5.83$, $P < 0.01$). Therefore, we eliminated from further analysis valleys with >50% unidentified individuals, and those sampled after 1 August because catkin phenology was too advanced by then to determine the sex for all individuals. This resulted in a final data set of 24 sampling transects from six valleys.

RESULTS

We observed significantly female-biased sex ratios at five of the six valleys included in the final analysis (Fig. 1). When valleys were pooled together, we found significantly female-biased sex ratios for four of the five most frequently sampled species, for individuals sampled between 1700 to 1800 m and 1800 to 1900 m, for individuals of patch sizes of less than 2.5 m³ or greater than 5 m³, and for individuals that were less than 20 and greater than 30 yr old (Fig. 1). The mean sex ratio across all valleys surveyed was 72% female, excluding valleys sampled in August and those where sex could not be determined for $\geq 50\%$ of individuals sampled. For the three transects where sex could be determined for all individuals sampled, the mean sex ratio was 67% female. Sampling elevation, shrub patch size, and stem age were not significantly associated with sex ratio (generalized linear model, $N = 24$ sampling transects, elevation: $z = 1.05$, $P = 0.30$; patch size: $z = 0.66$, $P = 0.51$; age: $z = -0.41$, $P = 0.68$).

DISCUSSION

We observed uniform female-biased sex ratios of ca. 2:1 in alpine willow species in our 2500-km² study area of the Yukon Territory, consistent with reports for other willow species (Faliński, 1980; Crawford and Balfour, 1983; Elmqvist et al., 1988; Alliende and Harper, 1989; Dawson and Bliss, 1989; Alstrom-Rapaport et al., 1997; Rottenberg, 1998; Predavec and Danell, 2001; Jong and Meijden, 2004; Dudley, 2006; Ueno et al., 2007; Hughes et al., 2009). The results of this study support our second hypothesis, that sex ratios in the surveyed willow species are determined by a process acting at an early life stage.

We found no evidence to support our first hypothesis that ecological factors influencing adult mortality determine sex ratios. This is the first time female-biased sex ratios for the species investigated in our study have been reported in the primary literature, with the exception of *Salix glauca* previously observed at one site in the Rocky Mountains of Colorado (Dudley, 2006). Perhaps surprisingly, we observed no significant difference in sex ratios between the dominant willow species sampled in this study (Fig. 1B), even though these species vary in ploidy level, reproductive phenology, and growth form (Table 2). Our data, and the accumulating reports of female-biased sex ratios for willows growing in a variety of different ecosystems, indicate that a female-biased sex ratio of 2:1 is a general phenomenon in the genus *Salix*.

Prezygotic mechanisms, such as gamete competition (Alstrom-Rapaport et al., 1997) could cause female-biased sex ratios in willows. Larger pollen loads could intensify gametophytic competition favoring selective fertilization by female-determining pollen tubes, resulting in more daughter progeny (Taylor et al., 1999; Stehlik et al., 2008; Barrett et al., 2010). For the willows we observed, many individuals at higher elevations exhibited reduced development of fertile seed capsules compared to individuals growing at lower elevations (I. H. Myers-Smith, M. M. Saunders [University of Alberta], and D. S. Hik, unpublished data). Pollen limitation could be a factor at higher elevations where populations of willows are less dense, and therefore the distance to the nearest male is greater. Resource limitation and phenological mismatch between male and female plants could also occur at high elevations sites near the elevational range edge. The fact that the sex ratios of these high elevation populations did not differ from those of denser populations at lower elevation suggests that pollen limitation or stressful growing conditions are not necessarily related to development of female-biased sex ratios in these willow species. In fact, the observed female-biased sex ratios could exacerbate any pollen limitation in high elevation populations, as the distance to the nearest males will be even greater in a female-biased population than if the population had an even sex ratio.

Another factor invoked to explain the development of female-biased sex ratios in several dioecious species is spatial segregation of the sexes (Bierzuchudek and Eckhart, 1988; Hughes et al., 2009). Niche differentiation between the sexes can result in variation in sex ratio across the landscape; however, we observed no difference in sex ratio with sampling elevation, aspect, or valley in our study. The plant traits of susceptibility to herbivory and stress tolerance could differ between male and female plants (Cornelissen and Stiling, 2005). Female willows have been found to allocate more resources to reproduction than males (Fox and Stevens, 1991; Ueno et al., 2007). The two sexes have also been found to respond differently to

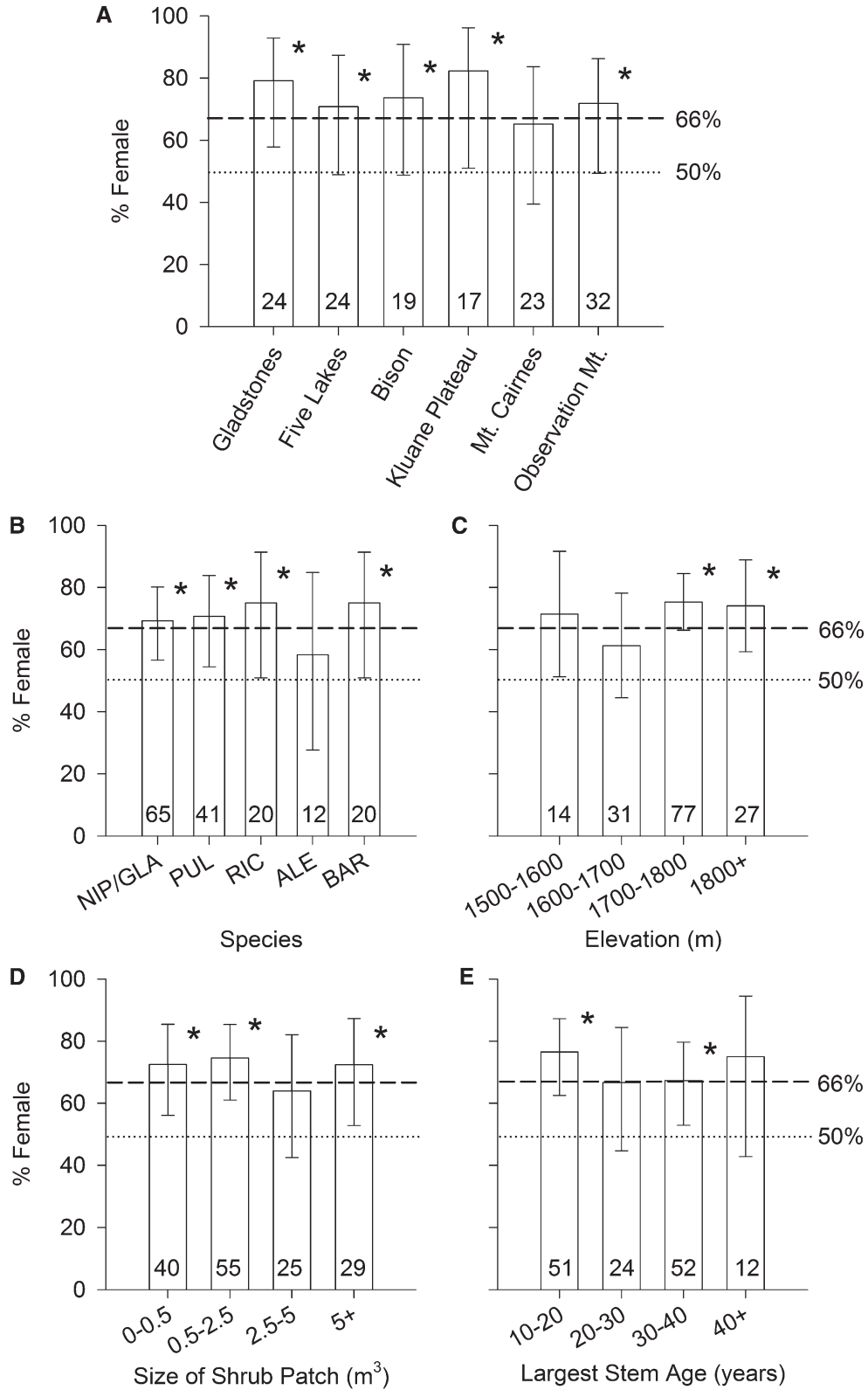


Fig. 1. Proportion of females of the species *Salix niphocladalgauca* (NIP/GLA), *S. pulchra* (PUL), *S. richardsonii* (RIC), *S. alaxensis* (ALE), and *S. barrattiana* (BAR) by (A) mountain valley, (B) species, (C) elevation of sampling, (D) patch size class, and (E) stem age. Species are pooled in plots A, C, D, and E. Error bars indicate binomial confidence intervals and the number in each bar indicates sample size. Asterisks indicate sex ratios that differ significantly from a 1♀:1♂ sex ratio (dotted line, χ^2 , $P < 0.05$); no sex ratios differ significantly from a 2♀:1♂ sex ratio (dashed line, χ^2 , $P < 0.05$).

ecological resources such as water availability (Dawson and Bliss, 1989; Dudley, 2006; Dudley and Galen, 2007; Hughes et al., 2009). We also did not observe significant sexual size dimorphism for the frequently sampled species (*S. niphoclada*/*glauca*, *S. pulchra*, *S. richardsonii*, and *S. barrattiana*, MANOVA, ns), with the exception of *S. alaxensis*, for which male individuals were taller in our survey (MANOVA, Pillai's trace = 0.61, $F_{2,10} = 7.91$, $P = 0.01$).

To further explore sex ratio development and better understand the ecological or evolutionary determinants of biased sex ratios in willow species, we first need to understand the genetic and developmental determinants of sex. Sex determination in the genus *Salix* is poorly understood (Alstrom-Rapaport et al., 1997, 1998) and since we currently lack the ability to genetically determine gender at early life stages, current investigations of sex ratios are limited to working with reproductive individuals. Gunter et al. (2003) speculated that key loci involved in sex determination could be conserved within the genus; however, the few sex-linked loci that have been identified do not always amplify when tested in multiple *Salix* species (Gunter et al., 2003; Semerikov et al., 2003; Temmel et al., 2007). Further research into the genetic or epigenetic basis for sex determination in the genus *Salix* will shed light on the ecological and evolutionary puzzle of female-biased sex ratios in willow species.

Conclusions—We observed uniform female-biased sex ratios of 2:1 for five species of tundra willows. Our data indicate that female-biased sex ratios develop at an early stage and do not vary across the landscape or with age or size of the willow patch. These female-biased sex ratios could have important implications for sexual reproduction of these species at their elevational or northern range edge where the greater abundance of female plants could exacerbate pollen limitation. Recent observations of widespread changes in abundance of arctic and alpine woody shrubs in tundra ecosystems (Myers-Smith et al., 2011) have renewed interest in the ecological mechanisms promoting growth and range expansion of these species. Therefore, studies that shed light on the reproductive ecology and life history strategies of willow species will be relevant to studies reporting changes in shrub abundance, experimental tests of climate change impacts on tundra ecosystems and model projections of range expansion of woody species in tundra ecosystems.

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