

1 **RESEARCH ARTICLE**

2 **Sexual dimorphism and sex ratio bias in the dioecious willow *Salix purpurea* L.**

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21 Running Title: *Salix* sexual dimorphism

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1 **Abstract**

2 **Premise** Sexual dimorphism in dioecious plant species is often not obvious or is
3 absent. Dioecious species populations also often exhibit deviations from expected sex ratios.
4 Previous studies on members of the Salicaceae family have shown strong, partial, and no sexual
5 dimorphism. Some studies have shown sex-biased ratios in several *Salix* spp., however, *S.*
6 *purpurea* has never been examined for evidence of sexual dimorphism or for the presence of sex-
7 ratio bias, and therefore a comprehensive phenotypic study is needed to fill this knowledge gap.

8 **Methods** This study examined a suite of morphological, phenological, physiological and wood
9 composition traits from multi-environment and multi-year replicated field trials in a diversity
10 panel of unrelated *S. purpurea* accessions and in full-sib F₁ and F₂ families produced through
11 controlled cross pollinations to test for sexual dimorphism and sex ratio bias.

12 **Key Results** Significant evidence of sexual dimorphism was found in vegetative traits with
13 greater means for many traits in male genotypes compared to females across three populations of
14 *S. purpurea*, measured across multiple years that were highly predictive of biomass yield. Male
15 plants exhibited greater nitrogen accumulation under fertilizer amendment as measured by SPAD
16 in the diversity panel, and males showed greater susceptibility to fungal infection by
17 *Melampsora* spp in the F₂ family. There were also consistent female-biased sex ratios in both the
18 F₁ and F₂ families.

19 **Conclusions** These results provide the first evidence of sexual dimorphism in *S. purpurea* and
20 also confirm the prevalence of female-biased sex ratios previously found in other *Salix* species.

21

- 1 **Keywords:** bioenergy; dioecy; *Melampsora*; nitrogen utilization; rust; *Salix purpurea*; sexual
- 2 dimorphism; sex ratio; willow; ZW sex determination
- 3

1 INTRODUCTION

2 Dioecy is found in 4-10% of all flowering plants and is therefore much less common than
3 the co-sexual states of monoecy and hermaphroditism (reviewed by Renner, 2014; Charlesworth,
4 2015; Sanderson *et al.*, 2019), even if one includes the instances of subdioecy, which has been
5 reported in 32 species in 21 families (Ehlers and Bataillon, 2007). Dioecy in flowering plants is
6 therefore thought to have evolved from an ancestral co-sexual state since the first flowering
7 plants evolved, approximately 124.6 million years ago (MYA) (Sun *et al.*, 2002). Dioecy has
8 evolved independently in many different plant families (reviewed by Renner, 2014) and even
9 with different species in the same genus (Westergaard, 1958; Feng *et al.*, 2020). In some plant
10 lineages, homomorphic or hetromorphic sex chromosomes have evolved (Ming *et al.*, 2007;
11 Chen *et al.*, 2016, reviewed by Charlesworth, 2015). Recent studies have identified sex-
12 determination regions (SDRs) in dioecious plants including grape (*Vitis*), papaya (*Carica*), and
13 persimmon (*Diospyros*), (Fechter *et al.*, 2012; Wang *et al.*, 2012; Akagi *et al.*, 2014), in which
14 the males are heterogametic, as well as other systems such as shrub willow (*Salix*) (Zhou *et al.*,
15 2018), in which females are heterogametic.

16 Species of the Salicaceae family are almost all dioecious, including the woody perennial
17 species in the genera *Populus* and *Salix*, which have clear morphological differences between
18 staminate and pistillate catkins (Dickmann and Kuzovkina, 2008). Dioecy in the Salicaceae is
19 thought to have evolved before the divergence of *Salix* and *Populus* approximately 65 MYA
20 (Collinson, 1992; Tuskan *et al.*, 2006). Multiple cytological studies of *Populus* suggest that
21 Salicaceae have homomorphic chromosomes (Peto, 1938; van Buijtenen and Einspahr, 1959),
22 and genetic mapping studies indicate that both male and female heterogametic systems are
23 present in *Populus* (Pakull *et al.*, 2009; Pakull *et al.*, 2011; Tuskan *et al.*, 2012; Kersten *et al.*,
24 2014, Geraldès *et al.*, 2015). The sex determining locus has been mapped to two different

1 positions on Chr19 in different *Populus* species (Kersten *et al.*, 2014; Geraldès *et al.*, 2015). In
2 willow species examined thus far, *S. viminalis*, *S. suchowensis*, and *S. purpurea*, females are the
3 heterogametic sex (Semerikov *et al.*, 2003; Hou *et al.*, 2015; Pucholt *et al.*, 2015; Chen *et al.*,
4 2016; Zhou *et al.*, 2018), in which the sex determination locus maps to Chr15 (Hou *et al.*, 2015;
5 Zhou *et al.*, 2018) suggesting that the sex determination locus has translocated during recent
6 evolution of *Populus* and *Salix*.

7 The relationship between sex chromosome evolution and sexual dimorphism and sex
8 ratios are not yet fully understood. Primary sexual dimorphism refers to differences in the
9 gametes produced, and in plants, this is expected to be controlled by sex-determining genes on a
10 single sex chromosome, at least a portion of which is non-recombining (Charlesworth and
11 Charlesworth, 1978). Secondary dimorphism includes other differences between males and
12 females, such as morphology, physiology, and phenology (Charlesworth, 1999; Dawson and
13 Geber, 1999; Delph, 1999), but in general sexual dimorphism in plants is less pronounced than in
14 many animal systems, and evidence for dimorphism in secondary characteristics is scarce. Such
15 differences may evolve when ecological and/or sexual selection lead to different fitness optima
16 between sexes, and/or when physiological trade-offs occur due to the unequal energy costs of
17 producing seeds versus pollen (Lewis, 1942; Arnold, 1994; Delph, 1999; Obeso, 2002). Females
18 of woody dioecious plants typically produce less biomass than males, due to slower vegetative
19 growth as a result of greater allocation to reproduction (Lewis, 1942; Lloyd and Webb, 1977;
20 Obeso, 2002). Traits such as primary growth, production of secondary metabolites, and water use
21 efficiency may be influenced by carbon resource allocation related to sex. Contrasting results
22 have been found in *Salix*. In *S. sachalinensis* (syn *S. udensis*), no differences were detected in
23 growth or mortality rates between males and females measured in a natural population over a
24 three-year period (Ueno *et al.*, 2007). Conversely, it was reported that drought tolerance and gas

1 exchange rates differed between sexes in *S. glauca*, indicating dimorphism in physiological
2 responses to abiotic stress with lower stomatal conductance (g_s) and transpiration rates in males
3 than in females when exposed to the same drought conditions (Dudley and Galen, 2007).
4 However, the possibility of dimorphism has been studied for only a limited number of
5 characteristics (Boecklen *et al.*, 1994; Boecklen *et al.*, 1990).

6 Another aspect of dimorphism in dioecious plants is sex ratio bias. Classical theories for
7 sex ratios predict a 1:1 ratio if the expense per progeny is the same for both sexes (Fisher, 1930;
8 Edwards, 2000). Theoretical models (reviewed by Delph, 1999; Obeso, 2002) suggest that sex
9 ratio bias in natural populations could be due to differences in pollen and seed dispersal leading
10 to different trade-offs between benefits and costs (Lloyd, 1982), other ecological factors (Barret
11 *et al.*, 2010), or to pollen competition in species with sex chromosomes (certation), such that X-
12 or Y-bearing pollen is slow growing, or genetic transmission bias due to distorters of the sex
13 chromosome segregation in meiosis (Taylor, 1999). Since dioecious species are typically
14 perennials (Field *et al.*, 2013a) and some can reproduce both clonally and sexually, the degree
15 and frequency of flowering and clonal propagation can also both influence sex ratios. Several
16 studies examining this in wild accessions of *Salix* have shown a female sex ratio bias (Barrett *et*
17 *al.*, 2010). Male bias is commonly seen in trees and often associated with biotic pollen dispersal
18 in contrast to female-biased ratios observed in shrubs and herbs that tend to be clonal, perennial
19 species (Field *et al.*, 2013). Several studies of wild accessions of *Salix* have found sex ratio
20 biases toward females (Ueno *et al.*, 2007; Che-Castaldo *et al.*, 2015), but male bias was
21 demonstrated in a controlled cross experiment in *S. viminalis* (Alström-Rapaport *et al.*, 1997).
22 Some studies of experimental populations (Mosseler and Zsuffa, 1989) have revealed greater
23 variability of sex ratio bias than in natural populations (Myers-Smith and Hik, 2012), but sex

1 ratio bias in progeny of controlled crosses depends on the nature of the cross (inter- or
2 intraspecific) as well as the ploidy levels of the parents.

3 The short generation time of *Salix* makes this genus suitable for studying sexual
4 dimorphism and its genetic control. This study examined *S. purpurea* L. (purple osier willow), a
5 naturalized species in North America and an important species in breeding shrub willow
6 bioenergy crops in North America as it has been used in over 30% of all intra- and interspecific
7 hybrids produced to date (Smart and Cameron, 2008). Critical traits to study for dimorphism
8 include pest and disease resistance, drought tolerance, nitrogen and water use efficiency, and
9 biomass yield.

10 This study describes the first investigation of sexual dimorphism in a *S. purpurea*
11 diversity panel and F₁ and F₂ populations. The objectives were to (1) evaluate the phenotypic
12 variability within naturally occurring and bred genotypes of *S. purpurea* (2) determine if there is
13 sexual dimorphism of secondary sex characteristics among natural and bred populations and (3)
14 test if observed sex ratios fit those expected based on a single locus sex determination system or
15 if there is evidence for an multigenic control of sex ratio bias within the species.

16

17 **MATERIALS AND METHODS**

18 *Germplasm and field trials*

19 Three populations of *S. purpurea* L. were used in this study: a diversity panel of
20 unrelated natural accessions, an F₁ family, and an F₂ family generated by crossing F₁ individuals.
21 The diversity panel contained 78 genotypes of *S. purpurea* natural accessions (Lin *et al.*, 2009 ,
22 Gouker *et al.*, 2019) (Appendix S1). In July 2012, all genotypes were hand planted using 20-cm
23 cuttings in a common garden design at three experimental sites (Appendix S2): Cornell AgriTech
24 in Geneva, NY the Cornell Lake Erie Research and Extension Lab (CLEREL) in Portland, NY;

1 and the West Virginia University (WVU) Agronomy Farm in Morgantown, WV. All sites were
2 planted in a randomized complete block design with six replicates of four-plant plots at each
3 location in single-row spacing with 1.82 m between rows and 0.40 m between plants within
4 rows. Border rows containing either genotype 94006 or cultivar ‘Fish Creek’ were planted on the
5 perimeter to avoid edge effects. At the end of the establishment year, all plants were coppiced
6 and trials were measured in 2013 and 2014 and subsequently harvested and weighed in early
7 2015. Prior to re-growth of the second rotation in 2015, 112 kg ha⁻¹ N-P-K fertilizer was applied
8 to half of the replicates at each location to test for nitrogen utilization. Rust was surveyed at two
9 locations at the end of the 2015 growing season.

10 The intraspecific F₁ *S. purpurea* family was generated from a cross between the female
11 genotype 94006 and the male genotype 94001, which were accessions collected near Syracuse,
12 NY and were also present in the diversity panel. Two F₁ siblings from this family were selected
13 and crossed (‘Wolcott’ × ‘Fish Creek’) to generate the F₂ population. A total of 100 F₁ and 482
14 F₂ progeny and their parents were hand planted using 20-cm cuttings at Cornell AgriTech in June
15 2014 (Appendix S2) in a randomized complete block design with four replicate blocks of three-
16 plant plots in the same single-row spacing described above (Carlson *et al.*, 2019). To avoid edge-
17 effects, border rows containing 94006 and ‘Fish Creek’ were planted along the perimeter of the
18 trial. At the end of the establishment year, all plants were coppiced, fertilized with 112 kg ha⁻¹
19 N, 67 kg ha⁻¹ P and K, and measurements were collected in 2015.

21 ***Phenotyping***

22 The diversity panel was evaluated for 26 biomass, morphological, phenological,
23 physiological and wood composition traits measured as described (Appendix S3) across three
24 sites in 2013 and 2014 and a subset of traits as well as rust incidence were measured in 2015.

1 The F₁ and F₂ populations were evaluated for all traits on one site in 2015 (Table 1) (Carlson *et*
2 *al.*, 2019). For growth measurements in the diversity panel, the inner two plants of each four-
3 plant plot were measured and the central plant in each three-plant plot were measured in the F₁
4 and F₂ families. Rust was surveyed by visually assessing percent uredospore pustule coverage
5 on leaves (0-100%) of all the plants in each plot. Sex was scored for clonally propagated plants
6 of each genotype growing in nursery beds and was confirmed in experimental plots.

7

8 ***Statistical analysis***

9 Statistical analyses were conducted using SAS[®] version 9.4 (SAS Institute, Cary, NC)
10 and R version 3.2.3 (R Core Development Team). Mixed linear models were used to analyze
11 phenotypic data implemented in SAS with the PROC MIXED statement and using the *lmer*
12 function within the *lme4* package in R (Bates *et al.*, 2015). All dependent variables were tested
13 for homogeneity of variances and normality using PROC UNIVARIATE using Kolmogorov-
14 Smirnov D and Shapiro-Wilk's K statistics. Non-parametric methods were used when
15 phenotypes that were not normally distributed could not be transformed to meet the assumptions
16 of parametric analyses using Box-Cox powers or log-transformation. Yield data were square-root
17 transformed to meet assumptions of normality. Pearson's product-moment correlations (*r*) were
18 calculated among all traits. To test for statistical differences between phenotypic traits based on
19 sex, a two-tailed Mann-Whitney *U*-test was conducted with hermaphrodite genotypes excluded.
20 To estimate the predictability and relationship of each trait and biomass yield, multiple linear
21 regression was performed with PROC REG using the stepwise regression method. Model
22 adequacy was checked with a general linear model to assess the global significance with PROC
23 GLM.

24

1 RESULTS

2 *Phenotypic variation*

3 In the diversity panel, all traits showed large differences among genotypes (Appendix
4 S4). Effects of genotype, location, and genotype \times location were highly significant for yield
5 ($P < 0.05$) (Table 2). Overall, the greatest differences in growth traits between genotypes were for
6 stem area (SA) and stem height (HT) (Fig. 1). The range of values across two growing seasons in
7 the diversity panel were 0.13 to 84.38 cm² for SA and 0.11 to 4.88 m in HT (Appendix S4).
8 There was also wide variability in stem number (STNo), which increased on average by 22%
9 from the first to the second year. Crown form (FORM), calculated from crown diameter (CDIA),
10 ranged from approximately 9 to 88° mean branching angle, but all genotypes had variable
11 FORM across sites. Of the four metrics obtained from leaf scans, the greatest variation was for
12 leaf perimeter (LFP). The same degree of variability was observed across sites for phenology and
13 physiology traits, where stomatal conductance (g_s) had the greatest variability with the maximum
14 value of 1164.2 mmol m⁻² s⁻¹ and the minimum value of 45.5 mmol m⁻² s⁻¹ in year 1
15 (Appendices S5, S6). The genotypic means for wood composition and specific gravity (SPGR)
16 also had wide variances. The largest variation was observed for cellulose content (CLS) with a
17 range of 37.94% difference between the highest and lowest value. On average, second year
18 measurements of hemicellulose content (HCL) and CLS were greater than first year values by
19 0.79% and 10.68%, respectively (Appendix S4). Lignin content (LIG) decreased by 5.48% in
20 year 2 compared to year 1 and ash content (ASH) declined by 27.76%, but SPGR only decreased
21 by 0.04 g cm⁻¹ in the second year.

22 In the F₁ and F₂ families, mean growth in Geneva was better than that of the diversity
23 panel (Table 3, Appendix S7, Appendix S8). The means for SA and HT for first-year coppice
24 growth were greater in the F₁ and F₂ families compared to the diversity panel. The first-year

1 mean SA in the diversity panel in Geneva was 14.95 cm², while it was 16.87 and 12.62 cm² in
2 the F₁ and F₂ families, respectively. Relative differences in first-year post-coppice HT were
3 similar, as the diversity panel in Geneva had mean HT of 2.30 m, the mean HT in the F₁ family
4 was 3.25 m and the mean HT of the F₂ family was 3.11 m. SPAD measurements and specific leaf
5 area (SLA) showed similar trends with the diversity panel with lower means for both traits
6 compared to the F₁ and F₂ families (Fig. 2).

7 In general, overall lower trait means were observed for biomass and physiological traits
8 in the F₂ family compared to the F₁ family (Table 3). For instance, SDIA, HT, STNo, and SA
9 were all significantly greater ($P < 0.01$) in the F₁ family with t -values ranging from 7.3 to 14.7,
10 as well as traits related to stem architecture, CDIA ($t = 12.6$) and FORM ($t = 12.1$) (Table 3).
11 Stem area was ~33% greater in the F₁ family compared to the F₂ family in 2015. Although
12 morphological leaf traits were significantly greater in the F₁ family, SLA and canopy color
13 (RGB) were the only two traits that were not significantly different between F₁ and F₂ families,
14 whereas SPAD was the only trait that was significantly greater in the F₂ family ($t = -3.97$, $P <$
15 0.01).

16 *Sexually dimorphic phenotypes*

17 Significant sex differences were found in *S. purpurea*, with males producing greater
18 growth and significantly greater means for most traits measured (Fig. 1, Table 4, Table 5). For
19 the diversity panel in the first year of growth (2013), two traits were significantly dimorphic
20 ($P < 0.05$, Table 4). Mean HT of females (1.95 m) across all three sites was 3.7% greater than
21 males (1.88 m). Yet males had significantly heavier leaf dry weight (LFDW) than females. The
22 number of dimorphic traits increased in year two (2014), when 11 of the traits were significantly
23 dimorphic (Table 4). There were greater trait means in males for CDIA, leaf length (LFL), leaf
24 width (LFW), leaf area (LFA), LFP, and LFDW. Crown form (FORM) was calculated from

1 CDIA and showed significantly lower branching angle in the males reflecting a greater crown
2 diameter in males than in females. Mean floral (FPHE) and vegetative (VPHE) phenology
3 measurements showed greater means for females indicating earlier bud break for males (Table
4 4). Six traits were sexually dimorphic in the F₁ family (Table 5). Male means for HT and specific
5 leaf area (SLA) were greater than for females, as was CDIA, meaning that the FORM angle was
6 lower in males than in females. In the F₁ family, SDIA of female progeny was greater than that
7 of males, whereas STNo was greater in males compared to females. In the F₂ family, HT was
8 significantly greater for males, as it was in the F₁ family. LFDW, LFP, and LFL means were
9 greater in females than males, while CDIA was greater for males, with a lower form angle, as
10 was observed in the diversity panel and F₁ family.

11 Leaf rust severity (RUST) was surveyed during the 2015 growing season in the diversity
12 panel and F₂ family. In the diversity panel in 2015, there were significant differences between
13 the two locations surveyed ($P < 0.05$). Based on percent disease severity using least square means,
14 for Geneva, NY, females had a greater mean score (25%) for RUST than males (22%) and in
15 Portland, NY least square means for RUST were 30% and 27% for females and males,
16 respectively, with overall significant differences of RUST averaged across locations between
17 males and females (Table 4, Fig. 3A). The male parents of the F₁ and F₂ families had
18 significantly greater mean RUST scores than the female parents (Fig. 3B). Similarly, the male F₂
19 progeny had significantly greater mean RUST than the F₂ female progeny ($P = 0.02$) (Table 5,
20 Fig. 3B). The overall F₂ progeny means for RUST were greater than that of the female parent,
21 ‘Wolcott’, but less than that of the male parent ‘Fish Creek’. Overall, there was a significant
22 negative correlation between RUST and both SA and HT ($P < 0.05$), with a significant positive
23 correlation between RUST infection and SPAD measurements ($P < 0.01$) (Appendix S9).

24

1 *Sex ratios*

2 There were 49 females and 29 males in the diversity panel, which were confirmed across
3 years and experimental locations based on documented sex phenotypes in nurse beds. The
4 diversity panel was not necessarily representative of the sex ratios in natural populations, since it
5 was assembled from only a few selected individuals from multiple sites, rather than a thorough
6 sampling of all individuals of a population (Gouker, *et al*, 2019). There was significant departure
7 from the expected 1:1 segregation ratio of males and females in both the F₁ and F₂ families. The
8 F₁ family consisted of 70 females and 30 males (ratio=2.33:1, $P < 0.01$) and the F₂ family
9 contained 266 females and 216 male genotypes (ratio=1.23:1, $P = 0.02$).

10

11 *Allometric model for yield*

12 All measured traits from the diversity panels were used as parameters in allometric
13 models to identify relationships between YLD and the yearly growth measurements using
14 multiple linear regression to predict second year biomass. Separating genotypes by sex and
15 examining allometric relationships with YLD revealed no significant difference ($P=0.15$) or
16 advantage of predicting YLD and therefore the data were not distinguished by sex in the
17 regression model. Variable inflation factors greater than 10 were observed between total SDIA
18 and total SA and indicated multicollinearity as indicated with the high correlation coefficient of
19 $r=0.95$ (Appendix S10). Since SA had a greater correlation with YLD and explained a greater
20 percentage of the variance in the model, it was kept and SDIA was removed. All other variables
21 that did not meet the $P < 0.05$ significance level were also removed. To test for global significance
22 of variables, a general linear model was fitted and revealed SLA in 2014 ($P=0.88$) and LFP
23 ($P=0.84$) were insignificant and were also removed as predictor variables. The best predictors for
24 the final multiple linear regression model were SA in 2013 and 2014, HT in 2014, and

1 AugSPAD in 2014 (Appendix S5), where yearly SA measurements gave the most accurate
2 estimates of YLD. A strong positive fit of predicted and observed biomass YLD resulted in an
3 overall $R^2 = 0.79$ (Appendix S10).

4

5 **DISCUSSION**

6 For dioecious species, genetic factors, selection pressures over time, and ecological
7 adaptation can lead to differential fitness between males and females and result in dimorphism of
8 secondary characteristics (Sakai and Weller, 1999). While such differential selection in males
9 and females may be expected to lead to sex ratio bias, very few published records of such bias
10 exist.

11 Sex ratio bias has been reported in *Salix* spp. and is most often biased towards females in
12 an approximate 2 female:1 male ratio (Alström-Rapaport *et al.*, 1997; Rottenberg, 1998; Dudley,
13 2006; Ueno *et al.*, 2007; Hughes *et al.*, 2009; Myers-Smith and Hik, 2012), though the closest
14 taxonomically related genus, *Populus*, has shown male-biased ratios (Tuskan *et al.* 2012). The
15 female bias observed in the F₁ and F₂ families of this study may be explained by the occurrence
16 of pollen competition (certation). Especially when pollen load is high, as in the case with
17 controlled crosses, the female determining pollen may be inherently more successful at
18 fertilization. We can speculate that there was relaxation of female:male ratios from the F₁ to F₂
19 generation as a result of slight inbreeding depression, differential mortality, or environmental
20 factors.

21 Despite the female-biased sex ratios in the F₁ and F₂ families, our results showed that
22 males were superior to females for many traits. Comparison of coefficients of variation (CV)
23 between sexes in each population showed consistently greater variation in males, which may
24 indicate that males have greater plasticity in response to environmental conditions, or

1 alternatively that females spend more resources on seed production and cannot vary growth
2 responses in relation to different environmental conditions. Yield and traits positively correlated
3 with yield also showed greater trait means in males. Direct measurement of YLD was not
4 significantly different, however. Within the F₁ family, all dimorphic traits except for DIA were
5 male-biased. It was shown that males have earlier bud break, which would extend their growing
6 season and may partly explain differences in leaf traits (Tharakan *et al.*, 2008). However, it has
7 been suggested that other phenological events (i.e. leaf unfolding and duration, growth cessation
8 and leaf abscission) affect annual biomass production, where growth cessation and late season
9 leaf retention may also impact aboveground growth as well as nutrient recycling and storage *in*
10 *planta* (Weih, 2009). An intensive study monitoring these additional traits could provide clues as
11 to whether sex-specific physiological patterns are seen across other dioecious species as well.

12 Sex dimorphism has also been studied extensively in the closely-related genus *Populus*.
13 Examination of phenotypic and gene expression data in *P. tremula* has shown no evidence of
14 sexual dimorphism for morphological or biochemical traits (Robinson *et al.*, 2014). Minor
15 differences across a set of growth traits observed in *P. euphratica* suggested that male trees are
16 more vigorous than females (Petzold *et al.*, 2012). Studies of *P. deltoides* and *P. tremuloides*
17 hybrids revealed significantly greater biomass production in males (Pauley, 1948; Farmer, 1964).
18 When examining the evidence for sexual dimorphism in *Salix*, there are also opposing
19 observations. In *S. planifolia*, it was reported that a larger allocation of resources is needed for
20 reproduction in females than in males (Turcotte and Houle, 2001), which may lead to the
21 assumption that females exhibit less biomass growth compared to males. Other studies have
22 shown that females have growth rates similar and sometimes greater, but not significantly
23 different, than males (Åhman, 1997; Sakai *et al.*, 2006).

1 Data from this study revealed consistent trends of dimorphism for CDIA and the
2 associated FORM showed a significant male bias for greater CDIA and subsequent shallower
3 branching angle for all years and populations. Specific gravity contributes to the mechanical
4 properties of wood and is known to scale positively with biomechanical strength and therefore
5 directly influences plant architecture (Chave *et al.*, 2009). This has practical importance for
6 shrub willow, because plants with a wide CDIA that expands into the alleys of a field will result
7 in biomass that may not be collected by a harvester. If specific cultivars have wide branching
8 angles, this will result in a loss of harvestable biomass and reduction in yield.

9 Another interesting result of sexual dimorphism observed in this study was the
10 significantly greater rust severity on male plants in the F₂ family and the male progenitors,
11 whereas in the diversity panel females exhibited greater rust severity. *Melampsora* leaf rust is the
12 most severe plant disease affecting short-rotation willow plantations where long-term stability of
13 yield will depend on host resistance. Resistance to *Melampsora* spp. has been mapped to
14 multiple linkage groups in backcross and full-sib families in *S. viminalis* (Rönnerberg-Wästljung *et*
15 *al.*, 2008; Hanley *et al.*, 2011; Samils *et al.*, 2011) with significant QTL for rust resistance on
16 Chr01, Chr05, and Chr10 in *S. purpurea* (Carlson *et al.*, 2019). Despite the sex dimorphism for
17 RUST from this study in the F₂ family, no resistance QTL have mapped to Chr15 near the SDR.
18 Among the studies of rust severity in willow, there are few that have provided information on
19 sex dimorphism. A study of largely unrelated commercial cultivars has indicated that there is
20 greater rust severity on female plants (Moritz *et al.*, 2016). Conversely, it has been observed in
21 two other *Salix* studies that male-biased rust severity also exists (McCracken and Dawson, 2003;
22 Pei *et al.*, 2008). Our results also present conflicting evidence between the diversity panel and F₂
23 family. The initial selection and cross of 94006 and 94001 had significantly different rust
24 susceptibilities, as well as the full-sib F₁ progeny ‘Wolcott’ and ‘Fish Creek’ which had greater

1 rust severity than the parental genotypes. The subsequent cross of these full-sibs was used to
2 generate the F₂ full-sib family which also showed significantly greater rust susceptibility in male
3 genotypes. Therefore, these differences may be a result of a genetic bottle neck through selective
4 breeding which skewed rust susceptibility towards male genotypes.

5 Differences in observed severity of males and females between the diversity panel and
6 the F₂ family could be due to the timing of rust assessment. This may be particularly important
7 for the higher level of rust incidence in Portland, NY, which was surveyed late in the season and
8 disease had already advanced causing extensive pre-mature defoliation and rendering phenotypic
9 differences that may exist between sexes indistinguishable. This suggests that differential rust
10 susceptibility could also be driven by phenological differences between male and female plants.
11 It is hypothesized that through life-history trade-offs of sexual morphs in dioecious species,
12 females typically allocate greater resources towards reproduction and defense against pests and
13 diseases (Seger and Eckhart, 1996; Vega-Frutis *et al.*, 2013), and males invest more resources
14 into primary growth (Delph, 1999; Obeso, 2002). Additionally, there may be differences in
15 mechanical or biochemical defense mechanisms that we did not measure and for which there are
16 limited studies examining this topic (Bañuelos *et al.*, 2004).

17 The SPAD values observed in this study, used as a non-destructive method to quantify
18 nitrogen status in the plant, showed significantly greater values in nitrogen amended versus
19 control plots, but also significantly greater values in males than females in the diversity panel.
20 The male-biased nitrogen capacity could possibly have been selected for by a greater nitrogen
21 requirement for pollen production (Carolyn and Rundel, 1979, Boecklen *et al.*, 1990) and the
22 greater resource allocation towards primary growth. It may also be that females require a larger
23 investment of nitrogen for seed production leaving more nitrogen available in males as observed
24 by the SPAD readings. Although, a review conducted by Hultine (2016) examining differential

1 resource acquisition between sexes of 22 species across multiple environments, concluded that
2 females generally do not have greater nutrient uptake or efficiency compared with males under
3 optimal growing conditions. Based on evidence so far, there tends to be greater nitrogen content
4 in males plants in willow and other plant species, however, it is uncertain of the exact
5 mechanisms contributing to these sexually dimorphic observations.

6

7 **CONCLUSIONS**

8 Shrub willows include very diverse species used in a number of horticultural applications
9 ranging from biomass crops, stream bank stabilization, living walls or snow fences, ornamental
10 landscaping, and riparian buffers (Kuzovkina & Quigley, 2005; Stott, 1992). Evidence of sexual
11 dimorphism for key traits suggests that sex of clones selected for particular uses can influence
12 performance. This study demonstrates that *S. purpurea* expresses secondary sexual dimorphism
13 for various traits where males in both a natural collection and in breeding populations responded
14 more positively to multi-environmental and multi-year growing conditions. These findings also
15 showed sex-specific differences in plasticity in response nitrogen amendments and disease
16 pressure providing insights into resource allocation for primary versus reproductive growth, but
17 whether there is sex-specific niche partitioning in shrub willow remains to be further evaluated.
18 This study also determined that there is evidence of female biased sex ratio in *S. purpurea*. The
19 results suggest that biased sex ratios in *S. purpurea* may be more strongly dependent on genetics,
20 but recent advances in genomics will lead to genetic markers for early sex determination to help
21 test this hypothesis.

22

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10

11 **AUTHOR CONTRIBUTIONS**

12 F.E.G., C.H.C. and L.B.S. planned and designed the research. F.E.G. and C.H.C. wrote the first
13 draft of the manuscript. F.E.G, C.H.C., L.B.S., J.Z., C.M.C., C.D.S., and L.M.E. performed the
14 experiments, collected data, and analyzed the data. All authors reviewed and revised the final
15 manuscript.

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28

Table 1. Phenotypic traits measured in the *S. purpurea* F₁, F₂, and diversity panel.

Trait	Abbreviation	Units
<i>Biomass</i>		
Height	HT	m
Stem number	STNo	#
Stem diameter	DIA	mm
Total stem area	SA	cm ²
Internode length	INL	cm
Biomass Yield	YLD	dry Mg ha ⁻¹
<i>Foliar</i>		
Leaf length	LFL	cm
Leaf width	LFW	cm
Leaf area	LFA	cm ²
Leaf perimeter	LFP	cm
Leaf dry weight	LFDW	g
Specific leaf area	SLA	cm ² g ⁻¹
<i>Architecture</i>		
Crown diameter	CDIA	cm
Crown form	FORM	degrees °
<i>Composition</i>		
Hemicellulose	HCL	%
Cellulose	CLS	%
Lignin	LIG	%
Ash	ASH	%
Specific gravity	SPGR	g cm ⁻³
<i>Physiology</i>		
August SPAD	SPAD1	SPAD units
September SPAD	SPAD2	SPAD units
Stomatal conductance	g_s	mmol m ⁻² s ⁻¹
Canopy color	RGB	RGB
Survival	SRV	%
<i>Phenology</i>		
Vegetative phenology	VPHE	day of year
Floral phenology	FPHE	day of year
<i>Pathology</i>		
Rust severity	RUST	%

Table 2. Mixed model test of effects of genotype and location on yield.

Source	DF	F Ratio	Pr > F
Location	2	155.23	<0.0001*
Genotype	77	11.51	<0.0001*
Genotype × Location	154	1.80	<0.0001*

*Significant differences at $P < 0.05$

1

Table 3. Means and standard deviations of phenotypic traits in the *S. purpurea* intraspecific F₁ family (n=100) and F₂ family (n=482) in Geneva, NY.

Trait ¹	F ₁ <i>S. purpurea</i> family		F ₂ <i>S. purpurea</i> family		<i>t</i> -value ²
	Mean ±SE	Min - Max	Mean ±SE	Min - Max	
<i>Biomass</i>					
HT	3.26 ±0.19	2.15 - 4.78	3.11 ±0.09	0.98 - 4.31	14.7*
STNo	21.8 ±0.32	6.00 - 41.0	18.7 ±0.16	1.00 - 44.0	8.51*
SDIA	9.44 ±0.05	6.36 - 12.4	8.81 ±0.02	5.00 - 12.4	12.9*
SA	16.9 ±0.28	4.48 - 33.3	12.6 ±0.12	2.80 - 37.7	7.30*
<i>Foliar</i>					
LFL	9.84 ±0.06	6.52 - 13.9	9.15 ±0.03	4.58 - 18.9	9.97*
LFW	2.18 ±0.02	1.40 - 4.93	2.04 ±0.01	1.10 - 12.7	4.90*
LFA	17.2 ±0.19	8.19 - 33.8	14.9 ±0.08	4.08 - 37.9	11.1*
LFP	22.4 ±0.28	13.8 - 57.8	21.0 ±0.12	9.54 - 58.2	4.80*
LFDW	0.13 ±0.002	0.06 - 0.28	0.11 ±0.001	0.03 - 0.41	9.74*
SLA	134 ±0.89	97.0 - 233	132 ±0.42	61.9 - 361	1.15
<i>Architecture</i>					
CDIA	36.9 ±0.43	18.1 - 84.7	31.3 ±0.19	3.10 - 77.0	12.6*
FORM	40.4 ±0.32	19.8 - 59.3	45.2 ±0.17	21.6 - 84.2	12.1*
<i>Physiology</i>					
SPAD1	55.9 ±0.29	11.1 - 76.3	57.2 ±0.13	26.2-91.9	-3.97*
RGB	112 ±0.78	55.5 - 158	111 ±0.37	48.3-168	0.90
SRV	99.7 ±0.20	33.3 - 100	99.1 ±0.15	0.00 - 100	2.70*
<i>Pathology</i>					
RUST	-	-	0.08 ±0.002	0.00-0.86	-

¹Phenotypic traits were measured in 2015. See Materials and Methods for trait definitions and Table 1 for abbreviations

²Student's *t*-test statistic, where * denotes significant differences among populations at a *P*<0.01 level-of-confidence, with positive values indicating greater means in the F₁ and negative value indicating greater means in the F₂.

Table 4. Comparison of phenotypic traits for female and male individuals in the *S. purpurea* diversity panel across three growing seasons.

Trait	Female (n=49)		Male (n=29)		P	Diff (%)
	Mean ± SE	CV (%)	Mean ± SE	CV (%)		
2013						
HT	1.95 ±0.02	28.04	1.88 ±0.02	28.81	0.05**	-3.29
STNo	18.14 ±0.34	54.76	17.63 ±0.45	58.43	0.35	-2.82
SDIA	7.3 ±0.06	22.62	7.38 ±0.07	21.62	0.38	1.22
SA	9.4 ±0.24	75.84	9.14 ±0.31	76.82	0.49	-2.75
INL	13.58 ±0.17	37.74	13.38 ±0.22	37.19	0.55	-1.47
LFL	6.91 ±0.07	23.58	7.02 ±0.09	23.68	0.35	1.62
LFW	1.69 ±0.05	72.05	1.85 ±0.07	67.64	0.06	9.31
LFA	8.86 ±0.18	49.53	9.42 ±0.24	47.89	0.08*	6.32
LFP	23.9 ±1	101.00	24.19 ±1.33	102.54	0.85	1.18
LFDW	0.0891 ±0.001	42.76	0.0896 ±0.003	58.78	0.01***	9.17
SLA	128.14 ±3.73	70.66	131.54 ±5.33	75.42	0.56	2.65
HCL	17.54 ±0.04	5.26	17.67 ±0.05	5.43	0.10*	0.72
CLS	37.55 ±0.13	8.53	37.55 ±0.18	9.14	0.98	0.02
LIG	28.9 ±0.09	7.43	28.89 ±0.11	7.37	0.96	-0.03
ASH	2.16 ±0.02	26.87	2.18 ±0.03	28.82	0.44	1.23
SPGR	0.45 ±0	15.97	0.45 ±0.004	14.69	0.96	0.01
SPAD1	45.85 ±0.32	20.50	45.88 ±0.42	20.69	0.94	0.08
SPAD2	41.2 ±0.35	20.34	42.29 ±0.53	23.36	0.06*	2.67
<i>g_s</i>	596.39 ±6.99	32.84	591.92 ±8.63	31.41	0.67	-0.75
2014						
HT	3.16 ±0.02	21.62	3.1 ±0.03	22.51	0.25	-1.84
STNo	22.14 ±0.39	52.00	21.55 ±0.53	56.01	0.39	-2.67
SDIA	239.42 ±5.13	63.58	172.35 ±4.69	62.12	0.07*	-28.01
SA	20.96 ±0.47	66.45	21.19 ±0.62	66.52	0.78	1.07
ILEN	13.59 ±0.24	42.07	12.98 ±0.28	40.60	0.05**	-4.45
YLD	2.71 ±0.06	69.25	2.79 ±0.08	67.81	0.56	2.81
LFL	6.6 ±0.06	28.18	6.92 ±0.07	24.54	<0.01***	4.88
LFW	1.81 ±0.06	96.01	2.03 ±0.11	118.12	0.05**	12.22
LFA	8.72 ±0.14	46.06	9.49 ±0.17	41.80	<0.01***	8.76
LFP	17.51 ±0.3	51.03	18.38 ±0.37	45.54	0.04**	4.97
LFDW	0.09 ±0.001	40.16	0.11 ±0.002	40.04	<0.01***	12.17
SLA	92.28 ±0.75	24.10	90.56 ±0.75	19.03	0.15	-1.86
CDIA	29.09 ±0.5	50.60	31.42 ±0.74	53.59	0.01***	8.01
FORM	49.36 ±0.46	27.69	47.61 ±0.64	30.84	0.03**	-3.55
HCL	17.69 ±0.03	4.36	17.79 ±0.04	4.34	0.09*	0.55
CLS	41.61 ±0.07	4.22	41.48 ±0.09	4.26	0.33	-0.32

LIG	27.29 ±0.05	4.42	27.36 ±0.06	4.37	0.42	0.26
ASH	1.55 ±0.02	24.89	1.58 ±0.02	25.22	0.34	1.84
SPGR	0.49 ±0.002	9.91	0.49 ±0.002	7.82	0.85	-0.14
SPAD1	44.93 ±0.24	12.84	46.15 ±0.33	13.29	<0.01***	2.73
SPAD2	42.45 ±0.28	18.35	43.72 ±0.36	17.66	<0.01***	2.99
g_s	474.51 ±5.7	35.66	483.68 ±7.18	33.88	0.39	1.93
VPHE	110.54 ±0.14	3.12	108.88 ±0.21	3.64	<0.01***	-1.51
FPHE	95.25 ±0.55	13.95	86.98 ±0.85	18.25	<0.01***	-8.69
2015						
RUST	28.33±0.53	43.70	25.45±0.72	50.49	0.01***	-10.17

All values are mean ± SE across three locations except for RUST which was evaluated across two locations.

Two-tailed Mann-Whitney U -test ($df=1$) results, where significant values ($P<0.05$) are denoted by bold font.

Additional levels significance are also shown denoted by $P<0.1$ (*), $P<0.05$ (**), and bold font, $P<0.01$ (***)

Positive values for dimorphism denote male-biased difference and negative values denote female-biased difference.

Table 5 Comparison of phenotypic traits for male and female individuals in an intraspecific F₁ *S. purpurea* family (n=100) and a F₂ *S. purpurea* family (n=482) measured in 2015 in Geneva, NY.

Trait	F ₁ <i>S. purpurea</i> family						F ₂ <i>S. purpurea</i> family					
	Female (n=70)		Male (n=30)		P	Diff (%)	Female (n=266)		Male (n=216)		P	Diff (%)
	Mean ±SE	CV	Mean ±SE	CV			Mean ±SE	CV	Mean ±SE	CV		
HT	3.24 ±0.22	11.42	3.29 ±0.34	10.94	0.02	1.54	3.11 ±1.15	12.22	3.13 ±1.35	12.78	0.02	0.64
STNo	21.2 ±0.4	33.33	23.3 ±0.53	26.09	0.03	9.52	18.4 ±0.21	38.89	19.0 ±0.25	36.84	0.11	5.56
SDIA	9.52 ±0.06	9.87	9.26 ±0.09	10.91	<0.01	-2.73	8.82 ±0.03	9.75	8.81 ±0.03	10.67	0.43	-0.11
SA	16.8 ±0.34	33.81	17.1 ±0.49	31.05	0.22	1.79	12.4 ±0.16	41.69	12.9 ±0.19	43.64	0.12	4.03
LFL	9.84 ±0.07	11.99	9.83 ±0.12	12.82	0.85	-0.10	9.19 ±0.04	13.38	9.09 ±0.04	14.52	0.03	-1.09
LFW	2.19 ±0.02	15.98	2.16 ±0.04	18.98	0.32	-1.37	2.05 ±0.01	21.95	2.04 ±0.02	27.94	0.13	-0.49
LFA	17.2 ±0.23	22.33	17.1 ±0.4	25.26	0.56	-0.58	15.1 ±0.11	23.71	14.8 ±0.12	24.73	0.07	-1.99
LFP	22.2 ±0.33	24.86	22.7 ±0.53	25.07	0.51	2.25	21.1 ±0.16	25.21	20.8 ±0.18	26.11	0.02	-1.42
LFDW	0.13±0.002	23.08	0.13±0.003	30.77	0.82	0.00	0.12 ±0.000	25.00	0.11 ±0.001	27.27	<0.01	-8.33
SLA	133 ±1.02	12.84	132 ±1.58	12.82	0.58	-0.75	131 ±0.56	13.89	134 ±0.59	13.04	<0.01	2.26
CDIA	36.3 ±0.49	22.59	38.7 ±0.88	24.44	0.02	6.61	30.6 ±0.24	25.23	32.1 ±0.3	27.32	<0.01	4.90
FORM	40.9 ±0.38	15.45	39.1 ±0.6	16.47	0.02	-4.40	45.8 ±0.22	16.05	44.6 ±0.27	17.96	<0.01	-2.62
SPAD1	56.2 ±0.3	8.52	54.9 ±0.68	12.28	0.11	-2.31	57.2 ±0.19	10.05	57.1 ±0.19	8.88	0.88	-0.17
RGB	112 ±0.94	14.11	111 ±1.42	13.78	0.38	-0.89	111 ±0.49	14.50	112 ±0.55	14.46	0.08	0.90
SRV	99.6 ±0.26	4.44	99.7 ±0.29	3.10	0.87	0.10	99.34 ±0.17	5.51	98.8 ±0.25	7.43	0.05	-0.54
RUST	-	-	-	-	-	-	7.9 ±0.002	88.61	8.8 ±0.003	90.91	0.03	11.39

Values are mean ± SE.

Two-tailed Mann-Whitney *U*-test (d.f.=1) results, where significant values ($P<0.05$) are denoted by bold font.

Positive values for dimorphism denote male-biased difference and negative values denote female-biased difference.

1

Table 6 Mixed model test for nitrogen utilization.

Source	DF	F Ratio	Pr > F
Location	2	65.26	< 0.0001 *
Treatment	1	170.00	< 0.0001 *
Sex	1	15.75	0.0001 *
Sex × Treatment	1	0.31	0.58
Location × Treatment	2	6.51	< 0.01 *

*Significantly different at $P < 0.05$

2

1 **SUPPORTING INFORMATION**

2 **Appendix S1.** Clone ID, sex, and source information for 78 genotypes in the diversity panel.

3 **Appendix S2.** Experimental site characteristics for all trial locations.

4 **Appendix S3.** Materials and methods detailing genotyping, phenotypic and statistical analysis
5 for *Salix purpurea*.

6 **Appendix S4.** Summary of phenotypic traits from the *Salix purpurea* diversity panel.

7 **Appendix S5.** Parameter estimates and significance values for multiple linear regression
8 predictors of second year yield.

9 **Appendix S6.** Matrix of all pair-wise comparisons between traits by location within each year.

10 The lower diagonal shows a scatter plot matrix with a LOESS smooth curve fitting, the main
11 diagonal is a histogram showing the distribution of each trait, and the upper diagonal indicating
12 the Pearson correlation coefficient (r) and P -value for each comparison. Locations: (A) Geneva,
13 NY 2013 (B) Portland, NY 2013, (C) Morgantown, WV 2013, (D) Geneva, NY 2014, (E)
14 Portland, NY 2014, and (F) Morgantown, WV 2014.

15 **Appendix S7.** Matrix of all pair-wise comparisons between traits measured in 2015 for the *Salix*
16 *purpurea* F₁ population.

17 **Appendix S8.** Matrix of all pair-wise comparisons between traits measured in 2015 for the *Salix*
18 *purpurea* F₂ population.

19 **Appendix S9** Correlation heatmap showing Pearson's correlation coefficients (r) for all *Salix*
20 *purpurea* accessions (n=78). Traits shown are divided by category as listed in Table 1. Colored
21 boxes indicate significant correlations at $P < 0.05$, where correlation coefficients of 1 are indicated
22 by dark red and -1 shown as dark blue. All pair-wise comparisons between traits by year and
23 location are shown in Appendix S6. Years: (A) 2013 and (B) 2014.

- 1 **Appendix S10.** Multiple linear regression model for estimating second year post-coppice
- 2 biomass yield from annual measurements.
- 3

1 **FIGURE LEGENDS**

2

3 **Figure 1.** Box plots of biomass and morphological traits that were significantly different
4 ($P < 0.05$) between females (grey boxes) and males (blue boxes). (A) Second year, first rotation
5 biomass yield from the diversity panel measured across three field trials. (B) First year,
6 significantly different traits from the diversity panel. (C) Second year, significantly different
7 traits from the diversity panel. (D) Significantly different traits from F_1 population. (E)
8 Significantly different traits from F_2 population.

9

10 **Figure 2.** SPAD values for monitoring nitrogen utilization in *Salix purpurea* diversity panel.
11 Box plots representing females are colored in green and males colored in blue. SPAD values for
12 control and fertilized plots for (A) Geneva, NY ($F_{1,105} = 15.73$, $P < 0.01$), (B) Portland, NY
13 ($F_{1,105} = 3.44$, $P = 0.06$), and (C) Morgantown, WV ($F_{1,105} = 5.96$, $P = 0.02$).

14

15 **Figure 3.** Least square means for leaf rust severity scores of female and male *Salix purpurea*.
16 (A) Rust severity scores and standard errors on females and males of the diversity panel at the
17 Geneva, NY and Portland, NY field sites. (B) Rust severity scores and standard error on F_1 , F_2
18 parents and female and male F_2 progeny in Geneva, NY. Significant differences between females
19 and males within each site and population are denoted by * $P < 0.10$, and ** $P < 0.01$, where n.s.
20 denotes no significant difference.

21





