Sex-Related Differences in Growth, Herbivory, and Defense of Two Salix Species

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Abstract: Sex-related differences in sex ratio, growth, and herbivory are widely documented in many dioecious plants. The common pattern is for males to grow faster than females and to be less well-defended against herbivores, but Salix is an exception. To study sex-related differences in the patterns of resource allocation for growth and defense in willows, we conducted a large-scale field experiment to investigate the flowering sex ratio, mortality, growth traits, insect herbivory and content of defensive substances in three Salix populations comprising two species. Results demonstrate that the two Salix suchowensis Cheng populations have a female bias in the sex ratio, whereas no bias is found in the S. triandra L. population. Male individuals in the S. suchowensis populations have significantly higher mortality rates than females. However, the mortality rate of S. triandra population has no gender difference. This finding may be one of the explanations for the difference in sex ratio between the two species. The females are larger in height, ground diameter, and biomass, and have a higher nutritional quality (N concentration) than males in both species. Nevertheless, slow-growing males have a higher concentration of the defense chemical (total phenol) and lower degrees of insect herbivory than females. Additionally, biomass is positively correlated with herbivory and negatively correlated with defense in the two willow species. It is concluded that the degrees of herbivory would have a great influence on resource allocation for growth and defense. Meanwhile, it also provides important implications for understanding the evolution of dioecy.

Keywords: Salix; sex ratio; growth; herbivory; chemical defense

1. Introduction

Dioecy is found in 175 flowering plant families, and 7% of flowering plant genera accounting for approximately 5% of all plants [1]. These species are present in a wide variety of habitat types, and many of them are of economic importance. They often show sexual differences in reproductive traits such as nectar production and flower longevity, and in vegetative characteristics, such as morphology, secondary chemistry, and phenology [2]. Dioecious plants often exhibit gender bias and have varying gender ratios and nutrition distributions. The principle of resource allocation [3] states that the allocation of plant resources for three main functions, i.e., growth, reproduction, and defense, is unequal. In other words, when more resources are allocated to a specific function, the allocation for other functions is correspondingly diminished [4]. Many studies have inspected gender differences in vegetative traits [5], reproductive costs [6,7], and demographic characteristics [8]. In particular, in dioecious species, the production of the reproductive structures of female plants (i.e., fruits and seeds) usually requires higher resource input than the male reproductive structures of plants, indicating a trade-off between reproduction, growth, and plant defense [9].
Herbivory has been considered a potential driver for sexual evolution [10]. Herbivores can change the morphology, productivity, and chemistry of preferred species, and these changes, in turn, affect the population dynamics of plants and herbivores [11]. The resource availability hypothesis predicts that slow-growing plant species have weaker defenses and higher herbivory rates than fast-growing plant species [12]. Male plants grow faster, invest less in chemical defense, and exhibit better nutritional quality tissue than female plants [13]. However, some studies documented no significant difference in herbivory between male and female plants [14], and female-biased herbivory [15]. Chemical defense is an adaptive response of plant species against selective pressure from herbivores [16]. If attacks by herbivores are severe, plants may invest more in chemical defenses, such as phenolics, which are often produced in large quantities and reduce digestibility. Some studies showed that if female plants allocate more resources for reproduction, a trade-off between chemical defense and vegetative growth occurs, and allocation for growth is increased at the expense of chemical defense [17,18]. However, male plants have more resources available for defense than female plants and thus have better defense capabilities [19]. Therefore, in dioecious species, different resource allocation patterns can affect plant growth, defense, and herbivory in the populations.

*Populus* and *Salix* belong to Salicaceae, which is mostly composed of dioecious plants. Male-biased sex ratios are often observed in *Populus* [20]. Conversely, predominantly female-biased sex ratios are found among the species of *Salix* [21–23]. In addition, equal or male-biased sex ratios have been observed in some natural willow populations [17,24,25]. The females of *Salix paraplesia* likely accumulate higher biomass included total leaf mass, total stem mass, and total root mass than males under either nutrient-rich or nutrient-poor conditions [22]. The bias is partly attributed to the differential feeding of herbivores [26] and the lower viability of males [27]. Recent studies reported that gender bias is related to sex-biased genes [28,29]. Thus far, the underlying reasons behind the predominance of females in *Salix* populations have not been determined in the literature.

*Salix suchowensis* and *Salix triandra* are shrub willows distributed in central and northern China, respectively. The two shrub willow species are considered promising biomass crops due to their excellent coppicing ability. Given their small size and early flowering characteristics, they are ideal plant materials to study the gender effects of dioecious plants. Compared with most studies on gender effects on resource allocation patterns in willows, our investigation has several advantages. We cultivated three whole sib families for these species and conducted a large-scale field experiment. The plants were nearly the same age and were grown in a fairly homogenous habitat where plant growth, insect herbivory, chemical defense, and nutritional quality were easily recorded. Furthermore, gender comparisons were made between sibling plants resulting from controlled hybridizations. The objectives of this study are as follows: (1) to determine whether responses in gender bias among the three full-sib pedigrees composed of two shrub willow species are consistent under a homogeneous environmental background, and (2) to determine whether differences in resource allocation translate into differences in growth, defense, and herbivory.

2. Materials and Methods

2.1. Plant Material and Study Design

In 2012, two full-sib families were established for *S. suchowensis*, by NF2 and XY12 crossed with LS7 separately. NF2 and XY12 are female stands collected from Nanjing and Xinyi in Jiangsu province of China, respectively, whereas LS7 was a male stand collected from Linshu in the Shandong province of China. For *S. triandra*, a full-sib pedigree was established by DB447 × DB134, both of the parents were collected from Miaoer mountain in the Heilongjiang province of China. The cutting orchard for these pedigrees was maintained at Sihong Forest Farm in Jiangsu province of China. In spring 2018, cuttings were collected from 319 progeny of NF2 × LS7, 334 progeny of XY12 × LS7, and 134 progeny of DB447 × DB134. The three willow populations were named *S. suchowensis* (NF), *S. suchowensis* (XY), and *S. triandra* (DB), respectively. Nine cuttings in the length of 20 cm were prepared for each individual.
In total, 7083 cuttings were prepared for carrying out the field trial. Each cutting was numbered (Table 1).

### Table 1. The individual ratio of males and females in three willow populations.

| Population          | Gender | Progeny Individual | Number of Cuttings | Female/Male | $\chi^2$ |
|---------------------|--------|--------------------|--------------------|-------------|---------|
| S. suchowensis (NF) | Female | 193                | 1737               | 1.53        | 14.07 ***|
|                     | Male   | 126                | 1134               |             |         |
| S. suchowensis (XY) | Female | 185                | 1665               | 1.24        | 3.88 *  |
|                     | Male   | 149                | 1341               |             |         |
| S. triandra (DB)   | Female | 70                 | 630                | 1.10        | 0.27    |
|                     | Male   | 64                 | 576                |             |         |

Notes: Chi-square test from a 1:1 ratio, * $p < 0.05$, ** $p < 0.001$.

The field trial was conducted at Baima Forest Farm in Nanjing of Jiangsu province, China (N 31°60′, E 119°17′), where the average annual temperature is 15.4 °C and average annual precipitation is 1009.7 mm. Rainfall was mainly concentrated in the growing season of willows from March to October. The study area was 0.45 ha in total. The soil on this site was mainly yellow-brown loam. Soil pH and chemical properties are listed in Table 2.

### Table 2. The main physical and chemical properties of soils

| Organic Matter (g kg$^{-1}$) | Total N Content (g kg$^{-1}$) | Alkaline Nitrogen (mg kg$^{-1}$) | Available Phosphorus (mg kg$^{-1}$) | Available Potassium (mg kg$^{-1}$) | Total Magnesium (g kg$^{-1}$) | Total Calcium (g kg$^{-1}$) | pH | Water Content (%) |
|------------------------------|-------------------------------|----------------------------------|-------------------------------------|------------------------------------|-------------------------------|-------------------------------|----|-------------------|
| 20.4 ± 0.5                   | 1.3 ± 0.1                     | 91 ± 2.9                         | 11.5 ± 0.2                          | 135.3 ± 6.3                       | 2.1 ± 0.1                     | 0.5 ± 0.1                     | 6.0 ± 0.2 | 18.8 ± 0.5 |

Notes: Values are expressed as the mean ± standard error (SE) in the table.

For field establishment, stem cuttings were stored at 4 °C for several days and then soaked in water for 48 h. The experimental land was deep-plowed and leveled before planting, and the black film was laid on each ridge for weed control. The cuttings were planted in a spacing of 0.5 m × 0.5 m. Three cuttings of the same genotype were planted in one plot, and each plot was replicated in three blocks according to the completely randomized block design. The cuttings were planted vertically by pushing them manually into the ground until approximately 3 cm of the cuttings protruded from the ground. Only one strong sprout was retained after 30 days of planting.

2.2. Growth Trait

The mortality of the cuttings was investigated, and the number of lateral branches was determined after deciduous. Meanwhile, the height and ground diameter of each plant were measured with a tower ruler and Vernier caliper at a precision of 0.1 mm. In January 2019, the harvest shoots were sent to Nanjing Senke Wood Drying Company, and the shoots were dried with GYB-D electric heating wood drying equipment (Senke, Nanjing, China) at 105 °C for 3 days. After being dried to a constant weight, the dry weight of each shoot was measured using an electronic balance with a precision of 0.1g. We used a leaf area meter (YMJ-B) to measure the leaf areas of the fresh leaves from the bottom, middle, and top of canopies at the end of September 2018.

2.3. Gender Assessment

All individuals reached sexual maturity one year after the willow cuttings were planted on the field. The flowers of S. suchowensis and S. triandra bloomed in early spring before the leaves appeared, and the male and female flowers were arranged in morphologically different catkins. In the middle of March 2019, the gender of each individual in the three willow populations was determined according to the distinct features of male and female flowers.
2.4. Herbivory

We observed that the larvae of Lepidoptera and Coleoptera caused the greatest harm to the two willow species in the field. Newly hatched larvae preferred to eat young shoots and young leaves, and only the central veins of the leaves were severely gnawed. A scale from 0 to 5 with a step size of 1 was used in quantifying larval attacks. This scale considers the proportion of attacked on top shoots and leaves as follows: A score of 0 denotes plants that were not attacked by insects. A score of 1 indicates that 20% of top shoots and leaves are affected by larvae. A score of 2 indicates 40% of top shoots and leaves are affected by larvae. A score of 3 indicates 60% of top shoots and leaves are affected by larvae. A score of 4 indicates 80% of top shoots and leaves are affected by worms. A score of 5 denotes that insects eat all the top buds and young leaves. We evaluated insect damage in each tree at the end of June 2018.

2.5. Leaf N Content

To quantify N content in leaves as a measure of nutritional quality. We measured SPAD-value, representing absorbance by chlorophyll, which is closely correlated to N concentration in *Salix* leaves [30,31] with a chlorophyll meter TYS-B (Zhejiang Top, Hangzhou, China). Three fresh leaves of each plant were selected for measurement at the end of June 2018.

2.6. Defense

Thirty individuals (fifteen males and fifteen females) were selected from each willow population, and the young leaves of willow trees were selected as materials at the end of September 2018. The leaves were oven-dried at 50 °C and weighed to the nearest 0.01 mg. The samples were then ground in a ball mill and analyzed colorimetrically for phenolic compounds and tannins.

Condensed tannins were measured using standard methods [32]. Briefly, 10 mg of leaf powder was weighed and washed with 500 µL of ether, then centrifuged at 3700 r.p.m for 4 min. Tannins were subsequently extracted four times with 200 µL of the solution containing acetone and water at 70:30 volume ratio and 1 mM ascorbate. The acetone in the final supernatant was removed by evaporation with Savant Speed-Vac. Distilled water was added until the final volume of 500 µL was obtained. The samples were analyzed using the *n*-butanol assay for proanthocyanidins [33]. Condensed tannin concentration (mg g⁻¹ dry leaf mass) was then calculated.

Total phenol content was measured by the Folin and Ciocateu method, which detects all compounds containing phenate ions [34]. Briefly, 15 mg of leaf powder was weighed into a 2 mL microfuge vial. Cold methanol (200 µL) was added to each vial, sonicated in a cold water bath for 12 min, and then centrifuged at 3200 r.p.m for 5 min. Catechin was used as a standard. Absorbance was measured with a spectrophotometer at a wavelength of 765 nm, and the extraction solvent was used as a control. Total phenol concentration (mg g⁻¹ dry leaf mass) was calculated.

2.7. Statistical Analyses

Experimental data were analyzed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Sex ratios and mortalities were compared to a 1:1 ratio by chi-square analysis. When more than one leaf was measured (e.g., leaf area, N concentration), the average mean was taken for statistical tests. Comparisons between females and males within each variable were performed using the pairwise t-test. Two-way ANOVA models were used in analyzing the factors, populations, and genders and their interactions for all the variables. Scatter plots, boxplots, and line charts were made by using the ggplot2 package in the R software (version 3.6.0). A principal component analysis (PCA) was performed for the detection of a dependent variable that was most affected by gender and species, and any correlation between the dependent variables was determined. PCA was carried out using the FactoMineR and factoextra packages in the R software. The results were presented as mean ± SE.
3. Results

3.1. Sex Ratio and Mortality

According to floral features, the two *S. suchowensis* populations studied were female-biased (Table 3). A chi-square test revealed that the sex ratio of the two *S. suchowensis* populations significantly departed from a 1:1 segregation ratio ($\chi^2_{NF} = 14.07, p < 0.001; \chi^2_{XY} = 3.88, p < 0.05$). The sex ratios of the two *S. suchowensis* populations sharing the paternal parent were not significantly different. However, the other population of *S. triandra* did not differ from a 1:1 sex ratio ($\chi^2_{DB} = 0.27, p > 0.05$).

| Population          | Gender | Number of Survival | Number of Death | Death Rate | $\chi^2$ |
|---------------------|--------|--------------------|-----------------|------------|----------|
| *S. suchowensis* (NF) | Female | 1649               | 88              | 0.051      | 4.97 *   |
|                     | Male   | 1053               | 81              | 0.071      |          |
| *S. suchowensis* (XY) | Female | 1529               | 136             | 0.082      | 9.38 **  |
|                     | Male   | 1186               | 155             | 0.12       |          |
| *S. triandra* (DB)  | Female | 577                | 53              | 0.092      | 0.036    |
|                     | Male   | 530                | 46              | 0.087      |          |

Notes: Chi-square test from a 1:1 ratio, * $p < 0.05$, ** $p < 0.01$.

The mortality survey revealed that the mortality of males in the two *S. suchowensis* populations was significantly greater than that of females ($\chi^2_{NF} = 4.97, p < 0.05; \chi^2_{XY} = 9.38, p < 0.01$). However, no difference in mortality was observed between the males and females in the *S. triandra* population ($\chi^2_{DB} = 0.036, p > 0.05$).

3.2. Gender Effects on Growth Traits

Growth traits, including tree height, ground diameter, number of lateral branches, leaf area, and dry weight, were measured (Table 4). The results of the two-way ANOVA indicated a significant effect of gender on the heights, ground diameters, and dry weights of the willows. The female trees were taller and had larger ground diameters and biomasses than the male trees (gender: $F_H = 24.9, p < 0.0001; F_{GD} = 9.6, p < 0.05; F_{DW} = 12.2, p < 0.01$). The results of the pairwise t-test revealed that the plant height, ground diameter, and dry weight of the female individuals of *S. suchowensis* (NF) were significantly greater than those of the male individuals. The plant height and dry weight of the female individuals in the *S. suchowensis* (XY) population were significantly higher than those of the male individuals, but no significant difference was observed in ground diameter. Significant differences were found between the sexes in terms of the growth traits of *S. triandra* (DB) included in the study (ground diameter, leaf area, and dry weight). Significant differences in growth traits were found between the populations (population: $F_H = 33, p < 0.001; F_{GD} = 21.2, p < 0.001; F_{NB} = 162.51, p < 0.001; F_{LA}=85.6, p < 0.001; F_{DW} = 68.1, p < 0.001$). Overall, these results showed that the plant height, ground diameter, number of lateral branches, leaf area, and dry weight of *S. triandra* (DB) were significantly greater than those of *S. suchowensis* (NF, XY). By contrast, the interaction between gender and population demonstrated no significant effect on the growth traits.
Table 4. Effects of gender on the growth of willow plants.

| Population     | Gender   | Height (cm) | Ground Diameter (mm) | Number of Lateral Branches | Leaf Area (cm²) | Shoot Dry Weight (g) |
|----------------|----------|-------------|----------------------|---------------------------|----------------|----------------------|
| **S. suchowensis** (NF) | Female   | 278.4 ± 1.6 | 17.7 ± 0.2           | 2.8 ± 0.1                 | 6.9 ± 0.09       | 114.8 ± 2.7          |
|                | Male     | 268.6 ± 2.2 | 17.1 ± 0.2           | 2.5 ± 0.2                 | 6.7 ± 0.1        | 104.0 ± 3.7          |
| **S. suchowensis** (XY) | Female   | 266.8 ± 2.4 | 18.1 ± 0.2           | 1.9 ± 0.1                 | 7.7 ± 0.1        | 87.8 ± 2.8           |
|                | Male     | 256.8 ± 2.5 | 17.7 ± 0.2           | 1.7 ± 0.1                 | 7.5 ± 0.1        | 78.0 ± 2.6           |
| **S. triandra** (DB)    | Female   | 286.1 ± 3.1 | 19.5 ± 0.3           | 5.6 ± 0.4                 | 9.2 ± 0.3        | 133.8 ± 7.0          |
|                | Male     | 278.3 ± 3.1 | 18.7 ± 0.3           | 5.4 ± 0.4                 | 8.7 ± 0.3        | 122.6 ± 7.1          |

Notes: The mean ± standard error (SE) in the table. Two-way ANOVA models were used in analyzing the factors, populations, and genders and their interactions for growth variables. Height (H), ground diameter (GD), number of lateral branches (NB), leaf area (LA), and shoot dry weight (DW) for the three Salix populations used in the trial. *p < 0.05; **p < 0.001.

3.3. Gender Effects on Herbivores

As shown in Figure 1, the degree of insect damage in the three willow populations varied between males and females, and the degree of females was higher than that of males (Gender: F = 21.1, p < 0.001; Table 5). In the population (XY), approximately 36.8% of the top shoots of the male plants were gnawed by insects, and 47.8% of the top shoots of female plants were damaged, which was significantly higher than that of male. In the other population (NF), similar findings were found, but the degree of overall damage in this group was higher than that of the population (XY). We also found that the females were more severely damaged in the S. triandra (DB) population. Approximately 26.1% of the top end was eaten, which is higher than that in the male individuals (13.5%). However, the degree of damage between the two willow species was quite different, and the data showed that the degree of damage to the S. triandra was much lower than that to S. suchowensis (Population: F = 56.8, p < 0.001).

Figure 1. Degrees of herbivory damage on male and female of willow plants. Notes: Bars represent SE. Comparisons between females and males within each variable were performed using the pairwise t-test. **p < 0.01; ***p < 0.001.
Table 5. ANOVA analysis of herbivory, N content, and chemical defense substance of willow plants.

| Factor             | Herbivory | Total Phenol | Condense Tannin | N Content |
|--------------------|-----------|--------------|-----------------|-----------|
| Population         | 56.8 ***  | 1.5          | 95.5 ***        | 227.1 *** |
| Gender             | 21.1 ***  | 7.7 **       | 4.0             | 40.3 ***  |
| Population × Gender| 0.3       | 0.1          | 0.9             | 0.9       |

Notes: Two-way ANOVA models were used in analyzing the factors, populations, and genders and their interactions for herbivory, N content, and chemical defense substance of willow plants. ** p < 0.01; *** p < 0.001.

3.4. Chemical Analyses between Genders

As shown in Figure 2 and Table 5, the chemical substances in the leaves of different willow species were different. The content of condensed tannins in the leaves of S. suchowensis was 38.9% higher than that in the leaves of S. triandra (Population: F_{CT} = 95.5, p < 0.001). We also found that the condensed tannin content of willow is only 5.7%–10% of the total phenolic content. However, no difference in total phenol content was found among the three willow populations. Meanwhile, we determined the leaf N content of the three willow populations. We found that the N content (1.2%) of S. suchowensis (XY) was significantly higher than that (0.8 %) of S. triandra (Population: F_{N} = 227.1, p < 0.001). Moreover, significant differences in leaf N concentration were found between females and males and among three willow populations (gender: F_{N} = 40.3, p < 0.001), and N concentration in the female leaves was 0.52%–6.1% higher than that of the males (Figure 3). Moreover, a significant difference in total phenol content was found between female and male willows (gender: F_{TP} = 7.7, p < 0.01). The content of total phenols in the leaves of the male individuals was 14.5%–22.6% higher than that of female individuals. No difference in tannin content was found between male and female individuals (Table 5). However, the condensed tannin content in the leaves did not differ between the females and males in the three willow populations.

Figure 2. Contents of defensive substances in leaves of willow plants. (a) Total phenol content in willow leaves. (b) Content of condensed tannin in willow leaves. Notes: Comparisons between females and males within total phenol (TP) and condensed tannin (CT) were performed using the pairwise t-test. ** p < 0.01.
Figure 3. Contents of N in leaves of willow plants. Notes: Comparisons between females and males within leaf N content (N) were performed using the pairwise t-test. ** p < 0.01. Filled dots represent outliers.

3.5. Relationship between Growth, Defense, and Herbivores

The model explained 73.3% of the total variance, which was loaded into a three-dimensional space. The first PCA axis explained 33.1% of the total variation. It showed a highly positive correlation with herbivory, plant height, ground diameter, leaf area, and dry weight, and a negative correlation with total phenol (Figure 4a). The second PCA axis explained nearly 29.2% of the variance, and the dominant trend was the positive relationship among herbivory and N content and condensed tannin (Figure 4a). Herbivory was negatively associated with total phenol. A high cos2 value indicates that the variable has a larger contribution to the principal component. Our data revealed that leaf area and insect herbivory are the two most important predictors of the two salix species. The score plot of the three willow families is shown in Figure 4b. The PCA results revealed that the growth, herbivory, and defense values collected from the two willow species were successfully distinguished but not according to the genders of the willows.

Figure 4. Principle component analysis (PCA) of the selected variables. (a) Relationships between all the variables; (b) under different species and genders. Notes: The variables are listed as follows: N, N concentration; CT, condense tannin; TP, total phenol; Herbivory, insect herbivory; DW, shoot dry weight; GD, ground diameter; H, plant height; NB, number of lateral branches; LA, leaf area. cos2 value indicates contribution of variables in PCA results.

4. Discussion

4.1. Sex Ratios

The sex ratios of different species have considerable differences, even among closely related species. Kaul and Kaul [16] found Salix amygdaloides has an equal number of male and female individuals,
and a 1.7:1.0 male-biased sex ratio was observed in *Salix exigua* [35]. However, sex ratios in the genus *Salix* are often more skewed toward females than males [36–39]. We have similar findings, and our data shows that the sex ratios of two *S. suchowensis* populations are an obviously female-biased sex ratio. By contrast, the *S. triandra* population has no gender bias. Female-biased sex ratios preferentially increase over time because males have lower viability than females in natural populations [27], and male plants are generally more herbivorous than females [14]. The population of *S. suchowensis* was established by our laboratory in 2012. One year since the establishment, Hou et al. [40] found that the sex ratio of the population was 1:1. However, the *S. suchowensis* population develops into a female-biased sex ratio group. Gender differences in mortality may lead to female bias, which is exacerbated during the life cycle to produce skewed sex ratios [41]. We found that the mortality of male individuals was significantly higher than that of female individuals. Therefore, survivorship may be a highly important factor in determining sex ratios.

Sex ratios in a given population are strongly modulated by environmental conditions [42]. The reproductive habit of willows may be an important factor affecting sex ratio bias. Some studies concluded that male and female willows respond differently to environmental factors, and females generally perform better than males under stress [26,27,38]. We demonstrated that female-biased ratios are related to the presence of sex chromosomes, suggesting that genetic factors play a role in changing sex ratios. Notably, the gender segregation rate in the analysis of the pedigree indicates that the sex of the willow is dominated by a single locus. This finding is consistent with the findings of earlier studies [29,43,44]. Therefore, willows provide a unique system for exploring sex effects.

4.2. Biomass

Our obtained data showed that gender significantly affected growth characteristics. The female plants in the three willow populations had larger biomass than the males. Our findings are consistent with previous reports on other *Salix* species. These reports suggested that female plants grow faster than male plants [22,40]. However, these results were contradictory to reports on *Salix* [17,24]. In addition, Maldonado-López et al. [18] found that physiological responses between the females and males of *Spondias purpurea* were different, and the water use efficiencies and photosynthetic rates of females were higher than those of males. These results indicated that input to plant growth was increased. This increase may be one of the reasons that females have faster growth than males.

4.3. Herbivory

Herbivory may play an important role in the evolution of plant reproductive systems. Male-biased herbivory appears to be the most common pattern [9,13]. However, some papers documented female-biased herbivory [15], suggesting that male-biased herbivory might not be universal. This finding is consistent with our findings, which showed that female plants have more serious insect herbivory than male trees. The effects of plant gender on herbivory rates might be related to environmental conditions (soil moisture and nutrition) and populations. Damage of *S. suchowensis* was much more severe than that of *S. triandra*. Herbivores may be affected by plant growth characteristics, such as biomass, number of branches, and arrangement of leaves [45]. An increase in above-ground biomass, number of leaves and stems, and sizes of leaves may lead to an increase in sensitivity to insect herbivory [17]. We found a positive correlation between plant biomass and insect herbivory in *S. suchowensis* and *S. triandra*. Plant individuals with large biomass or more vigorous growth may be more conducive to insect spawning, foraging, and survival.

4.4. Defense

Many studies provide more evidence that female plants have better chemical defenses than male plants. Palo et al. [46] found that male willows generally have lower concentrations of phenolic glycosides in the leaves. Furthermore, Danell et al. [47] suggested that female willows contain more tannin in the bark than male willows. High concentrations of quercetin-glucoside-related compounds
are found in the females of *Salix myrsinifolia* [48]. However, Moritz et al. [31] found no difference in total phenolic acid or lignan content between genders in *Salix viminalis*. Conversely, we found substantial intersexual differences in defensive compounds among the three willow populations. The leaves of the female willows had higher total phenol contents than those of the male trees. This result implied that phenols are a major deterrent of herbivores, as shown by Boeckler et al. [49]. Environmental variations may have a great impact on the content of defensive compounds in male and female willows. Jiang et al. [22] documented that females produce more condensed tannins than males under nutrient-poor conditions and produce fewer condensed tannins under nutrient-rich conditions. Therefore, our study does not support the idea that female plants have better chemical defenses than males in dioecious species [18]. Low nutritional quality is a potentially active defense against herbivorous insects [50]. We found that the leaves of females contain high N concentrations in *S. suchowensis* and *S. triandra*. Therefore, we conclude that nutritional value is limiting the feeding decisions of insects. We acknowledge that other nutritional variations exist between male and female plants. For example, the concentrations of phosphorous and potassium differ between male and female *Salix lasiosepsis* [17]. We realize that other aspects should be considered, such as leaf toughness [51] and diet maximizing soluble sugars [52]. Further study of these variables in relation to browsing and secondary metabolite profiles can broaden our understanding of generalist herbivore feeding decisions.

4.5. Resource Allocation Principle

In this study, we revealed that gender significantly affects the biomass, herbivory, and defense of *S. suchowensis* and *S. triandra*, and the females generally have significantly greater biomass, higher defensive substance content, and a lower rate of insect herbivory than males. Our findings are consistent with the results of a previous study [9]. However, our observations are inconsistent with the hypothesis of Myers-Smith and Hik. [38], who believed that female willows consume more sexual reproduction resources than male willows and thus delay plant growth. Our data showed that biomass is positively correlated with insect damage and negatively correlated with defensive compound content. When females allocate more resources to reproduction, the main reduction in allocation may be observed in defense, and the resource allocation for vegetative growth does not necessarily decrease. In this case, there would be no adverse effect on growth. Maldonado-López et al. [18] found that gender with great demand for reproductive resources generally tends to garner more resources than other factors. The relative differences in reproductive costs between females and males can be reduced through some mechanisms such as increased photosynthesis rates, increased canopy area, increased mineral nutrient uptake rates, increased root branching, and enhanced mycorrhizal associations [53]. Our results show that female willows have more branches, more leaves, higher leaf N content, and probably higher photosynthetic rates, which is expressed as greater investment in plant growth. Furthermore, browsing inhibited apical dominance and activated axillary and adventitious buds for the production of new vegetative shoots and reduced reproductive growth [54].

5. Conclusions

The sex ratios of two *S. suchowensis* populations are obvious female-biased sex ratios. By contrast, the *S. triandra* population has no sex ratio bias. Female-biased sex ratios preferentially increase over time because males have lower viability than females in natural populations and male plants are generally more herbivorous than females. The mortality of male individuals of *S. suchowensis* is significantly higher than that of female individuals. Survivorship may be a highly important factor in determining sex ratios of willow populations. In this study, Females generally have greater biomass, higher defensive substance content, and a lower rate of insect herbivory than males. Leaves of females contain high N concentrations in two willow species. The nutritional value may be limiting the feeding decisions of insects. PCA data shows that biomass is positively correlated with insect damage and negatively correlated with defensive compound content. When females allocate more resources to reproduction, the main reduction in allocation is observed in defense, and the resource
allocation for vegetative growth does not necessarily decrease. In this case, there would be no adverse effect on growth. Future work should focus on genetic data for the analysis of sex-biased gene expression patterns.

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