Gender-specific carbon discrimination and stomatal density in the dioecious tree of *Hippophate rhamnoides*

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Abstract

For a long time dioecious plants have played an important role in examination of the reproduction cost and determination of the sexual dimorphism evolution in life history. *Hippophae rhamnoides sinensis*, a dioecious, outcrossing plant, occurs mainly in the arid regions of northwest China, contributes to maintain the local ecosystems in these regions. In this study, δ^{13}C and the stomatal density of herbarium sheet were measured in *Hippophae rhamnoides sinensis*. It was found that the plants showed gender-difference in carbon isotope compositions: the males maintained higher δ^{13}C values and thus higher water use efficiency as well as slightly higher stomatal density than the females, moreover, the between-sex difference is stable in different contexts through carbon isotope discrimination. Although we found that the stomatal density had being reduced and is sensitive to atmospheric CO2 level, δ^{13}C value and thus water-use efficiency did not significantly change from 1978 to 2001. Consequently, gender-specific δ^{13}C could play a decisive role in explaining gender differences in the developmental growth.

Keywords: Carbon isotope discrimination; Dioecious plant; *Hippophate rhamnoides sinensis*; Stomatal density

1. Introduction

In dioecious plants, substantial differences in the demand for resources are usually observed between females and males, which might be due to difference in reproduction cost (Lloyd and Webb, 1977). The male allocates resources to a flower for a short period (the flowering time only) whereas the female allocates resources not only to flowers but also to fruits, and the allocation in reproduction occurs for a longer period during fruit maturation. Thus, the female generally incurs greater reproduction cost than the male does (Gross and Soule, 1981; Lovett Doust and Lovett Doust, 1988; Allen and Antos, 1988, 1993; Dawson and Ehleringer, 1993; Kohorn, 1994; Obeso, 1997; Nicotra, 1999). It has been suggested that the different cost for reproduction should impose a very different resource demand on the plants and, consequently, gender specialization has been interpreted as an evolved response that may allow each gender to meet the specific resource demands associated with reproduction (Cox, 1981; Dawson and Bliss, 1989; Dawson and Ehleringer, 1993). Gender differences have been well documented in growth (Ramp and Stephenson, 1988; Jing and Coley, 1990), survival (Lovett Doust and Lovett Doust, 1988; Allen and Antos, 1993), reproductive patterns (Bullock and Bawa, 1981; Cipollini and Whigham, 1994), spatial distribution (Bierzychudek and Eckhart, 1988; Iglesias and Bell, 1989), resource allocation (Wallace and Rundel, 1979; Gross and Soule, 1981; Ramp and Stephenson, 1988; Vitale et al., 1987; Delph, 1990), shoot structure and function (Ueno and Seiwa, 2003), canopy and leaf display (Wallace and Rundel, 1979; Kohorn, 1994) and patterns of defense and herbivory (Jing and Coley, 1990; Krischik and Denno, 1990). However, because of the greater difficulties in gathering data on eco-physiological processes, much less has been known about gender specialization in physiology (Bourdeau, 1958; Crawford and Balfour, 1983; Dawson and Bliss, 1989, 1993; Dawson and Ehleringer, 1993; Hill et al., 1996; Obeso et al., 1998; McDowell et al., 2000). This is a crucial issue, as variation in ecophysiological
attributes may, by itself or in combination with factors such as allocation, determine the performance of each sex in different micro habitats and, ultimately, it may have consequences for growth, survival and population structure. Furthermore little is known about the physiological differentiation between sexes that may influence habitat partitioning between the sexes (Freeman and McArthur, 1982; Crawford and Balfour, 1983; Dawson and Bliss, 1989; Dawson and Ehleringer, 1993) and about how gender-specific physiology could account for the differences in growth and size distributions of both sexes (Dawson and Ehleringer, 1993; Obeso et al., 1998; McDowell et al., 2000; Retuerto et al., 2000).

Most of the characteristics used to investigate the physiological differentiation between sexes, such as photosynthetic activity, transpiration rate and other water-related parameters, are instantaneous measurements that do not allow integration of ecophysiological processes (Retuerto et al., 2000). The use of stable isotope techniques in plant ecological research has grown steadily during the past two decades and this trend will continue as researchers realize that stable isotopes can serve as valuable non-radioactive tracers and nondestructive integrators indicating how plants today and in the past have interacted with and responded to their abiotic and biotic environments. Plant δ\(^{13}\)C has been used in ecological research as a long-term integrator of ecophysiological processes such as leaf conductance, hydraulic capacity, potential water-use efficiency and photosynthetic capacity (Körner et al., 1988; Farquhar et al., 1989). One of its uses has been to compare the physiological performance between male and female individuals (Dawson and Bliss, 1989; Dawson and Ehleringer, 1993; Retuerto et al., 2000).

**Hippophae rhamnoides sinensis** is an important resource plant in China and exists as a pioneer plant with significant value for water and soil conservation. It grows mainly on sandy soils by riverbanks or along riverbeds, mountain slopes, and valleys. It is found on the eastern edges ranging from Qinghai Province in the west to Hebei Province in the east, from Sichuan Province in the south to Inner Mongolia and Hebei Province in the north, with an altitude from 400 to 3900 m (Lian, 2000).

Here, we used herbarium sheet and explored the differences between sexes of the dioecious tree *Hippophae rhamnoides sinensis* in \(^{13}\)C/\(^{12}\)C discrimination in different habitats and years. Our main interest was to know if gender made a difference in the long-term potential water-use efficiency, as estimated by carbon isotope discrimination and if physiological differences between sexes could be dependent on the environmental context. Such information would contribute to a better understanding of the ecological and evolutionary significance of sex differences in *Hippophae rhamnoides sinensis*.

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**Fig. 1.** Map of China showing locations of collecting areas for samples of *H. rhamnoides sinensi*. 

![Map of China](image-url)
| Gender | Collecting time | Collecting location | Habitat          | $\delta^{13}$C value (%) | Stomatal density(mm$^{-2}$) |
|--------|----------------|---------------------|------------------|--------------------------|---------------------------|
| 1. Female | Sep. 1976 | Kangle, Gansu | Hillside | -28.7 | 246.42±15.2 |
| 2. Male | Sep. 1976 | Kangle, Gansu | Hillside | -27.2 | 252.49±7.1 |
| 3. Female | Jun. 1986 | Lingtang, Gansu | Riverside | -28.2 | 203.6±14.9 |
| 4. Male | Jun. 1986 | Lingtang, Gansu | Riverside | -27.3 | 226.55±8.99 |
| 5. Female | Jun. 1986 | Diebu, Gansu | Hillside | -26.6 | 213.43±7.32 |
| 6. Male | Jun. 1986 | Diebu, Gansu | Hillside | -25.9 | 232.13±8.16 |
| 7. Female | Sep. 1986 | Xiahe, Gansu | Hillside | -27.2 | 207.43±21.21 |
| 8. Male | Sep. 1986 | Xiahe, Gansu | Hillside | -27.6 | 225.34±13.1 |
| 9. Female | Oct. 1986 | Tianzhu, Gansu | Riverside | -28.4 | 215.21±20.42 |
| 10. Male | Oct. 1986 | Tianzhu, Gansu | Riverside | -27.2 | 252.49±6.48 |
| 11. Female | Sep. 1987 | Xiahe, Gansu | Hillside | -27.9 | 208.21±7.54 |
| 12. Male | Sep. 1987 | Xiahe, Gansu | Hillside | -27.5 | 219.23±8.42 |
| 13. Female | Sep. 1987 | Xiahe, Gansu | Hillside | -29.5 | 202.63±20.21 |
| 14. Male | Sep. 1987 | Xiahe, Gansu | Riverside | -28.3 | 226.79±18.58 |
| 15. Female | Sep. 1987 | Xiahe, Gansu | Riverside | -28.4 | 213.35±5.23 |
| 16. Male | Sep. 1987 | Xiahe, Gansu | Riverside | -28.1 | 221.54±13.76 |
| 17. Female | Sep. 1987 | Zhaoni, Gansu | Riverside | -28.5 | 198.62±10.63 |
| 18. Male | Sep. 1987 | Zhaoni, Gansu | Riverside | -27.2 | 214.71±19.32 |
| 19. Female | Sep. 1987 | Diebu, Gansu | Hillside | -28.8 | 209.25±13.44 |
| 20. Male | Sep. 1987 | Diebu, Gansu | Hillside | -27.4 | 235.56±12.37 |
| 21. Female | Sep. 1987 | Diebu, Gansu | Riverside | -28.1 | 216.35±13.24 |
| 22. Male | Sep. 1987 | Diebu, Gansu | Riverside | -26.9 | 226.76±7.67 |
| 23. Female | Sep. 1987 | Diebu, Gansu | Hillside | -27.4 | 208.43±15.32 |
| 24. Male | Sep. 1987 | Diebu, Gansu | Hillside | -26.8 | 224.53±7.20 |
| 25. Female | Sep. 1987 | Diebu, Gansu | Hillside | -27.6 | 215.44±9.82 |
| 26. Male | Sep. 1987 | Diebu, Gansu | Hillside | -27.4 | 225.56±4.78 |
| 27. Female | Sep. 1987 | Zhaonxiang, Gansu | Hillside | -27.5 | 205.63±6.76 |
| 28. Male | Sep. 1987 | Zhaonxiang, Gansu | Hillside | -27.6 | 222.45±11.40 |
| 29. Female | Sep. 1987 | Diebu, Gansu | Hillside | -28.1 | 221.84±15.22 |
| 30. Male | Sep. 1987 | Diebu, Gansu | Hillside | -27.8 | 233.64±13.96 |
| 31. Female | Sep. 1987 | Luqu, Gansu | Riverside | -27.3 | 209.88±6.31 |
| 32. Male | Sep. 1987 | Luqu, Gansu | Riverside | -26.0 | 221.17±23.27 |
| 33. Female | Sep. 1987 | Zhaonxiang, Gansu | Hillside | -29.0 | 201.59±16.11 |
| 34. Male | Sep. 1987 | Zhaonxiang, Gansu | Hillside | -28.6 | 214.56±12.37 |
| 35. Female | Oct. 1987 | Qingshui, Gansu | Hillside | -27.9 | 204.52±10.55 |
| 36. Male | Oct. 1987 | Qingshui, Gansu | Hillside | -27.1 | 238.66±9.77 |
| 37. Female | Sep. 1987 | Linxia, Gansu | Hillside | -27.5 | 196.43±20.60 |
| 38. Male | Sep. 1987 | Linxia, Gansu | Hillside | -27.7 | 218.46±13.54 |
| 39. Female | Oct. 1987 | Qinian, Gansu | Hillside | -26.8 | 205.79±8.23 |
| 40. Male | Oct. 1987 | Qinian, Gansu | Hillside | -25.9 | 224.41±17.88 |
| 41. Female | Oct. 1992 | Kangding, Sichuan | Riverside | -29.4 | 167.63±6.76 |
| 42. Male | Oct. 1992 | Kangding, Sichuan | Riverside | -26.6 | 213.97±11.40 |
| 43. Female | Sep. 2000 | Chengdu, Qinghai | Riverside | -29.5 | 163.52±8.99 |
| 44. Male | Sep. 2000 | Chengdu, Qinghai | Riverside | -27.3 | 165.25±13.44 |
| 45. Female | Sep. 2000 | Linxia, Gansu | Hillside | -29.3 | 155.64±17.48 |
| 46. Male | Sep. 2000 | Linxia, Gansu | Hillside | -26.6 | 183.87±9.87 |
| 47. Female | Sep. 2001 | Weixian, Hebei | Hillside | -28.1 | 176.98±13.90 |
| 48. Male | Sep. 2001 | Weixian, Hebei | Hillside | -27.1 | 182.28±20.10 |
| 49. Female | Sep. 2001 | Wu'ai, Shanxi | Hillside | -27.9 | 166.82±7.52 |
| 50. Male | Sep. 2001 | Wu'ai, Shanxi | Hillside | -26.7 | 186.44±13.63 |
| 51. Female | Sep. 2001 | Longde, Ningxia | Hillside | -27.8 | 164.31±20.12 |
| 52. Male | Sep. 2001 | Longde, Ningxia | Hillside | -27.1 | 177.91±7.32 |
| 53. Female | Sep. 2001 | Zhangyue, Gansu | Hillside | -28.2 | 167.27±11.67 |
| 54. Male | Sep. 2001 | Zhangyue, Gansu | Hillside | -26.2 | 174.56±12.37 |
| 55. Female | Sep. 2001 | Wuxiang, Shanxi | Hillside | -28.9 | 159.87±11.05 |
| 56. Male | Sep. 2001 | Wuxiang, Shanxi | Hillside | -25.7 | 181.51±15.12 |
| 57. Female | Sep. 2001 | Liangcheng Neimenggu | Hillside | -26.5 | 168.42±13.40 |
| 58. Male | Sep. 2001 | Liangcheng Neimenggu | Hillside | -24.1 | 175.25±10.23 |
| 59. Female | Sep. 2001 | Zongyang, Shanxi | Hillside | -27.8 | 157.35±11.43 |
| 60. Male | Sep. 2001 | Zongyang, Shanxi | Hillside | -28.0 | 166.38±7.50 |
| 61. Female | Sep. 2001 | Daning, Shanxi | Hillside | -27.0 | 153.49±13.40 |
| 62. Male | Sep. 2001 | Daning, Shanxi | Hillside | -28.2 | 182.57±10.23 |
understanding of the population genetics of this subspecies and facilitate its conservation and utilization as an important plant resource.

2. Materials and methods

2.1. Plant material

All samples used in this study were supplied by the School of Life Sciences, North-west Normal University. The geographical sites of the samples are given in Fig. 1.

2.2. Carbon isotope composition

To avoid position effects (Farquhar et al., 1989), we chose sampled leaves from “pairing specimen” (in the course of collecting samples, we collected leaves from those plants whose females and males live together in the same position). Each sample consisted of four individuals sampled from the same location on the same occasion and was stored very well. Sampled leaves were ball-milled to a homogenized fine powder to pass through a 40-mesh screen. Following the methods reported in our previous paper (Gao et al., 2006), δ13C determination was conducted using an isotope ratio mass spectrometer (Finnigan MAT-252, Cold and Arid Region Environmental and Engineering Research Institute, Lanzhou, China). The sample was prepared and the analysis error between repeated analyses of the same ground tissue was less than 0.1%.

2.3. Stomatal density and leaf morphological traits

We chose 3 to 4 healthy and mature leaves (bigger ones and not bitten by bugs), put them in boiling water for 20 min and then put them in 30 °C, 5% KClO3 for 1.5 h. The superﬁcies inﬁders were torn by forceps (both plants have bifacial leaves and only there are stomata in superﬁcial inﬁders), dyed by methyl blue, mounted on a slide and viewed using a Olympus microscope (made in Japan) at 100 magniﬁcation. Ten randomly selected fields of view (300–300 mm each), ﬁve on either side of the midrib, were counted per leaf and averaged (Zheng et al., 2001). Ten leaves were selected in the same position of the “pairing specimen”, then measured and averaged for length and width. Finally, the ratio of length/width was calculated.

2.4. The data of meteorology and atmospheric CO2 concentration

Meteorological data was provided by the Meteorological Bureau of Gansu Province. Atmospheric CO2 concentrations from NOAA were also used for the analysis.

3. Results

The male showed higher δ13C than the female in H. rhamnoides sinensi (Table 1). Furthermore, the between-sex difference is stable in different parts of the plant. δ13C has been proposed by several authors as an index of long-term water use efficiency and, in general, the higher the δ13C value, the higher the water use efficiency (Farquhar et al., 1989; Smedley et al., 1991; Dawson and Ehleringer, 1993; Zheng et al., 2001). As a consequence, the male maintained higher long-term water use efficiency than the female in the plant.

No significant relationship between δ13C values and temperature was detected, while the δ13C values were significantly and positively correlated with precipitation (Fig. 2).

Table 1 showed that stomatal density of the male was slightly higher than the female, but the difference is not significant (p<0.5). Moreover, the stomatal density of the male was reduced from 236.43 mm⁻² in 1976 to 180.20 mm⁻² in 2001 (r²=0.9166, p<0.001) and the stomatal density of the female...
was reduced from 242.49 mm$^{-2}$ in 1976 to 165.15 mm$^{-2}$ in 2001 ($r^2=0.898$, $p<0.001$). However, the $\delta^{13}$C value for both genders did not change in different times (Fig. 3, Table 1).

While measuring length and width of leaves and calculating length/width, we found that: 1) the variety of length and width could not serve as a rule to discern gender. 2) In most circumstances, leaves of the female were more confined and leaves of the male were wider, which can be used as an assisting tool for discerning the gender. 3) As for the length-to-width (F/W) ratio of leaves, the value of the female was always higher than the value of males and, therefore, it can be as a signal for discerning the gender (Fig. 4). The gender-difference in the length-to-width ratio was in contrast to the gender difference in $\delta^{13}$C value.

4. Discussion

Foliar $\delta^{13}$C is influenced by many environmental factors such as precipitation, temperature, humidity, irradiance, air CO$_2$ concentration, etc. (Farquhar et al., 1989). Thus plants with different geographic distributions have different $\delta^{13}$C values. Many studies have shown that foliar $\delta^{13}$C values are negatively correlated with precipitation, and the negative correlation is more distinct in arid areas than in moist areas (Farquhar et al., 1989, Williams et al., 1996; Gao et al., 2006; Zheng and Shangguan, 2007). In our study, the $\delta^{13}$C values were significantly and positively correlated with precipitation (Fig. 2), which was contradictory to the above viewpoints. Other researchers have similarly reported that leaf $\delta^{13}$C values were positively correlated to precipitation for Nothofagus and Quercus species (Read and Farquhar, 1991; Williams et al., 1996). The reason might be that these species had evolved greater hydraulic conducting capacity, allowing stomatal conductance and assimilation rate to remain comparatively high during drought periods (Read and Farquhar, 1991).

Our results showed that the males always maintained higher carbon isotope composition than females in _H. rhamnoides sinensis_ and the difference was stable in different habitats and times. Since a strong correlation between water use efficiency and stable carbon isotope composition was discovered (Farquhar et al., 1989; Smedley et al., 1991; Dawson and Ehleringer, 1993; Zheng et al., 2001), the male individuals showed higher water use efficiency. An early investigation by Sivstev and Sizov (1972) showed that among a variety of dioecious species, male plants were more drought-resistant than females. Freeman and McArthur (1982) reported that males of six dioecious desert shrub species demonstrated lower $\psi$ and thus greater water stress than females.
and Bliss (1989) documented difference in tissue elasticity and/or the ability to osmotically adjust in response to drought and showed that it was this physiological difference that allowed male plants of *Salix arctica* to maintain higher rates of stomatal conductance and carbon fixation in drier sites. So, these physiological differences contributed to the dominance of male in dry habitats and female in wet habitats along extremely sharp soil moisture gradients (Cox, 1981; Dawson and Ehleringer, 1993).

Although we found that males exhibited slightly higher stomatal densities than females, the difference was not significant and was in agreement with a report (Retuerto et al., 2000). However, Dawson and Ehleringer (1993) observed that males of *Acer negundo* exhibited lower stomatal densities than females. These authors and their colleagues (Woodward, 1987; Woodward and Bazzaz, 1988) have reported positive relationships between stomatal densities and conductance’s. However, it is unclear how much significance we can assign to the density of stomata, because stomatal diffusion resistance also depends on other critical variables such as pore width. In contrast, Dawson and Ehleringer (1993) reported that males maintained a lower stomatal conductance to net carbon assimilation, transpiration and water vapor and higher water-use efficiency than females. Crawford and Balfour (1983) reported that in arctic environments females of *Salix polaris* had greater leaf stomatal resistance than males, suggesting that female plants restricted water loss more than males did. Dawson and Bliss (1989) also suggested a greater potential water-use efficiency of female *Salix arctica* in xeric habitats compared with that of the male. Retuerto et al. (2000) found males maintained significant higher δ13C than females of *Ilex aquifolium* under more xeric conditions. These findings, together with our observations that the between-sex difference in carbon discrimination is unstable in different environments, has led us to conclude that environmental context is crucial for getting to know between sex differences in the physiology of some plants, but it is not very important for some plants such as *H. rhamnoides sinensis*, because the between-sex physiological differences do not change in different environments.

Differences in growth between the sexes have been well documented and it has usually been found that gender-specific growth rates depend on plant reproductive status (Ramp and Stephenson, 1988; Nicotra, 1999) and on environment (Dawson and Ehleringer, 1993). Lu (1995) observed males of *H. rhamnoides sinensis* showed significantly more plant height, stem diameter and above-ground biomass than females. Wu and Yu (1991) found more female plants in the streamside than in a non-streamside habitat and there were a greater number of large female trees (>1.5 m), compared with the small female saplings (<1.5 m) in drier habitats. Their observation supported our results: males maintain a higher water use efficiency and thus a more conservative water-use strategy than females.

In our study, the leaves of female are more confined and those of males are wider in *H. rhamnoides sinensis*. The length-to-width (F/W) ratio of female leaves is always higher than that of males, which can be used as a symbol to discern the gender (Fig. 2). The gender-difference in length-to-width ratio is in contrast to gender difference in δ13C value. Tuomi et al. (1983) considered that compensatory mechanisms existed to compensate for the cost of reproduction. A lot of experiments have demonstrated mechanisms enabling compensation of the cost in many different dioecious species (see a recently review by Obeso and Retuerto, 2002). Since male leaves are wider and their F/W is less than females, it will lead to more closed canopies in male trees, compared with the more open canopies of females that result in greater self-shading and perhaps a reduction in the total canopy carbon fixation in male trees relative to female ones. Therefore, we think the difference of F/W, and even δ13C value, is a special strategy of the plant to compensate for higher cost in females.

Atmospheric CO2 concentration can influence directly or indirectly stomatal differentiation and development through influencing cell division and physiological metabolisms, thus resulting in variation of plant stomatal density (Zheng et al., 2001). Direct measurement of atmospheric CO2 shows a mean decrease of ca 55 ppm for the period 1956–2000 (Francely et al., 1999; McCarroll and Loader, 2004). Our results showed that the stomatal density of *H. rhamnoides sinensis* was reduced from 1976 to 2001 and a significant relationship between stomatal density and atmospheric CO2 concentration existed (Fig. 3c). Other researchers have similarly reported that stomatal density decreases along with the increase of atmospheric CO2 level (Woodward, 1987; Beierling and Chaloner, 1992; Woodward and Kelly, 1995; Zheng et al., 2001). However, Körner et al. (1988) investigated the relationship between stomatal density and atmospheric CO2 level and found the two were not consistent with each other. In addition, evidence for the response of the plant in the field to CO2 change is presented in the form of a significant change in stomatal density and water use efficiency (Woodward, 1987; Beierling and Chaloner, 1992; Woodward and Kelly, 1995; Zheng et al., 2001). Many experiments showed that plants maintained higher photosynthesis and lower transpiration in high CO2 level, which improved water-use efficiency (Beierling and Woodward, 1995; Leymarie et al., 1999). In the present paper, foliar δ13C value and thus water-use efficiency did not significantly change from 1976 to 2001 (Fig. 3). Eamus (1991) found the water-use efficiency of *Andropogon virginicus* and *Pinus taeda* did not change in high CO2 level. Li and Kang (2002) showed that the effect of enriched CO2 concentration on water use efficiency depended on the soil nitrogen level. Therefore, we suggest that these differences might be due to the self-trats of different plant species and other environmental factors (Apel, 1989; Ferris and Taylor, 1994), on the other hand, changes in stomatal density should be used to indicate the change of CO2 level.

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