Sexual dimorphism in ecological and physiological traits of the subdioecious dune plant *Honckenya peploides* (L.) Ehrh.

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INFORMA:

Que a presente memoria titulada **“Sexual dimorphism in ecological and physiological traits in the subdioecious dune plant *Honckenya peploides* (L) Ehrh”** presentada por **Dª. Julia Sánchez Vilas** para optar ó Grao de Doutor en Bioloxía, foi realizada baixo a miña dirección na Área de Ecoloxía do Departamento de Bioloxía Celular e Ecoloxía da Universidade de Santiago de Compostela.

E considerando que representa traballo de Tese de Doutoramento, autorizo a súa presentación ante o Tribunal correspondente.

E para que así conste, firmo a presente en Santiago de Compostela a 17 de Maio de 2007.

Visto e Prace do Director,	A Doutoranda,
Fdo. Dr. Rubén Retuerto Franco	Fdo. Dª Julia Sánchez Vilas

A meus pais

A Xerardo

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General Introduction

SEXUAL DIMORPHISM

Dioecy and related breeding systems

The plant kingdom contains a variety of reproductive systems, with hermaphroditism being the most common. Hermaphroditism is a condition where each individual has the ability to transmit genes through male (pollen) and female (seed) function. Yampolsky & Yampolsky (1922), using Engler and Prantl's classification system, estimated that ca. 72% of the world's angiosperms species were hermaphrodites. The rest were dioecious, or comprised mixtures of unisexual and bisexual individuals (*i.e.* gynodioecious, androdioecious, trioecious, or subdioecious). A more recent survey of 240 000 angiosperm species by Renner & Ricklefs (1995) found that 6% were dioecious. In part due to this relative rarity, there has been a lot of interest in dioecy and in the selective factors that may have led to its evolution. Dioecy is the most extreme of the sexual polymorphisms above mentioned, and it is characterized by well differentiated males and females that produce only pollen and seed, respectively. Although there is not a single universal answer to the question of dioecism, it is recognized to have evolved for two main reasons (Bawa 1980; Thomson & Brunet 1990). First, dioecy is a way to avoid auto-fecundation and the consequent inbreeding depression (Charlesworth & Charlesworth 1978). Second, the division of labour between sexes that occurs in dioecious species may eventually lead to greater reproductive efficiency (Lloyd 1982) via a more efficient use of resources.

One common evolutionary pathway from hermaphroditism to dioecy is via gynodioecy (Charlesworth & Charlesworth 1978; Ross 1982; Webb 1999; Delph & Wolf 2005). Gynodioecy is a breeding system in which separate female and hermaphrodite plants coexist. Within a gynodioecious species, females produce only ovules, while hermaphrodites produce both ovules and pollen (Sakai & Weller 1999). A decrease in female function in the hermaphrodites along the gynodioecy pathway can occur, which causes a breeding system that is not strictly dioecious, but is near the dioecy end of the gynodioecy-dioecy continuum (Delph & Wolf 2005). In this sense, subdioecy is considered a condition close to dioecy in the evolutionary pathway from hermaphroditism to dioecy. Subdioecious refers to populations that regularly contain imperfectly differentiated individuals (of either or both sexes) in addition to strictly unisexual individuals (Ross 1978). In subdioecious species has been mostly demonstrated that the imperfectly differentiated or inconstant morph is the male morph (Westergaard 1958; Charlesworth & Charlesworth 1978; Delph &Wolf 2005; Ehlers & Bataillon 2007), which is expected in a species evolving towards dioecy via the gynodioecy pathway (Lloyd, 1976; Charlesworth & Charlesworth, 1978). Apart of subdioecy a variety of terms have been used to describe this breeding system, including leaky dioecy, polygamodioecy or near dioecy. We used the term to describe a breeding system in which at least some pollen-producing morphs produce seeds, but female are constant in that they never produce pollen (Delph & Wolf 2005). In some studies, authors consider that this system is composed by three sexual morphs, making a differentiation between the pollen-producing morphs that produce seeds (named as hermaphrodites, inconstant males or fruiting males) and those that does not (named as males). This situation is also defined as trioecy, where the three sexual morphs have stable frequencies rather in a subdioecious state (Flemming *et al.* 1994). In other studies the distinction is less clear and only two sexual morphs are described: female and pollen-producing morph also called polleniferous morph, hermaphrodite or male. Resource-dependent gender plasticity is documented, with males being more prone to produce fruits in moist, resource-rich environments than in drier, less resource-rich ones (Olson & Antonovics 2000; Delph & Wolf 2005). This gender-plastic expression in males was interpreted to be advantageous as it lowered the cost of producing seeds in resource poor habitats, hence reducing the possible trade-off between male and female functions (Delph & Wolf 2005). In this thesis pollen-producing morphs are named as males what emphasizes the functional gender rather morphological gender of plants and follows the convention proposed by Lloyd (1974, 1976 and 1980).

Cost of reproduction

Dioecious plants provide an excellent opportunity to determine sex-specific costs of reproduction, exploiting the fact that the sex functions are housed in separate individuals. Female and male sex functions incur different costs. As general pattern, the investment in male flowers is greater than in female flowers, but total investment in female reproductive structures is greater because of cost of producing not only flowers but also fruits (Allen & Antos 1988). Thus, fruit-producing plants generally incur in greater reproductive investment than nonfruiting plants (reviewed in Obeso 2002). As consequence of greater reproductive investment, they are expected to pay greater cost of reproduction, manifested as lower survival, lower frequency of flowering, and/or slower vegetative growth. Evidences of such sexual dimorphism (in ecological and morphological traits) have been found in a wide variety of taxa and growth forms by several authors in dioecious and also in some subdioecious species (reviewed in Delph 1999; Obeso 2002), although such differences can be offset when females acquire more resource than males (Delph *et al.* 1993; Delph & Meagher 1995). It is also expected that sex differences in reproductive effort may increase physiological stress in the gender with the highest reproductive investment (Dawson & Ehleringer 1993), but data are still scarce to test this prediction at a general level. Available data have shown that differences in physiology may aid each sex in meeting different resource demands associated with reproduction. However, there is not a general trend in the patterns of sexual dimorphism in physiological traits and it is found to be variable both in degree and in direction within species from site to site and also between related species (for a review, see Dawson & Geber 1999).

There are several physiological mechanisms that may help to mitigate the costs of reproduction (for review, Obeso 2002; Case & Ashman 2005). For example, the photosynthetic capacity of reproductive structures is reported by several authors (e.g. Bazzaz *et al.* 1979; Jurik 1985; Galen *et al.* 1993; Hogan *et al.* 1998; McDowell *et al.* 2000). Although positive net photosynthesis is rare, since it is achieved only at early stages of development (Goldstein *et al.* 1991) or under certain environmental conditions (Cipollini & Levey 1991), flowers and fruits can contribute up to 60% to their own carbon maintenance. In addition to this supply of carbon to the plant's resource pool, developing fruits can also act as carbon sinks and increase the photosynthesis in nearby leaves (*e.g.* Wardlaw 1990; Marshall 1996). Increase of resource uptake or increase of resource use efficiency to respond to the greater nutrient demand may also help to alleviate the reproductive costs. However, examples of a reduction in photosynthetic capacity in leaves on fruiting branches are also reported (Obeso *et al.* 1998; Karlsson 1994), which has been attributed to the depletion of the leaf nitrogen content due to reproductive nitrogen demand. The costs of reproduction may be also reduced if some of the resources invested in reproduction can be recovered from senescing reproductive structures. Evidences of such nutrient reabsorption from senescent floral structures have found by several authors (Goldman & Willson 1986; Chapin 1989; Ashman 1994). Habitat niche partitioning or spatial segregation of the sexes, where sexes occupy those habitats where they can best meet specific demands associated with reproduction, is also a possible mechanism that may help to reduce the costs of reproduction (Freeman *et al.* 1976; Bierzychudek & Eckhart 1988; Dawson & Ehleringer 1993). Therefore, physiology is a crucial aspect and variation in physiological attributes may determine the performance of each sex in different environments, and consequently, the gender-differential allocation to growth, reproduction and defence (Retuerto *et al.* 2000). Although studies examining sexual dimorphism in physiological traits are increasing, they are heavily biased towards dioecious species (see

Case & Ashman 2005, for review). As sex functions lead to different costs, the cost of reproduction is expected to vary among the sexual morphs in relation to their relative investment in male versus female function. In this sense, "intermediate" stages of the evolutionary pathway from hermaphroditism to dioecy could provide important clues about when and how sex-specific physiological traits originate (Case & Ashman 2005).

Spatial segregation of the sexes

The spatial distribution of the sexes of dioecious plants has been explored by more than 30 yr (Freeman *et al*. 1976). Distribution of male and female plants follows a random pattern in some dioecious species (Bawa & Opler 1977; Melampy & Howe 1977; Hancock & Bringhurst 1980; Armstrong & Irvine 1989), whereas in others, sexes are spatially segregated (*i.e.*, males tend to be found around other males, and females around other females) (Freeman *et al*. 1976; Lovett Doust & Cavers 1982; Sakai & Oden 1983; Freeman & Vitale 1985; Dawson & Bliss 1989). A random distribution of sexes has been suggested to be optimal for distribution of pollen and dispersal of seeds (Bawa & Opler 1977), because facilitates that pollen reaches female flowers and reduces mass predation on concentrated seeds. Spatial segregation of the sexes, SSS, (Bierzychudek & Eckhart 1988) is a surprising phenomenon because the separation of the sexes is expected to reduce the sexual reproductive success of individuals. Nevertheless, SSS occurs in more than 30 plant species from 20 families (reviewed by Bierzychudek & Eckhart 1988; Iglesias & Bell 1989; Korpelainen 1991; Shea *et al*. 1993; Lokker *et al*. 1994). It has been suggested that spatial segregation of the sexes might optimize the use of resources in space and time, increasing the fitness of males and females in comparison with cosexuals (Freeman *et al*. 1976; Lloyd 1982; Lovett Doust & Lovett Doust 1988; Pannell & Barrett 1998; Charlesworth 1999). Spatial segregation of males and females is usually found along environmental gradients (Freeman *et al*. 1976; Grant & Mitton 1979; Freeman *et al*. 1980;

Bierzychudek & Eckhart 1988; Sakai & Weller 1991; Dawson & Ehleringer 1993). The general pattern is females occupying preferentially resource rich-habitats, which might alleviate, at least in part, their higher reproductive cost (Freeman *et al.* 1976; Lloyd & Webb 1977; Cox 1981). Several mechanism have been proposed to explain a non-random distribution of the sexes including differences between the sexes in germination requirements (Bierzychudek & Eckhart 1988; Purrington 1993; Lyons *et al.* 1995), seed differential mortality in stressful habitats (Cox 1981; Lloyd & Webb 1977; Krischik & Denno 1990), competitive abilities (Freeman *et al.* 1976; Meagher 1980; Cox 1981; Ågren 1988), and flowering phenology (Conn 1981; Conn & Blum 1981; Purrington 1993). Regardless what initially generates patterns of spatial segregation of the sexes, it appears that gender specialization may help to maintain them. In particular, gender-specific physiological traits can help to explain the spatial segregation of male and female trees of *Acer negundo* is explained by Dawson & Ehleringer (1993). These authors pointed that such specialization might confer growth and perhaps even fitness benefits for each gender within a particular habitat type and may be the reason why spatial segregation of the sexes in dioecious plants is so commonly observed.

COASTAL DUNE ENVIRONMENTS

Coastal dune environments are very dynamics systems that are continually disturbed and reworked by tides and the wind, and that display several abiotic stresses that affect establishment, growth, survival and consequently the distribution of plants. One of the well known critical factors is salt spray, which has often been suggested as a primary factor determining the distribution or the growth form of plants growing in coastal areas (Wilson & Sykes 1999; Griffiths & Orians 2003). A proportion of salt spray is intercepted by plants, but much of it is also taken into the sand surface what contributes to increase the soil salinity. Differences between species in levels of tolerance to salt spray may result in zonation of vegetation, so that the most tolerant plants grow closer to the coastline, and are progressively replaced by less tolerant plants towards the interior (Oosting & Billings 1942; Oosting 1945; van der Valk 1974; Barbour 1978; Parsons 1981; Yura 1997). Other primary stress in the coastal dune ecosystem is the lack or limited availability of water and nutrients, which is explained by the poor water-holding capacity of sandy substrates that leads to rapid percolation of water precipitation and nutrient leaching. Low soil moisture coupled with the high temperatures achieved in these systems creates rather inhospitable conditions for plant's growth.

Sand movement, that includes loss of substrate by erosion and burial by sand accretion, is characteristic of sandy dunes. Erosion of sand from the bases of plants exposes the root systems to desiccation and therefore killing them (Maun 1981). Sand accretion alters the normal microenvironment of the plants, increasing moisture, nutrients but decreasing soil temperature, aeration and light intensity (Maun 1994). Only adapted species to live under burial conditions are able to survive in such conditions. Several authors concluded that the difference in the tolerance to sand movement determines the distribution of plant species in the area where accumulation or erosion of the substrate frequently occurs (van der Valk 1974; Avis & Lubke 1985; Moreno-

Casasola 1986). Sand particles transported by winds are also an important source of damage to the plants due to leaf abrasion.

Salt spray, nutrient deficiency, lack of moisture, sand movement, high temperatures are stressful factors that impose important selective pressures that shape the characteristic plant communities of coastal areas (Wilson & Sykes 1999; Griffiths & Orians 2003). Vegetation on coastal dune systems is composed of various species which are different from those distributing in inland areas of the same region and display a wide variety of adaptations and responses to cope with critical abiotic factors that allow them to live in these environments (Hesp 1991). For example, coastal plants may present some strategies as flexible patterns of biomass allocation to roots in response to water and nutrient deficiencies, morphological adaptations to reduce evaporation (*e.g.* leaf rolling, leaf hairiness), increases in water-use and nitrogen-use efficiency, redistribution of nutrients from vegetative to reproductive organs and succulence in response to salt spray or substrate salinity (see Hesp 1991, and references therein). As the level of abiotic stresses decrease from the upper beach to the stabilized dunes (Hundt 1985; Imbert & Houle 2000), abiotic conditions become less restrictive to plant growth with distance from the coast. In this sense, upper beach and embryo dunes are the most physically stressful places for plants, and plant adaptations and responses to these factors are particularly expected in these zones. The different tolerance and plasticity of the plants to stressful factors influence their distribution and dominance along the sequence upper beach-stabilised dunes (Hesp 1991).

Honckenya peploides

Honckenya peploides (Linnaeus) Ehrhart, sea sandwort, is a hemicryptophyte, succulent, yellow-green littoral plant in the family Caryophyllaceae. It is usually found at the upper beach, where it re-grows each spring from long rhizomes that produce compact groups of aerial shoots, forming vegetative clumps or mats. These clumps origin small mounds called embryo dunes.

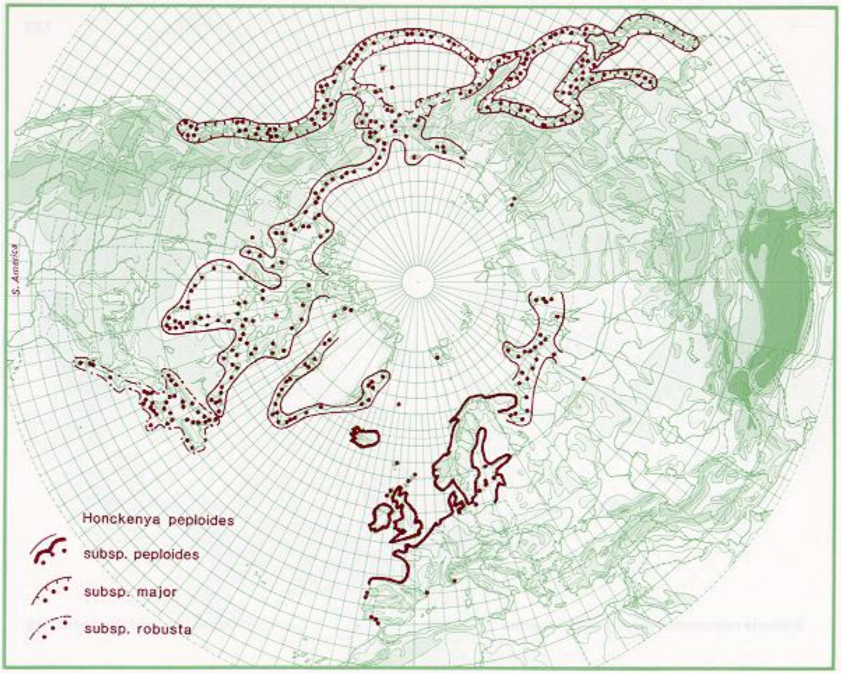

Fig. 1 Global distribution of *Honckenya peploides* (Hultén 1971). According to Hultén subsp. *diffusa* is only a variety of subsp. *peploides*. Subsp. *diffusa* occurs in the Northern parts of Norway, Svalbard, Iceland, Greenland (Kurtto 2001) and in subarctic Canada (Houle 1996).

Four subspecies are currently accepted (Kurtto 2001): subsp. *peploides* found on the coasts of Europe, from Northern (non-artic) Norway to Northern Portugal, subsp. *diffusa* (Hornem.) Hultén, with circumpolar distribution, mainly in arctic and Northern Boreal zones; subsp. *major* (Hook.) Hultén in

North Pacific area (reaching arctic West Alaska); and subsp. *robusta* (Fernald) Hultén in Northeast America. The species has a circumpolar distribution, from temperate to artic zones. It is also found in South America (anthropochorous origin), occurring in a small area in Chile and Argentina (Hultén 1971). In the Iberian Peninsula occurs the subsp. *peploides*, which grows in Cantabrian and in Atlantic coast at north Lisbon (Portugal). Figure 1.

Leaves arrangement (opposite), morphology (thick, from ovate to elliptic) and orientation (angle of 90º with stem) help to reduce incident radiation. Flowers are axillary, solitary and/or flowered terminal cymes. Small flowers are actinomorphic, composed by 5 sepals, 5 white petals, 10 stamens and ovary with 3 (-5) styles. Stamens have a large nectary at the base, and exude nectar during flowering period. Flowers are strongly honey-scent, which attract insects (Tsukui & Sugawara 1992). Two types of flowers can be found in *H. peploides* such as it has been reported in ssp. *major* by Tsukui & Sugawara (1992). One type has long styles, short petals and non-functional anthers and it is denoted as pistillate flower. While the other has short styles, long petals and long stamens that produce pollen grains and is denoted as staminate. Individuals with pistillate flowers are denoted as females and those with staminate flowers as males in consonance with Tsukui & Sugawara (1992) and following arguments of Lloyd (1976) and Delph (1990). Females never produce pollen and are constant in its expression. Males (pollen-producing morphs) rarely produce seeds and when they do it, the number of seeds is very low compared to female flowers. This complex and uncommon breeding system is described as subdioecy, being close to the dioecy in the evolutionary pathway from hermaphroditism to dioecy via gynodioecy (see Delph & Wolf 2005, for a review). Male seeds develop into female and male plants in the approximate ratio 1:3. Seeds of female flowers produce about as many males as female plants. This implies that the sex determination system is: female = XX, male = XY or YY (Malling 1957). Plants reproduce sexually by seeds, which are spread by sea currents or clonally by rhizomes. *H.*

peploides is an early colonizer, contributing to stabilisation and anchorage of the soil and facilitating the establishment of the other species (Houle 1997; Gagné & Houle 2001).

Fig. 2 Flowers of female (left) and male (right) *Honckenya peploides* photographed in natural populations in Galicia, Northwest Spain.

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OBJECTIVES AND OUTLINE OF THIS THESIS

The aim of this thesis was to examine the extent of sexual dimorphism in ecological, morphological and physiological traits of the sexes in the dune plant *Honckenya peploides*. In Northwest Spain (Galicia), sexes of this species can be found spatially segregated, which has prompted us to ask about the causes of this spatial segregation. In order to address this aim field data and samples for laboratory analysis were collected and glasshouse manipulative experiments were designed:

In **chapter 1** the genetic structure and genetic variability of unisexual clumps of *H. peploides* were analysed using two kinds of molecular techniques: isozyme analysis and AFLP (amplified fragment-length polymorphism).

Chapter 2 investigates the differences in ecophysiological traits between the sexes of *H. peploides* growing in natural conditions and whether possible sex differences depended on reproductive status and plant's position (edge or centre) in the clump. Aspects of population structure and differences in the reproductive investment between the two sexes were also evaluated.

Chapter 3 explores the seasonal patterns of biomass allocation of the sexes of *H. peploides* growing in natural conditions. Habitat quality of male and female plants was also investigated.

Chapter 4 examines if the sexes of *H. peploides* have physiological differences in terms of integrated water-used efficiency, $\Delta^{13}C$, at three moments over the season. Seasonal variation in leaf nitrogen content was also estimated.

In chapters 5 and 6, the physiological responses of the sexes to different stressful factors were studied under controlled conditions. In particular, **chapter 5**, studies the differential performance of the sexes (in terms of physiology and growth) under different conditions of water availability. **Chapter 6** investigates if sexes of *H. peploides* differ in their physiological responses to nutrients, salt spray and their interactions. It also explores whether nutrient availability and salt spray differentially affect phenological, reproductive and growth parameters in the sexes, and consequently plant fitness.

Photographs showing female (above) and male (below) clumps of *H. peploides* at the locality of Lariño in Galicia (Northwest Spain).

Chapter 1

1

Genetic variation in large unisexual clumps of *Honckenya peploides* (L.) Ehrh.

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ABSTRACT

In dioecious plant species with clonal growth, spatial segregation of the sexes may be measured at ramet and genet level. At ramet level, different vegetative propagation is an important factor affecting sex-ratio. *Honckenya peploides* is a subdioecious dune plant, which reproduces both sexually and by clonal growth. This species exhibits at the localities of this study an extreme spatial segregation of the sexes and our objective was investigate the genetic variation in unisexual clumps (three of them composed exclusively by males and three exclusively by females). In total, 193 samples were analysed using isozyme analysis and 80 samples were analysing using two AFLP primer combination. Both techniques revealed high genetic diversity (average values for proportion of distinguishable genotypes: 0.25 for isozymes and 0.41 for AFLP; average values for Simpson's D: 0.65 for isozymes and 0.68 for AFLP). Our results show that each unisexual clump consists of different genotypes, finding a high proportion of the genetic variation within clumps (according AMOVA analysis). We did not find differences between the clonal diversity of the sexual morphs. The causes for the spatial segregation point to different ecophysiological responses of the sexes.

Keywords: *Honckenya peploides*, AFLP, isozymes, subdioecy, spatial segregation.

INTRODUCTION

Clonal growth is widely occurring in plants and about the 65.5% of the central European plant species studied can be described as clonal (Klimes *et al.* 1997). Two levels of organization can be distinguished in clonal plants, genets and ramets. A genet is originating from one zygote, whereas a ramet is a potentially independent part of a genet (Richards 1986; Eriksson 1993). Different ramets within one genet are therefore genetically identical. For clonal species that also produce seeds, frequency of asexual propagation relative to sexual reproduction can affect genetic diversity. It was assumed that genetic diversity was lower for clonal than for non clonal plants (Harper 1977). More recently, however, with the development of new molecular approaches, this assumption has been challenged by studies reporting high degree of genetic diversity in plants with vegetative propagation (Ellstrand & Roose 1987; Parker & Hamrick 1992; Widén *et al.* 1994; Stehlik & Holderegger 2000). The molecular genetic methods also allow reliable, accurate and reproducible clone identification and hereby provide the opportunity to obtain an improved understanding of the population ecology of clonal plants.

In dioecious plants with clonal growth, spatial segregation of the sexes may be measured at ramet and genet level. Different rate of vegetative propagation in males and females has been postulated as one of the factors affecting ramet sex-ratio. Usually males have a higher expansion rate than females (Lovett Doust & Lovett Doust 1988; Popp & Reinartz 1988; Escarre & Houssard 1991; Korpelainen 1992). The lower female ramet production has been attributed to higher sexual reproduction costs. However, sometimes the capacity of expansion of females is higher than in males (Sakai & Burris 1985; Williams 1995). In order to investigate the sex-ratio at genet level molecular genetic methods are an indispensable tool.

Our aim in this study was *Honckenya peploides* (L.) *Ehrh.* (Caryophyllaceae) which has the capacity to reproduce both sexually and asexually by an effective rhizomatous system. The sexual system includes male-sterile morphs that completely lack the ability to produce pollen ("females"), and polleniferous morphs that retain a slight bisexual capability but achieve most of their fitness through pollen ("males"). This system is known as subdioecy, occupying an intermediate position in the evolution from gynodioecy to dioecy (Sakai & Weller 1999; Delph & Wolf 2005) and is supposed to be a very flexible reproductive system (Delph & Wolf 2005). In our three localities of the coast of Galicia (Northwest of Spain) (Lariño, O Bao and San Román), *H. peploides* exhibits a fascinating spatial segregation of the sexes. It is found in clumps composed by ramets of one sex separated by tens or hundreds of meters from clumps composed by ramets of the other sex. The observation of such pronounced spatial segregation has prompted us to ask whether each clump is the result of clonal propagation of only one or a few founder genets. Clonal growth is known to affect the genetic diversity of populations, and we hypothesized that the genetic diversity in clumps of this clonal plant is low due to extensive clonal growth. We also hypothesized that the genetic diversity may be higher in female clumps than in males, due to recruitment of new genets by seeds. To investigate the genetic variation we contrasted two kinds of molecular techniques: isozyme analysis and AFLP (amplified fragment-length polymorphism). This last technique potentially gives a much higher number of markers than do isozyme analysis and it is now a well established method for detecting genetic diversity (Mueller & Wolfenbarger 1999).

MATERIAL AND METHODS

Study species

Sea sandwort, *Honckenya peploides*, is a subdioecious perennial plant with a circumpolar distribution (from temperate to arctic zones; anthropochorous in South America). On the Iberian Peninsula, *H. peploides* extends from the Atlantic coast of northern Portugal and northwards and eastwards along the Bay of Biscay (Cantabrian Sea) to France. It is a hemicryptophyte regrowing each spring from buds on long rhizomes that produce compact groups of aerial shoots, forming vegetative clumps or mats. These clumps are typically found on the upper beach, forming small mounds called embryo dunes. *H. peploides* also shows sexual reproduction. Flowers are axillary and solitary, and/or in 1- to 6-flowered terminal cymes, strongly honey-scented. Two types of flower can be found in *H. peploides*, as reported for the subspecies *major* by Tsukui & Sugawara (1992): one type ("pistillate") has long styles, short petals and non-functional anthers, while the other ("staminate") has short styles, long petals and long stamens that produce pollen grains. This latter type rarely produces seeds, and when it does the number of seeds is very low compared to female flowers. Both types of flower have nectaries at the base of the stamens, which attract pollinators. In line with Tsukui & Sugawara (1992), and following Lloyd (1976) and Delph (1990), we will here refer to plants with pistillate flowers as females and to plants with staminate flowers as males.

Sampling

H. peploides samples were collected in August 2005 in three localities of the coast of Galicia: O Bao (42º32'N, 8º51'W), (Lariño: 42º45'N, 9º6'W,) and San Román (43º43'N, 7º37'W). In each locality (Fig. 1), we sampled two spatially separated clumps, one composed by females and the other by males.

Within each clump a grid was laid out and samples were taken at each point of intersection. The sampling points for electrophoresis are showed in Fig. 2. For AFLP, we randomly selected some of these points to collect the samples. The grid size was dependent on clump size, in such manner that in the biggest clumps we increased the minimal distance between grid points. In O Bao we collected 26 male and 28 female samples for electrophoresis and 14 male and 11 female for AFLP, in Lariño 32 male and 38 female samples for electrophoresis and 11 male and 15 female samples for AFLP and in San Roman 45 male and 24 female samples for electrophoresis and 18 male and 11 female samples for AFLP. The samples for electrophoresis were kept in closed zip-lock bags and were transport with block-ices to University of Copenhagen, where they were stored in a refrigerator until grinding. Samples for AFLP were immediately preserved in silica gel to prevent DNA degradation after collecting.

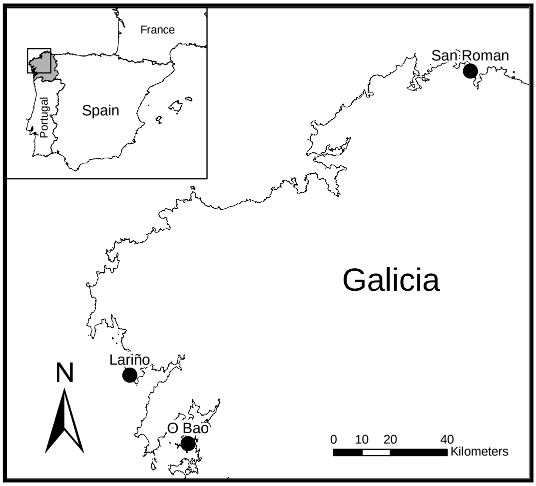

Fig. 1 Location of Galicia in Northwest Spain and location of the sampling sites.

Electrophoresis

Fresh green leaves, preferably young apical leaves were homogenised in a grinding buffer (Soltis *et al.* 1983) within two weeks after sampling. The extracts were absorbed on filter paper wicks and stored at - 80º C until analysis. The wicks were loaded on a 12% starch gel (Reppin PSG 1000 and StarchArt (1:3)). An initial screening on 12 isozyme systems was performed. Interpretable bands were obtained for four systems. Two buffer systems were used: Tris-citrate (pH 7.0) (Meizel & Markert 1967) and Poulik-Torres (pH 7.8) (Torres & Bergh 1978). On Tris-citrate buffer the following systems were examined: UTP-glucose-1-phosphate Uridylyltransferase (UGPP; E.C. 2.7.7.9), Isocitrate dehydrogenase (IDH; E.C. 1.1.1.42) and Phosphogluconate dehydrogenase (PGD; E.C. 1.1.1.44). On Poulik-Torres buffer, phosphoglucoisomerase (PGI; E.C. 5.3.1.9) was examined. Staining recipes were taken from Wendel & Weeden (1989) except for UGPP which was resolved following the procedure from Harris & Hopkinson (1976).

Gels were run in a refrigerator with ice trays on top of them to avoid a possible temperature gradient inside the gels during the running (Wendel & Weeden 1989). Gels were run for 4 hours at 60 mA for Tris-citrate and 75 mA for Poulik Torres.

Amplified Fragment Length Polymorphism (AFLP)

We crushed 15 mg silica gel dried leaf material in a mill and DNA was isolated with a Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) following supplied instructions with minor modifications. Quality of the extracted DNA was estimated by measuring the 260nm and 280nm UV absorbance and the integrity was verified by electrophoresis on a 0.7% agarose gel. For AFLP we used 0.250 µg of DNA per sample. The AFLP reactions were performed using the PE Applied Biosystems AFLP^{TM} plant mapping kit for average sized genomes. The method is based on Vos *et al.* (1995) but uses primers that are labelled with non-radioactive fluorescent dyes. DNA was digested with the restriction enzymes EcoRI and MseI to generate the template DNA fragments, and then the respective adapters (EcoRI and MseI) were ligated onto the ends of the restriction sites creating primer-binding sites. A preselective amplification was performed with PCR primers with a single nucleotide included at the 3’ end, which implies the amplification of only a subset of the restriction fragments. To verify the amplification, we ran 1.5% agarose gels. For the second amplification (the selective one) an initial screening using 25 selective primer combinations (composed of EcoRI and MseI-based primers with 3 selective bases) was performed on individuals of both sexes across all localities sampled. From that analysis, the two primer combinations MseI-CTA/EcoRI-ACT Fam and MseI-CTC/EcoRI-AGG Joe appeared to be sufficiently polymorphic to discriminate clones within populations. PCR amplifications were done in a thermal cycler (PTC-200; Peltier Thermal Cycler). The samples were denatured at 95ºC for 2 min before electrophoresis and then, they were loaded on a preheated 5% denaturing acrylamide gels on a ABI 377 DNA automatic sequencer together with a size standard (Genescan-500 ROX, Applied Biosystems). Fragment sizes were automatically calculated by GeneScan analysis software 3.1.2 (PE Applied Biosystems). Data were imported to the analysing software Genotyper 2.1 (PE Applied Biosystems) and a preliminary matrix with 1 corresponding to the presence and 0 to the absence of a band at each locus was automatically produced. This preliminary matrix was checked manually and only clear bands were recorded.

Data analysis

Electrophoresis

H. peploides is a polyploidy plant, so genetic interpretation may be difficult due to dosage differences. For this reason, the number of multilocus phenotypes (MLP) was determined from the registered presence or absence of bands for each clump in each locality. Samples of the same clump (same sex) and identical MLP were grouped as one unique genet (genet level). All samples were included in the calculations at the ramet level. To describe the genotypic diversity different methods were used. The proportion of distinguishable MLP, PD, was calculated dividing the number of MLP by the sample number (N) (Ellstrand & Roose 1987):

$$PD = \frac{MLP}{N}$$

PD approach 0 if all individuals in a sample have the same MLP and 1 if each individual has a unique MLP. Simpson's index (D) of diversity corrected for finite sample size (Pielou 1969) was calculated as:

$$D = 1 - \sum_{i=1}^{k} \frac{n_i(n_i - 1)}{N(N-1)},$$

where n_i is the number of individuals with the *i'th* MLP, and N is the number of individuals sampled. Simpson's index of diversity is bounded by 0 and D_{max} ($D_{max}=[N(G-1)]/[G(N-1)]$), where G is the number of unique MLP. This index gives the probability that two individuals randomly selected from a population have different MLP (Montalvo *et al*. 1997). D ranges from 0, in a population composed of a single clone to D_{max}, in a population where each sample has a unique MLP. The distribution of MLP was assessed using Fager's evenness index (Fager 1972):

$$E = \frac{D - D_{\min}}{D_{\max} - D_{\min}},$$

where $D_{\min} = [(G-1)(2N-G)]/[N(N-1)]$. The values of E ranges from 0 to 1, where 0 correspond to a sample with a skewed distribution of multilocus phenotypes, and 1 correspond to a sample with evenly distributed multilocus phenotypes (all MLPs are replicated the same number of times in a population).

AFLP

To test the reproducibility of the AFLPs, the fingerprints obtained with two independent DNA extractions from 12 plants were compared. We used CLONES (function developed by Ehrich 2006) within R 2.4.0 (Anon 2004) to know the number of different genotypes. This program uses the error rate of AFLP procedure to determine the maximum number of differences allowed among individuals to still be "ramets" with the "same" multilocus genotype. CLONES also estimates genotype diversity according to Nei's formula (1987): $D = n/(n-1)*[1-\mathrm{sum}(\text{genotype frequencies}^2)]$ and the effective number of genotypes according to Parker (1979) as Effective nb = 1 / sum (genotype frequencies^2). Two analyses of molecular variance (AMOVA; Excoffier *et al.* 1992) were applied to partition variance between and within sex and among and within localities using ARLEQUIN (Schneider *et al.* 2000). We arranged the data in two groups to test the effects of different sex and conducted a first AMOVA, and in three groups to test the effects of different locality and conducted a second AMOVA. Only genet data were used to test for differentiation. For each analysis, 10000 permutations were performed to obtain significance levels. AMOVA analyses were based on the pairwise squared Euclidean distances.

The relation of clonal diversity between the two molecular techniques used was calculated as Spearman's Rho (r_s) correlation.

RESULTS

Electrophoresis

PD values, number of clones per sample, ranged from 0.11 to 0.42 in females and from 0.13 to 0.42 in males (Table 1). The values of Simpson's index corrected for finite sample size, D, ranged from 0.56 to 0.87 in females and from 0.25 to 0.88 in males (Table 1). The values of genotypic evenness, E, ranged from 0.61 to 0.79 in females and from 0.06 to 0.78 in males. These genetic diversity indicators did not differ significantly between sexes (Mann-Whitney *U* test P=0.658 for PD; P=0.827 for D; P=0.275 for D_{max} and P=0.827 for E) or localities either (Kruskal Wallis's test, P=0.620 for PD, P=0.651 for D and P=0.651 for D_{max} and P=1 for E).

The minimal distance between two different genets was 50 cm at O Bao in both clumps (male and female), 1 meter at Lariño and in females of San Roman, and 2 meters in males at San Roman. The spatial distribution of the different genets detected by electrophoresis is shown in Fig. 2.

Table 1 Summary of genotypic diversity indices estimated by isozyme analysis at clump level for male and female plants of *Honckenya peploides* in three localities of Galicia (NW Spain). N = number of samples; MLP = number of multilocus phenotypes; PD = the proportion of distinguishable MLP (MLP/N); D = Simpson´s index of diversity; Dmax = maximum value for D; E = Fager´s eveness index.

Sex	Locality	N	MLP	PD	D	Dmax	E
Female	O Bao	28	5	0.18	0.61	0.83	0.61
Female	Lariño	38	4	0.11	0.56	0.77	0.66
Female	San Román	24	10	0.42	0.87	0.94	0.79
Male	O Bao	26	11	0.42	0.88	0.95	0.78
Male	Lariño	32	7	0.22	0.71	0.88	0.67
Male	San Román	45	6	0.13	0.25	0.85	0.06
Average		32.17	7.17	0.25	0.65	0.87	0.59

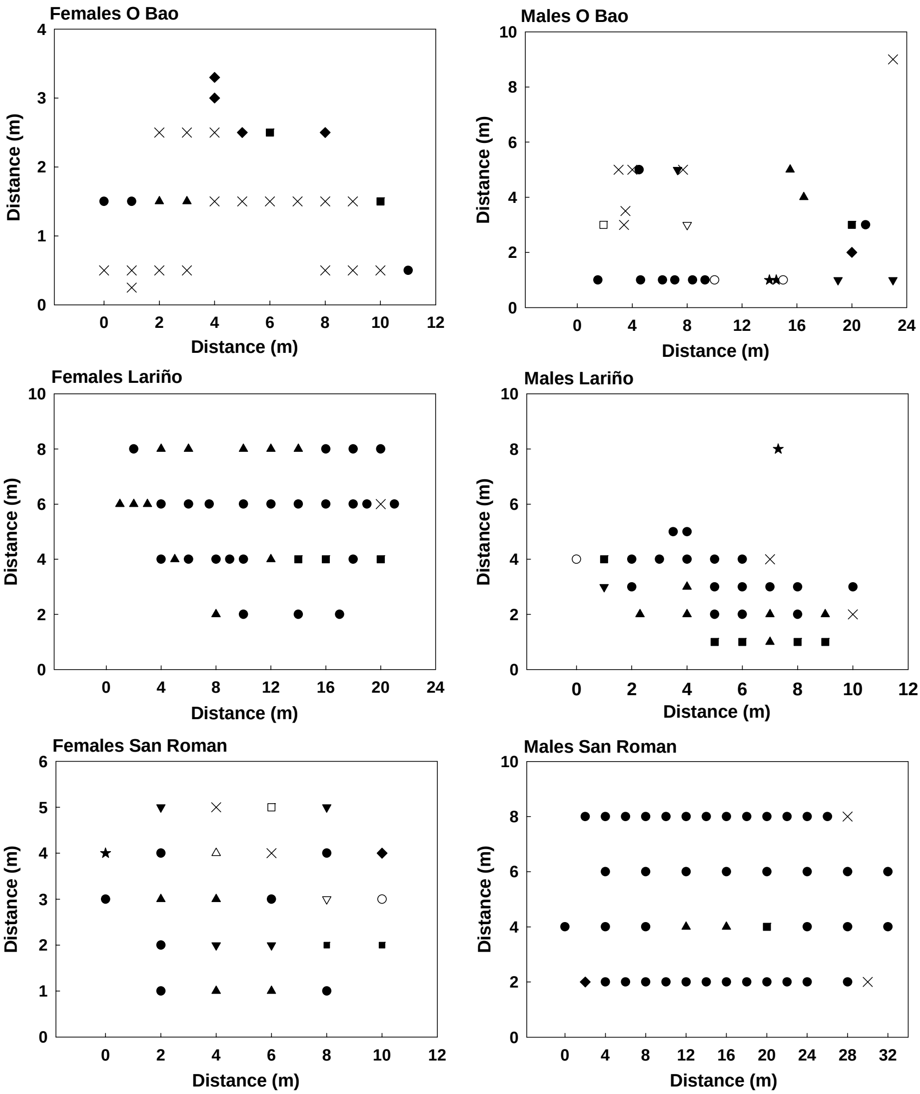

Fig. 2 Sampling points at female and male patches of *Honckenya peploides*. Different genets detected by electrophoresis are represented by different symbols.

AFLP

From the two AFLP primer combinations 51 polymorphic markers were generated from a total of 127 bands recorded which represents a 40.2% of polymorphism. Analysis of two different DNA extractions from 12 plants indicated that 94.2% of the markers were reproducible. As consequence a polymorphism exceeding 5.8% was taken as threshold value for distinguishing the genets. A total of 32 genets were detected among 80 samples studied. All clumps studied consisted of several genets, although the proportion of genets was different in each of them (Table 2). Nei's (1987) genetic diversity corrected for sample size (or Simpson's diversity index) values for all clumps studied ranged from 0.89 for males from O Bao to 0.31 for males from San Román. Mean value for all clumps was 0.68. These genotype diversity indicators did not vary significantly among localities (Kruskal-Wallis' tests, $P = 0.867$ for G/N, D and effective nb) neither between sexes (Mann-Whitney U test, $P = 0.827$ for G/N, D and effective nb).

Arranging data by sex, the hierarchical AMOVA partitioned 89.50% of the genetic variation between genets and 12.79% between clumps. No variation was attributed to sex. When data where arranged by locality into three groups (O Bao, Lariño and San Román), AMOVA partitioned 88% of the genetic variation between genets, 8.33% among clumps and 3.56% among localities (Table 3).

We observed a significant correlation between clonal diversity (D) calculated using AFLP technique and using electrophoresis ($r_s = 0.943$, $P = 0.005$).

Table 2 Summary of genotypic diversity as estimated by AFLP procedure in male and female clumps of *Honckenya peploides* at three localities of Galicia (NW Spain). N = number of samples, G = number of unique genotypes indentified, G/N = proportion of distinguishable genotypes, D = clonal diversity and Effective number of genotypes.

Sex	Locality	N	G	G/N	D	Effective nb genotypes
Female	O Bao	11	3	0.27	0.47	1.75
Female	Lariño	15	6	0.40	0.77	3.57
Female	San Román	11	6	0.54	0.85	4.48
Male	O Bao	14	8	0.57	0.89	5.76
Male	Lariño	11	5	0.45	0.78	3.46
Male	San Román	18	4	0.22	0.31	1.42
Average		13.3	5.3	0.41	0.68	3.41

Table 3 Summary of analysis of molecular variance. The analyses were based on AFLP phenotypes. (SS: sum of squares, Va: variance components, %: proportion of genetic variability, *P*: level of significance).

Source of variation	d.f.	SS	Va	%	*P*
With sex grouping					
Between sexes	1	8.82	-0.16	-2.29	0.693
Among clumps within sex	4	43.07	0.89	12.79	<0.001
Within clumps	26	161.86	6.22	89.50	<0.001
Total	31	213.75	6.96		
With localities grouping					
Among localities	2	24.61	0.25	3.56	0.326
Among clumps within localities	3	27.28	0.59	8.33	0.004
Within clumps	26	161.86	6.22	88.11	<0.001
Total	31	213.75	7.06		

DISCUSSION

Within each of the six unisexual clumps a high number of genets were identified by both techniques. Our results revealed that the two marker systems tested, isozymes and AFLP, were useful procedures for obtaining information on levels of genetic diversity in *Honckenya peploides*. Allozyme variation showed to be an efficient method to distinguish genets, giving similar values for diversity indices as AFLP. Mean values of diversity indices found with AFLP were, however, slightly higher than those found with isozymes even though we included an error rate of 5.8. The reproducibility of AFLP markers found in this study (94.2%) falls in the range reported by Lindqvist-Kreuze *et al.* (2003) who also performed two different extractions of DNA to test reproducibility, and found that 93.5% of the markers were reproducible. Winfield *et al.* (1998) found a similarity between 96% and 100% assessed on duplicate samples. Values of 98% of reproducibility were found by Arens *et al.* (1998), after checked six independent DNA isolations and by Kjølner *et al.* (2004), after having checked three samples with different initial DNA concentration.

In this study, *H. peploides* showed high clonal diversity (mean value PD = 0.25 and Simpson's D = 0.65 for electrophoresis, and G/N = 0.41 and Simpson's D = 0.68 for AFLP). Mean values obtained with both techniques were higher than average values reported by Ellstrand & Roose (1987) for 21 clonal plant species (G/N = 0.17 and Simpson's D = 0.62). Despite clonal growth within unisexual clumps, both molecular methods revealed that all studied clumps contained more than one genet. Examples of genetically polymorphic unisexual populations, or with highly biased sex-ratios, have also been reported by other authors (Eppley *et al.* 1998; Rottenberg *et al.* 1999, 2000; Torimaru & Tomaru 2005).

A plausible explanation for clonal diversity found in the present study, is the recruitment of genets from seeds. Seed production was observed in *H. peploides*, not only in females but also in males, since these can produce seeds although in less number than those produced by females. This input of genets due to sexual reproduction does not seem to be the unique factor modulating the clonal diversity. Other factors, as for example genet competition or differential vegetative growth may account for differences in clonal diversity found among clumps. Further, the genetic diversity found within clumps, may be explained by the existence of somatic mutations accumulated for a very long time (Tuskan *et al*. 1996). In this way, unisexual clumps will be composed by genetically different ramets originating from a single founder through vegetative propagation. Since, however, seedling recruitment has been observed in these populations of *H. peploides*, establishment of different genotypes through seeds is the more convincing hypothesis to explain the clonal diversity found. In addition, according to the AMOVA analysis, a high proportion of the genetic variation was observed within clumps and only a low proportion of the genetic variation was distributed between clumps. The high variability among individuals is associated with outcrossing, and the lower genetic diversity across clumps could have arisen by gene flow. These results imply that sexual reproduction plays an important role within clump.

We did not find differences between the clonal diversity of the sexual morphs. This is in contrast to Torimaru & Tomaru (2005) who found a higher clonal diversity in female patches compared to male patches. Our result could, at least in part, be due to the capacity of males to produce seeds. Although we have found that seed set of *H. peploides* males may be nine times lower than the seed set of females (data unpublished), it has been postulated, that even a low rate of seedling recruitment is high enough to maintain or even increase local genetic variation (Soane & Watkinson 1979).

The causes for unisexuality of the clumps which are composed by several different genotypes are not known. Malling (1957) reported that seeds from males in a wild population of *Honckenya peploides* gave males in a 3:1 ratio, suggesting a male heterogamety. Seeds from females gave 44.4%

females (N = 178). These results influence the distribution of the sexual morphs but do not explain the unisexuality. Gender-specific responses to ecological factors may be involved in skewed sex ratios. In general, it is assumed that females prefer high quality habitats to meet the specific resource demands associated with their higher reproductive effort (Freeman *et al*. 1980; Lloyd & Bawa 1984). So, the spatial segregation found in *H. peploides* may be related to different habitat preferences of males and females. At the localities of this study we did not observe any obvious differences among habitats of the two sex morphs. We can, on the other hand, not exclude the existence of such differences. In addition, different vegetative growth rate of sexes may imply the colonization of an area by the sexual morph with the greater vegetative propagation. In line with this, different competitive abilities may displace one gender, favouring the establishment of the other. Also, different mortality rates between genders have been proposed as cause of skewed sex ratios (Lloyd & Webb 1977; Lovett Doust *et al*. 1987). However, all these explanations for differential responses of the sexes as cause of the spatial segregation need to be supported by field and greenhouse experiments. In a parallel study we found under greenhouse-conditions, sex-specific differences in photochemical efficiency and proportion of total biomass invested in leaves in *Honckenya peploides.* Differences in morphological attributes at leaf level, as degree of succulence and stomatal density were also found, suggesting sex-specific strategies to cope with water availability (Sánchez-Vilas *et al*., unpublished). These differences may explain habitat-related between-sex differences in performance, and therefore the spatial segregation of the sexes.

In conclusion we found high genetic variation within clumps of *Honckenya peploides*. Clones were found to vary in size from 50 cm to several meters. The clumps were unisexual despite establishment from seeds occur and despite consisting of different genotypes. Several reasons for the unisexuality are discussed and most of the available evidences indicate that ecophysiological differences between sexes are responsible for the fact that clumps are composed by different genotypes but nevertheless unisexual.

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Chapter 2

2

Reproductive status and plant position in the clump influence ecophysiological responses of the subdioecious *Honckenya peploides* (L.) Ehrh.

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ABSTRACT

As a consequence of the different reproductive functions performed by the sexes, sexually dimorphic/polymorphic plants may exhibit gender-related variations in the energy and resources allocated to reproduction, and in the physiological processes that underlie these differences. This study investigated whether the sexes of *Honckenya peploides* (L.) Ehrh. differ in ecophysiological traits and whether possible sex differences depend on reproductive status and on the plant's position (edge or centre) in the population. In three sites in NW Spain we registered, in two segregated clumps of plants, the sex and density of individuals, number and dry mass of flowers and fruits. In two sites we measured photosynthetic efficiencies, chlorophyll contents, and specific leaf areas. We found extreme cases of sex-ratio variation, with some clumps composed exclusively of male-sterile morphs (females) and others of polleniferous morphs (males). Sex-differences in reproductive costs depended on time in season. The results show sex-specific effects of reproduction on SLA and that the reproductive status of a shoot, its position within the clump and site conditions affect its photosynthetic efficiency and chlorophyll content. These findings cast doubt on our capacity to generalize about gender responses within or across environments, and argue for a greater consideration of gender-specific interactions with the environment in future studies of dioecious species. The results also suggest that the examination of sex reproductive costs at different times in season may lead to quite different conclusions as regards the relative resource costs of reproduction in male and female plants.

Keywords: Chlorophyll fluorescence, chlorophyll contents, sexual dimorphism, photochemical efficiency, reproductive investment, sex-ratio

INTRODUCTION

Sexual reproduction in plants is costly in terms of resources required for flowering and fruit set. In sexually dimorphic/polymorphic plants, females and males may incur in different reproductive costs. In dioecious plants, females typically invest more in reproduction than males over the course of the growing season, although for some species, resource investment in flowers alone may be considerably higher for males (Gross & Soule 1981; Allen 1986). The greater allocation towards reproduction by a sex may result in a cost that should be measurable, for example, as a lower vegetative growth rate, less frequent flowering, reduced longevity or greater physiological stress in this sex (Lloyd & Webb 1977; Dawson & Bliss 1989; Dawson & Ehleringer 1993; Gehring & Monson 1994; Laporte & Delph 1996; Geber *et al.* 1999). However, sex divergence in physiology may counterbalance differences in allocation to reproduction (Laporte & Delph 1996). In this respect, many studies have suggested that physiological specialization of the sexes may be an evolved response to allow each sex to meet the specific resource demands associated with reproduction (Cox 1981; Dawson & Bliss 1989; Dawson & Ehleringer 1993; Retuerto *et al*. 2000). Thus, sexual dimorphism may involve not only differences in the amount of energy and resources allocated to reproductive function but also in the physiological processes that underlie these differences. Sexual variation in physiological attributes may be a crucial factor determining the performance of each sex in different habitats, and consequently, promoting the spatial segregation of the sexes, a phenomenon often observed in dioecious species, commonly along environmental gradients (Freeman *et al.* 1976; Cox 1981; Bierzychudek & Eckhart 1988).

Studies of sex differences in physiology are increasing, although heavily biased towards dioecious species. Intermediate sexual systems, where the scale and history of differentiation may be reduced, have been suggested to provide clues as to the origins and selective pressures favoring sex-specific physiology (Case & Ashman 2005). This study investigated the particular ecology and physiology of the sexes of *Honckenya peploides*, which presents a complex and unusual sexual system with male-sterile morphs that completely lack the ability to produce pollen ("females"), and polleniferous morphs that retain a slight bisexual nature but achieve most of their fitness through pollen ("males"). This system is known as subdioecy, occupying an intermediate position in the evolution of gynodiecy to dioecy (Sakai & Weller 1999). In our territory, *H. peploides* exhibits a surprising segregation of the sexes, with clumps composed of individuals of one sex separated by tens or hundreds of meters from clumps composed of individuals of the other sex. The observation of such pronounced spatial segregation has prompted us to ask whether sexes of *H. peploides* differ in ecophysiological traits in ways that make them better adapted to different habitats. Because previous studies have reported that females typically invest more in reproduction than males (Obeso 1997; Delph 1999; Nicotra 1999) and that differences between sexes may appear or become more evident during the reproductive period (Freeman & McArthur 1982; Dawson & Bliss 1989; Antos & Allen 1990), in this study we also tested whether possible sex differences in physiology were dependent on reproductive status, as suggested by Obeso *et al.* (1998). On the basis of findings by Karlsson (1994) and Nicotra *et al.* (2003) suggesting that the reallocation of nitrogen from photosynthetic enzymes or chlorophyll to seeds during fruit maturation may result in lower photosynthetic rates in females, we predicted that in a nutrient-limited system, such as a dune habitat, photosynthesis will not be sink-regulated (Laporte & Delph 1996) and females will experience a decline in photosynthetic efficiency during the reproductive period. Previous studies reporting higher photosynthetic rates in males relative to those in females justify this prediction (Correia & Díaz Barradas 2000; Nicotra *et al.* 2003). In addition, considering that significant changes in abiotic conditions may occur on very short distances in dune environments (Houle 1997; Gagné & Houle 2001), and that *H. peploides* forms large clumps measuring several meters across, we investigated whether individuals growing at the edge and at the centre of the clumps differed in performance and ecophysiological traits. Gagné & Houle (2001), suggested that although plant establishment in dune systems facilitates recruitment, plant growth conditions can be more restrictive in vegetated areas than in bare areas, because of the accumulation of sand and higher salinity found in vegetated areas of coastal dunes. Assuming these results, we expected better performance in *H. peploides* individuals growing at the edge of clumps than in those at the centre. Finally, we investigated various aspects of population structure and evaluated differences in the reproductive investment between the two sexes.

MATERIAL AND METHODS

Study species

Sea sandwort, *Honckenya peploides* (L.) Ehrh. (Caryophyllaceae), is a subdioecious perennial plant with a circumpolar distribution (from temperate to Arctic zones; also anthropochorous in South America). On the Iberian Peninsula, *H. peploides* extends from the Atlantic coast of northern Portugal and northwards and eastwards along the Bay of Biscay (Cantabrian Sea) to France. It is a hemicryptophyte regrowing each spring from long rhizomes that produce compact groups of aerial shoots, forming vegetative clumps or mats. These clumps are typically found on the upper beach, forming small mounds called embryo dunes. Plants reproduce sexually by seed or clonally by rhizomes. This species is an early colonizer, contributing to stabilization and anchorage of the soil and facilitating the establishment of other species (Houle

1997; Gagné & Houle 2001). Two types of flower can be found in *H. peploides*, as reported for the subspecies *major* by Tsukui & Sugawara (1992): one type ("pistillate") has long styles, short petals and non-functional anthers, while the other ("staminate") has short styles, long petals and long stamens that produce pollen grains. This latter type rarely produces seeds, and when it does the number of seeds is very low compared to female flowers. Tsukui & Sugawara (1992) reported that fruit set percentage varied from 12.0 – 76.5% in females to 0 – 2.2% in males. Both types of flower have nectaries at the base of the stamens, which attract pollinators. In line with Tsukui & Sugawara (1992), and following Lloyd (1976) & Delph (1990), we will here refer to plants with pistillate flowers as females and to plants with staminate flowers as males.

Study sites

Fieldwork was conducted from May to August of 2003 at three sites on the coast of Galicia (Northwest Spain). At each site, we studied two segregated clumps of plants, one composed of female and the other of male individuals. Individuals from the three sites were sexed on the basis of their floral morphology, and the study was conducted during the flowering and fruiting seasons. The presence of single-sex clumps might suggest that all the stems within a clump are the product of clonal growth and thus represent one genotype. However, a study using amplified fragment length polymorphism (AFLP) as well as isozyme analysis has found high values of genetic variation, revealing several genets within each of these unisexual clumps (mean values for proportion of distinguishable genotypes: 0.25 for isozymes and 0.41 for AFLP; mean values for Simpson`s diversity index: 0.65 for isozymes and 0.68 for AFLP; N = 193 for isozymes and N = 80 for AFLP, Sánchez-Vilas *et al.*, unpublished data). In all three sites *H. peploides* grows forming big clumps, in which it is the dominant species; other species including *Cakile maritima*, *Eryngium maritimum* or *Ammophila arenaria* are sometimes present, but at low density in the clump.

Population structure and reproductive investment

We mapped the clumps in order to calculate the total area occupied by this species in each site. Sampling was done at the end of the months of May (at Lariño) and June (at O Bao), or in the second week of July (at San Román). In each clump, we established regularly spaced transects perpendicular to the coast line; along each transect, 50 × 50 cm quadrats were randomly selected; within each quadrat we recorded the number of individuals of *H. peploides*, defined as number of aerial shoots, as well as plant cover, number of flowers and number of fruits. The number of quadrats selected was proportional to the total area of the clump, with the area sampled being approximately 12% of the total clump area. Dry flower mass was estimated from flower number and mean flower dry mass of about 50 randomly selected flowers (5.2 ± 0.2 mg, N = 46 for females; 6.0 ± 0.2 mg, N = 59 for males; mean ± SE). Likewise, we estimated fruit mass from fruit number and mean fruit dry mass (51.2 ± 2.1 mg, N = 52 randomly selected fruits; mean ± SE). Flower and fruit dry mass per unit area of plant cover (mg cm^{-2}) was used as an estimate of reproductive investment: due to the different sizes of aerial shoots, we consider that plant cover is a better estimate of above-ground vegetative biomass than number of shoots.

Measurements of ecophysiological traits

In two of the sites, Lariño and O Bao, we randomly selected 37 individuals for each combination of sex (males and females), reproductive status (reproductive and nonreproductive) and position (edge and centre), giving a total of 296 individuals per site. We considered reproductive individuals to be those bearing flowers or fruits. The individuals selected were separated by at least 30 cm. We measured photosynthetic efficiencies, chlorophyll contents and specific leaf areas (SLA) as response variables. Efficiency of photosynthesis was evaluated by chlorophyll fluorescence parameters recorded on the upper surface of one expanded leaf per plant, using a portable pulse-amplitude-modulated fluorometer (Mini-PAM, Walz, Effeltrich, Germany). Chlorophyll fluorescence measurements were taken at a photosynthetic photon flux density (PPFD) of 1400 µmol m^{-2} s^{-1}, with the fluorometer fiberoptic (active cross section 6 mm) placed at a 60º angle to the leaf blade and positioned so as not to shade the leaf surface. Following the exposure to a modulating light, a 0.8 s saturating pulse of 4000 µmol m^{-2} s^{-1} PPFD was applied to the leaf. Steady-state light-adapted fluorescence yield (Ft) and maximum light-adapted fluorescence yield (Fm') were recorded with the fluorometer. The effective quantum yield of PSII, ΦPSII = (Fm'-Ft)/Fm', calculated from the measured data, is a measure of the overall efficiency of PSII reaction centres in light (Genty *et al.* 1989). Several studies have demonstrated that this parameter can be used to predict CO_2 assimilation rates accurately and quickly (Genty *et al.* 1989; Demming-Adams *et al.* 1990; Edwards & Baker 1993). All measurements were made at midday on sunny days at the end of the months of May (at Lariño) and June (at O Bao).

On the same leaves used to record chlorophyll fluorescence parameters we estimated chlorophyll contents with a hand-held chlorophyll meter (SPAD-502, Minolta Camera Co., Osaka, Japan), which calculates an index based on absorbances at 650 and 940 nm. SPAD values are well correlated with the chlorophyll content of leaves (Markwell *et al.* 1995; Wood *et al.* 1993). To calculate SLA (leaf area / unit leaf dry mass, cm^2 g^{-1}), we first measured the leaf areas and then determined biomass after drying at 70ºC for 72 h.

Data analysis

To test for the effects of the different factors on fluorescence parameters, chlorophyll contents and SLA, we used a split-plot analysis of variance (SYSTAT 11, Evanston, Illinois). We treated sex (male and female), position within the clump (centre and edge) and reproductive status (reproductive and nonreproductive) as fixed factors and site (Lariño and O Bao) as a random factor nested within sex. We tested the main and interaction effects over the appropriate error term when the latter was significant. If not, the corresponding effects were tested over the within error term to maintain power in the analysis (Sokal & Rolf 1995; Pigliucci 2002). Effects were considered significant in all statistical calculations for $P < 0.05$. When significance among means was detected, multiple comparisons were performed using LSD tests (Fisher's least significance difference; Sokal & Rolf 1995). Prior to analyses, data were examined for normality and homoscedasticity and no significant violation of assumptions were found. The significance of differences between the sexes in the structural characteristics of their clumps (area, plant cover and density), and in parameters related to reproductive investment was determined by Mann-Whitney *U* tests.

RESULTS

Population structure and reproductive investment

In the three sites studied, we observed complete spatial segregation of the sexes of *H. peploides*, with the distance between the clumps of the different sexes varying from a few meters to several hundred meters. We did not find individuals of different sex sharing the same clump in any of the sites, although at O Bao and San Román we observed some males producing a few fruits.

The structural characteristics of the clumps and parameters related to reproductive investment are shown on Table 1. The total area of the clumps studied varied from a minimum of 21.4 m^2 to a maximum of 224.7 m^2. At two sites, O Bao and San Román, males occupied more extensive areas than females. The density of individuals was significantly higher for males than for females at Lariño and San Román ($P < 0.004$, Mann-Whitney U test), but not at O Bao ($P = 0.459$). At Lariño, males showed higher plant cover than females ($P < 0.001$, Mann-Whitney U test), but we did not find significant differences at O Bao and San Román ($P = 0.65$ and 0.087, respectively). Males always displayed more flowers per square meter than females (Mann-Whitney U tests: $P < 0.001$ for O Bao and Lariño, and $P = 0.055$ for San Román). In May, at Lariño, females allocated significantly more biomass to flowers and to flowers-plus-fruits than males (Mann-Whitney U tests: $P = 0.014$, and $P = 0.009$, respectively). However, one month later, at O Bao, this trend was reversed and males allocated significantly more biomass to flowers and to flowers-plus-fruits than females (Mann-Whitney U tests: $P = 0.002$, and $P = 0.003$, respectively). By mid July, in San Román, females had stopped flowering, but showed a considerable number of fruits, while males still maintained a large number of flowers. At this time, males allocated significantly more biomass to flowers than females (Mann-Whitney U tests: $P < 0.001$). Total reproductive biomass (flowers plus fruits) did not differ significantly between the sexes (Mann-Whitney U test: $P = 0.320$).

Table 1 Structural characteristics and parameters related to reproductive investment in the populations of *Honckenya peploides* studied. Values are means (± SE), except for total area. The number of quadrats sampled (n) in each clump was proportional to the total area of the clump. Plant cover was used as an estimate of above-ground vegetative biomass.

	Lariño		*O Bao*		*San Román*	
	females (n=15)	males (n=16)	females (n=25)	males (n=47)	females (n=21)	males (n=59)
Total area (m^2)	25.37	21.37	37.9	95.48	35.84	224.72
Plant cover (%)	4.8 (± 1.3)	22.7 (± 3.5)	24.5 (± 4.3)	16.8 (± 2.1)	39.6 (± 5.1)	49.0 (± 3.6)
δ individuals (aerial shoots per m^2)	91.2 (± 22.6)	567.0 (± 80.5)	243.0 (± 35.4)	118.5 (± 13.4)	286.7 (± 38.2)	404.3 (± 23.1)
δ flowers (flowers per m^2)	73.6 (± 20.8)	163.0 (± 38.9)	275.7 (± 66.5)	573.9 (± 89.6)	0	963.2 (± 127.4)
Flower dry mass (mg) per unit plant cover (cm^2)	0.97 (± 0.24)	0.43 (± 0.07)	0.43 (± 0.07)	1.77 (± 0.24)	0	0.88 (± 0.11)
Fruit dry mass (mg) per unit plant cover (cm^2)	0.02 (± 0.02)	0	0.17 (± 0.05)	0.18 (± 0.08)	1.23 (± 0.38)	0.17 (± 0.09)

Effects on photosynthetic efficiencies

Nonreproductive individuals had significantly higher effective quantum yields (ΦPSII) than reproductive individuals, with these differences depending on the position of the individuals within the clump (Table 2; Fig 1), so that the effect of reproductive status was greater for individuals growing at the edge of the clumps (LSD tests: $P < 0.001$) than for those growing at the centre (LSD tests: $P = 0.003$).

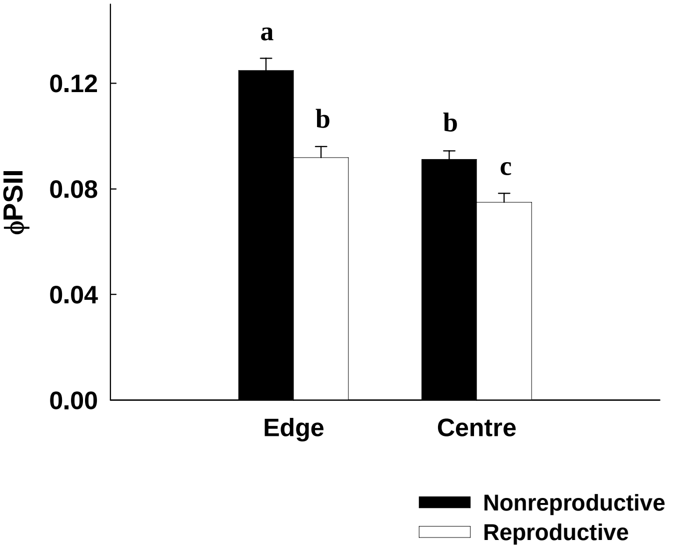

Fig. 1 Mean values (± SE) of effective quantum yield (Φ PSII) for nonreproductive and reproductive individuals growing at the edge or at the centre of clumps (N = 148). Means with the same letter do not differ significantly at the 5% level (Fisher's LSD tests).

We detected significant spatial variation in the effects of position: the effect of the position on ΦPSII values was different for all replicate sites of each sex (significant Position × Site(Sex) interaction; Table 2), although individuals growing at the edge of the clumps always had greater ΦPSII than individuals growing at the centre (Table 3). We did not detect effects of sex in effective quantum yield.

Table 2 Results of split-plot analysis of variance for effective quantum yield (ΦPSII), chlorophyll content and specific leaf area (SLA). Terms used as error are labelled with letters. Letters following *F values* indicate the denominator used to test each effect. *P* < 0.005 is marked in bold.

		ΦPSII			Chlorophyll content			SLA		
Source of variation	*df*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Sex	1	0.0704	3.50^{A}	0.202	7568	5.26^{A}	0.149	15787	6.60^{A}	0.124
Site(Sex)^{A}	2	0.0201	10.09^{E}	**<0.001**	1439	42.74^{E}	**<0.001**	2393	7.65^{E}	**<0.001**
Reproductive (R)	1	0.0897	44.94^{E}	**<0.001**	1148	5.33^{B}	0.147	3852	12.31^{E}	**0.001**
R × Sex	1	0.0003	0.13^{E}	0.719	1011	4.69^{B}	0.163	1952	6.24^{E}	**0.013**
R × Site(Sex)^{B}	2	0.0045	2.25^{E}	0.106	215	6.40^{E}	**0.002**	256	0.82^{E}	0.445
Position (P)	1	0.0948	7.47^{C}	0.112	308	0.28^{C}	0.650	4111	2.78^{C}	0.236
P × Sex	1	0.0298	2.35^{C}	0.265	251	0.22^{C}	0.685	472	0.32^{C}	0.629
P × Site(Sex)^{C}	2	0.0127	6.35^{E}	**0.002**	1117	33.18^{E}	**<0.001**	1479	4.73^{E}	**0.009**
R × P	1	0.0104	5.23^{E}	**0.023**	19	0.57^{E}	0.439	540	1.73^{E}	0.189
R × P × Sex	1	0.0022	1.12^{E}	0.290	13	0.40^{E}	0.527	334	1.07^{E}	0.301
R × P × Site(Sex)^{D}	2	0.0004	0.20^{E}	0.827	53	1.56^{E}	0.203	463	1.48^{E}	0.224
Within error^{E}	576	0.002			34			313		

Table 3 Effective quantum yield (ΦPSII), chlorophyll content (Chl. content) and specific leaf area (SLA) of male and female plants, growing at two sites (Lariño *vs.* O Bao), in different positions (edge *vs.* centre) within a clump, and with different reproductive status (nonreproductive *vs.* reproductive). Values are means ± SE of 37 plants.

Sex	Site	Position	Reproductive status	ΦPSII	Chl. content	SLA
Female	Lariño	Edge	Nonreproductive	0.111 ± 0.008	42.01 ± 1.04	99.69 ± 2.94
			Reproductive	0.092 ± 0.007	37.92 ± 1.39	112.31 ± 3.73
		Centre	Nonreproductive	0.089 ± 0.005	40.08 ± 0.86	100.77 ± 2.57
			Reproductive	0.078 ± 0.006	36.69 ± 1.01	110.87 ± 3.58
	O Bao	Edge	Nonreproductive	0.100 ± 0.008	40.64 ± 0.86	99.73 ± 3.22
			Reproductive	0.059 ± 0.006	33.81 ± 0.99	105.39 ± 3.85
		Centre	Nonreproductive	0.091 ± 0.007	42.18 ± 0.98	106.43 ± 1.69
			Reproductive	0.059 ± 0.006	34.88 ± 1.19	112.98 ± 3.74
Male	Lariño	Edge	Nonreproductive	0.127 ± 0.007	46.41 ± 0.66	105.58 ± 2.91
			Reproductive	0.091 ± 0.008	48.48 ± 0.86	106.87 ± 2.12
		Centre	Nonreproductive	0.089 ± 0.008	49.46 ± 0.71	117.77 ± 1.92
			Reproductive	0.084 ± 0.008	50.55 ± 0.46	119.10 ± 1.96
	O Bao	Edge	Nonreproductive	0.161 ± 0.011	48.52 ± 0.97	115.18 ± 2.76
			Reproductive	0.126 ± 0.009	44.78 ± 1.10	123.66 ± 2.78
		Centre	Nonreproductive	0.096 ± 0.007	38.65 ± 0.85	123.93 ± 2.51
			Reproductive	0.079 ± 0.005	38.55 ± 0.97	118.71 ± 3.04

Effects on chlorophyll content

There was significant spatial variation in the effects of the reproductive status on chlorophyll content: nonreproductive individuals had higher chlorophyll content than reproductive individuals, except the males growing at the site of Lariño, (Reproductive status × Site(Sex) interaction; Table 2 and Table 3). Chlorophyll content was also affected by the position of the individuals within the clump, with the effect varying between sites without a clear trend (Position × Site(Sex) interaction; Table 2 and Table 3). Sexes did not differ significantly in chlorophyll content (Table 2).

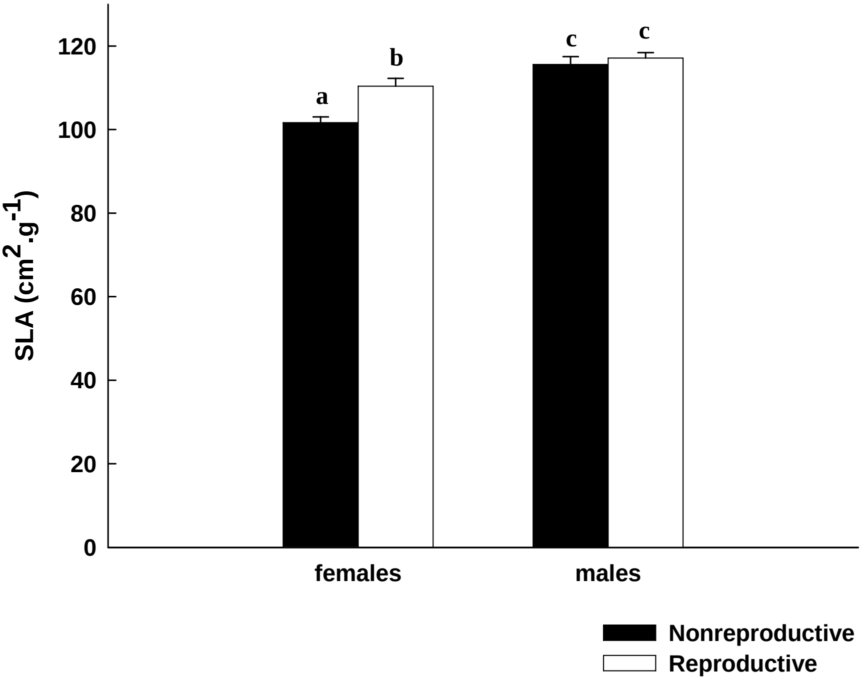

Fig. 2 Mean values (±SE) of specific leaf area (SLA, *cm^2 g^{-1}*) in reproductive and nonreproductive males and females (N = 148). Means with the same letter do not differ significantly at the 5% level (Fisher's LSD tests).

Effects on specific leaf areas

Specific leaf areas of males and females were differentially affected by reproductive status (Table 2). Reproductive females had significantly higher SLA than nonreproductive females (LSD test: $P < 0.001$), but reproductive status did not significantly affect SLA in males (LSD test: $P = 0.488$; Fig. 2). The effect of the position of the individuals within the clump on specific leaf areas varied significantly among sites, as follows: individuals growing at the centre of the clumps had greater SLA values than those growing at the edge, except the females growing in the site of Lariño, which did not differ in SLA (Position × Site(Sex) interaction; Table 2 and Table 3).

DISCUSSION

Analysis of the structure of our *H. peploides* populations showed that clumps were composed exclusively of individuals of one or other sex, providing clear evidence of spatial segregation of the sexes. Thus, the populations investigated in this study represent an extreme case of sex ratio variation across space; though note that we have also observed other populations of this species in Northwest Spain in which male and female individuals coexist in the same clump (although one sex is generally strongly predominant). Spatial segregation of the sexes is a common phenomenon in dioecious species (see Bierzychudek & Eckhart 1988), though its ultimate cause (disruptive selection or competitive character displacement) is a subject of debate (see Geber 1999). Some previous studies have reported that when recognizable habitat differences exist, sex ratios are often male-biased in stressful or resource-poor habitats and female-biased in favourable habitats (Freeman *et al.* 1976; Bierzychudek & Eckhart 1988; Dawson & Bliss 1989). In the present study, some female clumps were no more than 20 or 30 m from the nearest male clump. Melampy (1981) has also reported spatial variations in sex ratio for *Thalictrum* species, without association with any particular habitat characteristics. We are currently investigating whether possible habitat differences may explain the extreme spatial segregation observed by us between the sexes of *H. peploides* plants.

At each site we only made a static estimation of reproductive costs, without consideration of some important aspects such as maintenance costs and fruit abortion (see Obeso 2002). However, our results can provide a dynamic picture over a 3-month period. Thus, in May, in Lariño, females had a greater number of flowers per unit of vegetative biomass (*i.e.* unit area of plant cover), and invested 2.3 times more biomass in reproduction than males. One month later, in O Bao, males showed a greater number of flowers than females and allocated 3.3 times more biomass to reproduction. Finally, by mid July, in San Román, males maintained a large number of flowers long after female flowering had ceased, and the reproductive effort of females in producing fruits was comparable to that of males in maintaining flowers. These results suggest first that the two sexes have different flowering phenologies, with males maintaining functional flowers later in the season. Second, these results suggest that the reproductive effort of males relative to that of females changes over the season, in turn indicating that static estimations of reproductive costs may not accurately reflect time-integrated costs. Differences in the timing of reproductive investment are an important consideration that may influence estimates of the impact of fruit production on vegetative growth (Delph 1990; Obeso 2002; Suzuki 2005). Third, our results suggest that males may incur direct costs (resources invested in pollen, nectar, respiration and transpiration) for the maintenance of functional flowers (Obeso 2002), and so their reproductive costs may not be so low relative to females as has been commonly shown (Lloyd & Webb 1977; Willson 1983; but see Sakai & Sharik 1988 and Davidson & Remphrey 1990). Moreover, the small green fruits of *H. peploides* may contribute through fruit photosynthesis to reduce the relative cost of fruit production, as has been demonstrated for other species (Bazzaz *et al.* 1979; Cipollini & Levey 1991; Ogawa 2002). According to Ogawa (2002), the net respiration of green fruits changes from negative to positive as the final dry mass of the fruit exceeded 0.481 g. The mean fruit dry mass for *H. peploides* (0.0512 ± 0.002 g; mean ± SE, N = 52) is far below this threshold, which raises the possibility that fruit photosynthesis in *H. peploides* may significantly contribute to carbon requirements.

Several studies have demonstrated increases in carbon assimilation rates during reproduction, which have been attributed to a mechanism of sink regulation of photosynthesis triggered by the increased demand for assimilates from developing fruits and flowers (Dawson & Bliss 1993; Laporte & Delph 1996). In contrast, our results showing significantly higher effective quantum yield (ΦPSII) in nonreproductive individuals, especially in those growing at the edges of the clumps, indicate that reproduction may also cause a decrease in photosynthetic efficiency. Similarly, we also found that, except for the males growing in Lariño, nonreproductive individuals had higher chlorophyll content than those reproductive. These results suggests that in our dune systems, under conditions of nitrogen limitation, the photosynthetic tissues may compete with reproductive tissues (flowers and fruits) for the scarce nitrogen available, resulting in decreased photosynthetic capacity. This conclusion is in line with previous studies reporting that in plants facing resource limitation, reproduction will result in decreased photosynthetic capacity (Karlsson 1994; Saulnier & Reekie 1995; Nicotra *et al.* 2003).

If females of dioecious plants usually expend proportionally more of their resources on reproduction (Lloyd & Webb 1977; Willson 1983), the impact of reproduction might be expected to be greater in females than in males. However, we did not detect any sex-differential cost of reproduction in terms of photochemical efficiency or chlorophyll content. In intermediate sexual systems, where gender dimorphism has only recently evolved and there has been little time or opportunity for selection, sex morphs may be more similar to each other with respect to physiology (Case & Ashman 2005). The literature on the differential effects of reproduction in the sexes of dioecious species reports conflicting result. For example, reproductive activity increased photosynthetic capacity in females of *Salix arctica* (Dawson & Bliss 1989) and *Silene latifolia* (Laporte & Delph 1996); reduced photosynthetic capacity to similar extent in the two sexes of *Siparuna grandiflora* (Nicotra *et al.* 2003); and decreased photosynthetic efficiency in females of *Ilex aquifolium* under low-light conditions though not under saturating light (Obeso *et al.* 1998). Even previous-year reproduction has been reported to reduce photosynthetic capacity in females of a neotropical dioecious species

(Wheelwright & Logan 2004). In the present study, we found some evidence that suggest a higher cost of reproduction in females. Although reproduction did not affect SLA in males, reproductive females had significantly higher SLA than nonreproductive females. This suggests that females are more responsive to the effects of reproduction than males. Increased SLA has been related to nitrogen limitations (Hunt *et al.* 1985), which is consistent with the hypothesis that reproductive tissues (flowers and fruits) may act as sinks for the scarce nitrogen available, though other studies have reported that nitrogen limitation reduced (van Arendonk *et al.* 1997) or had no effect on SLA (van der Werf *et al.* 1993). Increased SLA in reproductive females may be interpreted as a cost bearing in mind that an increased ratio of leaf area to leaf dry mass has been associated with a lower photosynthetic capacity per unit leaf area (Dijkstra 1989) and with a lower tolerance to drought (Givnish 1979; Retuerto & Woodward 1993).

Although the effect of the position within the clumps on photosynthetic efficiencies was significantly influenced by the reproductive status of the plants and also varied significantly between the replicate sites of each sex, our results showed that, irrespective of reproductive status or site, individuals growing at the edge of the clumps had higher effective quantum yields and electron transport rates, supporting our expectation of better performance of *H. peploides* individuals growing at the edge compared to those growing at the centre. The interpretation of a better performance in individuals growing at the edge is reinforced by the lower specific leaf areas in these individuals. Lower SLA, *i.e.* a larger amount of photosynthetically active material per unit leaf area, may be especially beneficial at the high levels of radiation experienced by these plants in dune habitats. We explain the better performance of plants at the edges of the clumps as a response to a less exploited environment, due to a lower density of individuals. This interpretation is in line with the findings of Gagné & Houle (2001), who reported more restrictive growth conditions in vegetated than in bare areas of coastal dunes.

In conclusion, the present study describes an extreme case of spatial segregation of the sexes with unisexual clumps of plants, and establishes that male and female specific leaf areas are differentially affected by reproduction. Our results also demonstrate that the reproductive status of a shoot, its position within the clump (edge or centre) and also site conditions affect its photosynthetic efficiency and chlorophyll content. These findings cast doubt on our capacity to generalize about gender responses within or across environments, and argue for a greater consideration of gender-specific interactions with the environment in future studies of dioecious species. Finally, the study indicated that the estimation of male and female reproductive costs at different times in the season may lead to quite different conclusions regarding the real relative resource costs of reproduction in male and female plants. Clearly, further studies are required to accurately assess whole-season reproductive costs in males and females of this species and to assess possible among-location or among-year variation in these costs.

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Chapter 3

3

Sex-specific differences in the seasonal allocation to biomass and reproduction and in habitat characteristics of the subdioecious dune plant *Honckenya peploides* (L.) Ehrh.

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ABSTRACT

Sex dimorphic plants often show sex-specific differences in growth and biomass allocation. These differences have been explained as a consequence of the different reproductive functions performed by the sexes. Empirical evidence strongly supports a greater reproductive investment in females. Sex differences in allocation may determine the performance of each sex in different habitats and therefore might explain the spatial segregation of the sexes described in many dimorphic plants.

Honckenya peploides is a subdioecious, perennial halophyte found on the upper beach and embryo dunes. At the studied site, *H. peploides* displays an extreme spatial segregation of the sexes, with unisexual clumps of plants. This study aimed to inquire into the causes of the niche segregation of the sexes by comparing their seasonal patterns of biomass allocation.

Three "female" and three "male" clumps of plants were studied during the flowering and fruiting seasons at Lariño (NW Spain). A cylindrical sampler was used for collecting soil and plant samples at randomly selected points in the clumps. Above (vegetative and reproductive) and below-ground mass, as well as the number of flowers and fruits, were determined for each sample.

Water and nutrient contents, conductivity and bulk density of the soil samples were also measured.

Females had significantly greater total and below-ground mass and higher below-/above-ground mass than males during the flowering period, but latter in the season these differences became non significant. At the flowering stage, males and females did not differ in the number of flowers but the mean weight of a male flower was significantly higher than that of a female flower, what brought about a marginally significant higher total flower dry mass in males than in females. There were no differences in reproductive traits at the end of the season. Significant differences in soil water content emerged as the season progresses and water becomes scarcer. Thus, in June and July, the water content in the first 10 and 20 cm soil layers was significantly higher in the habitats of females than in those of males. In August, significant differences were only maintained in the first 10 cm of soil. This study demonstrates that the examination of male and female reproductive costs at different times may lead to quite different conclusions as regards the relative resource costs of reproduction in male and female plants. The study demonstrated that reproductive effort is not always higher in females than in males in dimorphic plants. We concluded that between-sex differences in allocation traits may explain the spatial segregation of the sexes.

Keywords: spatial segregation, biomass allocation, reproductive effort, *Honckenya peploides.*

INTRODUCTION

Polymorphic or dimorphic plant species, in which different individuals perform different sexual functions, often show sex-specific differences in morphological and physiological traits (Dawson & Ehleringer 1993; Obeso *et al.* 1998; Dawson & Geber 1999; Retuerto *et al.* 2000; Correia & Díaz Barradas 2000; Verdú 2004), in ecological characters, as patterns of defence and herbivory (Krischik & Denno 1990; Retuerto *et al.* 2006), survival (Allen & Antos 1988) or spatial distribution (Iglesias & Bell 1989), and in patterns of growth and resource allocation (Ågren 1988; Ramp & Stephenson 1988; Nicotra 1999; Leigh *et al.* 2006). These intersexual differences have commonly been explained as a consequence of the different reproductive functions performed by the sexes. Because females produce seeds and fruits in addition to flowers, many studies have found that females expend proportionally more of their resources on reproduction and less on maintenance and growth compared to males (Lloyd & Webb 1977; Wilson 1983; Delph 1999). However, some studies have challenged this generalization, reporting similar or even higher reproductive investment in males (Sakai & Sharik 1988; Davidson & Remphrey 1990; Delph *et al.* 1993; Leigh *et al.* 2006). Sexual dimorphism in allocation might also arise as result of selection for traits that may allow each gender to meet the specific resource demands associate with reproduction (Cox 1981; Cipollini & Stiles 1991; Nicotra *et al.* 2003).

Honckenya peploides is a dimorphic species typically found on the upper beach and embryo dunes. It presents a breeding system in which male and female flowers are borne on separate plants. Female plants never produce pollen and are constant in their sex expression, whereas "male" plants, all of which produce pollen, may also produce a small number of seeds. We will name this second sexual morph as male since it attains most of its fitness via pollen export. This system, called subdioecy, is close to dioecy in the evolutionary pathway from hermaphroditism to dioecy via gynodioecy (see

Delph & Wolf 2005 for a review). At the location of our study, *H. peploides* displays an extreme spatial segregation of the sexes, with monomorphic clumps composed exclusively of individuals of either one sex or the other.

In dimorphic flowering plants like *H. peploides*, biased sex ratios have been widely reported (Dawson & Geber 1999, and references therein; Eppley 2006). Within-population sex ratio variation, or spatial segregation of the sexes, may occur if the sexes respond differentially to given environmental conditions. Differences in competitive abilities between the sexes (Cox 1981; Eppley 2006), morphological or physiological specialization of the sexes to different habitats (Dawson & Ehleringer 1993; Dawson & Geber 1999) or intersexual differences in reproductive biology (Lloyd 1973; Bierzychudek & Eckhart 1988) have been suggested as possible causes of spatial segregation.

Sexual differences in resource allocation may be an important determinant of the performance of each sex in different habitats and therefore could help to explain why some populations of dioecious species show habitat-specific sex ratio biases (Freeman *et al.* 1976; Bierzychudek & Eckhart 1988). If the reproduction is more costly for females, as it has usually been argued, the relative fitness of females will increase as environmental quality improves and consequently, the sex ratio will become female-biased in high-quality environments and male-biased in stressful or resource poor habitats. In fact, this is the prevailing pattern emerging in the literature on spatial segregation of the sexes (Freeman *et al.* 1976; Lloyd & Webb 1977; Freeman *et al.* 1980; Bierzychudek & Eckhart 1988; Geber 1999; Dawson & Geber 1999).

This study focussed on sexual dimorphism in biomass and reproductive allocation in *H. peploides*. Most studies on patterns of biomass allocation in dimorphic species have examined sexual differences in biomass distribution at a single point in time (Wallace & Rundel 1979; Bullock 1984; Hemborg & Harlsson 1999). However, because sexes may differ in their timing of development (Lloyd & Webb 1977), static, or single point estimation of patterns of allocation may not reflect real or time-integrated patterns. The few studies that have examined seasonal patterns of biomass distribution have found significant differences between the sexes in the amount of resources allocated, and in the timing of allocation, to reproductive and vegetative structures (Gross & Soulé 1981; Ågren 1988; Hemborg & Karlsson 1999; Ehlers & Thompson 2004). More research is required for a thorough understanding and realistic modelling of the quantitative relationships between male and female reproductive effort over the whole season. Due to the difficulties in measuring below-ground biomass in plants growing in natural conditions, there have been few or no field studies considering this component in the analysis of the patterns of biomass allocation in dimorphic plants. Most of the field studies on allocation have expressed the reproductive effort as the ratio of the dry mass of reproductive tissues to the total dry mass of the above ground tissues (Korpelainen 1992; Leigh *et al.* 2006; Zunzunegui *et al.* 2006). Although the extent and vertical distribution of the root system has been found to be critical for water uptake and drought tolerance (Moroke *et al.* 2005; Yu *et al.* 2007), we know no studies addressing if sexes of dimorphic species differ in vertical root distribution. However, allocation to below-ground biomass may be especially crucial for dune plants. Due to the low capacity of sandy soils to buffer against drought, dune plants, as *H. peploides*, may experience severe water deficits, especially in spring and summer, when high temperatures lead to high evapotranspiration rates. Under these conditions, sex-specific allocation of biomass to structures specialized in the uptake of water and the scarce nutrients available in the dune systems might result in a differential survival of the sexes in different habitats with the result of a spatial segregation of the sexes.

In this study, we aimed to inquire into the causes of the extreme niche segregation of the sexes of *H. peploides*. Specifically, we set out to answer the following questions: 1) Do male and female differ in their seasonal patterns of biomass allocation? 2) Do they differ in the extent and timing of biomass allocated to sexual reproduction? and 3) Do habitats of “male” and “female” plants differ in quality, as estimated by water availability, bulk density, nutrient content or conductivity?

MATERIAL AND METHODS

Study species

Sea sandwort, *Honckenya peploides* (L.) Ehrh. (Caryophyllaceae), is a subdioecious perennial plant with a circumpolar distribution (from temperate to arctic zones; anthropochorous in South America). In the Iberian Peninsula, *H. peploides* extends from the Atlantic coast of northern Portugal and northwards and eastwards along the Bay of Biscay (Cantabrian Sea) to France. It is a hemicryptophyte regrowing each spring from long rhizomes that produce compact groups of aerial shoots, forming vegetative clumps or mats. These clumps are typically found on the upper beach, forming small mounds called embryo dunes. Flowers are axillary and solitary, and/or in 1- to 6-flowered terminal cymes, strongly honey-scented. Two types of flower can be found in *H. peploides*, as reported for the subspecies *major* by Tsukui & Sugawara (1992). One type (hereinafter “female flower”) has long styles, short petals and non-functional anthers; the other (hereinafter “male flower”) has short styles, long petals and long stamens that produce pollen grains; this definition of male and female flowers is as per Tsukui & Sugawara (1992), and based on the arguments of Lloyd (1976) and Delph (1990). Male flowers rarely produce seeds, and when they do the number of seeds produced is very small compared to female flowers. Both types of flower have nectaries at the base of the stamens which attract pollinators. Plants reproduce sexually by seed or clonally by adventitious shoots produced by root buds. *Honckenya peploides* is an early colonizer, contributing to stabilization and anchorage of the soil and facilitating the establishment of other species (Houle 1997; Gagné & Houle 2001).

Study site

Fieldwork was conducted from April to the end of August of 2006 at the Lariño site (42º45'N, 9º6'W), on the coast of Galicia (Northwest Spain). We studied the six segregated clumps of *H. peploides* plants existing at that site, three composed exclusively of females and the other of male individuals. Individuals from the six monomorphic clumps were sexed on the basis of their floral morphology, and the study was conducted during the flowering and fruiting seasons. We have been studied this population during the last five years and we have not observed inconstancy in sex-expression. In Lariño, *H. peploides* grows forming well-delimited clumps, in which it is the dominant species; other species including *Cakile maritima*, *Eryngium maritimum* or *Ammophila arenaria* are sometimes present, but at very low density in the clump. Clumps did not differ in topographic position nor distance to the sea.

Soil and plant samples were collected in the early morning, at different points in the clumps. Given the small size of the clumps, in April and May we collected two samples in each of the six clumps. In June, July and August, the size of four of the clumps (two male and two female) had increased so much that we decided to increase the number of samples per clump to four, maintaining two samples in each of the two smallest clumps. We used a cylindrical steel sampler for collecting soil and plant samples at the same point, in order to determine above and below-ground plant mass, and the moisture and bulk density of the soil. The sampler maintained the structure of its samples of soil, so that the volume of the sampler can be assumed to be the volume of the soil samples. The cylindrical steel sampler (10 cm diameter, 45 cm length) was driven into the soil and carefully removed to preserve the plant and soil sample as it existed *in situ*. Samples were taken from the 0-10 cm, 10-20 cm, 20-30 cm and 30-40 cm depths, by cutting the soil cores at these predetermined depths. The 0-10 cm samples included the above-ground biomass. Each sample was put into a sealed plastic bag (with tight-fitting lids) and this into a zip plastic bag. Each sample was identified and carried to the laboratory, where samples were weighed at a 0.01 g precision to obtain their wet mass. Then, from each sample, we separated the biomass of *H. peploides* and determined its fresh mass. Fresh biomass was subtracted from the total sample mass to obtain soil sample mass. From the 0-10 cm samples we separated above (shoot) and below-ground (rhizomes) mass of *H. peploides*. We split the above-ground mass into vegetative and reproductive (flower and fruits). In these samples, we also counted the number of fruits and in April, at the peak of the flowering, the number of fresh flowers. Below-ground mass was washed and all plant material was oven-dried at 55ºC for 6 days and weighed at a 0.0001 g precision (Mettler AJ100, Switzerland) to calculate dry mass of rhizomes, shoots, flowers and fruits. Biomass allocation patterns were assessed by calculating below-/above-ground ratios and reproductive effort, estimated as the ratio of reproductive to vegetative dry mass. Mean flower and fruit dry mass were estimated from dry flower and fruit mass and the number of flowers and fruits, respectively. After separating *H. peploides* biomass, soil samples were placed in an oven at 75ºC for a minimum of 72 hours for drying. Soil water content at each predetermined depth was obtained by dividing the difference between wet and dry soil masses by the mass of the dry sample to obtain the ratio of the mass of water to the mass of dry soil (g water kg^{-1} dry soil). When multiplied by 100, this becomes the percentage of water in the sample on a dry-weight basis. Soil bulk density at each predetermined depth was calculated as the ratio of the mass of dry solids (the below-ground dried mass of *H. peploides* was added to get the dry soil mass) to the bulk volume of the soil occupied by those dry solids. The bulk volume includes the volume of the solids and the pore space.

Soil carbon and nitrogen contents were measured in 15 cm depth soil samples collected in June at 6 different points in each of the six clumps. Samples were stored at < 10°C until analysis. Samples were dried to a constant weight at 75°C, sieved to < 2 mm and analyzed for C and N content via dry combustion in a CHN elemental analyzer (LECO CHNS-932), using three subsamples of ca. 2 mg from each sample. In the same samples we measured conductivity (conductivity meter 524, Crison, Barcelona, Spain). Soil electrical conductivity (µS cm^{-1}) provides a means for rapidly mapping variations in soil properties such as salinity and water potential (Greenberg *et al.* 1992).

Statistical analysis

We performed five (one per month) two-way nested analysis of variance by nesting the random-effects factor (plot or clump) within the main fixed factor (sex). Plot within sex was used as the error term to test for the effect of sex. In order to detect possible significant temporal trends in data, we also carried out a multivariable analysis of variance, with time and sex as fixed factors and plot nested within sex. The level of significance accepted was set at $P < 0.05$. Prior to analyses, the distribution of variables was examined for non-normality and heteroscedasticity and when significant violations were found data were $\log_e$ transformed.

RESULTS

Biomass and reproductive traits

Results of ANOVAs on series of monthly data showed that male and female differed in biomass and reproductive traits. Most of these differences were detected in April and disappeared as the season progresses, so that in July and August we did not detected any significant between-sex differences in these traits (Table 1). Although females always invested a higher percentage of biomass to below-ground structures than males, differences in total below-ground dry biomass (biomass in the 0-40 cm soil layer) were only statistically significant in April and May (Table 1; Fig. 1).

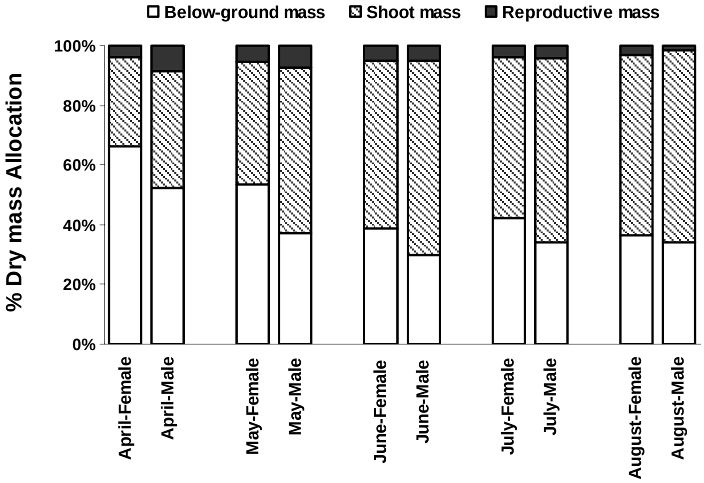


Fig. 1 Seasonal variation in the percentage of dry mass allocated to below-ground structures, shoots and reproductive structures for each sex in samples of *Honckenya peploides*. ANOVAs results indicated significant sex-differences for below-ground mass in April ($F_{1,4}$ = 18.529, P = 0.013) and May ($F_{1,4}$ = 7.964, P = 0.048).

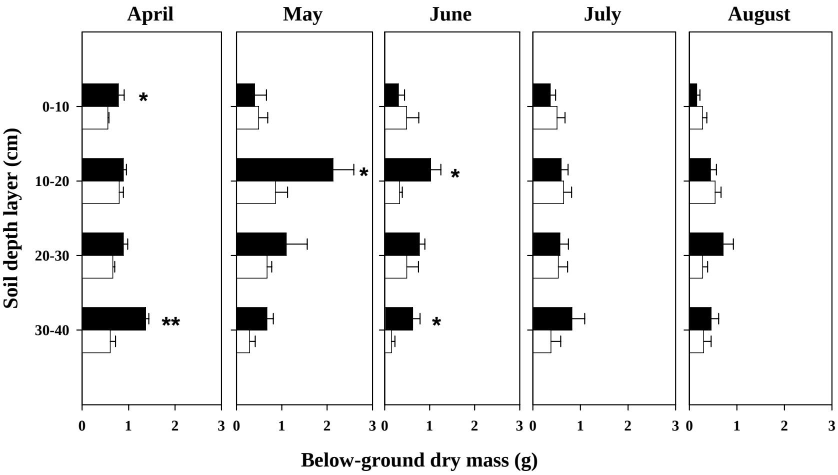


Fig. 2 Vertical distribution profiles from April to August of mean (±SE) below-ground dry mass in male (open bars) and female (solid bars) habitats of *Honckenya peploides* .

Analyses of differences in below-ground dry mass in the different soil layers also showed significant sex effects at different depths in April, May and June (Table 1; Fig. 2), with females investing significantly more biomass to below-ground structures than males. Late in the season, in July and August, this trend was somewhat reversed: males and females did not significantly differ in biomass allocation to below-ground structures, but males showed more biomass in the two shallowest soil layers than females. In line with all these results, females also showed significantly higher below-/above-ground mass ratios than males in April and May (Table 1). Between-sex differences in total dry mass were only statistically significant in April, when females had greater biomass than males (Table 1). We did not detect significant between-sex differences in the biomass invested to reproductive structures, estimated as absolute reproductive dry mass (flowers plus fruits) or as reproductive effort (reproductive / vegetative dry mass), although in April differences were marginally significant for both variables (Table 1; Fig. 3).

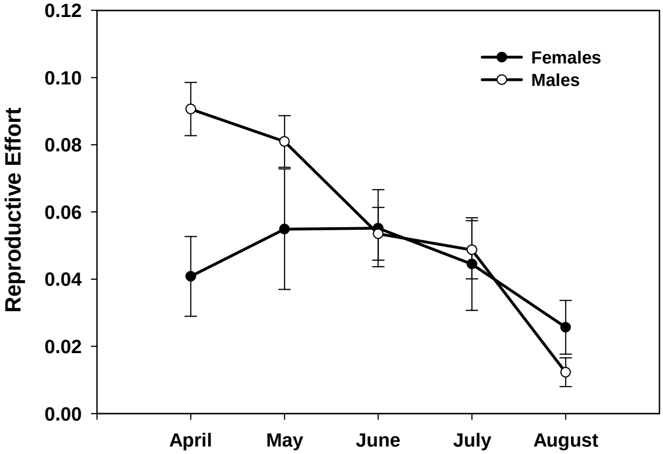


Fig. 3 Temporal variation in Reproductive Effort (reproductive / vegetative dry mass) of males and females of *Honckenya peploides*.

In April, males and females did not differ in the number of flowers (69.83 ± 6.03, for males vs. 40.50 ± 8.69, for females) but the mean mass of a male flower (0.0059 ± 0.0003 g) was significantly higher than that of a female flower (0.0050 ± 0.0003 g), what brought about a marginally significant higher total flower dry mass in males than in females ($P = 0.053$; Table 1).

MANOVA analysis showed that the reproductive and biomass parameters changed significantly with time (Table 2), with the exception of below-ground dry mass in the 20-30 cm, 30-40 cm and 20-40 cm soil layers. This analysis also revealed that throughout the studied period females always allocated a significantly higher amount of below-ground dry mass in the 30-40 cm soil layer than males (Fig. 4a), and confirmed some temporal trends suggested by the results of the ANOVAs performed on the series of monthly data (Table 2). Early in the season, females tended to allocate a significantly higher dry mass to below-ground structures but later, in July and August, between-sex differences in allocation disappear. The analysis also detected that the relative allocation of males and females to above-ground dry mass and below-/above-ground ratios changed significantly with time (Table 2). Although above-ground dry mass in males and females changed with time without a consistent pattern, there was a clear temporal trend in below-/above-ground ratios (Table 3; Fig. 4b), similar to that mentioned above for below-ground dry mass.

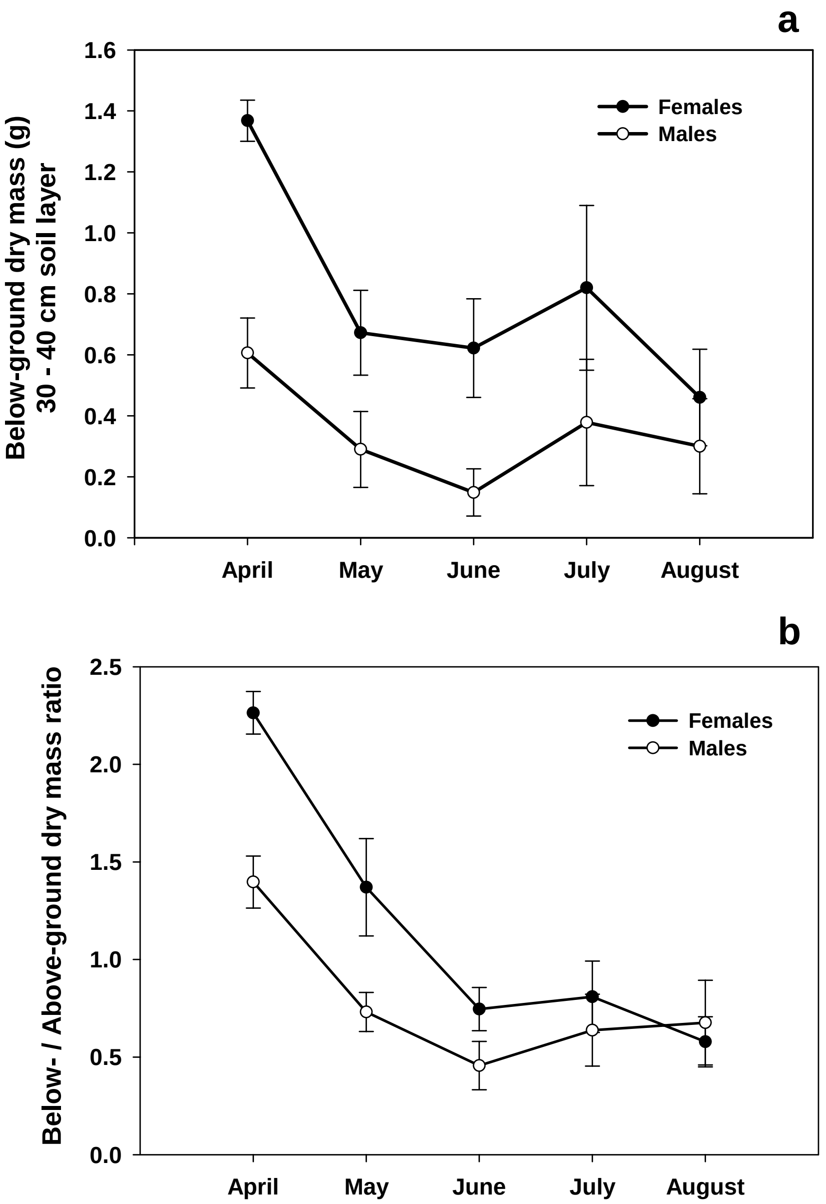


Fig. 4 Temporal variation in below-ground dry mass (a) and below/above-ground dry mass ratios (b) of males and females of *Honckenya peploides*.

Habitat characteristics

ANOVAs carried out on series of monthly data did not detect significant differences in April and May soil water content or bulk density between the habitats of the two sexes, except for the 0-20 cm soil layer in April (P = 0.050), when female habitats had more water available than male habitats in that soil layer. More significant differences emerged as the season progresses and water becomes scarcer. Thus, in June and July, the water content in the firsts 10 and 20 cm soil layer was significantly higher in the habitats of females than in those of males (Table 3; Fig. 5). In August, these significant differences were only maintained in the first 10 cm of soil. The bulk density of the first 30 cm of soil was significantly higher in June in the habitats of males, compared to females, and also in July for the 20-30 cm, 10-40 cm and 20-40 cm soil layers (Table 3; Fig. 5).

According to the results of the MANOVA analysis, soil water content in all the soil layers examined changed significantly with time, and also the soil bulk density for most of the soil layers examined (Table 4). The analysis also showed that throughout the growing season the 0-10 cm and 0-20 cm soil layers in the female habitats had significant greater water content than these same soil layers in the male habitats (Table 4; Fig. 6).

Male and females habitats did not differ in soil characteristics as carbon, carbon/nitrogen ratios or salinity, as estimated by soil electric conductivity (P always greater than 0.355, *d.f.* 1,4).

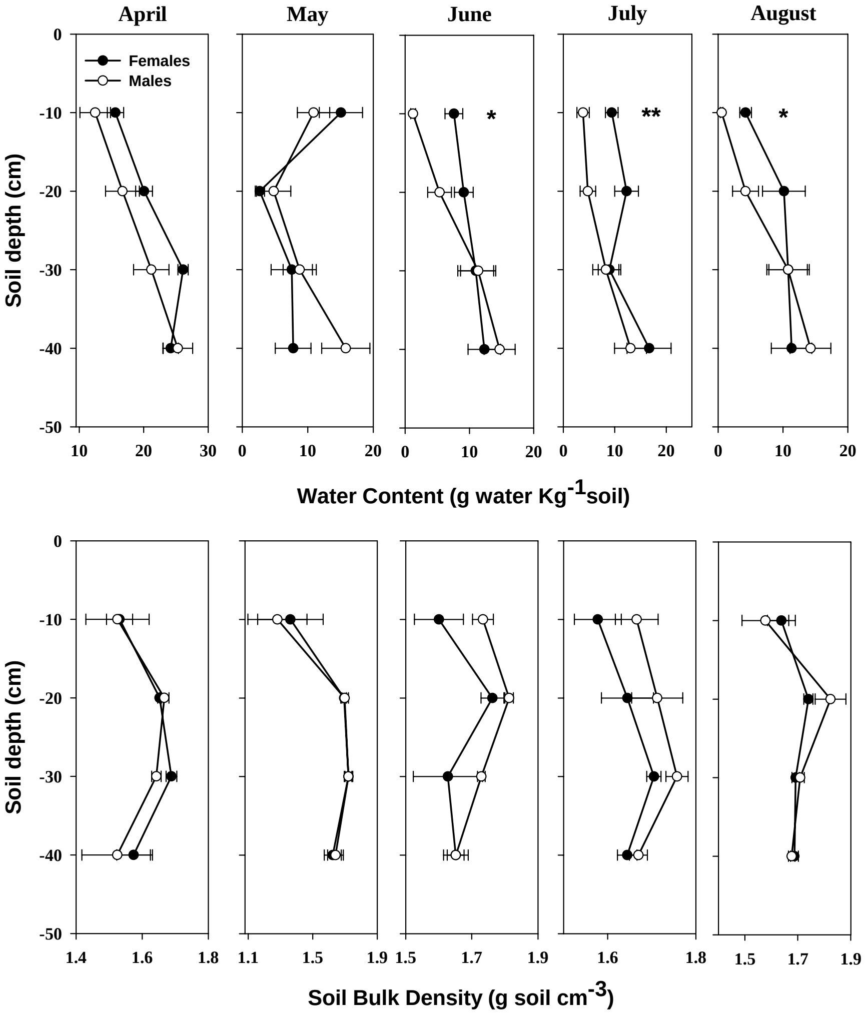
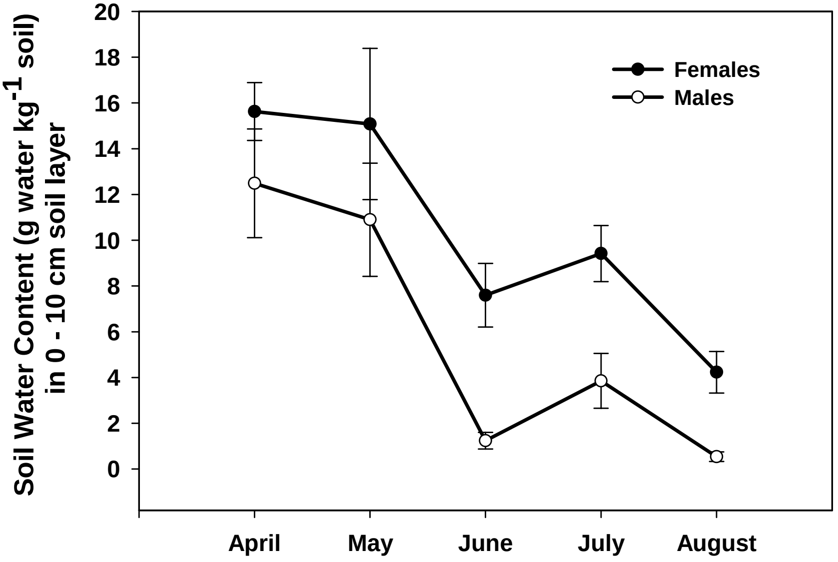


Fig. 5 Vertical distribution profiles from April to August of mean (± SE) soil water content and bulk density in male and female habitats of *Honckenya peploides*.



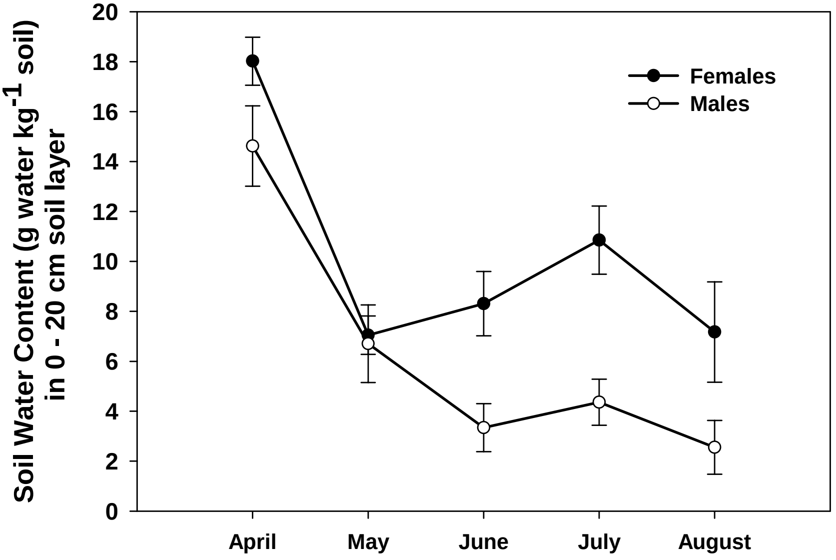


Fig. 6 Temporal variation in soil water content of 0 - 10 cm and 0 - 20 cm soil layers of male and female habitats of *Honckenya peploides.*

Table 1 Sex effects from the Nested ANOVAS on monthly data of biomass and reproductive attributes of *Honckenya peploides* plants. Values of $P < 0.005$ are shown in bold type.

	April			May			June			July			August		
Sex Effects (*d.f.* 1, 4)	MS	F	P	MS	F	P	MS	F	P	MS	F	P	MS	F	P
No. Flowers	1.197	4.091	0.113												
Below dry mass 0-10	0.245	17.704	***0.014***	0.024	0.057	0.822	0.007	0.053	0.829	0.045	0.130	0.736	0.016	0.189	0.686
Below dry mass 10-20	0.022	0.306	0.609	4.853	10.601	***0.031***	0.535	11.458	***0.028***	0.052	0.146	0.721	0.069	0.205	0.674
Below dry mass 20-30	0.154	2.202	0.212	0.217	0.669	0.459	0.397	1.817	0.249	0.003	0.009	0.93	0.496	0.913	0.393
Below dry mass 30-40	1.741	30.623	***0.005***	0.439	4.083	0.113	0.969	15.720	***0.017***	0.447	0.383	0.57	0.076	0.188	0.687
Below dry mass 0-20	0.296	4.884	0.092	4.200	4.258	0.108	1.057	1.436	0.297	0.194	0.340	0.591	0.149	0.393	0.565
Below dry mass 0-30	0.877	5.979	0.071	7.763	5.238	0.084	3.256	1.653	0.268	0.249	0.175	0.697	0.101	0.063	0.814
Below dry mass 0-40	5.089	18.529	***0.013***	11.895	7.964	***0.048***	7.777	2.879	0.165	0.029	0.007	0.939	0.352	0.099	0.769
Below dry mass 10-30	0.294	1.601	0.274	8.643	7.519	0.052	4.381	4.917	0.091	0.082	0.056	0.825	0.196	0.117	0.750
Below dry mass 10-40	3.467	10.887	***0.030***	12.977	11.046	***0.029***	9.469	6.775	0.060	0.146	0.032	0.867	0.515	0.140	0.728
Below dry mass 20-40	2.932	13.111	***0.022***	1.959	2.042	0.226	1.162	3.962	0.117	0.373	0.146	0.722	0.959	0.525	0.509
Above-ground dry mass	0.123	0.784	0.426	0.026	0.021	0.892	2.412	1.402	0.302	1.565	1.433	0.297	0.630	0.745	0.437
Total dry mass	2.482	8.846	***0.041***	10.411	1.815	0.249	0.533	2.702	0.176	0.029	0.129	0.738	2.394	0.284	0.623
Flower dry mass	0.133	7.356	0.053	0.040	0.506	0.516	0.003	0.044	0.844	0.037	0.994	0.375	0.022	0.401	0.561
Mean Flower dry mass	0.000	35.174	***0.004***												
Reproductive dry mass	0.083	6.219	0.067	0.004	0.025	0.883	0.047	0.699	0.450	0.013	0.393	0.565	0.026	0.470	0.531
Below-/Above-ground	2.253	11.951	***0.026***	1.225	32.376	***0.005***	0.421	2.604	0.182	0.031	0.097	0.771	0.050	0.155	0.714
Reproductive Effort	0.007	5.410	0.081	0.002	0.861	0.406	0.000	0.001	0.981	0.000	0.031	0.869	0.001	0.527	0.508

Table 2 Results of the MANOVA analysis for biomass and reproductive traits of *Honckenya peploides.* Values of $P < 0.005$ are shown in bold type.

	Time (*d.f.* 4, 16)			Sex (*d.f.* 1, 4)			Time * Sex (*d.f.* 4, 16)			Plot (Sex) (*d.f.* 4, 54)			Time * Plot (Sex) (*d.f.* 16, 54)			Error (*d.f.* 54)
	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS
Below dry mass 0-10	0.415	3.320	***0.037***	0.018	0.026	0.880	0.07	0.560	0.695	0.682	3.114	***0.022***	0.125	0.571	0.892	0.219
Below dry mass 10-20	2.326	21.339	≤ ***0.001***	2.292	2.428	0.194	1.247	11.440	≤ ***0.001***	0.944	3.266	***0.018***	0.109	0.377	0.983	0.289
Below dry mass 20-30	0.501	1.444	0.265	1.312	1.915	0.239	0.133	0.383	0.818	0.685	2.231	0.078	0.347	1.130	0.353	0.307
Below dry mass 30-40	0.930	2.6271	0.073	3.162	13.3983	***0.022***	0.192	0.5424	0.707	0.236	1.0489	0.391	0.354	1.5733	0.109	0.225
Below dry mass 0-20	3.746	22.703	≤ ***0.001***	1.901	0.931	0.389	1.164	7.055	***0.002***	2.041	4.476	***0.003***	0.165	0.362	0.986	0.456
Below dry mass 0-30	6.918	14.413	≤ ***0.001***	6.372	1.551	0.281	1.807	3.765	***0.024***	4.109	4.461	***0.003***	0.48	0.521	0.924	0.921
Below dry mass 0-40	10.432	7.9573	***0.001***	18.51	3.1720	0.150	2.397	1.8284	0.173	5.836	4.1012	***0.006***	1.311	0.9213	0.551	1.423
Below dry mass 10-30	4.817	10.849	≤ ***0.001***	7.071	2.320	0.202	1.903	4.286	***0.015***	3.048	3.848	***0.008***	0.444	0.561	0.899	0.792
Below dry mass 10-40	7.167	5.4543	***0.006***	19.69	4.3116	0.106	2.338	1.7793	0.178	4.567	3.5736	***0.012***	1.314	1.0282	0.444	1.278
Below dry mass 20-40	2.108	1.9775	0.147	8.548	5.3626	0.082	0.328	0.3077	0.869	1.594	2.1986	0.081	1.066	1.4703	0.146	0.725
Above-ground dry mass	6.806	16.600	≤ ***0.001***	0.011	0.003	0.959	1.582	3.859	***0.022***	3.999	2.930	***0.029***	0.41	0.300	0.995	1.365
Total dry mass	15.079	7.2635	***0.002***	19.42	1.0330	0.367	4.873	2.3473	0.098	18.797	5.7343	***0.001***	2.076	0.6333	0.842	3.278
Flower dry mass	0.177	8.429	***0.001***	0.077	0.535	0.505	0.047	2.238	0.111	0.144	6.857	≤ ***0.001***	0.021	1.000	0.471	0.021
Reproductive dry mass	0.229	6.361	***0.003***	0.008	0.047	0.839	0.05	1.389	0.282	0.172	6.880	≤ ***0.001***	0.036	1.440	0.159	0.025
Below-/Above-ground	2.709	14.1832	≤ ***0.001***	2.144	10.995	***0.029***	0.58	3.037	***0.049***	0.195	0.890	0.476	0.191	0.872	0.602	0.219
Reproductive Effort	0.608	6.1414	***0.003***	0.340	1.339	0.312	0.209	2.111	0.127	0.254	3.681	***0.010***	0.099	1.435	0.161	0.069

Table 3 Sex effects from the Nested ANOVAs on monthly data of soil water content and bulk density of the different soil layers considered. Values of $P < 0.005$ are shown in bold type.

	April			May			June			July			August		
Sex Effects (*d.f.* 1, 4)	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Water content 0-10	29.513	1.177	0.339	52.383	2.554	0.185	202.847	20.133	***0.011***	135.737	49.863	***0.002***	74.180	13.771	***0.021***
Water content 10-20	33.840	0.996	0.375	13.430	0.349	0.586	104.562	2.199	0.212	334.090	4.326	0.106	200.271	2.763	0.172
Water content 20-30	73.235	1.952	0.235	4.423	0.043	0.846	13.591	0.072	0.802	9.813	0.189	0.686	6.831	0.038	0.855
Water content 30-40	3.573	0.093	0.775	193.781	1.720	0.260	2.664	0.026	0.881	156.349	0.570	0.492	14.202	0.067	0.808
Water content 0-20	34.571	7.671	***0.050***	0.352	0.025	0.881	145.313	9.907	***0.035***	223.249	9.692	***0.036***	123.156	4.331	0.106
Water content 0-30	48.826	5.463	0.080	0.041	0.002	0.968	90.880	1.825	0.248	119.276	5.266	0.083	68.202	1.360	0.308
Water content 0-40	23.346	2.392	0.197	14.610	0.406	0.559	47.514	0.797	0.423	130.363	2.656	0.178	28.368	0.387	0.568
Water content 10-30	52.784	1.497	0.288	8.282	0.164	0.706	48.243	0.489	0.523	111.879	2.288	0.205	71.456	0.752	0.435
Water content 10-40	18.400	0.608	0.479	41.533	0.642	0.468	17.614	0.178	0.695	126.996	1.591	0.276	20.263	0.169	0.702
Water content 20-40	11.742	0.363	0.579	61.450	0.578	0.489	1.406	0.010	0.925	61.193	0.710	0.447	0.353	0.002	0.968
Bulk Density 0-10	0.012	0.002	0.963	1.939	0.077	0.795	14.137	2.816	0.169	3.696	3.811	0.123	0.344	0.050	0.834
Bulk Density 10-20	0.060	0.408	0.558	0.002	0.011	0.923	0.407	0.728	0.442	2.009	0.803	0.421	2.067	0.907	0.395
Bulk Density 20-30	0.621	3.208	0.148	0.000	0.000	0.987	3.562	0.861	0.406	1.469	17.632	***0.014***	0.233	0.592	0.485
Bulk Density 30-40	0.735	0.181	0.692	0.088	0.045	0.843	0.000	0.000	0.985	0.280	0.654	0.464	0.003	0.007	0.938
Bulk Density 0-20	0.006	0.006	0.941	0.402	0.060	0.819	4.835	7.395	0.053	2.789	2.114	0.220	0.181	0.284	0.622
Bulk Density 0-30	0.049	0.099	0.769	0.176	0.054	0.828	4.389	12.493	***0.024***	2.302	3.617	0.130	0.198	0.571	0.492
Bulk Density 0-40	0.148	0.292	0.617	0.056	0.021	0.892	2.483	5.815	0.073	1.613	6.538	0.063	0.102	0.423	0.551
Bulk Density 10-30	0.074	0.504	0.517	0.000	0.001	0.972	1.594	1.150	0.344	1.728	2.517	0.188	0.922	0.935	0.388
Bulk Density 10-40	0.218	0.270	0.631	0.012	0.026	0.879	0.719	0.741	0.438	1.108	7.980	***0.048***	0.387	0.547	0.501
Bulk Density 20-40	0.677	0.504	0.517	0.020	0.024	0.885	0.908	0.486	0.524	0.758	8.024	***0.047***	0.046	0.126	0.741

Table 4 Results of the MANOVA analysis for soil water content and bulk density of the different soil layers considered. Values of $P < 0.005$ are shown in bold type.

	Time (*df* 4, 16)			Sex (*df* 1, 4)			Time * Sex (*df* 4, 16)			Plot (Sex) (*df* 4, 54)			Time * Plot(Sex) (*d.f.* 16, 54)			Error (*d.f.* 54)
	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS
Water Content 0-10 cm	362.142	29.9217	< ***0.001***	417.058	19.842	***0.011***	7.610	0.629	0.649	21.019	1.134	0.350	12.103	0.653	0.825	18.539
Water Content 10-20 cm	363.714	10.014	< ***0.001***	341.890	2.920	0.163	57.750	1.590	0.225	117.096	3.308	***0.017***	36.322	1.026	0.445	35.393
Water Content 20-30 cm	506.278	13.028	< ***0.001***	50.101	0.144	0.723	14.502	0.373	0.824	347.428	7.128	< ***0.001***	38.862	0.797	0.682	48.741
Water Content 30-40 cm	329.811	5.636	***0.005***	25.055	0.052	0.831	90.697	1.550	0.235	480.893	7.955	< ***0.001***	58.516	0.968	0.503	60.455
Water Content 0-20 cm	257.288	19.820	< ***0.001***	352.999	12.909	***0.023***	23.098	1.779	0.182	27.346	1.838	0.135	12.981	0.873	0.602	14.876
Water Content 0-30 cm	312.038	27.551	< ***0.001***	228.450	2.419	0.195	14.118	1.247	0.331	94.428	4.700	***0.003***	11.326	0.564	0.897	20.093
Water Content 0-40 cm	308.946	26.027	< ***0.001***	103.113	0.634	0.471	26.530	2.235	0.111	162.761	6.886	< ***0.001***	11.870	0.502	0.935	23.635
Water Content 10-30 cm	411.317	18.059	< ***0.001***	163.800	0.774	0.429	22.755	0.999	0.437	211.741	6.170	< ***0.001***	22.776	0.664	0.816	34.318
Water Content 10-40 cm	371.749	18.217	< ***0.001***	50.044	0.174	0.698	37.075	1.817	0.175	287.149	8.237	< ***0.001***	20.407	0.585	0.881	34.862
Water Content 20-40 cm	381.718	14.345	< ***0.001***	1.510	0.004	0.954	32.700	1.229	0.338	409.937	8.935	< ***0.001***	26.609	0.580	0.885	45.880
Bulk Density 0-10 cm	0.236	3.688	***0.026***	0.018	0.083	0.788	0.042	0.656	0.631	0.217	4.094	***0.006***	0.064	1.208	0.293	0.053
Bulk Density 10-20 cm	0.050	4.545	***0.012***	0.023	2.875	0.165	0.003	0.273	0.891	0.008	0.500	0.736	0.011	0.688	0.793	0.016
Bulk Density 20-30 cm	0.011	1.100	0.390	0.012	0.923	0.391	0.010	1.000	0.436	0.013	0.722	0.581	0.010	0.556	0.902	0.018
Bulk Density 30-40 cm	0.031	2.583	0.077	< 0.001	0.001	0.974	0.003	0.250	0.905	0.034	3.400	***0.015***	0.012	1.200	0.298	0.010
Bulk Density 0-20 cm	0.090	6.429	***0.003***	0.020	0.345	0.589	0.012	0.857	0.510	0.058	3.625	***0.011***	0.014	0.875	0.599	0.016
Bulk Density 0-30 cm	0.034	4.857	***0.009***	0.018	0.600	0.482	0.010	1.429	0.270	0.030	2.727	***0.039***	0.007	0.636	0.840	0.011
Bulk Density 0-40 cm	0.029	5.800	***0.004***	0.010	0.370	0.576	0.007	1.400	0.279	0.027	3.375	***0.015***	0.005	0.625	0.850	0.008
Bulk Density 10-30 cm	0.011	1.571	0.230	0.016	2.667	0.178	0.004	0.571	0.687	0.006	0.667	0.618	0.007	0.778	0.702	0.009
Bulk Density 10-40 cm	0.016	3.200	***0.041***	0.007	0.583	0.488	0.003	0.600	0.668	0.012	2.000	0.108	0.005	0.833	0.644	0.006
Bulk Density 20-40 cm	0.015	2.500	0.084	0.002	0.100	0.768	0.005	0.833	0.524	0.020	2.222	0.079	0.006	0.667	0.813	0.009

DISCUSSION

The results of the analyses comparing the amount of resources allocated to growth and reproduction in male and females plants of *H. peploides* and the extent to which sexes differed in their timing of allocation support the hypothesis of intersexual differences in the seasonal patterns of biomass allocation.

Vegetative Allocation

Differences in resource allocation patterns between the sexes of dimorphic species have been documented in many studies, from grasses to trees (see Dawson & Ehleringer 1993 for references). This was expected since the sexes of dioecious plants play different roles in reproductive biology and consequently experienced different demand of resources. However, a lesser amount of studies have considered seasonal patterns of biomass distribution (Gross & Soulé 1981; Ågren 1988; Eckhart & Seger 1999; Zunzunegui *et al.* 2006). An original result of our study is that differences in the total amount and proportion of dry mass that sexes allocated to below-ground structures followed a clear seasonal pattern, which was more marked for female plants. Early in the growing season, females allocated to below-ground growth a significantly greater proportion of their biomass than did males, what brought about significant differences between the sexes in total biomass. At that time, males allocated to sexual reproduction a greater proportion of their biomass than did females, what probably constrained the mass that males allocated to below-ground growth. The greater dry mass that females allocated to below-ground structures at the early growing season is likely to give them a competitive advantage through a greater ability to capture limiting resources and to cope with stresses, relative to males. The differences in below-ground allocation between males and females narrowed gradually as the season progresses, until disappear at the end of the growing season. The decrease in female below-ground mass is somewhat surprising, considering that a larger proportion of assimilates allocated to root production may be particularly significant in maintaining an adequate water balance (Aronson *et al.* 1992; Retuerto & Woodward 1993; Fitter & Hay 2002). Although we know no field studies examining seasonal changes in below-ground allocation in dimorphic or polymorphic plants, some authors have reported results consistent with our findings. Escarrré *et al.* (1990) reported decreased root/shoot ratios in water-stressed females as compared to well-watered conditions. Similarly, Cibils *et al.* (2005) found that females of *Atriplex canescens* produced the least amount of shoots, compared to males, when soil moisture was adequate, and the least amount of roots when soil moisture was deficient. These authors suggested that females were constrained by their relative inefficient use of water and were unable to produce sufficient photosynthates to sustain an adequate root system.

Since the energetic requirements for producing fruits are generally greater than for flower production, and the maturation of fruits usually extends for a considerable period after flowering, females must continue to expend resources longer than do males. For these reasons, many studies have found higher vegetative growth in males of dioecious plants at the end of the growing season, which is consistent with the existence of a trade-off investment between reproductive and vegetative biomass (Herrera 1988; Vasiliauskas & Aarsen 1992; Gibson & Menges 1994; Watson 1995; Díaz Barradas & Correia 1999; Hemborg & Karlsson 1999). However, we have not found the predicted intersexual differences in vegetative growth: female growth was not reduced relative to males. One plausible explanation is that in our study females did not increase their proportional contribution in dry mass to reproductive structures at the end of the season. Consequently, the lower female investment to below-ground growth at this stage, relative to that at the early growing season, can not be directly interpreted as a reproductive cost.

Reproductive allocation

Several studies have shown that the female reproductive cost exceed that of the males's to the detriment of vegetative growth, provided that certain threshold of fruit set is achieved (Wallace & Rundel 1979; Gross & Soulé 1981; Ågren 1988; Allen & Antos 1988; Popp & Reinartz 1988). Although this threshold is generally exceeded in natural populations some of these studies also suggested that possible fruit set failure could result in interyear variation in the female reproductive effort. In line with these findings, our results suggest that females may have had a lower fruit set than in a normal year. Nicotra (1999) has noted that differences in reproductive allocation may be sufficient to set up a selective environment favouring the evolution of sexual differentiation even if female reproductive allocation only exceeds that of males on an individual episodic basis.

Although we did not detect significant intersexual differences in reproductive allocation when examined at a single point in time, our results showed some temporal trend. Early in the season, differences between male and female reproductive effort, estimated as the amount of biomass allocated to the male flower and the female flower/fruit, respectively, were marginally significant, with a greater reproductive effort in males. As we noted above, this greater reproductive allocation in males could constrain the mass that males allocated to below-ground growth at the early season. At that time, females allocated less biomass to flowers than males, although differences were only marginally significant, and produced less and significantly smaller flowers than males. At the end of the growing season, there were no intersexual differences in reproductive traits. In dioecious plants, females typically have been found to have a higher investment in reproduction than males over an entire growing season (Lloyd & Webb 1977; Gross & Soulé 1981; Willson 1986; Popp & Reinartz 1988). Leigh *et al*. (2006) recorded 44 studies that documented reproductive effort in dimorphic species and found that in no case female reproductive effort was lower than that of male, although, unexpectedly, in their study, they found that males of *Gynatrix pulchella* allocated significantly more resources to reproduction than females. However, males frequently invest more resources than females in flowers alone, sometimes at the expense of vegetative growth (Ågren 1988; Popp & Reinartz 1988; Pendleton *et al*. 1992). For instance, Ramp & Stephenson (1988) found that female reproductive allocation was ¼ of male's at anthesis, and similar differences have been reported for *Simmondsia chinensis* (Wallace & Rundel 1979) and for *Silene alba* (Gross & Soulé 1981). Nicotra (1999) has suggested that lifetime reproductive allocation may be similar for the sexes, since selection to maximize fitness should lead to maximal possible allocation to reproduction in both sexes. As reported in other dioecious species (Lloyd & Webb 1977; Stephenson & Bertin 1983; Bell 1985), in addition to more flowers, males of *H. peploides* also produced larger flowers than females. One explanation for the evolution of this sexual difference in the allocation of biomass of dioecious plants is that intrasexual competition for mates is likely to be more intense among males than among females, which may potentially explain a greater male investment in floral display (Bawa 1980; Stephenson & Bertin 1983). Furthermore, if reproductive functions were more seriously affected by flower herbivory in females than in males (Cox 1982), a reduced floral display in females may be an evolved response to selection (Ågren 1988).

There is a wealth of evidence that support the hypothesis that temporal displacement of male and female allocation may increase the effective cost (an opportunity cost) of male function because they invest in reproductive structures (pollen and flowers) early in the flowering season (Popp & Reinartz 1988; Delph 1990; Eckhart & Seger 1999; Case & Ashman 2005). Supporting this view, Eckhart & Chapin (1997) found that the opportunity costs of male function were more pronounced under nutrient-limited conditions, as those prevailing in our dune system. Some authors have suggested that an early investment of nutrient in reproduction diverts them away from investment in photosynthetic machinery that might otherwise contribute to increased growth, confirming that the timing of resource investment in reproduction may be as, or more, important than the amount invested (Gross & Soulé 1981; Eckhart & Seger 1999; Case & Ashman 2005). Our results showed that the higher reproductive cost incurred by males in April was measurable as a lower below-ground growth. In our dune system, where nutrient and water availability are more limiting for plant growth than radiation or CO_2, the lower allocation to below-ground structures may be more limiting for subsequent growth than a lower investment in photosynthetic machinery.

Differences in habitat characteristics

Our study revealed higher water content in the two shallowest soil layers of the habitats of females when compared to those of males. Therefore, our findings are in line with previous studies reporting that males dominate in the poorest quality sites and females in the less stress-prone habitats (Bierzychudek & Eckhart 1988; Dawson & Bliss 1989; Dawson & Ehleringer 1999). In 17 of 18 instances referred by Bierzychudek & Eckhart (1988), females were more common under less stressful conditions, sites that were moister, less exposed, less saline, or at higher nutrient concentrations. In our study, differences between male and female habitats appeared as the season progresses, at the more stressful time, when climatic conditions were more adverse for plant growth (higher temperatures, intense radiation and lower precipitation), specially for females because of the higher costs of fruit production. In a spatially heterogeneous environment, as our studied system, female success may be relatively higher on patches where soil is relatively moister during seed and fruit maturation, such as previous studies have found among a variety of dioecious species (Freeman *et al*. 1976; Dawson & Ehleringer 1993). Dawson & Ehleringer (1993) stated that in the absence of gender differences in physiology (and in other traits) we could account for male-biased sex ratios in dry sites, but could not explain female-biased sex ratios in wet sites. In addition to physiology, differences in allocation can influence overall performance and ultimately affect habitat distribution of male and female plants in relation to environmental gradients. Our study suggests that differences in allocation might explain the spatial segregation of the sexes. Males are likely to meet their reproductive costs in both wet and dry sites, but they would be at a considerable disadvantage relative to females at the early growing season. At that time, when males are making a greater investment to reproduction than females, at the expense of below-ground growth, females out-competed males through a greater allocation to below-ground structures, what determine a higher ability to capture limiting resources. On the other hand, males out-competed, and potentially exclude females from the drier sites because the most stressful water restrictions occurred when females are maturing fruits and seeds and are investing less mass to below-ground growth. In addition, it has been argued that males may actually gain higher fitness in the drier sites because pollen can move much greater distances (Dawson & Bliss 1989; Dawson & Ehleringer 1993). Bierzychudek & Eckhart (1988) have suggested that differences in the reproductive biology of the two sexes, rather than competition between them can be the source of a differential mortality of males and females in different environmental patches what would cause spatial segregation of the sexes. These authors stated that spatial segregation of the sexes would be favoured only if the deleterious effects of competition between males and females were more severe than those of competition between individuals of the same sex. According to these authors, competition between the sexes seems a priory an unlikely ultimate cause of spatial segregation, given the ecological similarity between individuals of the same sex. They referred some studies demonstrating greater interference between plants of the same sex than between plants of opposite sex (Putwain & Harper 1972; Wade 1981). Our study, however, did not dismiss the hypothesis that competition may be important to explain why males and females are spatially segregated. Other studies have pointed out that intraspecific competition between the sexes is the selective force for spatial segregation of the sexes (Freeman *et al*. 1976, 1980; Cox 1981). In support of our interpretation, other studies have suggested that noticeable differences in allocation can lead to increased spatial separation of the sexes along environmental gradients, or over different microhabitats (Korpelainen 1992; Dawson & Ehleringer 1993)

In conclusion, our study has shown contrasting patterns of resource allocation in male and female plants of *H. peploides*, what reveals the necessity of making dynamic estimations of reproductive and biomass allocation if we really want to know the real cost of the male and female functions in dimorphic or polymorphic species. The study demonstrated that reproductive effort is not always higher in females than in males in dioecious species. The seasonal timing of resource investment in flowering and in fruit set may cause male reproductive effort to be as, or even more, costly than that of females. The study also suggest that between-sexes differences in the seasonal patterns of reproductive and biomass allocation may play an important role in explaining the spatial segregation of the sexes.

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Chapter 4

4

Seasonal variations in carbon isotope discrimination and leaf nitrogen content in males and females of the subdioecious plant *Honckenya peploides* (L.) Ehrh.

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ABSTRACT

Males and females of dimorphic plants may often differ in ecolophysiological traits and show spatial segregation. These differences have been interpreted as an evolved response of the sexes to meet their specific resource demands associated with reproduction. *H. peploides* is a subdioecious plant growing in coastal dunes, with two distinguishable sexual morphs that are spatially segregated. Since flowering and fruiting patterns change along the time of the season, we expected that sex-specific patterns of water use efficiency depended on time. This study explores if seasonal variations in the water use efficiency of males and females might explain the spatial segregation of the sexes observed in this species. At four locations of the coast of Galicia, water use efficiency, as inferred from carbon isotope discrimination (Δ^{13}C), and carbon and leaf nitrogen content of males and females were measured in April, June and August 2005. Sexes did not differ in Δ^{13}C or leaf nutrient content, but there were some significant intersexual differences in the relationships between Δ^{13}C and foliar nitrogen content. Soil water content decreased as the season progresses more steeply in male than in female habitats. We concluded that the lack of sex-related differences in integrated water use efficiency and leaf nitrogen content found here, make it difficult to establish a physiological basis for the spatial segregation of the sexes. We suggest that males and females through a selection of habitats might meet with their different demand of resources for reproduction, with each sex achieving the best performance in the habitat in which it predominates.

Keywords: carbon isotope discrimination, *Honckenya peploides*, subdioecy, leaf nitrogen content.

INTRODUCTION

Females of dioecious plants usually expend more resources in reproduction than males because they produce fruits in addition to flowers (Wallace & Rundel 1979; Correia *et al*. 1992; Antos & Allen 1994; Jonasson *et al*. 1997; Obeso 1997; Hogan *et al*. 1998). It has been postulated that to meet the specific demand of resources associated with reproduction, males and females may have evolved specific morphological and ecophysiological traits (Cox 1981; Dawson & Bliss 1989; Dawson & Ehleringer 1993; Geber *et al*. 1999 and references therein). Several authors have also stated that the spatial segregation of the sexes exhibit by a significant number of dioecious species may be the result of adaptations to the different reproductive demands of males and females (Freeman *et al*. 1976; Bierzychudek & Eckhart 1988; Dawson & Geber 1999). In support of this assertion, field evidence from a number of species has shown that along gradients of resource availability the most commonly observed pattern of segregation is females occurring at high resource sites, and males predominating at low resource sites (Bierzychudek & Eckhart 1988; Dawson & Bliss 1989; Dawson & Ehleringer 1993). Intersexual differences in physiology may play a crucial role in explaining this pattern since, as pointed out by some authors, sexual differences in resource demands might lead to greater physiological stress in the sex with the higher reproductive investment (Dawson & Bliss 1989; Gehring & Monson 1994;

Laporte & Delph 1996). Variation in physiological attributes may, by itself or in combination with other factors as biomass allocation, determine the performance of each sex in different microhabitats, and consequently, influence habitat distribution of male and female plants in relation to environmental gradients (Dawson & Bliss 1989; Dawson & Ehleringer 1993; Retuerto *et al.* 2000). Although the literature on physiological aspects of sexual dimorphism in dimorphic or polymorphic plants is rapidly increasing (see Dawson & Geber 1999; Case & Ashman 2005) there is still an important gap in our knowledge because of the difficulties in gathering data on ecophysiological processes. Most of the studies on gender specialization in physiology have considered instantaneous rather than integrated measurements of physiological traits, in spite of being less informative. A long-term integrator of ecophysiological processes such as leaf conductance, hydraulic capacity, potential water-use efficiency and photosynthetic capacity is the $^{13}C/^{12}C$ discrimination ($\Delta^{13}C$) (Ehleringer *et al.* 1993; Farquhar *et al.* 1989; Körner *et al.* 1988). For example, $\Delta^{13}C$, has been used to compare integrated water use efficiency between males and females of dioecious species (Dawson & Bliss 1989; Dawson & Ehleringer 1993; Retuerto *et al.* 2000). Carbon isotope discrimination has also been negatively related to leaf nitrogen, due to the high photosynthetic demand and steep carbon gradients between the leaf and the atmosphere at high leaf N content (Farquhar *et al.* 1989; Sparks & Ehleringer 1997). However, other studies have found no correlation (Damesin *et al.* 1997) or context-dependent relationships (Retuerto *et al.* 2000).

Most of the available data on sex differences in physiology comes from dioecious species (see Dawson & Geber 1999 for a review). There is a lack of research on intermediate sexual systems, where the scale and history of differentiation may be reduced. Studies filling this gap will provide clues as to the origins and selective pressures favouring sex-specific physiology. Here, we report the results of a study on some aspects of the physiological ecology of the subdioecious dune plant *Honckenya peploides*. The sexual system of *H. peploides*, known as subdioecy, is characterized by the presence of females, and hermaphrodites strongly biased toward male function (denoted as males). This sexual system has been interpreted as a transitory stage, near to dioecy, along a pathway between cosexuality and dioecy (Delph & Wolf 2005). *H. peploides* exhibits in the localities of the study an extreme spatial segregation of the sexes, with males and females forming unisexual clumps of plants clearly separated. In this study, we aimed to find some physiological explanation for habitat partitioning between the sexes. Specifically, we explored if the sexes of *H. peploides*, growing under natural conditions, in a context of spatial segregation, differed in terms of seasonal patterns of integrated water-used efficiency, as estimated by carbon isotope discrimination, $\Delta^{13}C$. Since flowering and fruiting patterns change along the time of the season, we expected that sex-specific patterns of water use efficiency depended on time. Seasonal changes in carbon isotope discrimination have been documented in species of arid environments (Winter *et al*. 1978). Assuming previous results on this species (*unpublished results*) showing that female habitats were moister than those of males, we hypothesized smaller carbon isotope discrimination or higher water use efficiency in males than in females. In addition, we explored the relationships between stable carbon isotope discrimination ($\Delta^{13}C$) and leaf nitrogen in the sexes of *H. peploides*.

MATERIAL AND METHODS

Study species

Sea sandwort, *Honckenya peploides* (L) Ehrh (Caryophyllaceae), is a subdioecious perennial plant with a circumpolar distribution (from temperate to arctic zones; also anthropochorous in South America). On the Iberian

Peninsula, H. peploides extends from the Atlantic coast of northern Portugal and northwards and eastwards along the Bay of Biscay (Cantabrian Sea) to France. It is a hemicryptophyte regrowing each spring from long rhizomes that produce compact groups of aerial shoots, forming vegetative clumps or mats. These clumps are typically found on the upper beach, forming small mounds called embryo dunes. Plants reproduce sexually by seed or clonally by rhizomes. This species is an early colonizer, contributing to stabilization and anchorage of the soil and facilitating the establishment of other species (Houle 1997; Gagné and Houle 2001). Flowers are axillary and solitary, and/or in 1- to 6-flowered terminal cymes, strongly honey-scented. Two types of flower can be found in H. peploides, as reported for the subspecies major by Tsukui and Sugawara (1992): one type ("pistillate") has long styles, short petals and non-functional anthers, while the other ("staminate") has short styles, long petals and long stamens that produce pollen grains. This latter type rarely produces seeds, and when it does it the number of seeds is very low compared to female flowers. Both types of flower have nectaries at the base of the stamens, which attract pollinators. In line with Tsukui and Sugawara (1992), and following Lloyd (1976) and Delph (1990), we will here refer to plants with pistillate flowers as females and to plants with staminate flowers as males.

Study sites

Sampling was carried out on April, June and August 2005, at four locations on the coast of Galicia (Fig. 1): San Román (43º43'N, 7º37'W), Lariño (42º45'N, 9º6'W), Esteiro (42º47'N, 8º58'W) and O Bao (42º32'N, 8º51'W). At each location, we randomly collected male and female shoots from segregated clumps of plants. Individuals from the four locations were sexed on the basis of their floral morphology, which was consistent in the two previous years. On April, we sampled individuals bearing reproductive structures and individuals non reproductively active. On June and August, most of the individuals were reproductive, so we only sampled reproductive individuals.

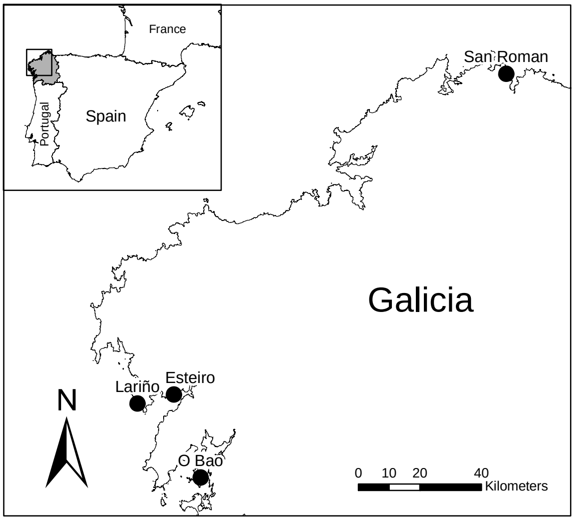

Fig. 1 Location of Galicia in Norhwest Spain and location of the sampling sites.

C:N and isotope analyses

Samples of about 3 mg were dried at 50ºC for over 5 days and finely ground. C and N concentrations and the molar $^{13}C/^{14}C$ ratio were measured using and elemental analyzer (FlashEA 1112 Series) coupled with an isotope ratio mass spectrometer (ThermoFinnigan MAT253, Bremen, Germany). The carbon isotope composition ($\delta^{13}C$) of tissues is expressed relative to the composition of the Pee Dee Belemnite (PDB) standard, using the following ratio: ($\delta^{13}C=[(R_{sam}/R_{std})-1]\times1000$, were R refers to the ratio of $^{13}C/^{12}C$ in the plant sample (sam) and standard (std), respectively. This ratio is expressed in parts per mil (‰). Values of $\delta^{13}C$ were converted into carbon isotope discrimination, $\Delta^{13}C$, using the expression $\Delta^{13}C = (\delta^{13}C_{air} - \delta^{13}C_{plant})/(1 + \delta^{13}C_{plant})$ (Farquhar *et al.* 1989), assuming a $\delta^{13}C$ air value of – 8.0 ‰ on the PDB scale.

Soil analysis

At two of the locations (Lariño and San Román), we took soil samples at each sampling date. Soil samples were collected in the early morning at three different points (ca. 15 cm in depth) in each of four clumps (one male and one female per each location). These soil samples were weighed before and after drying to constant weight at 75ºC in order to estimate soil water content (g water kg^{-1} dry soil).

Soil nitrogen content was measured on soil samples collected in June. Three subsamples of about 2 mg from each sample were dried to a constant weight at 75ºC, sieved to < 2 mm and analyzed for N content via dry combustion in a CHN elemental analyzer (LECO CHNS-932).

Data analysis

Variation in $\Delta^{13}C$ and leaf nutrient content was examined by analysis of variance (ANOVA) using the general linear model (GLM) procedure of Systat (SYSTAT 11, Evanston, Illinois). We performed a repeated measure GLM design with sex (male and female) and time (April, June and August) as fixed factors and location (Lariño, O Bao, San Román and Esteiro) as a random factor nested within sex. For the first date, April, we also performed a GLM design with sex and reproductive status (reproductive and nonreproductive) as fixed factors and location as a random factor nested within sex.

Standardized major axis (SMA) regression was used to estimate the significance of the relationships between $\Delta^{13}C$ and leaf N content for each sex at each sampling date. Correlations coefficients and SMA slopes were calculated using a DOS-based computer package, SMATR (Warton *et al.* 2006). In this program, heterogeneity between SMA slopes is tested via a permutation test.

RESULTS

Carbon isotope discrimination

Carbon isotope discrimination was not significantly affected by the sex of the plants, by the reproductive status or by their interaction. Time and the second order interaction Time × Sex had neither a significant effect. However, we detected significant spatial variation, as indicated by the Location(Sex) and Time × Location(Sex) effects (Table 1 and Table 2).

Carbon to nitrogen ratio and foliar nitrogen content

The carbon to nitrogen ratio and foliar nitrogen content were significantly affected by the reproductive status of the plants (Table 1). Reproductive individuals showed higher C:N ratio and lower foliar N content than nonreproductive plants (Fig. 2). Both parameters also differed significantly with time, increasing the C: N ratio and decreasing the foliar N content (Fig. 3) as growth season progresses. Sex, and the second order interactions, Sex × Reproductive status, and Time × Sex, had no effects on C:N ratio or foliar N content. Significant spatial variation was also detected (Table 1 and Table 2; Location(Sex) and Time × Location(Sex)).

Table 1 Results of general linear model of carbon isotope discrimination ($\Delta^{13}C$), carbon to nitrogen ratio (C:N) and leaf N (%DW) for male and female plants of *Honckenya peploides* examined at different reproductive status (Rep). *Site(Sex)* was the error term for *Sex*, and *Rep × Site(Sex)* was the error term for *Rep* and *Rep × Sex.*

		$\Delta^{13}C$			C:N			N (% DW)		
Source	*df*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Sex	1	14.554	0.242	0.642	31.394	0.647	0.452	4.412	0.866	0.388
Site (Sex)	6	60.112	5.183	< **0.001**	48.525	37.332	< **0.001**	5.095	25.952	< **0.001**
Rep	1	19.743	0.917	0.375	42.254	16.801	**0.006**	7.821	22.156	**0.003**
Rep × Sex	1	13.277	0.617	0.462	3.542	1.408	0.280	0.414	1.173	0.320
Rep×Site(Sex)	6	21.520	1.855	0.090	2.515	1.935	0.077	0.353	1.800	0.101
Error	208	11.599			1.300			0.196		

Table 2 Results of repeated-measures analysis of carbon isotope discrimination ($\Delta^{13}C$), carbon to nitrogen ratio (C:N) and leaf N (%DW) for male and female plants of *Honckenya peploides*. *Site(Sex)* was the error term for *Sex*, and *Time × Site(Sex)* was the error term for *Time* and *Time × Sex.*

		$\Delta^{13}C$			C:N			N (%DW)		
Source	*df*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Sex	1	1.31	0.023	0.884	65.88	0.078	0.789	0.08	0.014	0.910
Site (Sex)	6	57.66	5.749	< **0.001**	844.20	18.218	< **0.001**	5.87	20.321	< **0.001**
Time	2	18.07	1.321	0.303	9792.35	23.625	< **0.001**	134.20	60.290	< **0.001**
Time × Sex	2	7.47	0.546	0.593	125.99	0.304	0.743	3.65	1.640	0.235
Time × Site (Sex)	12	13.69	1.364	0.182	414.48	8.945	< **0.001**	2.23	7.711	< **0.001**
Error	312	10.03			46.34			0.29		

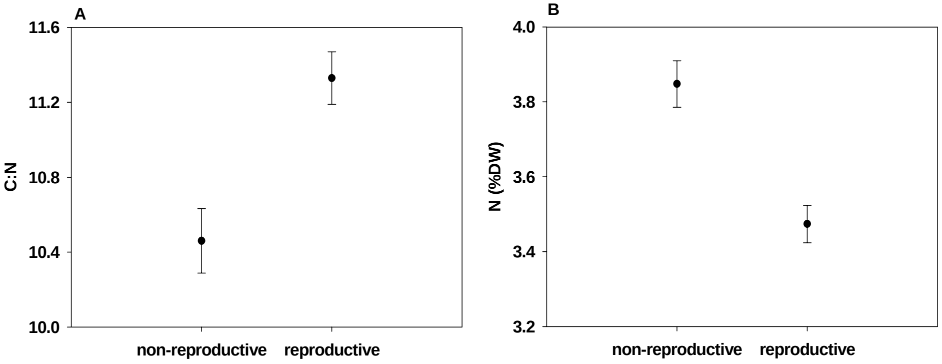

Fig. 2 Mean values (± SE) of carbon to nitrogen ratio (C:N) and leaf nitrogen content (N, %DW) for reproductive and non-reproductive individuals (N = 112).

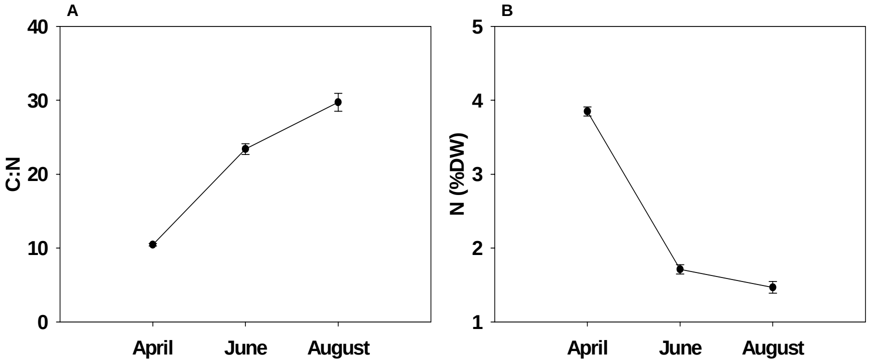

Fig. 3 Temporal patterns of variation for carbon to nitrogen ratio and for leaf nitrogen content (N, %DW). Values are means of 112 plants (±SE).

Relationship between carbon isotope discrimination and foliar N content

At the beginning of the season (April), there was a significant negative relationship between $\Delta^{13}C$ and foliar N content for nonreproductive males. In April and June, no relationship was found between $\Delta^{13}C$ and foliar N for plants bearing reproductive structures, but at the end of the season (August), we found in males a significant correlation between both parameters. On August, there was a significant difference in slope between the two sexes of *H. peploides*. For female plants, there was no relationship between $\Delta^{13}C$ and foliar N content at any time of the season (Fig. 4).

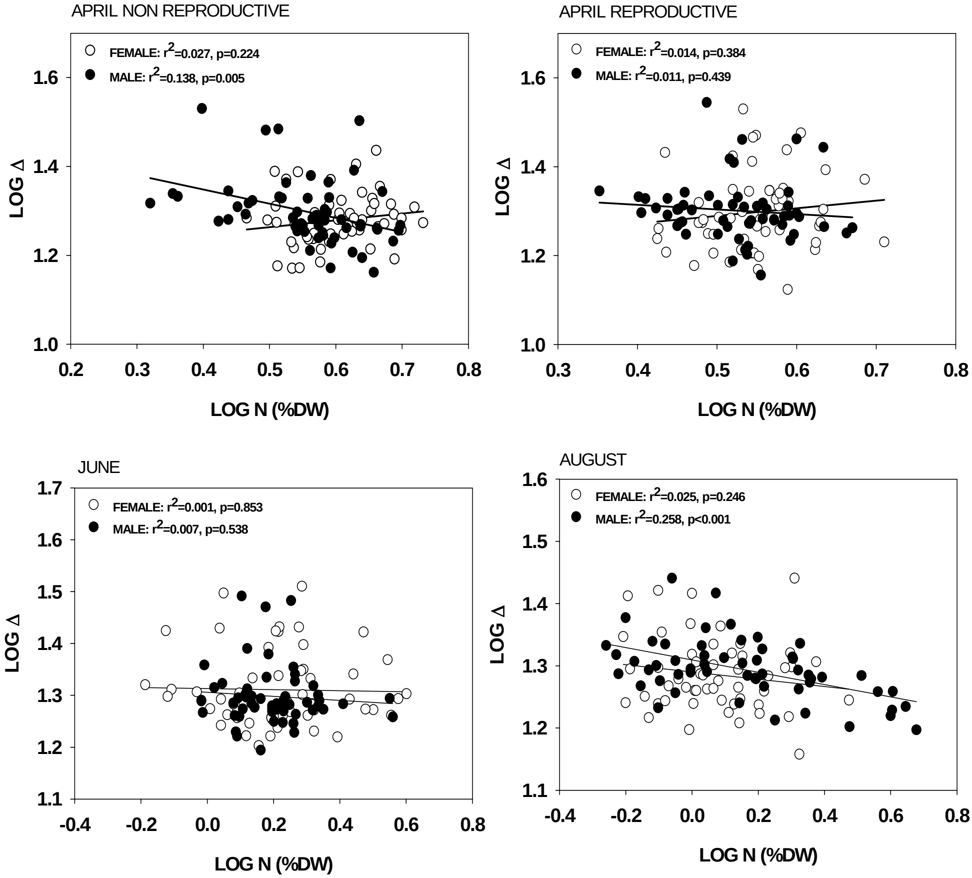

Fig. 4 Relationships between carbon isotope discrimination (Δ) with leaf N (%DW) of male and female plants of *Honckenya peploides* at different reproductive status and at three dates (April, June and August).

Soil analysis

Soil water content decreased as the growth season progresses, a decrease that was significantly more important in males than in females (Time × Sex: $F_{2,4}$ = 16.00, P = 0.012, Fig. 5).

No differences were detected on the soil nitrogen content between male and female clumps ($F_{1,2}$ = 1.12, P = 0.350).

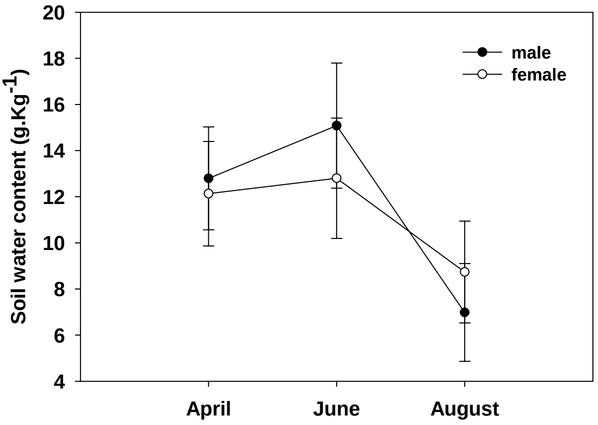

Fig. 5 Soil water content at three sampling dates for male and female clumps (N = 6).

DISCUSSION

In the present study of the subdioecious species *H. peploides* we did not find intersexual differences in the carbon isotope discrimination, which suggest similar time integrated water use efficiencies in both sexes. Gender specific differences in carbon isotope discrimination have been found in several dioecious species, although there is no a general pattern in these differences. Males may have lower isotope discrimination rates than females in certain species (Dawson & Ehleringer 1993; Marshall *et al*. 1993; Ward *et al*. 2002), higher in others (Dawson & Bliss 1989; Jones *et al*. 1999), or may not differ from females (Kohorn *et al*. 1994; Leigh & Nicotra 2003). Furthermore, males and females may show environmental context dependent differences in carbon discrimination (Retuerto *et al*. 2000). Studies in other sexually dimorphic, non-dioecious, species involving carbon isotope discrimination are scarce. Case & Barret (2001) found higher carbon isotope discrimination in cosexuals than in unisexuals (especially males) of *Wurmbea dioica*. However, Verdú (2004) found that males of the androdioecious *Fraxinus ornus* showed higher carbon isotope discrimination than hermaphrodites. In intermediate sexual systems, sex morphs may be more similar to each other if gender dimorphism has only recently evolved and there has been little time and opportunity for selection (Case & Ashman 2005).

Sex specific differences in carbon isotope discrimination are associated with soil moisture, which may change across space (dry or wet habitats) or along time. For example, Dawson & Ehleringer (1993) observed that the greatest differences in leaf $\Delta^{13}C$ between male and female *Acer negundo* plants were on dry, not wet, habitats. This context-dependent response of the sexes is relevant in order to explain the maintenance of habitat-specific sex ratio biases in dimorphic species. In this way, the spatial segregation of the sexes observed in some dioecious species has been explained by gender-specific differences in physiological attributes, particularly, carbon isotope discrimination (Dawson & Bliss 1989; Dawson &

Ehleringer 1993). In the present study, no significant differences were detected in soil water content of males and females habitats, although, late in the season, we observed a higher significant decrease in soil moisture in male habitats than in those of females.

In addition to the relevance of the environmental context, the patterns of differences between sex-morphs in water use efficiency may be affected by growth stage, changing during flowering and fruiting (Case & Ashman 2005). However, we did not find any difference in the seasonal patterns of carbon isotope discrimination of male and female plants. Differences between sex-morphs may be influenced by the time scale for the integration of leaf Δ^{13}C. Leigh & Nicotra (2003) have reported that the greater reproductive allocation found in females of *Maireana pyramidata* had an immediate impact on their capacity for conservative water use (low instantaneous water use efficiency, photosynthesis/transpiration) but did not lead to long-term differences in water use efficiency (time-integrated water use efficiency, Δ^{13}C). These authors suggested that females have specialized in overcoming the demands of reproduction and water stress over the lifetime of the plant. Other authors, as Case & Ashman (2005), have suggested that for some species, sex-specific life history strategies or spatial segregation may buffer changes in the physiological environment of the plants, maintaining homeostasis within photosynthetic organs, in such a way that no physiological dimorphism should be expected.

The leaf N content was lower for reproductive individuals (irrespective of the sex), suggesting a translocation from vegetative to reproductive structures (Karlsson 1994; Saulnier & Reekie 1995; Poot *et al.* 1996). Costs of reproduction at the beginning of the season (April) are attributable mainly to the resource requirements for flower production, since fruit developing and maturation occurs later in the season. There was also a decrease in leaf N content from the beginning to the end of the time of the season (considering reproductive individuals only). The magnitude of this decrease was the same in males and females, although it could be expected to be higher in females, which carry the burden of fruit maturation (especially later in the season). Nevertheless, pollen is a nitrogen-rich inversion, and flower production could incur considerable cost to males (Goldman & Willson 1986). Our results showed that the reproductive costs of males and females of *H. peploides* did not produce intersexual differences in leaf N content.

Negative correlations between foliar $\Delta^{13}C$ and N concentrations, as we found for nonreproductive males at the beginning of the season and for post-reproductive males at the end of the season, have been explained to occur because high leaf N concentrations lead to increased photosynthetic capacities, decreased internal leaf CO_2 and increased water use efficiency (Mooney *et al.* 1978; Sparks & Ehleringer 1997).

In conclusion, the lack of consistent sex-related differences in integrated water use efficiency and leaf nitrogen content found here, make it difficult to establish a physiological basis for the spatial segregation of the sexes. We suggest that males and females through a selection of habitats might meet with their different demand of resources for reproduction, with each sex achieving the best performance in the habitat in which it predominates. Experimental manipulations might make it easier to draw conclusions about if physiological responses of male and female individuals of *H. peploides* might explain the spatial segregation.

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Chapter 5

5

Sex-specific physiological and growth responses to water availability are important in explaining the spatial segregation of the sexes observed in the subdioecious plant *Honckenya peploides* (L.) Ehrh.

Julia Sánchez Vilas and Rubén Retuerto

ABSTRACT

Dioecious plant species often show differences in the habitat distribution of male and female plants. Sex-specific variation in ecophysiological traits may determine the performance of each sex in different habitats, and therefore promote the spatial segregation of the sexes. Our objective was to inquire into the causes of the extreme niche segregation of the sexes of the subdioecious dune plant *Honckenya peploides.* To this end, we designed a manipulative experiment to investigate whether males and females of this species differ in physiological traits (photochemical efficiency and leaf spectral properties) and/or components of relative growth rate, and whether possible dimorphism in such traits is dependent on water availability. Photochemical efficiencies integrated over time were significantly higher in males than in females. The time-course of maximum quantum yield differed between the sexes, and depending on water availability: water deficit decreased maximum quantum yield in females more rapidly than in males, but females (unlike males) recovered initial values by the end of the experiment. Maximum quantum yield in males was more affected by severe drought than in females, indicating that males are more susceptible to photoinhibition. The sexes did not differ in relative growth rates, but males invested a higher proportion of their total biomass in leaves, and had higher leaf area per unit biomass than females. Females had higher water content and were more succulent than males. Abaxial stomatal density was differentially affected in males and females by water availability: under well-watered or mild drought conditions males had higher stomatal density, but females had higher stomatal density under severe drought. Our results suggest that the sexes of *H. peploides* follow different strategies to cope with water stress, with females being more tolerant of severe drought conditions. The study also provides evidence of differences in allocation traits between male and female plants. We conclude that between-sex differences in ecophysiological and allocation traits may explain habitat-related between-sex differences in performance, and therefore the spatial segregation of the sexes.

Keywords: Chlorophyll fluorescence, dioecy, *Honckenya peploides*, relative growth rate, spatial segregation, spectral reflectance, water stress.

INTRODUCTION

Among the many factors that influence plant performance, water availability is certainly one of the most important: on the global scale, water availability restricts terrestrial plant productivity more than any other single environmental factor (Turner & Kramer 1980; Boyer 1982). On local scales, competition for water among neighbouring plants can be very severe, and an important cause of mortality (McLeod & Murphy 1983; Ehleringher 1984; Maun 1985, 1994). This is especially true in coastal habitats, where the low water-retention capacity of sandy substrates leads to rapid percolation of rainwater and nutrients. Due to the low capacity of sandy soils to buffer against drought, plants that live on dunes may experience severe water deficits, especially in the spring and summer when high temperatures lead to high evapotranspiration rates. Water deficits affect a wide variety of physiological processes in higher plants, including leaf expansion and photosynthetic carbon assimilation (Lambers *et al.* 1998; Lu & Zhang 1998), and these effects may ultimately determine plant abundance and distribution (Schulze *et al.* 1987).

Honckenya peploides is a perennial halophyte typically found on the upper beach and embryo dunes, and therefore susceptible to suffer severe water deficits. It presents an unusual and complex breeding system with two distinguishable sex morphs: females, which never produce pollen and are constant in their sex expression, and "males", all of which produce pollen, but at least some of which also produce seeds. This system, called subdioecy, is close to dioecy in the evolutionary pathway from hermaphroditism to dioecy via gynodioecy (Lloyd 1976; Webb 1979; see Delph & Wolf 2005 for a review). In dimorphic flowering plants like *H. peploides*, biased sex ratios are widely reported (Dawson & Geber 1999 and references therein; Eppley 2006). It is usually argued that sex ratio will be female-biased in high-quality environments, because reproduction is more costly for females, so that the relative fitness of females will improve as environmental quality improves (Freeman *et al.* 1976; Lloyd & Webb 1977; Freeman *et al.* 1980). Within-population sex ratio variation, or spatial segregation of the sexes, may occur if the sexes respond differentially to given environmental conditions. Differences in competitive abilities between the sexes (Cox 1981; Eppley 2006) and morphological or physiological specialization of the sexes to different habitats (Dawson & Ehleringer 1993; Dawson & Geber 1999) are also possible causes of spatial segregation.

At the location from which we obtained the experimental plant material for this study, *H. peploides* displays a fascinatingly extreme degree of spatial segregation of the sexes, with clumps composed exclusively of individuals of either one sex or the other. This extreme spatial segregation may be the result of a differential response of the sexes to key abiotic factors in the habitat, such as water deficit. In fact, the sexes of other dioecious plant species have previously been shown to respond differently to water stress (Fox & Harrison 1981; Freeman & McArthur 1982; Freeman & Vitale 1985; Vitale *et al.* 1987; Retuerto *et al.* 2000).

This study focuses on sexual dimorphism in physiology and components of growth rate in *H. peploides*. Gender dimorphism in plants has been an important topic of research (Geber *et al.* 1999 and references therein). However, in spite of its potential for explaining differences in growth, allocation or gender-specific habitat preferences, relatively little work to date has investigated gender differences in physiology (see Dawson & Geber 1999 for a review; Verdú *et al.* 2004). Specifically, in this study we searched for sex differences in photochemical efficiencies, something that only rarely has been made (Obeso *et al.* 1998; Correia & Díaz Barradas 2000; Retuerto *et al.* 2000, 2006), and go beyond what has previously been shown by exploring sex differences in leaf reflectance characteristics related to physiological performance. Since possible differences in the relative growth rates (RGR) of the sexes are not necessarily due to variation in the efficiency with which the sexes acquire carbon we also aimed to understand sex variation in growth by exploring differences in the most crucial component of RGR (Poorter 1989), the leaf area ratio (LAR), *i.e.* the relative amount of biomass a sex invest in leaf area. We further analysed if variation in LAR may be due to a difference in the investment of each sex in leaf biomass (*i.e.* leaf mass ratio; LMR) or to a difference in leaf morphology (specific leaf area; SLA). Although a considerable number of studies have dealt with gender dimorphism in growth and mass partitioning (Ågren 1988; Ramp & Stephenson 1988; Nicotra 1999; Leigh *et al.* 2006) we know of no studies examining sex-specific variation in components of RGR. In the present study, we designed a manipulative experiment in which we specifically addressed the following questions: (1) Do male and female *H. peploides* individuals differ in physiological traits as photochemical efficiencies and leaf reflectance?, (2) Do they differ in RGR or in a key factor in growth as the leaf area ratio?, and (3) If sexual dimorphism in such traits does occur, are the differences dependent on water availability? These are crucial issues, since sex-specific variation in ecophysiological attributes may determine the performance of each sex in different habitats, and are therefore decisive for ascertaining the ultimate causes of the niche segregation of the sexes of *H. peploides*. The cost of reproduction has been commonly considered as a cause of differential responses of the sexes to environmental factors (Dawson & Ehleringer 1993; Obeso *et al.* 1998). In the present experiment, the individuals under study were not reproductively active. Thus, any sex-specific differences in performance can be viewed as independent of sex differences in investment in reproduction.

MATERIAL AND METHODS

The species

The sea sandwort, *Honckenya peploides* (L.) Ehrh. (Caryophyllaceae), has a circumpolar distribution (from temperate to Arctic zones; also anthropochorous in South America). In the Iberian Peninsula, *H. peploides* extends from the Atlantic coast of northern Portugal to the eastern end of the Cantabrian coast of Spain. It is a hemicryptophyte, re-growing each spring from long rhizomes that produce compact groups of aerial shoots, forming vegetative clumps or mats. These clumps are typically found on the upper beach, giving rise to small mounds called embryo dunes. Plants reproduce sexually by seeds or clonally by buds produced on rhizomes. This species can be considered an early colonizer, contributing to stabilization and anchorage of the soil and facilitating the establishment of other species (Houle 1997; Gagné & Houle 2001). Flowers are axillary, solitary and/or in 1-6 flowered terminal cymes, strongly honey-scented. Two types of flower can be found, as reported in the ssp. *major* by Tsukui & Sugawara (1992): one type ("pistillate") has long styles, short petals and non-functional anthers, while the other ("staminate") has short styles, long petals and long stamens that produce pollen grains. This latter type rarely produces seeds, and when it does it the number of seeds is very low compared to pistillate flowers. Both types of flower have nectaries at the base of the stamens, which attract pollinators. In line with Tsukui & Sugawara (1992), and following Lloyd (1976) and Delph (1990), we will here refer to plants with pistillate flowers as females and to plants with staminate flowers as males.

Experimental design

Plant material was collected at the beginning of February of 2005 from the six existing clumps of *H. peploides* in the locality of Lariño, on the coast of Galicia (42º45'N, 9º6'W; Northwest Spain). Three clumps were composed exclusively of females and the other three of males, the distance between clumps varying from a few tens of meters to hundreds of meters. Clumps were sexed on the basis of their floral morphology in the three previous years and the sex was corroborated in the year of the study. Plant material was collected from several points within each clump, to sample as many genotypes as possible. An ongoing study using amplified fragment length polymorphism (AFLP) as well as isozyme analysis has found high values of genetic variation, revealing several genets within each of these unisexual clumps (mean values for proportion of distinguishable genotypes: 0.25 for isozymes and 0.41 for AFLP; mean values for Simpson`s diversity index: 0.65 for isozymes and 0.68 for AFLP; N = 193 for isozymes and N = 80 for AFLP, Sánchez-Vilas *et al.*, unpublished data).

After washing, plant material was weighed in order to determine initial fresh mass, then individually planted in 1.3 L pots filled with dune sand and fertilized (KB Abono Universal, 7-5-6 NPK applied as a solution at ca. 7 mg per pot). Then, plants were left to grow for three months in an open-end greenhouse at the field station of the University of Santiago de Compostela (42º53'N, 8º32'W, 260 m a.s.l.) and watered as needed. At the start of the experiment, plants were assigned randomly to one of the three watering treatments: well-watered, mild drought stress and severe drought stress. Well-watered plants received as much water as needed, mild drought-stressed plants were watered twice a week for four weeks then once a week until the end of the experiment, and severe drought-stressed plants were watered once a week for 4 weeks then once every 2 weeks until the end of the experiment. Nine plants of each sex served as replicates for each treatment (a total of 27 plants of each sex). The experiment was conducted in the greenhouse of the field station of the University of Santiago de Compostela, between 30th May and 8th August 2005. All pots were rearranged weekly to minimize the possible effects of position within the greenhouse.

Chlorophyll fluorescence measurements

Throughout the experiment, we measured *in vivo* chlorophyll fluorescence on leaves at the top of the plant (three per plant) using a pulse-amplitude-modulated fluorometer (MiniPam, Walz, Effeltrich, Germany). Measuring light and saturating light pulses (> 4000 µmol photons m^{-2} s^{-1}, 0.8 s pulse length, actinic white light) were applied through a fiberoptic at a 60° angle relative to the sample and at 12 mm from the leaf. The maximum quantum yield of photosystem II (PSII) was assessed by the ratio Fv/Fm = (Fm-F_0)/Fm (see Bolhàr-Nordenkampf *et al.* 1989), where F_0 and Fm are defined as minimal and maximal fluorescence yield of a dark-adapted sample, with all PSII reaction centres fully open. This parameter was measured at predawn, with plants in a dark-adaptation state ensuring that all their PSII reaction centres were open. The maximum quantum yield estimates the efficiency of excitation energy capture by open PSII reaction centres (Butler & Kitajima 1975) and is correlated with the amount of carbon gained per unit of light absorbed (Bolhàr-Nordenkampf & Öquist 1993). Measurements of Fv/Fm were made bi-weekly, from the 2nd of June to the 4th of August 2005. The effective quantum yield of PSII was calculated as ΦPSII = (Fm'-Ft)/Fm' (see Genty *et al.* 1989), where Fm' is the maximal fluorescence yield reached in a pulse of saturating light with an illuminated sample and Ft is the fluorescence yield of the leaf at a given photosynthetic photon flux density (PPFD). This parameter, which estimates the proportion of the light absorbed by the chlorophyll that is used in photochemistry (Maxwell & Johnson 2000), was measured on the same dates (plus or minus one day) as Fv/Fm, under a PPFD of 851 ± 5 µmol m^{-2} s^{-1} (N = 972). Several studies have demonstrated that ΦPSII can be used to predict CO_2 assimilation rates precisely and quickly (Genty *et al.* 1989; Edwards & Baker 1993; Andrews *et al.* 1995).

Spectral reflectance measurements

Reflectance parameters were measured on three leaves of each of the 54 plants, using a portable spectrometer (UniSpec, PP Systems, Haverhill, MA, USA) on the same dates (plus or minus one day) as the fluorescence measurements, bi-weekly from the first week of June to the first week of August. Reflectance spectra (wavelengths 306 - 1136 nm) were calculated by dividing the spectral radiance of the leaf by the radiance of a reflective white standard (Spectralon Reflectance Standard, Labsphere, North Sutton, NH, USA). Reflectance indices were determined as follows. The photochemical reflectance index (PRI) was determined as $(R_{531}-R_{570})/(R_{531}+R_{570})$, where R_{531} is reflectance at 531 nm (a wavelength sensitive to changes in the xanthophyll cycle epoxidation state) and R_{570} is reflectance at 570 nm (a reference wavelength unaffected by xanthophyll activity). PRI is indicative of xanthophyll cycle energy dissipation and radiation-use efficiency (mol CO_2 mol^{-1} photons) (Peñuelas *et al.* 1995; Filella *et al.* 1996; Gamon *et al.* 1997). The chlorophyll content index (CHL) was calculated as R_{750}/R_{700}. Lichtenthaler *et al.* (1996) have demonstrated that this index allows estimation of the chlorophyll content of leaves with an error of less than 2.1 µg cm^{-2}. Lastly, we computed the normalized difference vegetation index (NDVI) as $(R_{900}-R_{680})/(R_{900}+R_{680})$. NDVI seems to reflect the process of chlorophyll degradation, and correlates empirically with photosynthetic activity (Garty *et al.* 1997).

Growth and relative water content measurements

Fresh weight (FW) was determined immediately after cutting leaves. Turgid weight (TW) was obtained after soaking leaves in distilled water in Petri dishes for 24 hours at 4ºC under darkness; they were then dried with tissue paper to remove any surface water, and weighed immediately. Leaves were scanned and their projected area (one side of the leaf) was determined with image analysis software (ImageJ 1.37h, Rasband 2005). Samples were then oven-dried at 70ºC for at least 4 days, and their dry weight (DW) was determined. Relative water content [RWC = 100 × (FW-DW)/(TW-DW)], water content [WC = (FW-DW)/DW], degree of succulence [(FW-DW)/leaf area, g cm^{-2}] and leaf dry matter content (LDMC = DW/FW) were calculated from three fully expanded leaves per plant.

Stomatal density was measured on the adaxial and abaxial surfaces of each of three fully expanded leaves per plant. We took leaf imprints by applying a thin layer of clear nail varnish on each side of the leaf, then stripped the dried imprints obtained from the leaves and mounted them on slides. We counted the number of stomata in five 500 × 500 µm fields of view on each imprint, using a Visopan microscope (Reichert, Austria) at 150× magnification.

At the end of the experiment all plants were harvested and the dry mass of all plant parts was determined after oven-drying at 70ºC for at least 4 days. We calculated leaf area ratio (LAR = plant leaf area per unit plant mass, cm^2/g), leaf mass ratio (LMR = leaf mass/plant mass), stem mass ratio (SMR = stem mass/plant mass), root mass ratio (RMR = root mass/plant mass), specific leaf area (SLA = leaf area per unit dry mass, cm^2/g) at the whole-plant level, and relative growth rate (RGR = [ln(harvest dry mass)-ln(initial dry mass)]/days). Initial dry mass was determined from a whole-plant harvest of 20 individuals before the experiment began. On the basis of data from this initial harvest we determined the initial dry mass of our experimental plants, and differences between sexes were not detected (ANOVA: $F_{1,52} = 2.9$; $P = 0.095$).

Data analysis

Two-way ANOVA was used to test for effects of water treatments and sex (both treated as fixed) on growth and structural attributes. Repeated-measures ANOVA was used to analyse the bi-weekly physiological measurements. For multiple comparisons, we used the Least Significant Difference (LSD) test. All statistical analyses were performed using SPSS v. 14.0 for Windows (SPSS Inc., 1989-2005), taking $\alpha = 0.05$ as the threshold for statistical significance.

RESULTS

Physiological measurements

Chlorophyll fluorescence. Mean maximum quantum yield (Fv/Fm) and effective quantum yield (ΦPSII) were significantly higher in males than in females. Both parameters were also significantly affected by water availability (between-subject effects in Table 1). Mild or severe drought-stressed plants showed significantly lower ΦPSII values than well-watered plants (LSD tests: $P = 0.001$ and $P = 0.018$, respectively). Fv/Fm values in severe drought-stressed plants were lower than in both well-watered plants (LSD test: $P \leq 0.001$) and mild drought-stressed plants (LSD test: $P = 0.002$). The effects of water availability on the time-courses of maximum quantum yield differed between male and females (within-subject effects on Table 1; Fig. 1). Specifically, water deficit decreased Fv/Fm values in females earlier than in males. However, females, in contrast to males, recovered initial Fv/Fm values at the end of the experiment. In addition, Fv/Fm in males was not affected by mild drought, but was more markedly affected by severe drought than in females (Fig. 1).

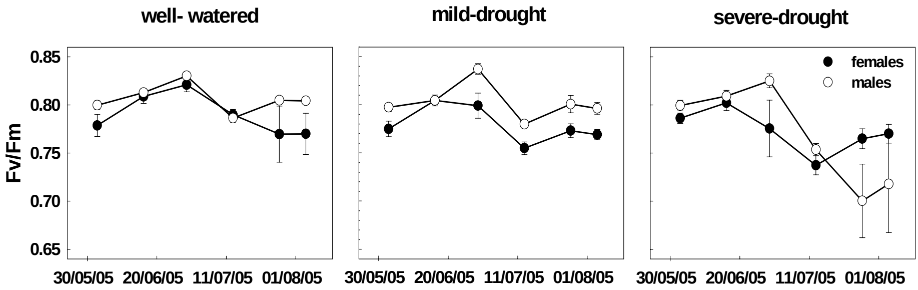

Fig. 1 Time-courses of maximum quantum yield (Fv/Fm) for males and females at different levels of water availability. Data are means of nine measurements per sex and water availability condition (error bars = SE).

Spectral reflectance. The photochemical reflectance index (PRI) decreased significantly with drought stress, whether mild or severe (between-subject effects in Table 1), with the differences between well-watered and drought-stressed plants increasing over time (within-subject effects in Table 1; Fig. 2a). At the end of the experiment, well-watered plants showed significantly higher PRI values than mild and severe drought-stressed plants (LSD test: P = 0.012 and P < 0.001, respectively). No between-sex differences in PRI were observed. Chlorophyll content index (CHL) and NDVI values were significantly affected by water availability (MANOVAR, between-subject effects: Table 1 and Figs. 2b and 2c, respectively). At the end of the experiment, well-watered plants showed significantly higher CHL values than drought-stressed plants (LSD tests: P = 0.032 for mild stress, P = 0.003 for severe stress). NDVI values for well-watered plants were likewise significantly higher than for drought-stressed plants (LSD tests: P = 0.026 for mild stress, P = 0.003 for severe stress).

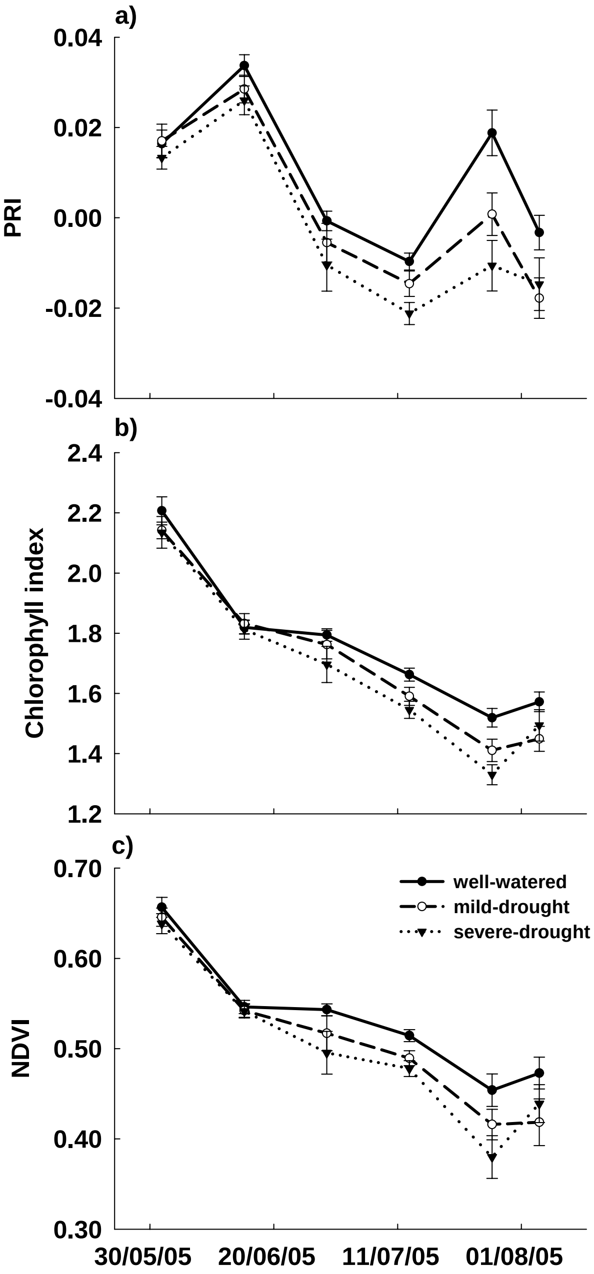

Fig. 2 Time-courses of photochemical reflectance index (PRI), chlorophyll index (CHL) and normalized difference vegetation index (NDVI) at different levels of water availability. Data are means of 18 measurements (error bars = SE).

Table 1 Results of repeated-measures ANOVA to assess the effects of sex, water and time on chlorophyll fluorescence (maximum, Fv/Fm, and effective, ΦPSII, quantum yield) and spectral reflectance parameters (photochemical reflectance index, PRI; chlorophyll index, CHL; and normalized difference vegetation index, NDVI). Significant *F* values ($P < 0.05$) are indicated in bold.

		Fv/Fm			ΦPSII			PRI			CHL			NDVI		
Within-Subjects	*df*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Time	5	231.2	12.50	**<0.001**	3277.6	62.28	**<0.001**	159.7	69.46	**<0.001**	3.844	177.49	**<0.001**	3597.1	95.59	**<0.001**
Sex × Time	5	20.7	1.12	0.351	69.1	1.31	0.259	5.0	2.19	0.056	0.045	2.08	0.069	80.4	2.14	0.062
Water × Time	10	33.6	1.82	0.058	78.8	1.50	0.141	4.6	2.00	**0.034**	0.024	1.12	0.345	38.3	1.02	0.429
Sex × Water ×Time	10	42.6	2.30	**0.013**	34.6	0.66	0.763	1.0	0.43	0.929	0.006	0.29	0.982	14.0	0.37	0.958
Error	240	18.5			52.6			2.3			0.022			37.6		
Between-Subjects																
Sex	1	109.9	4.90	**0.032**	422.3	6.59	**0.013**	21.1	2.77	0.103	0.017	0.35	0.554	6.5	0.09	0.764
Water	2	226.8	10.12	**<0.001**	396.1	6.18	**0.004**	13.4	8.45	**0.001**	0.249	5.21	**0.009**	371.9	5.22	**0.009**
Sex × Water	2	60.1	2.68	0.079	76.7	1.20	0.311	40.9	1.26	0.294	0.041	0.86	0.428	24.8	0.35	0.708
Error	48	22.4			64.1			6.1			0.048			71.3		

Growth and relative water content

Males and females did not differ in relative growth rates. Likewise, neither stem mass ratio, root mass ratio nor specific leaf area differed between the two sexes. Males invested a greater proportion of their total biomass in leaves, and dedicated more leaf area per unit of plant mass than females (Tables 2 and 3).

Females showed significantly higher water content and lower leaf dry matter content than males. Females also showed a higher degree of succulence than males (Tables 2 and 3). The number of stomata on abaxial leaf surfaces of both males and females was affected by water availability. Under no drought or mild-drought conditions, males had higher abaxial stomatal density than females. Nevertheless, under severe drought abaxial stomatal density decreased in males and increased in females (see Fig. 3). Stomatal density on adaxial leaf surfaces was not affected by sex or water availability (Tables 2 and 3).

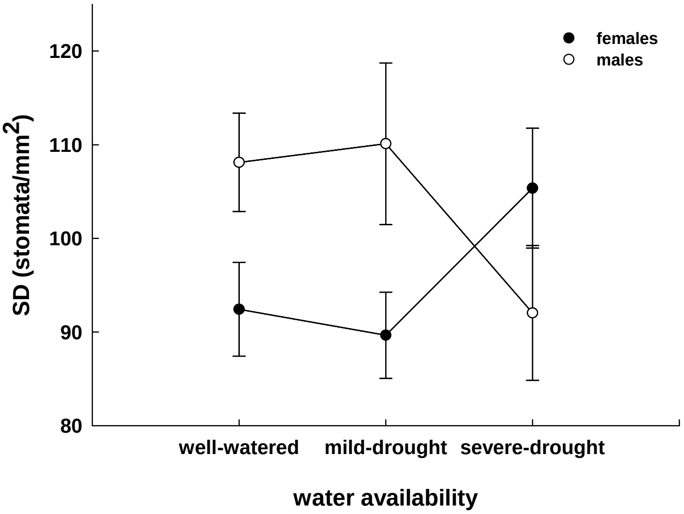

Fig. 3 Stomatal density in each sex under well-watered, mild drought and severe drought conditions. Data are means of 9 measurements (error bars = SE).

Water treatments significantly affected relative growth rate (Tables 2 and 3). Severe drought reduced relative growth rate by 21%.

In contrast to specific leaf area and to root and stem mass ratios, which were not affected by water treatments, leaf mass ratio and leaf area ratio significantly decreased with water deficit (Tables 2 and 3). Severe water deficit decreased the proportion of total biomass dedicated to leaves by 22% compared to well-watered plants (LSD test: $P = 0.013$) and by 19% compared to plants under mild drought (LSD test: $P = 0.043$). Well-watered plants allocated 1.15 and 1.30 times more leaf area per unit of plant mass than mild drought-stressed plants (LSD test: $P = 0.025$) and severe drought-stressed plants (LSD test: $P < 0.001$), respectively.

Drought stress significantly reduced relative water content, water content of leaves, and leaf dry matter content (Tables 2 and 3). Multiple comparison tests showed major differences between well-watered and severe drought-stressed plants both in water content and in leaf dry matter content (in both cases $P = 0.003$, LSD tests), and between well-watered and mild drought-stressed plants in relative water content (LSD test: $P = 0.001$). Succulence decreased as drought stress increased, with greater and more significant differences between well-watered and severe drought-stressed plants (LSD test: $P = 0.001$) than between mild drought-stressed and severe drought-stressed plants (LSD test: $P = 0.022$).

Table 2 Mean values ± SE (N = 9) for growth and leaf traits of female and male plants of *Honckenya peploides* grown at three different levels of water availability.

	females			males		
	well-watered	mild drought	severe drought	well-watered	mild drought	severe drought
Relative Growth Rate (g.g^{-1}.day^{-1})	0.0219±0.0023	0.0215±0.0019	0.0190±0.0016	0.0215±0.0010	0.0185±0.0014	0.0153±0.0015
Leaf mass ratio	0.261±0.037	0.266±0.0198	0.244±0.026	0.363±0.014	0.331±0.014	0.239±0.040
Stem mass ratio	0.172±0.026	0.184±0.011	0.194±0.018	0.164±0.005	0.175±0.013	0.141±0.025
Root mass ratio	0.567±0.054	0.551±0.029	0.562±0.040	0.473±0.016	0.494±0.022	0.619±0.064
Specific leaf area (cm.g^{-1})	89.61±3.60	86.48±3.12	85.20±3.92	84.40±3.81	80.16±3.47	82.32±4.75
Leaf area ratio (cm.g^{-1})	25.51±2.19	22.67±1.50	20.38±1.94	30.74±1.57	26.34±1.06	23.00±1.55
Relative water Content (%)	91.17±0.10	88.60±0.55	90.03±1.32	92.46±1.11	87.51±0.91	89.73±1.21
Water Content	5.33±0.18	5.06±0.14	4.66±0.16	4.70±0.22	4.33±0.21	4.15±0.24
Succulence degree (g.cm^{-2})	0.072±0.003	0.071±0.001	0.068±0.002	0.071±0.002	0.067±0.001	0.058±0.003
Leaf Dry Matter Content (mg.g^{-1})	160.58±4.51	166.78±4.26	178.68±5.07	177.95±6.55	190.34±7.19	198.71±8.97
Abaxial SD (stomata.mm^{-2})	92.43±5.01	89.66±4.60	105.36±3.28	108.12±5.25	110.10±8.63	92.04±7.20
Adaxial SD (stomata.mm^{-2})	104.43±13.99	103.41±16.60	125.81±23.99	109.24±14.13	118.52±20.10	113.37±20.88

Table 3 Effects of sex and water treatments on growth and leaf traits of *Honckenya peploides*, as determined by two-way ANOVA. Significant *F* values ($P < 0.05$) are indicated in bold.

		Sex			Water Treatment			Sex × W Treatment		
	Error MS (*df* = 48)	MS (*df* =1)	*F*	*P*	MS (*df* = 2)	*F*	*P*	MS (*df* =2)	*F*	*P*
Relative Growth Rate ($g.g^{-1}.day^{-1}$)	0.250	0.774	3.102	0.085	0.919	3.679	**0.033**	0.138	0.551	0.580
Leaf Mass Ratio	0.007	0.040	5.944	**0.019**	0.025	3.720	**0.031**	0.013	1.975	0.150
Stem Mass Ratio	0.003	0.007	2.510	0.120	0.001	0.296	0.745	0.003	1.070	0.351
Root Mass Ratio	0.015	0.013	0.876	0.354	0.029	1.916	0.158	0.028	1.843	0.169
Specific Leaf Area ($cm.g^{-1}$)	107	311	2.893	0.095	73	0.678	0.512	14	0.129	0.879
Leaf Area Ratio ($cm.g^{-1}$)	22	199	9.062	**0.004**	187	8.524	**0.001**	8	0.351	0.706
Relative Water Content (%)	9.829	0.014	0.001	0.970	63.725	6.484	**0.003**	6.691	0.681	0.511
Water Content	0.343	5.199	15.176	**<0.001**	1.672	4.882	**0.012**	0.055	0.160	0.853
Succulence Degree (g. cm2)	0.472	3.446	7.300	**0.010**	3.306	7.002	**0.002**	1.046	2.214	0.120
Leaf Dry Matter Content (mg.g)	359	5573	15.519	**<0.001**	1700	4.735	**0.013**	43	0.121	0.886
Abaxial Stomatal Density ($stomata.mm^{-2}$)	361	780	2.160	0.148	12	0.033	0.967	1503	4.160	**0.022**
Adaxial Stomatal Density ($stomata.mm^{-2}$)	325	84	0.258	0.614	762	2.341	0.107	872	2.679	0.079

DISCUSSION

The significant differences in ecophysiological and allocation traits seen in the present study between male and female plants of *H. peploides* may lead to differences in the performance of the two sexes under different environmental conditions, and therefore be decisive in explaining the extreme spatial segregation of the sexes observed in this species.

Analysis of the allocation traits provides evidence that males have better light acquisition potential. Although we did not find significant differences in relative growth rate (RGR) between the two sexual morphs of *H. peploides*, males showed a higher leaf area ratio, the overriding component in explaining variation in RGR (RGR = LAR × net assimilation rate (NAR); Poorter & Remkes 1990; Reich *et al.* 1991). The between-sex differences in LAR (= LMR × SLA) were attributable to variation in LMR rather than to variation in SLA, since we did not detect sex differences in the latter. This result suggests that females may be compensating for the lower allocation to autotrophic tissues by maintaining a higher NAR. Although the contributions of NAR and LAR to RGR are not necessarily opposite (Poorter 1989), many studies have reported a negative correlation between NAR and LAR (see Konings 1989 and references therein). NAR, the physiological component of RGR, is a complex parameter determined by the balance between the plant's carbon gains through photosynthesis and carbon losses due to respiration, exudation and volatilization (Poorter 1989). Considering that maximum and effective PSII quantum yields are well correlated with the amount of carbon gain per unit of light absorbed (Bolhár-Nordenkampf & Öquist 1993) and with CO_2 assimilation rates (Demmig-Adams *et al.* 1990; Edwards & Baker 1993), respectively, the higher photochemical efficiencies seen in males strongly suggest higher photosynthetic rates. However, the effect of the higher photosynthetic rate on the NAR of males seems to be counterbalanced by higher respiration costs. This is in line with findings by Poorter *et al.* (1990) suggesting that the apparent lack of relation between RGR and potential photosynthetic capacity could be due to differences in maintenance respiration and tissue construction costs. Intersexual differences in growth have generally been attributed to a higher resource allocation to reproduction in females, due to the need not only to flower but also to mature fruits (Popp & Reinartz 1988; Dawson & Ehleringer 1993; Obeso *et al.* 1998; Nicotra 1999; Rocheleau & Houle 2001), though note that these differences may be counterweighted in cases in which females acquire more resources than males (Delph *et al.* 1993; Delph & Meagher 1995). Our results, like those of Eppley (2006), provide evidence that differences in allocation traits between male and female dioecious plants may be present in individuals that are not bearing the cost of reproduction.

Differences between male and female plants in the time-course of the maximum quantum yield response to water deficit suggests that the sexes of

H. peploides have different strategies for coping with water stress. Females seem to be more tolerant of drought conditions, maintaining reasonably high photochemical efficiency even under conditions of severe soil water stress. By contrast, Fv/Fm values for males under severe water stress were well below the range of 0.75-0.85 given by Björkman & Demming (1987) and Schreiber *et al.* (1995) for unstressed plants. This result suggests that females are less susceptible to photoinhibition than males, given that a reduction in Fv/Fm in dark-adapted plants has been considered symptomatic of stress-dependent photoinhibition (Long *et al.* 1994). This is particularly important for female plants, because fruit maturation takes place in summer, when water stress is most severe (in our study region as in many others). Females may benefit from a higher photosynthetic capacity than males under such conditions, since they must allocate a higher proportion of resources (*i.e.* carbon) to reproductive tissues (*i.e.* fruits). The higher water content and higher succulence of females than males might explain our observation that females maintained higher photosynthetic efficiency under severe drought stress. Water storage mechanisms are of course important for desiccation avoidance, with stored water protecting the plant from sudden wilting and severe leaf shrinkage. Succulence is a plant trait for coping with water stress, supporting growth when soil moisture is scarce or unavailable: it may reflect an increase in the thickness of the palisade layer of the mesophyll, where gas exchange actually occurs, and it may thus increase the potential rate of gas exchange (Welch & Rieseberg 2002). In coastal dunes, where dryness is a common abiotic condition, selection may force females to preserve water late into the growing season, when soil moisture is likely to decrease but females need to complete fruit maturation.

Males showed higher abaxial stomatal density than females under well-watered and mild drought conditions, but lower abaxial stomatal density under severe drought. Higher stomatal density presumably enables better control of transpiration (Paoletti & Gellini 1993). The pattern of between-sex differences in stomatal density is coincident with the pattern of between-sex variation in photochemical efficiency (see Fig. 1, last day), with males showing higher photochemical efficiencies than females under well-watered or mild drought conditions, but lower efficiencies than females under more severe water deficits. There is no simple relationship between stomatal density and photosynthesis: depending on the plant species, previous studies have observed positive correlation, negative correlation, or no correlation. Our results are in agreement with those previous studies that have found a positive correlation, *i.e.* increasing stomatal density with increasing photosynthesis and yield (Walton 1974; Araus *et al.* 1986; Retuerto & Woodward 1993; Kundu & Tigerstedt 1998).

Although not in direct relation to our starting initial hypothesis, this study also found significant effects of the water treatments on several growth and ecophysiological traits, indicating that the *H. peploides* plants experienced considerable water stress under our experimental conditions. As expected from previous research on the effects of water stress (Ludlow 1989), all ecophysiological parameters directly related to the photosynthetic performance of plants (such as maximum and effective quantum yield, and the CHL, NDVI and PRI indices) significantly decreased with declining water availability. Drought decreased the RGR of *H. peploides* by reducing LAR (due basically to a reduction in LMR).

In conclusion, this study shows that male and female plants of *H. peploides* differ significantly in important ecophysiological and allocation traits such as, photochemical efficiency, susceptibility to photoinhibition, and proportion of total biomass invested in leaves. The study also demonstrates sex-specific differences in morphological attributes at the leaf level, such as degree of succulence and stomatal density, suggesting sex-specific strategies to cope with water availability. The interplay among these physiological, allocational and morphological growth determinants may contribute to explaining between-sex differences in performance responses to environmental stress, and therefore the maintenance of the marked spatial segregation of the sexes seen in this species. Our study also demonstrates that sexual dimorphism in ecophysiological traits is present in individuals that are not bearing reproductive structures, and therefore is not solely a consequence of between-sex difference in resources invested in reproduction.

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Chapter 6

6

Competition between sexes for nutrients under different salt spray conditions: a mechanistic approach to sexual-niche-partitioning in the subdioecious *Honckenya peploides* (L.) Ehrh.

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ABSTRACT

Biased sex ratio occurs in a wide variety of dimorphic flowering species. Spatial segregation of the sexes may be related to their differential response to environmental conditions, and is found in diverse species. In coastal dune ecosystems, several factors affect growth, survival and establishment of plants. In this study, we tested the response of the different sexes of the subdioecious *Honckenya peploides* to salt spray and nutrient availability. In an attempt to simulate realistic conditions, males and females were grown in the same container, so that we could evaluate, at least indirectly, the intraspecific competitive effects. We did not find any differential responses between the sexes to salt spray, which caused an increase in root mass production and a decrease in reproductive effort. Addition of nutrients increased growth of the plants, and the male plants accumulated more total and above-ground dry mass than the female plants provided with nutrients. The values for photochemical efficiency were higher in the fertilized female than in the fertilized male plants. Females allocate more to reproduction and less to growth than males, which is interpreted as a trade-off between reproductive and vegetative growth. These differences between the sexes may help to explain the spatial segregation observed. The limited growth of females may affect their competitive abilities, with males taking advantage to colonize the same space. However, the possible improvement in competitiveness under nutrient-rich conditions as well as the greater reproductive effort in females, suggest that growth of female plants may be favoured under such conditions.

Keywords: chlorophyll fluorescence, spectral reflectance, photosynthetic efficiency, sexual dimorphism, coastal dunes, competition.

INTRODUCTION

Amongst the many factors that determine plant performance and distribution in coastal dune ecosystems, nutrient availability and salt spray are generally assumed to be of major importance (Wilson & Sykes 1999). Coastal plants can obtain essential elements for growth from seawater salt spray, precipitation, or even from seaweeds and deposition of dead animals, but the low water-holding capacity of sandy substrates leads to rapid leaching of precipitation water, which implies an important loss of nutrients. The lack of a large reservoir of nutrients in the soil may be substituted by large annual meteorological inputs (van der Valk 1974). In response to nutrient deficits, coastal plants may present some strategies of flexible patterns of biomass allocation to organs such as roots, with proliferation of lateral roots in zones of higher nutrient availability, redistribution of nutrients from vegetative to reproductive organs or nitrogen fixation by rhizosphere bacterial activity or endomycorrhization (see Hesp 1991, and references therein).

Salt spray is an important selective force that shapes the characteristic plant communities of coastal areas (Wilson & Sykes 1999; Griffiths & Orians 2003). Differences between species in levels of tolerance to salt spray may result in zonation of vegetation, so that the most tolerant plants grow closer to the coastline, and are progressively replaced by less tolerant plants towards the interior (Oosting & Billings 1942; Oosting 1945; van der Valk 1974; Barbour 1978; Parsons 1981; Yura 1997). The effect of salt spray on plant growth is species-specific. Some dune species, such as *Cakile maritima* and *Salsola kali*, have been shown to be resistant to, and also stimulated in their growth by salt spray, especially under conditions of low soil fertility (Rozema *et al.* 1982). Nevertheless, in some other species such as *Solidago nemoralis*, *Myrica pensylvanica* and *Quercus ilicifolia*, salt spray can reduce survival and inhibit growth by reducing the leaf area, which implies a smaller area available for photosynthesis (Griffiths & Orians 2003). Salt spray susceptible species, such as e.g. *Pinus taeda*, may be completely absent from areas with high levels of salt spray (Wells & Shunk 1938).

Plants growing in embryo dunes, such as *Honckenya peploides*, are generally exposed to the simultaneous effects of low nutrient availability and salt spray. *H. peploides* presents a complex and uncommon breeding system with two distinguishable morphs: females, which never produce pollen and are constant in their expression, and pollen-producing morphs, named males, at least some of which produce seeds. This system is described as subdioecy, and is close to the dioecy in the evolutionary pathway from hermaphroditism to dioecy via gynodioecy (see Delph & Wolf 2005, for a review). At the location where we obtained the experimental plant material for this study, *H. peploides* displays a fascinating case of extreme spatial segregation of the sexes, with clumps composed exclusively by individuals of either one sex or the other. A wide variety of sexually dimorphic flowering plants exhibit habitat-related sex ratio biases. The prevalent pattern is a predomination of males in stressful or resource poor habitats and that of females in more favourable habitats (Crawford & Balfour 1983; Bierzychudek & Eckart 1988; Webb 1992; Allen & Antos 1993; Dawson & Geber 1999; Ortiz *et al.* 2002). Habitat-related sex ratio biases may be a consequence of differential reproductive costs (the commonly assumed higher reproductive cost of females should result in increased female mortality in poorer habitats) (Bierzychudek & Eckhart

1988), differences in competitive abilities between the sexes (Cox 1981; Eppley 2006; Nanami *et al.* 2005) and/or physiological or morphological specialization of the genders to different habitats (Dawson & Ehleringer 1993; Dawson & Geber 1999; Retuerto *et al.* 2000).

Despite the large body of literature regarding habitat-related sex ratios, and the importance of gender competition as a major determinant of natural distribution patterns, very little manipulative research has been carried out under controlled conditions to determine how the mechanisms of competition could explain or predict sex ratio variation across space (but see Conn & Blum 1981; Lovett-Doust *et al.* 1987; Bertiller *et al.* 2002; Eppley 2006). The different resource requirements of the sexes associated with male vs. female sexual reproduction may determine the outcome of competition. According to the resource competition theory the sex with the lowest requirement for the resource should be the superior competitor (Stewart & Levin 1973; Tilman *et al.* 1981).

The extreme spatial segregation of sexes that we have observed in *H. peploides*, may be a consequence of the differential responses of the sexes to key abiotic factors found in their environments, which in turn may determine their competitive interaction. We specifically designed the present study to investigate the outcome of sex-related competition for nutrients under different, controlled, seawater salt spray conditions. Since nutrient availability and salt spray are claimed to be important selective forces in coastal dune environments, our study represents a test of a mechanistic approach to sexual-niche-partitioning. Thus, the general objective of this study was to improve our understanding of the proximate (ecological) causes of spatial segregation of sexes in *H. peploides*. More specifically, the objectives were to investigate, under competitive conditions: 1) whether sexes differed in their physiological responses to nutrients, salt spray and their interactions; 2) whether nutrient availability and salt spray differentially affected phenological, reproductive and growth parameters in the sexes, and consequently plant fitness.

MATERIAL AND METHODS

The species

Sea sandwort, *Honckenya peploides* (L.) Ehrh. (Caryophyllaceae), has a circumpolar distribution, extending from temperate to Arctic zones, and is also anthropochorous in South America). In the Iberian Peninsula, *H. peploides* spreads from the Atlantic coast of northern Portugal to the eastern end of the Cantabrian coast of Spain. It is a hemicryptophytic plant, which re-grows each spring from long rhizomes that produce compact groups of aerial shoots to form vegetative clumps or mats. These clumps are typically found on the upper beach where they help to accumulate small mounds of sand called embryo dunes. This species may be considered an early colonizer because it contributes to stabilisation and anchorage of the soil and facilitates the establishment of other species (Houle 1997; Gagné & Houle 2001). Flowers are axillary, solitary and/or in 1-6 flowered terminal cymes, and are strongly honey-scented. Two types of flower can be found, as reported in the variety *major* by Tsukui & Sugawara (1992): one type ("pistillate") has long styles, short petals and non-functional anthers, whereas the other ("staminate") has short styles, long petals and long stamens that produce pollen grains. The latter type rarely produces seeds, and when it does it the number of seeds is very low compared with those produced by pistillate flowers. Both types of flowers have nectaries at the base of the stamens, which attract pollinators. In accordance with Tsukui & Sugawara (1992), and following Lloyd (1976) and Delph (1990), we will hereafter refer to plants with pistillate flowers as females and to plants with staminate flowers as males.

Experimental design

Plant material was collected in mid-summer in 2003 from the six existing clumps of *H. peploides* in the locality of Lariño, on the coast of Galicia (42º45’N, 9º6’W; Northwest Spain). Three clumps were composed exclusively of females and the other three of males, the distance between clumps varied from tens of meters to hundreds of meters. Plant material was collected from several points within each clump, to obtain as many genotypes as possible. An ongoing study of these plants with amplified fragment length polymorphism (AFLP) and also isozyme analysis, has revealed high values of genetic variation and several genets within each of these unisexual clumps (mean values for proportion of distinguishable genotypes: 0.25 for isozymes and 0.41 for AFLP; mean values for Simpson`s diversity index: 0.65 for isozymes and 0.68 for AFLP; N = 193 for isozymes and N = 80 for AFLP, Sánchez-Vilas *et al.*, unpublished data).

Selected plants of similar size were washed, weighed and planted at random in 65 L plastic containers filled with dune sand. Five male and five female plants were interspersed at regular distances in each container (Fig. 1).

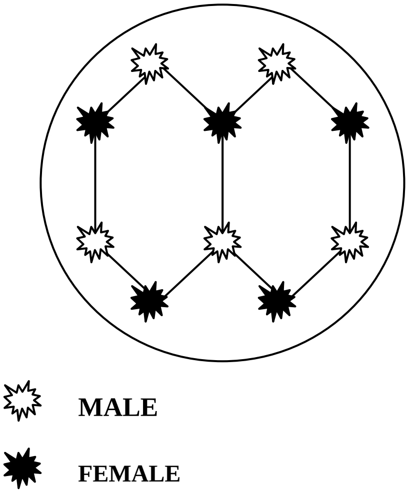

Fig. 1 Schematic diagram of the experimental set-up, showing the position of male and female plants of *Honckenya peploides* in each pot.

Plants were acclimatized gradually over four months to natural conditions at the University of Santiago field station (42º53’N, 8º32’W, 260 m a.s.l.). Response of the sexes to nutrient availability and salt spray was studied between November 2003 and November 2004. The treatments were randomly assigned to a total of eight containers. For the salt spray treatment, ~ 3 mL of sea water (collected from the Atlantic Ocean, on the coast of Galicia; ca. 34 g salts/L of water) was sprayed homogeneously on to each plant every week. At the same time, control plants were sprayed with the same volume of tap water. Before the third consecutive application of spray, all the plants were rinsed with tap water to prevent excessive accumulation of salt spray on the plants; otherwise plants were watered (at ground level) every day or two days in spring-summer and weekly in autumn-winter. For nutrient addition, a commercial fertilizer (KB Abono Universal, 7-5-6 NPK) was applied as a solution (6 mL of product in 2 L of water, providing 487 mg of nitrogen per container) every 15 days. Plants that did not receive nutrients were watered with 2 L of tap water on the same dates.

Chlorophyll fluorescence measurements

Throughout the experiment, *in vivo* chlorophyll fluorescence was measured on leaves at the top of the plant (one per each of the 80 plants) with a pulse-amplitude-modulated fluorometer (MiniPam, Walz, Effeltrich, Germany). Measuring light and saturating light pulses (> 4000 μmol photons m^{-2} s^{-1}, 0.8 s pulse length, actinic white light) were applied through a fiberoptic cable at an angle of 60º relative to the sample and at 12 mm from the leaf. The maximum quantum yield of photosystem II (PSII) was assessed by the ratio Fv/Fm = (Fm- F_0)/Fm (see Bolhàr-Nordenkampf *et al.* 1989), where F_0 and Fm are defined as minimal and maximal fluorescence yields of a dark-adapted sample, with all PSII reaction centres fully open. This parameter was measured at predawn, with plants in a dark-adaptation state, to ensure that all the PSII reaction centres were open. The maximum quantum yield estimates the efficiency of excitation energy capture by open PSII reaction centres (Butler & Kitajima 1975) and is correlated with the amount of carbon gained per unit of light absorbed (Bolhàr-Nordenkampf & Öquist 1993). A decrease in Fv/Fm has been considered symptomatic of stress-dependent photoinhibition (Long *et al.* 1994). Measurements of Fv/Fm were made monthly between May and November 2004, both inclusive. The effective quantum yield of PSII, was calculated as ΦPSII = (Fm'-Ft)/Fm' (see Genty *et al.* 1989), where Fm' is the maximal fluorescence yield reached in a pulse of saturating light with an illuminated sample and Ft is the fluorescence yield of the leaf at a given photosynthetic photon flux density (PPFD). This parameter, which measures the proportion of the light absorbed by the chlorophyll used in photochemistry (Maxwell & Johnson 2000), was measured on the same dates as Fv/Fm, with an external halogen lamp (wavelength < 710 nm) and provided a PPFD of 845 ± 13.45 μmol m^{-2} s^{-1} (N = 476). Several studies have demonstrated that ΦPSII can be used to predict CO_2 assimilation rates precisely and quickly (Genty *et al.* 1989; Edwards & Baker 1993; Andrews *et al.* 1995).

Spectral Reflectance Measurements

On 24th October 2004, we measured reflectance parameters on one leaf in each of the 80 plants, using a portable spectrometer (UniSpec, PP Systems, Haverhill, MA, USA). Reflectance spectra (wavelength range from 306 to 1136 nm) were calculated by dividing the spectral radiance of the leaf by the radiance of a reflective white standard (Spectralon Reflectance Standard, Labsphere, North Sutton, NH, USA). Reflectance indices were measured as follows. The photochemical reflectance index (PRI) was calculated as $(R_{531}-R_{570})/(R_{531}+R_{570})$, where R indicates reflectance and the numbers indicate nanometers. Previous studies with diverse species, functional types and nutrient conditions have demonstrated that PRI correlates significantly with both net CO_2 uptake and photosynthetic radiation-use efficiency (mol CO_2 mol^{-1} photons) (Peñuelas *et al.* 1995; Filella *et al.* 1996; Gamon *et al.* 1997) and also with the ratio of carotenoids (involved in the dissipation of excess light energy) to chlorophylls (Guo & Trotter 2004). The chlorophyll content index (CHL) was calculated as R_{750}/R_{550}. Lichtenthaler *et al.* (1996) have demonstrated that this index allows estimation of the chlorophyll content of leaves with an error of less than 2.1 μg cm^{-2}. Lastly, we computed the

Normalized Difference Vegetation Index (NDVI) as $(R_{900} - R_{680})/(R_{900}+R_{680})$. NDVI appears to reflect a process of chlorophyll degradation and correlates empirically with photosynthetic activity (Garty *et al.* 1997).

Phenology and reproductive parameters

The phenological pattern of flowering in male and female plants was characterized by flowering duration and synchrony (Rathcke & Lacey 1985). The number of flowers produced by each plant was counted every 7 days and flowering duration was noted. Open flowers and flowers that showed no signs of wilting were included in the counts. Flowering synchrony was calculated by estimating the number of days that the flowering of an individual overlaps with the flowering of every other in the sample (Augspurger 1983). The index of synchrony (X) for an individual plant (i) is defined as:

$$X_i = (1/n-1)(1/f_i)\sum_{j=i}^{n} e_{j\neq i}$$

Where e_j is the number of days during which individuals *i* and *j* flower synchronously, f_i is the number of days on which individual *i* is flowering and *n* is the number of individuals in the sample. When the value of the index equals one, this indicates that the flowering time of an individual overlaps completely with all other individuals, and a value of zero indicates that there is no overlap in an individual's flowering time.

All the flowers and fruits produced by each plant were recorded. The fruits were weighed and fruit set and the proportion of flowers setting fruit were calculated. The reproductive effort was estimated as the ratio of reproductive dry mass (flowers and fruits) to vegetative dry mass. Flower dry mass was determined from flower number and the mean flower dry mass, which was estimated from a set of randomly selected flowers (5.2 ± 0.2 mg, N = 46 for females and 6.0 ± 0.2 mg, N = 59 for males; mean ± SE).

Growth parameters

To evaluate the effects of the treatments on growth parameters, we harvested all the plants at the end of the experiment. At harvest, plants were separated into below- (hereafter roots) and above-ground (shoot) mass. Roots were washed and all plant material was oven-dried at 55ºC for 6 days and weighed to the nearest 0.0001 g (Mettler AJ100, Switzerland). Root mass ratio (RMR = root mass/plant mass) and relative growth rate (RGR = [ln (harvest dry mass)-ln (initial dry mass)]/days) were calculated. Before the experiment began, initial dry mass was determined from a whole-plant harvest of 20 randomly selected individuals.

Statistical analysis

A complete randomized split-plot experimental design was applied, with nutrient and salt spray as whole plot factors and sex as subplot factor. The entire experiment was replicated twice. For chlorophyll fluorescence and spectral reflectance parameters, repeated measurements were made on the same individual plants; these measurements cannot be considered independent from each other and thus we employed a split-plot analysis of variance with repeated measures. We tested the main and interaction effects over the appropriate error term when this was significant. Otherwise, the effects were tested over the within error term to maintain power in the analysis (Sokal & Rolf 1995; Pigliucci 2002). The analysis was carried out with SYSTAT 11 (Systat software, Inc. 2004, CA, USA). The alpha level was set at $P = 0.05$. Prior to analysis, data were examined for normality and homogeneity and transformed when necessary to improve the assumptions of the analysis of variance. Relative growth rate, synchrony index and reproductive effort were log transformed.

RESULTS

Chlorophyll fluorescence

Fv/Fm values were always higher than 0.804, although nutrients had a significant effect on this parameter, with effect being dependent on salt spray and time (Table 1; Fig. 2). At the end of the experiment (28th September and 20th October), plants that did not receive any salt spray or nutrients showed significantly lower Fv/Fm values than plants assigned to the other treatments (LSD test: $P < 0.05$). Throughout the experiment, the effective quantum yield (ΦPSII) of the sexes did not differ in response to nutrient addition, salt spray, or their interactions.

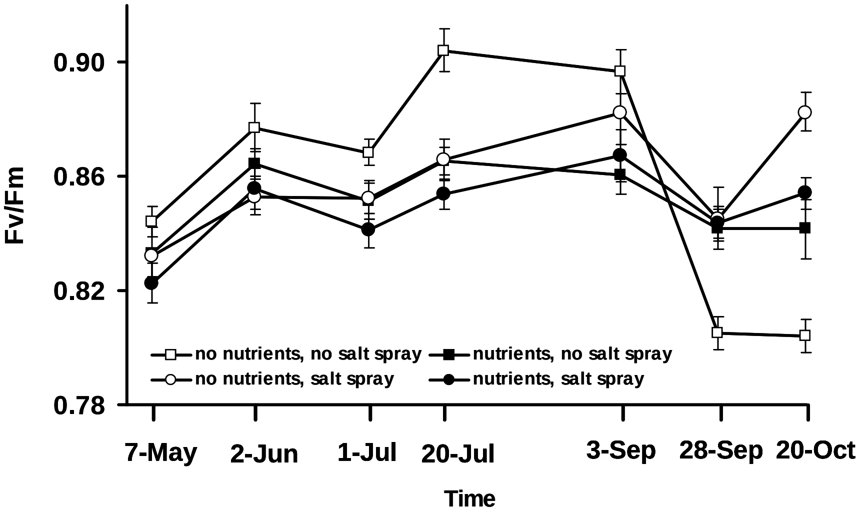

Fig. 2 Time course of maximum quantum yield (Fv/Fm) (means ± SE) for *Honckenya peploides* plants, as a function of nutrient and salt spray treatments (N = 20).

Table 1 Results of repeated measures split-plot analysis for maximum quantum yield (Fv/Fm) and effective quantum yield (Φ PSII). Terms used as errors are labelled with letters. Letters following *F* values indicate the denominator used to test each effect. Values of $P < 0.005$ are shown in bold type.

		Fv/Fm			Φ PSII		
Source of variation	*df*	MS	*F*	*P*	MS	*F*	*P*
Nutrient(N)	1	0.960	8.333^{e}	**0.004**	0.691	0.605^{e}	0.437
Spray(S)	1	0.003	0.029^{e}	0.865	4.324	3.786^{e}	0.052
S × N	1	0.081	0.702^{e}	0.403	0.073	0.064^{e}	0.801
Plot (S × N)	4	0.200	1.737^{e}	0.141	1.374	1.203^{e}	0.309
Sex	1	0.056	0.484^{e}	0.487	0.191	0.167^{e}	0.683
Sex × N	1	0.012	0.102^{e}	0.749	0.022	0.019^{e}	0.889
S × Sex	1	0.012	0.104^{e}	0.747	0.021	0.018^{e}	0.893
N × S × Sex	1	0.039	0.335^{e}	0.563	0.006	0.005^{e}	0.944
Sex × Plot (S × N)	4	0.061	0.532^{e}	0.712	1.947	1.705^{e}	0.148
Time(T)	6	2.465	21.391^{e}	**<0.001**	38.741	17.952^{a}	**<0.001**
N × T	6	0.490	4.249^{e}	**<0.001**	1.757	0.814^{a}	0.570
S × T	6	1.241	10.767^{e}	**<0.001**	4.275	1.981^{a}	0.108
S × N × T	6	0.587	5.089^{e}	**<0.001**	2.215	1.026^{a}	0.433
T × Plot (N × S)^{a}	24	0.174	1.512^{e}	0.058	2.158	1.889^{e}	**0.007**
Sex × T	6	0.067	0.578^{e}	0.748	1.245	1.09^{e}	0.367
N × Sex × T	6	0.065	0.560^{e}	0.762	0.698	0.611^{e}	0.722
S × Sex × T	6	0.091	0.792^{e}	0.577	1.188	1.04^{e}	0.399
S × Sex × N× T	6	0.063	0.549^{e}	0.771	1.029	0.901^{e}	0.494
Sex × T× Plot(S× N)	24	0.091	0.792^{e}	0.748	0.895	0.783^{e}	0.759
Within error^{e}	448	0.115			1.142		

Spectral reflectance

The photosynthetic radiation-use efficiency of sexes, as estimated by PRI index, differed significantly in response to nutrient addition, with fertilized females, but not males, showing greater PRI values than unfertilized females

(LSD test: P = 0.021; Table 2; Fig. 3). The chlorophyll index and the normalized difference vegetation index were not significantly affected by the experimental treatments or interactions.

Table 2 Results of split-plot analysis for photochemical reflectance (PRI), chlorophyll content (CHL) and normalized difference vegetation (NDVI) indices. Terms used as errors are labelled with letters. Letters following *F*values indicate the denominator used to test each effect. Values of $P < 0.005$ are shown in bold type.

		PRI			CHL			NDVI		
Source of variation	*df*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Nutrients(N)	1	4.968	0.166^{a}	0.705	320.396	4.678^{a}	0.097	0.217	0.042^{a}	0.848
Spray(S)	1	1.436	0.048^{a}	0.837	191.878	2.802^{a}	0.170	2.888	0.565^{a}	0.494
N × S	1	1.734	0.058^{a}	0.822	35.040	0.512^{a}	0.514	6.057	1.185^{a}	0.338
Plot(N × S)^{a}	4	29.91	8.664^{e}	< **0.001**	68.491	12.902^{e}	< **0.001**	5.111	65.952^{e}	< **0.001**
Sex	1	0.428	0.124^{e}	0.726	8.304	1.564^{e}	0.216	0.216	2.782^{e}	0.100
N × Sex	1	25.104	7.272^{e}	**0.009**	2.305	0.434^{e}	0.512	0.082	1.054^{e}	0.309
S × Sex	1	8.176	2.368^{e}	0.129	0.364	0.069^{e}	0.794	0.002	0.021^{e}	0.884
N × S × Sex	1	0.211	0.061^{e}	0.806	4.815	0.907^{e}	0.345	0.034	0.437^{e}	0.511
Sex × Plot (N × S)	4	1.688	0.489^{e}	0.744	8.087	1.523^{e}	0.206	0.079	1.025^{e}	0.401
Within error^{e}	64	3.452			5.309			0.077		

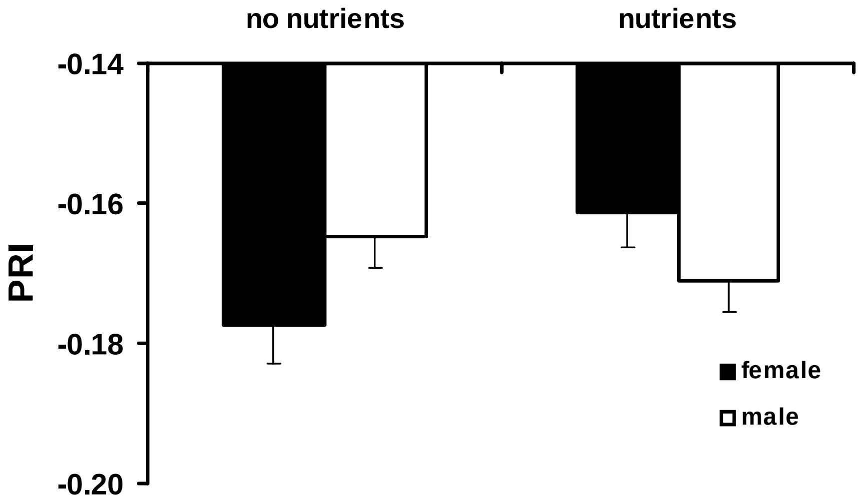

Fig. 3 Mean values (± SE) of Photochemical reflectance index (PRI) for male and female plants of *Honckenya peploides* growing with and without addition of nutrients (N = 20).

Reproductive and phenology parameters

Females produced a significantly greater number of fruits, allocated significantly more biomass to reproduction and flowered for a significantly longer period than males (Tables 3 and 4). The effect of addition of nutrients in decreasing flowering synchrony was lower for salt-sprayed plants (Tables 3 and 4). Fertilized plants flowered for a significantly longer period than unfertilized ones and the reproductive effort in plants sprayed with salt was half that of non-sprayed plants (Tables 3 and 4).

Table 3 Results of split-plot analysis of variance for reproductive effort (RE), flowering duration (days), flowering synchrony and fruit set (%). Since the terms Plot(N × S) and Sex × Plot(N × S) were non significant, the main and interaction effects were tested over the within error term to maintain power in the analysis. Values of *P* < 0.005 are shown in bold type.

		RE			Flowering duration			Flowering synchrony			Fruit set		
Source	*df*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Nutrients (N)	1	0.01	0.001	0.982	9131.40	38.750	**<0.001**	815.95	45.864	**<0.001**	7.03	1.906	0.172
Spray(S)	1	220.48	9.003	**0.004**	0.74	0.003	0.955	13.54	0.761	0.386	12.96	3.515	0.065
N × S	1	3.86	0.158	0.692	105.11	0.446	0.507	92.63	5.207	**0.026**	11.55	3.131	0.082
Plot (N × S)	4	8.97	0.366	0.832	266.20	1.130	0.351	14.93	0.839	0.506	8.72	2.364	0.062
Sex	1	1253.25	51.178	**<0.001**	1127.25	4.784	**0.032**	0.49	0.027	0.869	414.70	112.436	**<0.001**
N × Sex	1	0.34	0.014	0.906	105.11	0.446	0.507	0.89	0.050	0.824	1.06	0.286	0.594
S × Sex	1	0.86	0.035	0.852	150.97	0.641	0.426	0.11	0.006	0.938	8.80	2.387	0.127
N × S × Sex	1	31.16	1.272	0.264	83.84	0.356	0.553	0.43	0.024	0.877	0.03	0.009	0.925
Sex × Plot (N × S)	4	17.98	0.734	0.572	149.73	0.635	0.639	16.77	0.943	0.445	5.71	1.549	0.199
Within error	64	24.49			235.65			17.79			3.69		

Table 4 Mean values (± SE) of reproductive effort (RE), flowering duration (FD, days), flowering synchrony (FS) and fruit set (%) for male and female *H. peploides* plants, as a function of salt spray and nutrient treatments (N = 10).

Nutrients	Salt Spray	Sex	RE	FD	FS	Fruit set
No nutrients	No salt spray	Female	0.112 ± 0.038	34.3 ± 6.3	0.628 ± 0.032	0.512 ± 0.050
		Male	0.023 ± 0.004	33.9 ± 3.2	0.632 ± 0.018	0.009 ± 0.006
	Salt spray	Female	0.121 ± 0.031	41.6 ± 6.1	0.611 ± 0.027	0.437 ± 0.083
		Male	0.017 ± 0.004	31.6 ± 4.1	0.610 ± 0.015	0.075 ± 0.075
Nutrients	No salt spray	Female	0.322 ± 0.091	62.3 ± 5.6	0.516 ± 0.013	0.666 ± 0.051
		Male	0.068 ± 0.043	53.2 ± 4.3	0.515 ± 0.012	0.125 ± 0.090
	Salt spray	Female	0.100 ± 0.028	60.9 ± 3.3	0.551 ± 0.005	0.447 ± 0.082
		Male	0.020 ± 0.004	50.4 ± 4.6	0.552 ± 0.012	0.031 ± 0.010

Growth parameters

The relative growth rate of males was significantly higher than that of females (0.0073 ± 0.0004 g g^{-1} day^{-1} compared with 0.0086 ± 0.0004 g g^{-1} day^{-1}; mean ± SE) (Table 5). Males also invested significantly more of their total biomass to root (RMR) than females (Table 5 and Fig 4). Addition of nutrients resulted in a 5.5-fold increase in total dry mass and an 8.7-fold increase in above-ground dry mass in male plants and a 4.5-fold increase in total dry mass and a 6.6-fold increase in above-ground mass in female plants. As result of this differential increase in dry mass in response to nutrient addition, we found significant differences between males and females in total dry mass and shoot dry mass under fertilized (LSD test: $P < 0.001$ for both) but not under unfertilized conditions (LSD test: $P = 0.662$ for total and $P = 0.902$ for shoot dry mass), with males showing the higher values (Table 5 and Fig. 4).

The magnitude of relative growth rate increased significantly with nutrient addition (from 0.0057 ± 0.0002 g g^{-1} day^{-1} for unfertilized plants to 0.0102 ± 0.0003 g g^{-1} day^{-1} for fertilized plants; mean ± SE), but was not affected by application of salt spray (Table 5). Addition of nutrients significantly increased biomass production, and decreased the proportion of total biomass invested in roots (Table 5 and Fig. 4). Salt spray significantly increased the root dry mass, especially in fertilized plants, and marginally increased the total dry mass (Table 5 and Fig. 4).

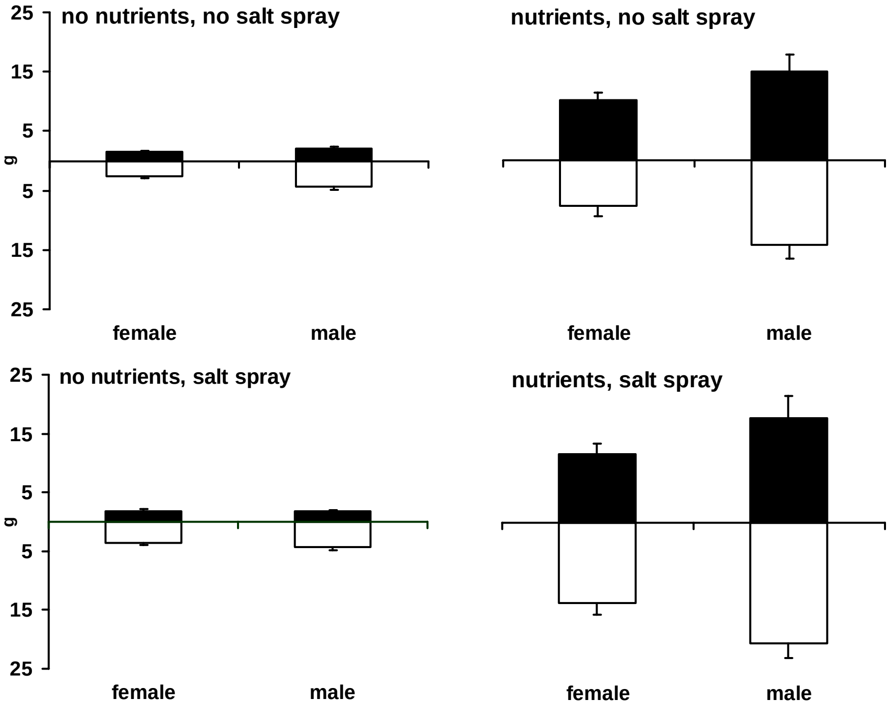

Fig. 4 Above (solid bars) and below-ground (open bars) dry mass (*g*) for male and female plants of *Honckenya peploides*, as a function of the different experimental conditions (N= 10). Error bars indicate standard error of the means.

Table 5 Results of split-plot analysis of variance for below-, above-ground and total dry mass (DM), and for root mass ratio (RMR) and relative growth rate (RGR). Terms used as error are labelled with letters. Values of $P < 0.005$ are shown in bold type

		Below-ground DM			Above- ground DM			Total DM			RMR			RGR		
Source	*df*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Nutrients(N)	1	2133.04	98.82^{c}	**<0.001**	2024.07	66.329^{c}	**<0.001**	9767.32	96.338^{c}	**<0.001**	59.448	22.083^{a}	**0.009**	734.313	144.169^{c}	**<0.001**
Spray(S)	1	221.74	10.27^{c}	**0.002**	47.52	1.557^{c}	0.217	380.95	3.757^{c}	0.057	8.912	3.310^{a}	0.143	10.066	1.976^{c}	0.165
N × S	1	156.60	7.25^{c}	**0.009**	46.06	1.509^{c}	0.224	285.08	2.812^{c}	0.098	3.086	1.146^{a}	0.345	0.05	0.01^{c}	0.921
Plot(N × S)^{a}	4	26.08	1.21^{c}	0.316	2.47	0.081^{c}	0.988	10.71	0.106^{c}	0.980	2.692	3.311^{c}	**0.016**	2.963	0.582^{c}	0.677
Sex	1	309.41	5.16^{b}	0.086	315.69	10.345^{c}	**0.002**	921.86	9.093^{c}	**0.004**	3.764	4.629^{c}	0.035	67.47	13.246^{c}	**0.001**
N × Sex	1	148.59	2.48^{b}	0.190	224.32	7.351^{c}	**0.009**	573.08	5.652^{c}	**0.020**	0.001	0.001^{c}	0.974	3.869	0.76^{c}	0.387
S × Sex	1	0.79	0.04^{b}	0.849	0.10	0.003^{c}	0.954	0.05	0.0005^{c}	0.983	1.122	1.380^{c}	0.244	3.461	0.679^{c}	0.413
N × S × Sex	1	1.74	0.08^{b}	0.778	0.33	0.011^{c}	0.917	10.73	0.106^{c}	0.746	0.483	0.594^{c}	0.444	5.393	1.059^{c}	0.307
Sex × Plot (N × S)^{b}	4	59.95	2.78^{c}	**0.034**	60.19	1.973^{c}	0.109	232.25	2.291^{c}	0.069	0.793	0.976^{c}	0.427	9.016	1.77^{c}	0.146
Within error^{c}	64	21.59			30.52			101.39			0.813			5.093		

DISCUSSION

This study represents a mechanistic approach to understanding the causes of spatial segregation of the sexes in *H. peploides*. Evaluation of the responses of the different sexes to important selective forces in dune environments from the understudied point of view of sexual competition, revealed significant between-sex differences as regards physiology, growth, reproduction and phenology.

Sex-differential responses

In this study, male plants grew significantly faster than female plants, and under nutrient rich conditions the shoot and total dry mass increased significantly more in males than in females. Moreover, male plants dedicated more biomass to roots than females. The advantages of a high RGR seem clear. Plants with higher RGR can occupy a larger space and would therefore have access to a larger portion of limiting resources (Poorter 1989). The sex-related differences in vegetative growth and biomass allocation that we found may be translated into sex-related differences in competitive abilities (Lloyd & Webb 1977). Thus, our findings suggest that because of their higher vegetative growth, males have a higher potential to exploit the substrate and colonize new sites. Competitive exclusion resulting from intersexual differences in sensitivity to neighbouring plants has been postulated as a possible cause of spatial segregation of the sexes (Meagher 1980; Sakai & Oden 1983; Bertiller *et al.* 2002). Other authors have proposed spatial segregation as a mechanism for prevention of intersexual competition (Freeman *et al.* 1976; Cox 1981; Wade *et al.* 1981).

Although the general trend in dimorphic woody species is for males to be larger and grow faster than females (Obeso *et al.* 1998; Obeso 2002) there are insufficient data available to establish whether this pattern exists in herbaceous species. Obeso (2002) reviewed 103 studies of dimorphic species (91 of which dioecious), but only two of these studies examined RGR in herbaceous species. One study found that males outperformed females, but the other found no significant differences between morphs. Case & Ashman (2005) referred two studies evaluating vegetative propagation in herbaceous dimorphic species, and therefore comparable to *H. peploides*: one study found lower vegetative propagation in females, but the other found no sex-related difference. Clearly, more studies are needed to reveal the existence of a consistent pattern.

We also found that the provision of nutrients significantly increased values of photochemical reflectance index in females but not in males. The different response between the sexes to nutrient provision suggests that photochemical efficiency is more limited by nutrients in females than in males, at least under competitive conditions. Higher PRI values for fertilized females may also result in higher tolerance to photoinhibition, since high PRI values have been related to higher contents of carotenoid components in the xanthophyll cycles involved in the mechanism of thermal dissipation of excess excitation energy (Gilmore & Yamamoto 1993).

Together the results show that the competitive ability of the sexes is environment dependent. Under natural conditions, with the high levels of radiation characteristic of dune environments, the higher growth rates of males that we found under nutrient-rich conditions may be counterbalanced by the higher photochemical efficiency and higher tolerance of females to photoinhibition. Previous studies have suggested that context-dependent variation in competitive effects between males and females of dioecious species may play a crucial role in maintenance of the patterns of spatial segregation of the sexes in these species (Bertiller *et al.* 2002; Eppley 2006).

Greater reproductive effort in females has been documented in many plant species (e.g., Delph 1990; Cipollini & Whigham 1994) and also in the present study for *H. peploides*. Because there may often be a trade-off between reproductive and vegetative growth, the greater reproductive allocation in females should result in females having less resources available for growth and survival. In this way, the greater allocation to reproductive structures in females of *H. peploides* may explain the differences between sexes in terms of growth. The cost of a high investment in reproduction is argued to be alleviated under good environmental conditions (Freeman *et al.* 1976; Lloyd & Webb 1977). If the higher reproductive effort of females is limiting their vegetative growth, we would expect mitigation of this under nutrient-rich conditions. However, under such conditions, we found that shoot and total dry mass increased significantly more in males than in females. As already mentioned, the situation may be different under nutrient-rich conditions in the field, where females may take advantage of their higher photochemical efficiency and higher tolerance to photoinhibition. The effect of disruptive selection acting on male-female populations with different reproductive costs has been related to the spatial segregation of the sexes (Freeman *et al.* 1976; Lovett-Doust *et al.* 1987; Bierzychudek & Eckhart 1988; Geber 1999). Sex ratios are often biased along a gradient of resource availability, with a preferential distribution of females in fertile and sheltered microsites as a result of their higher reproductive cost than in males (Freeman *et al.* 1976; Bierzychudek & Eckhart 1988; Dawson & Ehleringer 1993; Dawson & Geber 1999).

It has been argued that to enhance reproductive fitness, males presumably need to flower longer than females. Because males cannot predict the timing of peak female flowering the probability of pollination is uncertain and extending male floral longevity reduces the risk of pollination failure (Rathcke & Lacey 1985; Abe 2001). Our study however showed longer flowering duration in females than in males. In many dioecious and gynodioecious species, the longevity of pistillate flowers is greater than that of flowers with male function, because pistillate flowers must wait for pollen to arrive, whereas males have completed their function as soon as pollen is shed (Primack 1985; Ashman & Stanton 1991). Abe (2001) demonstrated how female flower lifespan increased in the absence of pollen.

Nutrient and Salt spray effects

This study shows that salt spray did not differentially affect growth, phenological or physiological responses of the sexes. However, as shown for other species (Boyd & Barbour 1986; Cheplick & Demetri 1999), salt spray significantly decreased biomass allocated to reproduction in *H. peploides*. In contrast, salt sprayed plants increased the allocation of biomass to roots, an increase that was significantly higher when plants also received nutrients. In addition, we observed a tendency to higher total dry mass in plants that received salt spray. These results suggest that reproductive structures are more sensitive to the effects of salt spray than vegetative ones and that salt spray is not only a stressful factor, but also a source of macro and micronutrients (Evans 1988). This may favour the growth of salt-tolerant plants (Rozema *et al.* 1982) such as *H. peploides*, a pioneer species in dune colonization, exposed to the highest levels of salt spray.

The values of chlorophyll fluorescence parameters, within the range considered normal in non-stressed plants (Lambers *et al.* 1998), demonstrated that this species is able to maintain good function of its photosystems, even when nutrients are not readily available. Only an extended period without nutrients or salt spray (at the two last measurements dates) appears to produce moderate impairment of the photosynthetic function. Such a situation is unlikely to occur in natural conditions. The tolerance of *H. peploides* to low nutrient availability was also reported by Gagné & Houle (2002). Natural environmental conditions impose limitations to growth, since sandy substrates tend to lose nutrients quickly, due to their low-retention capacity. Therefore as expected, fertilization stimulated growth. The low nutrient availability in dune habitats therefore limits the growth of *H. peploides*.

Although most studies have tried to explain phenological patterns in terms of selection exerted by several biotic agents (Augspurger 1981; Dieringer 1991; Gómez 1993) or by climatic constraints (Galen & Staton 1991; Herrera 1992; Buide *et al.* 2002), we found that abiotic factors, such as salt spray and nutrient availability, may also constrain phenological patterns through effects on flowering synchrony and duration.

In conclusion, in this study we found that sexes differed in growth rate, biomass allocation, phenology and also in physiological traits related to photochemical efficiency and tolerance to photoinhibition in response to nutrient availability. The differential responses of the sexes to such an important selective force in coastal environments probably determine the relative competitiveness of the sexes. Thus, nutrient availability is a potentially crucial determinant of the outcome of interactions between sexes. The environmentally dependent differences in the competitiveness of the sexes revealed in this study may help to explain the extreme spatial segregation of the sexes that we have observed under field conditions. Further research on the effects of nutrient availability on the performance of the sexes in natural conditions should be a priority for future studies. In addition, the results also confirm some general trends observed in dimorphic species, such as the higher proportion of biomass allocated to reproduction in females than in males.

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Conclusions

CONCLUSIONS

Chapter 1

This study finds high genetic variation within the clumps of *Honckenya peploides*. The clumps are unisexual despite establishment from seeds occur and despite consisting of different genotypes. Several reasons for the unisexuality are discussed and most of the available evidence indicate that ecophysiological differences between the sexes may be responsible for the fact that clumps are composed by different genotypes but nevertheless unisexual.

Chapter 2

The findings of this study establish that male and female specific leaf areas are differentially affected by reproduction.

The reproductive status of a shoot, its position within the clump (edge or centre) and also site conditions affect its photosynthetic efficiency and chlorophyll content.

The estimation of male and female reproductive costs at different times in the season may lead to quite different conclusions regarding the real relative resource costs of reproduction in male and female plants.

Chapter 3

The male and female plants of *H. peploides* show contrasting patterns of resource allocation, what reveals the necessity of making dynamic estimations of reproductive and biomass allocation if we really want to know the real cost of the male and female functions in dimorphic and polymorphic species.

This study demonstrates that reproductive effort is not always higher in females than in males in dioecious species. The seasonal timing of resource investment in flowering and in fruit set may cause male reproductive effort to be as, or even more, costly than that of females.

The between-sex differences in the seasonal patterns of reproductive and biomass allocation may play an important role in explaining the spatial segregation of the sexes.

Chapter 4

The lack of sex-related differences in integrated water use efficiency and leaf nitrogen content make difficult to establish a physiological basis for the spatial segregation of the sexes. The study suggests that males and females through a selection of habitats might meet with their different demand of resources for reproduction, with each sex achieving the best performance in the habitat in which it predominates.

Chapter 5

Male and female plants of *H. peploides* differ significantly in important ecophysiological and allocation traits, such as photochemical efficiency, susceptibility to photoinhibition, and proportion of total biomass invested in leaves.

Sexes differ in morphological attributes at the leaf level, such as succulence degree and stomatal density, suggesting sex-specific strategies to cope with water availability.

The presence of sexual dimorphism in ecophysiological traits in individuals that are not bearing reproductive structures indicates that dimorphism is not only a consequence of between-sex difference in resources invested in reproduction.

Chapter 6

Sexes differ in growth rate, biomass allocation, phenology and physiological traits related to photochemical efficiency and tolerance to photoinhibition in response to nutrient availability. The differential responses of the sexes to such an important selective force in coastal environments may determine the relative competitiveness of the sexes. The environmentally dependent differences in the competitiveness of the sexes that reveal this study may help to explain their extreme spatial segregation observed under field conditions.

The results of this study confirm some general trends observed in dimorphic species, such as the higher proportion of biomass allocated to reproduction in females than in males.

As a **general conclusion**, sexual dimorphism in *H. peploides* depends on environmental conditions where sexes grow and the causes of the spatial segregation of the sexes are complex, requiring more than one explanation. The interplay among physiological, allocational and morphological growth determinants may contribute to explaining between-sex differences in the responses to environmental stress, and therefore the maintenance of the marked spatial segregation observed in this species.

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RESUMO

ANTECEDENTES

Dioecia e sistemas relacionados

No reino vexetal existe unha ampla variedade de sistemas reprodutivos, sendo o máis común o hermafroditismo, representando o 72% das anxiospermas, onde cada individuo ten a capacidade de transmitir os seus xenes a través das funcións feminina (sementes) e masculina (polen). O resto son dioicas ou con sistemas sexuais compostos por individuos unisexuais e bisexuais (*i.e.* xinodioicas, androdioicas, trioicas ou subdioicas). A dioecia, é o máis extremo dos polimorfismos mencionados, e caracterizase pola presenza de individuos masculinos e femininos ben diferenciados, que producen só polen e sementes, respectivamente. En parte debido a súa rareza, en torno a un 6% do total das anxiospermas segundo Renner e Ricklefs (1995), existe moito interese arredor da dioecia e nos factores selectivos responsables da súa evolución. Aínda que non hai unha resposta universal, recoñécense dúas razóns principais para a súa evolución (Bawa 1980; Thomson e Brunet 1990). Por un lado, a dioecia permite evitar a auto-fecundación e a conseguinte depresión endogámica (Charlesworth e Charlesworth 1978). Por outro lado, nas especies dioicas existe unha “división do traballo” entre os sexos, o cal podería contribuír a unha maior eficiencia reprodutiva grazas a un máis eficiente uso dos recursos.

Custos da reprodución

As plantas dioicas supoñen unha excelente oportunidade para determinar os custos asociados cas funcións de reprodución masculina e feminina, posto que estas dispóñense en individuos separados. Como patrón xeral, as femias inverten máis en reprodución que os machos, xa que os machos destinan recursos só á floración, mentres as femias teñen que producir tanto flores como froitos (Allen e Antos 1988). Como consecuencia deste maior investimento, as plantas produtoras de froitos pagarán maiores custos, en termos de menor supervivencia, menor frecuencia de floración e/ou crecemento vexetativo máis lento. Asemade, esperase un incremento no estres fisiolóxico no sexo co maior esforzo reprodutivo (Dawson e Ehleringer 1993). A maioría dos estudos sobre a especialización dos sexos en plantas dioicas centráronse en carácteres morfolóxicos e propiedades ecolóxicas, encontrando en moitos casos diferenzas entre os sexos (Allen e Antos 1993). Debido ás dificultades na recollida de datos sobre procesos fisiolóxicos, os aspectos de diferenciación fisiolóxica entre os sexos son moito menos coñecidos. Diferentes mecanismos fisiolóxicos poden axudar a aliviar os custos da reprodución (ver revisións Obeso 2002; Case e Ashman 2005). A fotosíntese en flores e froitos, incrementos na taxa fotosintética foliar en resposta á demanda de carbono motivada polo desenvolvemento do froito, ou a reabsorción de nutrientes desde estruturas florais son algúns exemplos destes mecanismos. A fisioloxía é sen dubida, un aspecto crucial que debe de abordarse, pois a variación nos atributos ecofisiolóxicos pode determinar o rendemento de cada sexo nos diferentes hábitats e, en última instancia, ter consecuencias sobre o crecemento, reprodución e supervivencia. Neste sentido, a segregación espacial dos sexos, onde cada sexo ocupa aquel hábitat onde mellor atopa as demandas específicas asociadas coa reprodución é un posible mecanismo que tamén pode axudar a aliviar os costos de reprodución.

Segregación espacial dos sexos

A distribución espacial dos sexos é obxecto de estudo dende fai máis de 30 anos (Freeman *et al.* 1976). A distribución das plantas masculinas e femininas segue un patrón aleatorio nalgunhas especies dioicas (Bawa e Opler 1977; Melampy e Howe 1977; Hancock e Bringhurst 1980; Armstrong e Irvine 1989), mentres noutras, os sexos están espacialmente segregados (Freeman *et al.* 1976; Lovett Doust e Cavers 1982; Sakai e Oden 1983; Freeman e Vitale 1985; Dawson e Bliss 1989). A segregación espacial dos sexos é un fenómeno sorprendente, xa que teoricamente unha maior separación dos sexos suporía un descenso no éxito de reprodución sexual nos individuos. Sen embargo, é un fenómeno relativamente frecuente, ocorrendo en máis de 30 especies de plantas de 20 familias (revisado por Bierzychudek e Eckart 1988; Iglesias e Bell 1989; Korpelainen 1991; Shea *et al.* 1993; Lokker *et al.* 1994). Diferentes autores suxiren que a segregación espacial dos sexos pode optimizar o uso de recursos no espazo e no tempo, incrementando a "fitness" de machos e femias en comparación a individuos hermafroditas (Freeman *et al.* 1976; Lloyd 1982; Lovett Doust e Lovett Doust 1988; Pannell e Barrett 1998; Charlesworth 1999). Normalmente a segregación espacial de machos e femias ocorre seguindo un gradiente ambiental (Freeman *et al.* 1976; Grant e Mitton 1979; Freeman *et al.* 1980; Bierzychudek e Eckhart 1988; Sakai e Weller 1991; Dawson e Ehleringer 1993), coas femias ocupando preferiblemente os sitios máis ricos en recursos, o cal pode aliviar en parte, os seus maiores custos reprodutivos (Freeman *et al.* 1976; Lloyd e Webb 1977; Cox 1981). Varios mecanismos poderían explicar a distribución non-aleatoria dos sexos, incluíndo diferenzas entre os sexos nos requirimentos de xerminación (Bierzychudek e Eckart 1988; Purrington 1993; Lyons *et al.* 1995), mortalidade diferencial das sementes en diferentes hábitats estresantes (Cox 1981; Lloyd e Webb 1977; Krischik e Denno 1990), e diferenzas nas habilidades competitivas (Freeman *et al.* 1976; Meagher 1980; Cox 1981; Ågren 1988). Pero, independentemente de cal sexa a causa de orixe, a especialización fisiolóxica dos sexos pode axudar a mantela.

A especie

Honckenya peploides (L.) Ehrh., arenaria do mar, é unha herbácea da numerosa familia das Cariofiláceas (Orden Cariofiliales) con 75 xéneros e unhas 2000 especies. É a única representante do xénero *Honckenya*. É moi variable nas súas características morfolóxicas, as cales son facilmente modificadas polas condicións locais recoñecéndose normalmente tres variantes morfolóxicas: subsp. *peploides* atopada nas costas europeas dende o Norte de Noruega ata o Sur de Portugal, subsp. *diffusa* cunha distribución circumpolar principalmente ártica e no norte da zona boreal e subsp. *major* (Hooker) Hultén que se distribúe pola área do Pacífico Norte (incluíndo a zona ártica do Norte de Alasca). Na Península Ibérica atopamos representantes da subsp. *peploides* nas costas cantábricas e atlánticas excepto no

SW, sendo as zonas costeiras de Galicia xunto cas do Norte de Portugal as máis ó Sur nas que se atopa dita subespecie.

É unha planta perenne tipicamente presente na duna embrionaria, na que forma manchas de ata 50 cm. en altura. As súas follas opostas son carnosas, anchas na base e forman un ángulo agudo co talo. Tanto a disposición, a forma como a orientación das follas reducen a radiación incidente. Presenta un sistema radicular de tipo rizomatoso, amplamente desenvolvido, xogando un papel fundamental en sistemas areosos ós que lles da estabilidade, permitindo a acumulación de area e creando un medio óptimo para o establecemento de novas plántulas. Presenta flores solitarias nas axilas foliares e na parte apical dos talos. As pequenas flores son brancas, actinomórficas e florecen desde finais de Marzo a Xuño. Cada flor está formada por cinco sépalos, cinco pétalos, dez estames e un ovario composto que consta de 2 a 4 estilos. Na base de cinco dos estames, alternando cos pétalos, están presentes cinco glándulas produtoras de néctar que exsudan dita sustancia no período de floración. As flores teñen un olor característico, recordando un pouco ao mel, que atrae a insectos (Tsukui e Sugawara 1992). A planta produce cápsulas que encerran varias sementes (xeralmente 3-8) de tipo piriforme e de aproximadamente 3-4 mm. en lonxitude.

Esta especie presenta un sistema sexual complexo, composto por femias, machos e hermafroditas (ou machos inconstantes) que algúns autores denominan subdioecia ou andro-xino-dioecia mentres que outros optan en denominar dioecia funcional. Este sistema sexual, considérase un paso intermedio no camiño evolutivo entre o hermafroditismo e a dioecia vía xinodioecia, un camiño evolutivo amplamente recoñecido na evolución da dioecia (Charlesworth e Charlesworth 1978; Ross 1982; Webb 1999; Delph e Wolf 2005). A xinodioecia é un sistema sexual no cal coexisten plantas femias, que producen só óvulos, e plantas hermafroditas, que producen tanto óvulos coma polen (Sakai e Weller 1999). Ó longo dese continuo evolutivo pode ocorrer un descenso na función feminina nos hermafroditas, causando sistemas sexuais que aínda que non son estritamente dioicos están moi próximos a esta condición. Estes sistemas coñécense como subdioicos, aínda que na bibliografía existe unha ampla variedade de termos para referirse a este estado. Nas especies subdioicas as plantas femia con frecuencia son constantes na súa expresión sexual, producindo só óvulos, mentres que as plantas produtoras de polen presentan labilidade sexual ou inconstancia de xénero, producindo tanto polen como óvulos en diferentes grados. Se ben en *H. peploides* a diferenciación entre hermafroditas e machos non é clara, precisando incluso de estudios de invernadoiro, o que está claro é que nas poboacións de Galicia, que corresponden coa subespecie *peploides*, hai 2 tipos ben definidos morfoloxicamente de flores. O primeiro cos pétalos e estames de menor tamaño e o segundo cos pétalos e os estames máis grandes que coinciden cos descritos por Tsukui e Sugawara (1992) para a subsp. *major* na zona de Xapón. Estes autores que describen á especie como funcionalmente dioica atopan que o primeiro tipo funciona como femia, mentres o segundo tipo non produce froitos e se o fai é en menor medida e cun éxito na xerminación menor que as femias, co cal optan por chamarlle machos. Ó longo desta tese as plantas de *H. peploides* produtoras de polen, desígnanse como machos tal e como foi adoptado para outras especies consideradas subdioicas descritas por diversos autores (Lloyd 1976; Olson e Antonovics 2000).

O medio dunar

As condicións medioambientais son especialmente duras nas dunas costeiras e van a restrinxir o establecemento, o crecemento e a supervivencia das plantas pioneiras (Maun 1994), explicando, en parte, a escasa diversidade vexetal existente. Primeiramente os substratos areosos teñen unha escasa capacidade de retención de auga o que significa unha rápida percolación da auga de choiva cun significativo lixiviado en nutrientes (Kellman e Roulet 1990). Outra característica destes medios é a alta mobilidade do substrato, con erosión e acumulación local, favorecido polos fortes ventos e o relativamente fino tamaño de partícula (Olson 1958; Hesp 1989). Ademais os ventos transportan gotículas de sal mariña ("sprai salino") que se acumulan sobre os tecidos das plantas e tamén contribúen a aumentala salinidade do substrato. As mareas tamén poden incrementar a salinidade do substrato, e en determinadas ocasións (ex. grandes tormentas) causan erosión na parte superior da praia. Todas estas condicións son xeralmente desfavorables para os diferentes estados vitais das plantas e en particular para as plántulas. A maioría das especies vexetais non poden tolerar tales condicións, sen embargo hai especies que evolucionaron e presentan unha serie de adaptacións particulares a estes medios dunares. As partes do sistema dunar máis afectadas por estas duras condicións van a ser ás máis próximas á praia. Así, a duna embrionaria sitúase en contacto directo coa parte máis elevada da praia, é a máis rica en sales solubles e a flora característica (*Cakile maritima, Atriplex prostata, Euphorbia peplis*, *Honckenya peploides*, etc.) aparece especialmente adaptada á alta salinidade existente e á inestabilidade do medio físico. Por detrás fórmase a crista da duna ou duna primaria, colonizada por gramíneas rizomatosas (*Elymus farctus, Ammophila arearia*), que desempeñan un papel crucial como fixadores da area.

Sen embargo, dende a duna embrionaria ata as dunas estabilizadas (secundaria, terciaria...) as condicións abióticas chegan a ser menos restritivas para o crecemento vexetal. Cambios significativos nestas condicións poden existir incluso en distancias relativamente curtas, por exemplo desde a duna embrionaria ata a duna primaria (aproximadamente 15 m; Houle 1997) existen diferenzas significativas en variables como o pH ou a salinidade do substrato. Así as especies máis tolerantes ós diferentes factores de estrés serán as situadas máis preto da praia. A zoazón vexetal nas dunas costeiras pode polo tanto reflectir tolerancia dunha especie a tales condicións ambientais (Oosting e Billings 1942; Barbour e de Jong 1977; Wilson e Sykes 1999). O movemento de area, o spray salino, a salinidade do solo e o pH tipicamente descenden mentres a concentración de macronutrientes e o contido en materia orgánica do solo increméntanse (Hundt 1985; Imbert e Houle 2000) posibilitando o asentamento dun elenco maior de especies. O maior contido en materia orgánica fai que o solo dunar adquira unha coloración gris característica que da nome á duna gris, con vexetación herbácea, con especies características como *Iberis procumbens, Armeria pungens, Artemisia crithmifolia*, etc. Nas partes da duna máis evolucionada poden desenvolverse especies arbóreas como a sobreira (*Quercus suber*), o érbedo (*Arbutus unedo*) ou o loureiro (*Laurus nobilis*). No interior da duna poden existir zonas que se manteñen permanentemente húmidas en profundidade por achegas freáticas de auga mariña ou continental, favorecendo a aparición de especies higrófilas como *Juncus acutus, Scirpus holoschoenus, Thypha*.

OBXECTIVOS E PRINCIPAIS RESULTADOS E CONCLUSIÓNS

O obxectivo principal desta tese é o estudio do dimorfismo sexual en carácteres ecolóxicos, morfolóxicos e fisiolóxicos dos sexos na planta dunar *Honckenya peploides*. En Galicia, nas localidades de estudio, os sexos desta especie atópanse espacialmente segregados, o cal nos leva a preguntarnos acerca das causas desta segregación espacial. En orde a acadar estes obxectivos, estudamos diferentes aspectos das poboacións no campo e deseñamos experimentos de invernadoiro para coñecer as respostas dos sexos a diferentes variables ambientais:

No **capítulo 1**, estudouse a estrutura e a variabilidade xenética de formacións unisexuais de *H. peploides*. Nas plantas dioicas con crecemento clonal, a segregación espacial dos sexos pode medirse tanto a nivel de "xenetos" (individuos xeneticamente diferenciados) como de módulos (froito do crecemento clonal). No segundo caso, a diferente taxa de propagación vexetativa é un importante factor que afecta á relación entre os sexos (sex-ratio). *H. peploides* pode reproducirse tanto sexualmente como por crecemento clonal. As grandes formacións compostas por un só sexo atopadas nas localidades de estudo, fan pensar que son principalmente o orixe do crecemento clonal. O noso obxectivo neste traballo foi investigar a variación xenética en 3 formacións de macho se 3 de femias, mediante o uso de dúas técnicas moleculares: isoenzimas e AFLP (amplified fragment length polymorphism) . En total, analizamos 193 mostras usando isoenzimas e 80 usando AFLP. Ambas técnicas revelaron unha alta diversidade xenética. Os nosos resultados demostran que as formacións unisexuais están compostas de diferentes xenotipos, cunha alta proporción de variabilidade xenética entre os individuos.

No **capítulo 2**, investigouse se os sexos de *H. peploides* difiren en carácteres ecofisiolóxicos e se as posibles diferenzas dependen do estado reprodutivo e da posición das plantas na formación (borde ou centro). En tres localidades da costa galega (Lariño- A Coruña, San Román - Lugo e O Bao - Pontevedra), rexistramos o sexo e a densidade dos individuos, o número e a biomasa de flores e froitos. En dúas das localidades medimos eficiencia fotosintética, contido en clorofilas e área específica foliar. Atopamos que as diferenzas entre os sexos no esforzo reprodutor dependeron do momento da estación, o cal suxire que o exame dos costos reprodutivos en momentos puntuais da estación pode resultar en diferentes conclusións á hora de valorar o esforzo reprodutor de machos e femias. Tanto o estado reprodutivo como a posición dos individuos na formación afectaron á eficiencia fotosintética e os contidos en clorofila. Neste traballo atopamos que o ambiente afecta de forma significativa ós parámetros estudados, co cal a consideración da interacción do sexo polo ambiente resulta importante a ter en conta en futuros estudos.

No **capítulo 3**, exploráronse os patróns estacionais de reparto de biomasa aos diferentes órganos (estruturas subterráneas, aéreas e reprodutoras) nos sexos de *H. peploides* en condicións naturais. Ao longo do período de floración e frutificación estudáronse tres formacións “macho” e tres “femia” na localidade de Lariño (Galicia). Mensualmente dende Abril a Agosto, ambos inclusive, tomáronse mostras de solo e de plantas en puntos aleatoriamente seleccionados dentro de cada formación. Para cada mostra determinouse biomasa aérea (vexetativa e reprodutiva) e a subterránea, así como o número de flores e froitos. Tamén se mediron os contidos en auga e nutrientes, condutividade. Os resultados indican que as femias tiveron, durante o período de floración, significativamente maior biomasa total e subterránea e unha maior proporción de biomasa subterránea con respecto á aérea que os machos. Sen embargo, avanzada a estación de crecemento estas diferenzas pasaron a ser non significativas. No momento da floración, machos e femias non diferiron no número de flores, pero o peso medio dunha flor foi significativamente maior para machos que para femias, supoñendo diferenzas salientables no peso das flores a favor dos machos. Non houbo diferenzas nos carácteres reprodutivos ó final da estación. As diferenzas significativas no contido en auga do solo apareceron avanzada a estación, cando a seca avanza e a auga chega a ser máis escasa. Este estudo demostra que o exame dos custos reprodutivos a diferentes momentos pode levar a moi diferentes conclusións con respecto ao custo de reprodución de machos e femias. Ademais, podemos concluír que as diferenzas nos patróns de reparto de biomasa poden axudar a explicar a segregación espacial dos sexos observada en *H. peploides*.

No **capítulo 4**, investigouse se os sexos de *H. peploides* teñen diferentes contidos en nitróxeno foliar e diferentes eficiencias no uso da auga, estimadas a partir do uso da técnica de discriminación isotópica de carbono ($\Delta^{13}C$) en mostras foliares. Posto que *H. peploides* medra nos sistemas dunares, nas dunas embrionarias, caracterizados por unha baixa capacidade de retención de auga, poderiamos esperar diferentes patróns no uso da auga que nos axuden a explicar a segregación espacial dos sexos. Sen embargo, os resultados non indican diferenzas entre os sexos no uso da auga, e tampouco no contido en nitróxeno nas follas. Esta falta de diferenzas entre os sexos pode ser interpretada como unha especialización espacial dos sexos, de tal forma que cada sexo acada a máxima eficiencia fisiolóxica no medio onde se atopa.

No **capítulo 5**, deseñouse un experimento para investigar se os machos e as femias de *H. peploides* diferían en características fisiolóxicas (eficiencia fotoquímica e propiedades espectrais) e en compoñentes da taxa de crecemento relativo e se as posibles diferenzas en tales características foron dependentes da dispoñibilidade hídrica. As eficiencias fotoquímicas integradas no tempo foron significativamente maiores para machos que para femias. Sen embargo, a resposta dos sexos en termos de eficiencia fotoquímica variou no tempo en función da dispoñibilidade hídrica. Así as femias víronse afectadas antes polos efectos da falta de auga, pero ao remate do experimento foron os machos ós mais afectados polos efectos da falta de auga severa. Os sexos non diferiron nas taxas relativas de crecemento, pero os machos investiron unha maior proporción da súa biomasa total en follas, e tiveron unha maior área foliar por unidade de peso. O contido hídrico das follas foi mais alto nas femias, as cales tamén tiveron un maior grado de suculencia. Estes resultados indican que os sexos desta especie seguen diferentes estratexias para vencer á seca, cas femias sendo máis tolerantes fronte a condicións de falta de auga severa. Ademais o estudio tamén aporta evidencias de diferentes patróns de reparto de biomasa entre os sexos. Tanto as diferenzas ecofisiolóxicas como nos patróns de reparto de biomasa, poden influír nas respostas específicas dos sexos a diferentes condicións ambientais e polo tanto axudar a explicar a segregación espacial dos sexos.

No **capítulo 6**, valorouse a resposta dos sexos ó spray salino e á dispoñibilidade de nutrientes, dous factores clave nos sistemas dunares. Nun intento por achegarnos a unhas condicións máis realistas, os machos e as femias medraron na mesma maceta, de tal forma que isto permite avaliar, polo menos de forma indirecta, ós efectos da competencia intraespecífica. Non houbo diferenzas nas respostas dos sexos fronte ó spray salino, o cal si que causou un incremento na biomasa subterránea e un descenso no esforzo reprodutivo. O aporte de nutrientes, incrementou o crecemento das plantas, acumulando unha maior biomasa total e aérea os machos que as femias. En condicións de fertilización, as femias tiveron valores de rendemento fotoquímico máis altos que os machos. As femias dedicaron unha maior proporción da biomasa á reprodución e menos a crecemento, o que se interpreta como un custo asociado ca reprodución. Este limitado crecemento das femias pode afectar ás súas capacidades competitivas, véndose os machos favorecidos para colonizar un mesmo espazo. Sen embargo, unha posible mellora na capacidade competitiva das femias en ambientes ricos en nutrientes pode favorecer o seu crecemento en tales situacións.

Como conclusion xeral, o dimorfismo sexual en *H. peploides* depende das condicións ambientais nas que medran ós sexos e as causas da súa segregación especial son complexas, podendo requerir máis dunha explicación. A interacción entre os atributos fisiolóxicos, morfolóxicos, e de crecemento pode contribuir a explicar as diferenzas nas respostas concretas ós diferentes factores de estrés, e polo tanto a marcada segregación espacial observada nesta especie.

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