

Environmental sex determination in ferns: effects of nutrient availability and individual density in *Woodwardia radicans*

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Summary

1. In environmental sex determination (ESD) gender is decided after conception, depending on the environment, rather than being genetically fixed. ESD in plants has been mainly studied in angiosperms, where the dominant form of ESD is sexual lability. Surprisingly, ESD has rarely been studied in homosporous ferns, the only plants in which ESD is the rule, rather than the exception.
2. Here, we address the mechanism underlying ESD for the fern *Woodwardia radicans* by experimentally manipulating nutrient availability and gametophyte density.
3. Stress (limited nutrient supply, crowding) affected sexual expression in *W. radicans* gametophytes in a way compatible with ESD. Under good growth conditions (low density or high nutrient), gametophytes matured sexually at a relatively large size and turned into females and subsequently into bisexuals. Under harsh growth conditions, gametophytes matured sexually at a smaller size and turned into males.
4. Interestingly, gametophyte sexual expression was consistent with the size-advantage model, because the number of archegonia increased with gametophyte size, but not the number of antheridia.
5. The sex switch threshold size was variable and decreased with increasing stress, as predicted by age and size to maturity models.
6. *Synthesis.* Sexual expression in fern gametophytes can be fruitfully studied within the ESD theoretical framework. Stress induced male expression in gametophytes in a way compatible with ESD. In addition, size-related patterns of sexual expression were consistent with the size-advantage model, because female function benefited more from a larger size than did male function. Finally, the sex switch threshold size was variable and decreased with increasing stress, a result predicted by age and size to maturity models but seldom empirically tested before.

Key-words: plant size, sequential hermaphroditism, sex switch threshold, stress conditions

Introduction

Environmental sex determination (ESD) is a mechanism by which sex is decided after conception, depending on the environment, rather than being genetically fixed (Bull 1981). Charnov & Bull (1977) predicted that selection favours ESD when three conditions are met: (i) the environment strongly influences lifetime fitness, (ii) the environment is patchy with females performing better than males in some patches and vice versa, and (iii) individuals have little control on the patch they land on. Among the relevant aspects of environment contributing to patchiness are local mate competition,

resource availability and sex-specific mortality (Charnov & Bull 1977). ESD is taxonomically scattered across animals (where the dominant form is sequential hermaphroditism) and plants (often via sexual lability) (Policansky 1982). Although far less frequent than genetic sex determination, ESD provides unique opportunities to understand the evolution of sex allocation patterns in variable environments (Charnov 1982).

ESD in plants has been mainly studied for angiosperms (Freeman *et al.* 1980; Schlessman 1988; Korpelainen 1998). The dominant form of ESD in angiosperms is sexual lability, in which sexual expression varies bidirectionally from male to female (or bisexual) through the plant's lifetime (Korpelainen 1998). Two models exist for sexual lability in angiosperms (Schlessman 1988). According to the 'patchy environment model', sex change towards maleness is mediated by conditions

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that cause stress, such as shading, drought, cold or a previous heavy fruit crop (Freeman *et al.* 1980). *Catasetum viridiflavum* (Zimmerman 1991) and *Atriplex canescens* (Freeman *et al.* 1984) fit this model. According to the 'size advantage model' (Ghiselin 1969; Warner 1975), sex change is mediated by changes in size, which entail changes in the fitness prospects as male or female. Most often, only plants above a threshold size become female, reproducing as males below that threshold, due to the higher resource investment needed to produce seeds compared to pollen (Delph 1999; see Day & Aarssen 1997 for an alternative mechanism based on size-dependent mortality and the longer time required for successful female reproduction, compared to the fast male reproduction). *Arisaema triphyllum* (Policansky 1981) and *Panax trifolium* (Schlessman 1991) fit this model. These two models are not mutually exclusive. For example, the threshold size for sex switch could differ among populations if environmental conditions affect the sex-specific relationship between size and fitness (Bierzychudek 1984a).

Surprisingly, the above mentioned conceptual framework has rarely been applied to homosporous ferns, which are the only plants in which ESD is the rule, not the exception (Korpelainen 1998). In homosporous ferns, sporophytes (the diploid generation) produce free-living gametophytes (the haploid generation) that are potentially bisexual, i.e. that can bear female (archegonia) and male (antheridia) reproductive organs (gametangia) on the same individual. Several environmental factors influence sexual expression, but maleness-inducing pheromones, known as 'antheridiogens', have received most attention (Schneller *et al.* 1990). Antheridiogens are gibberellin-like compounds secreted by large, female or bisexual gametophytes that reduce growth and induce maleness in nearby asexual gametophytes. A profuse literature has dealt with the taxonomic distribution of antheridiogens (Chiou & Farrar 1997), as well as their chemical composition (Yamane 1998), effects on gametophytes (Strain *et al.* 2001) and evolutionary implications (Willson 1981; Haig & Westoby 1988; Korpelainen 1998).

Other environmental factors affecting gender in ferns, such as nutrients, have received very little attention in most reviews of sex expression in fern gametophytes (Cousens 1988; Korpelainen 1998; see however Raghavan 1989). Notwithstanding this, three well-known facts from gametophyte biology make a strong case for the importance of ESD. Firstly, ontogenetic changes in gametophyte gender have long been known and indeed entail sequential hermaphroditism, i.e. unidirectional sex change along development. Klekowski (1969a) distinguished four types: (A) male to bisexual, (B) male to female to bisexual, (C) female to bisexual, and (D) male to bisexual to female. Secondly, coexistence of gametophytes of different gender within a population has been repeatedly reported (Klekowski 1969b; Hamilton & Lloyd 1991). Thirdly, harsh growth conditions, such as poor substrate or high density, are known to lead to small, male gametophytes (Miller 1968; Rubin & Paolillo 1983; Rubin *et al.* 1985; Korpelainen 1994, 1995; Huang *et al.* 2004). Although reference to such environmental influences on gametophyte gender are

scattered in the literature on gametophyte biology, it is only recently that an ESD-based interpretation has gained momentum. To our knowledge, the only study addressing sexual expression in ferns from an ESD perspective is Guillon & Fievet (2003), who analysed the effect of light on horsetail gametophytes. As horsetails are not known to produce antheridiogens (Hauke 1977), it remains to be seen if Guillon and Fievet's findings hold for antheridiogen-producing ferns.

In the present study, we address the ESD for the antheridiogen-producing fern *Woodwardia radicans* (L.) Sm. We experimentally manipulated nutrient availability and gametophyte density to answer the following question: Is the ontogenetic development of gametophytes fixed or is it sensitive to environmental factors? We predict that environmental factors aside from antheridiogen should influence sexual expression. According to ESD theory and previous empirical evidence we expected an association between stressing conditions (high density, low nutrients), small size and male gender. In addition, we asked two questions relevant to ESD and not previously addressed with ferns. Firstly, do larger gametophytes produce more archegonia and/or antheridia? Answering this question can provide clues about the relative fitness advantages of each gender in relation to size, which is a fundamental part of ESD theory. Secondly, is the threshold size for sex switch fixed or variable according to environmental conditions as shown for angiosperms (Bierzychudek 1984a)? Preliminary evidence in *W. radicans* also indicates that the threshold for female expression is variable (Quintanilla *et al.* 2007b).

Material and methods

STUDY SYSTEM

Woodwardia radicans (Blechnaceae) is an evergreen fern found in riparian forests of the northern Iberian Peninsula, Macaronesia and some Mediterranean localities. Leaves are > 2 m long and arranged in crowns. Sporophytes can propagate asexually via adventitious buds formed on the rachis. In the northern Iberian Peninsula, sporangium dehiscence occurs around February-March, when temperatures are suitable for spore germination (Quintanilla *et al.* 2000). Following germination, gametophytes, as in most homosporous ferns (Nayar & Kaur 1971), go through a short filamentous stage because of one-dimensional growth, but soon develop into a spatulate, bidimensional shape, and eventually adopting a heart shape, when a notch meristem appears in the middle of the upper margin. Under rich growing conditions heart-shaped gametophytes become several cell-layers thick in the centre – i.e. below the meristem notch – and develop as females, before turning into bisexuals (Quintanilla *et al.* 2005). Thus, the changes in gender described for *W. radicans* fit type C of Klekowski's (1969a) classification. Archegonia invariably develop below the meristem notch, on both surfaces of the thickened central pad of tissue. In bisexuals, antheridia are produced on the whole surface or on proliferating, spatulated lobes. In males, the position of antheridia is variable. In experimental cultures, female and bisexual gametophytes release an antheridiogen that inhibits vegetative growth and induces antheridium production in asexual gametophytes (Quintanilla *et al.* 2007b). Because of the effects of antheridiogen, gametophyte populations in nature are presumed to be composed of female, bisexual and male individuals (Quintanilla *et al.* 2005). *Woodwardia radicans*

has the generic base number of chromosomes, $2n = 68$ (Löve *et al.* 1977), and is isozymically diploid (Quintanilla *et al.* 2007a).

SPORE COLLECTION

Spores were obtained in Fragas do Eume Natural Park (Northwest Spain, 43°24' N, 8°03' W) in December 2004. Fragments of leaves with mature sporangia were collected from 10 ramets. To increase the probability of sampling different genets, sampled ramets were more than 20 m apart. Spore release was promoted by drying the fragments on sheets of smooth paper for 1 week in the laboratory. Spores from the 10 ramets were then pooled prior to the culture experiments.

EXPERIMENTAL TREATMENTS

Spores were sown on agar containing the mineral medium of Dyer (Dyer 1979) in 5.5-cm-diameter Petri dishes. To minimize the risk of fungal contamination, the culture medium contained nystatin (100 U/mL). The dishes were sealed with Parafilm (American National Can, Chicago, IL) and incubated in a growth chamber (20 °C, PAR 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 16 h photoperiod). Five weeks after sowing, the resulting gametophytes, still asexual at that time, were transplanted to square Petri dishes divided into 25 square cells of 4 cm² each (Bibby Sterilin; Barloworld Scientific, Stone, Staffordshire, UK), each cell containing 3 mL of culture medium.

Two independent experiments were carried out: nutrient or density manipulation. For the nutrient experiment, five nutrient levels were established by diluting the Dyer medium 0, 10, 100, 1000 or 10 000 times. For each nutrient level, one gametophyte was transplanted to the centre of individual cells. In total, 225 gametophytes per nutrient level were transplanted. For the density experiment, undiluted Dyer medium was used and four density levels were tested: 1, 3, 5 and 9 gametophytes per cell. For each density level, one focal gametophyte was placed in the centre of individual cells and the remaining 0, 2, 4 or 8 gametophytes regularly arranged around it at a distance of 5 mm. For each density level, 200 central gametophytes, with their corresponding neighbours, were transplanted. All square dishes corresponding to the two experiments were then sealed with Parafilm and the gametophytes of each experimental level were equally divided between two growth chambers. Temperature and light conditions were the same as for gametophyte production. Petri dishes were rotated weekly within the growth chamber to avoid position effects.

To study size and gender, gametophytes were sampled 10, 14 and 25 weeks after spore sowing. At each time, seven gametophytes in each level-chamber combination were harvested. In the density experiment, only the central gametophytes were harvested. Gametophytes were fixed in a mixture of acetocarmine and chloralhydrate (Edwards & Miller 1972), heated in a water bath at 50 °C for 2 h, and rinsed with distilled water. To observe both gametophyte surfaces under the light microscope, they were mounted between two cover-glasses. For each gametophyte, four variables were gathered: gender (asexual, female, male or bisexual), number of archegonia, number of antheridia, and gametophyte size. For the latter variable, images of mounted gametophytes were obtained with a high resolution scanner (0.008 mm/pixel) and their area was determined with the program ImageJ (Abramoff *et al.* 2004).

STATISTICAL ANALYSIS

The effects of nutrient and density on gametophyte gender and size were separately analysed by fitting generalized linear models

(GLMs) using the GENMOD procedure of SAS 9.0 (SAS Institute 2002). These models were chosen because of unbalanced sample sizes, empty cells and variables departing from a normal distribution. Likelihood Ratio Statistics for type 3 analysis were utilised to determine deviance (G^2) for each term in the models that does not depend on the order in which the terms are specified (Quinn & Keough 2002). A multinomial distribution with cumulative logit link function was used for gender, and a normal distribution with identity link was set for size. Models included the effects of treatment level, growth chamber and harvest time nested into chamber; all of these were considered as fixed effects. Growth chamber was treated as a fixed effect due to the complex computational analysis for a multinomial distribution of response variable with random predictors. To consider as a fixed effect one that is random is more desirable than transforming the variable distribution and should not affect our conclusions. Gender was considered as a fixed effect in the models for size.

The relationship between gametophyte size and number of gametangia was assessed by Pearson correlation coefficient within each gender. This analysis was performed with SPSS 13.0 (SPSS 2003). ANCOVA was used to test for differences between sexes in the slopes of the relationship between the number of gametangia and gametophyte size. Two separate analyses were performed, one comparing male vs. female gametophytes, and the other comparing antheridia and archegonia within bisexual gametophytes. To improve normality, the number of gametangia (dependent variable) was squared root transformed and gametophyte size (the covariate) was log transformed. This analysis was performed with STATISTICA 6.0 (www.statsoft.com).

Results

NUTRIENT EXPERIMENT

Woodwardia radicans gametophytes exhibited successful vegetative and reproductive development overall. Only 3% individuals died during the experiment, and most produced gametangia even at the lowest nutrient levels (Fig. 1a). Gender was significantly affected by nutrient level, time and their interaction (Table 1). After 10 weeks, most gametophytes became female at high nutrient levels ($\geq 1/100$) whereas at 1/1000 and 1/10 000 levels, males outnumbered females (Fig. 1a). After 14 and 25 weeks, male percentages decreased and females became predominant even at the lowest nutrient levels. The abundance of bisexuals also increased over time and, after 25 weeks, reached ca. 50% at 1/10 and undiluted levels.

For size, all main effects and the interactions nutrient \times time and gender \times time were significant (Table 1). Size increased in the order: male < bisexual < female, the only exception being 25-week gametophytes at the richest nutrient levels, for which female < bisexual (Fig. 2). The size of females and bisexuals increased with increasing nutrient level and over time within levels, with highest means of $\sim 100 \text{ mm}^2$ for both genders. Males did not show clear size patterns across nutrient levels and mean sizes were < 5 mm² after 10 and 14 weeks, reaching a maximum of 19 mm² after 25 weeks (1/1000 level).

DENSITY EXPERIMENT

As in the nutrient experiment, mortality was very low and most gametophytes reached sexual maturity in all experimental

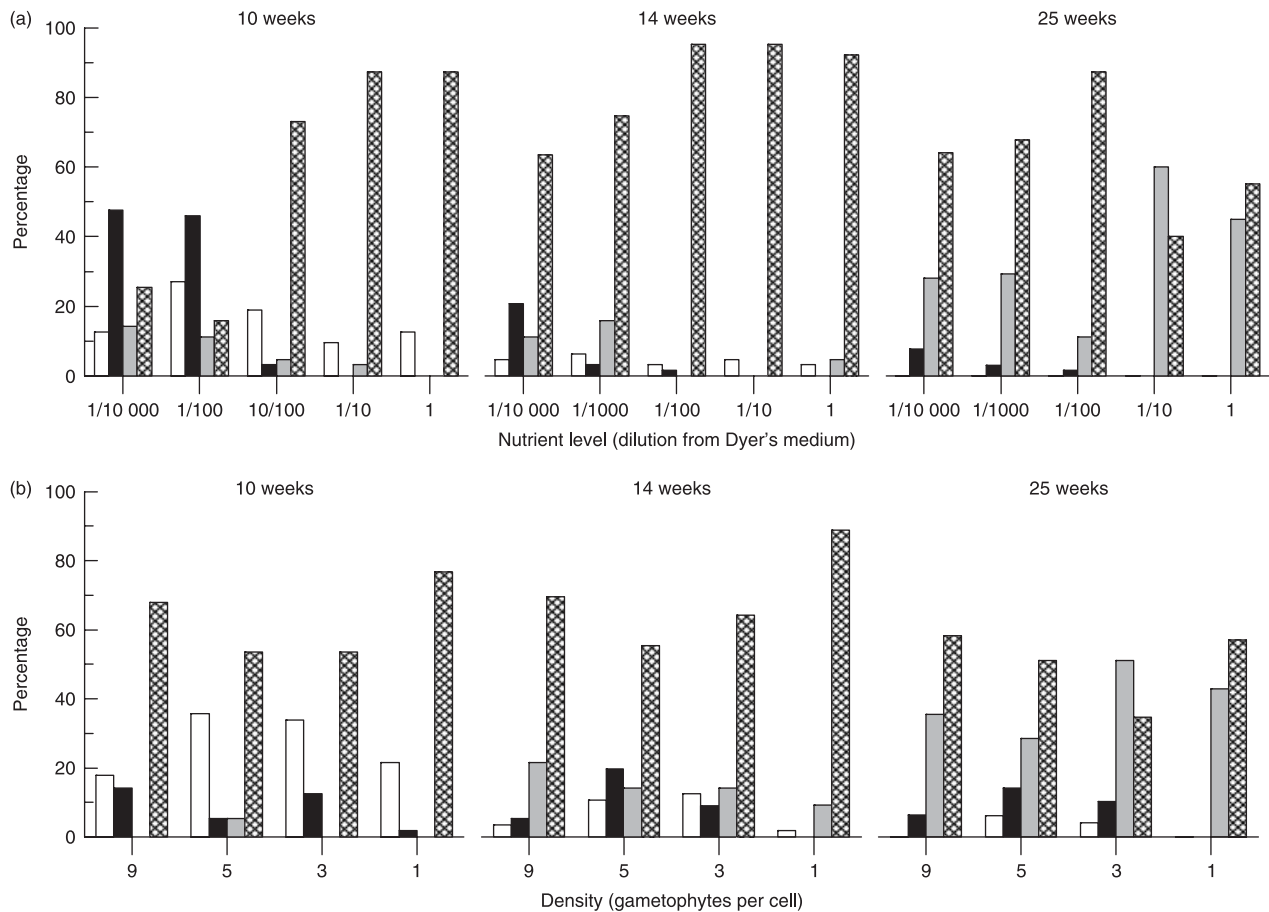


Fig. 1. Relative frequencies of gametophyte genders at the three harvest times in (a) each of the five nutrient levels and (b) each of the four density levels. White bars: asexual; black bars: male; grey bars: bisexual; double hatched bars: female. In all plots, stress decreases from right to left.

Experiment	Variable	Effect in model	d.f.	G ²	P
Nutrients	Gender	Nutrients	4	41.08	< 0.0001
		Chamber	1	1.33	0.2482
		Time (chamber)	4	220.85	< 0.0001
		Nutrient × time (chamber)	20	57.31	< 0.0001
	Size	Nutrients	4	378.84	< 0.0001
		Gender	3	43.19	< 0.0001
		Chamber	1	6.81	0.0091
		Time (chamber)	4	355.57	< 0.0001
		Nutrient × time (chamber)	20	229.74	< 0.0001
		Gender × time (chamber)	13	37.04	0.0004
Density	Gender	Density	3	13.89	0.0031
		Chamber	1	0.01	0.9037
		Time (chamber)	4	135.86	< 0.0001
		Density × time (chamber)	15	20.11	0.1678
	Size	Density	3	239.73	< 0.0001
		Gender	3	57.54	< 0.0001
		Chamber	1	2.39	0.1222
		Time (chamber)	4	37.3	< 0.0001
		Density × time (chamber)	15	181.67	< 0.0001
		Gender × time (chamber)	15	29.81	0.0126

Table 1. Summary of GLMs for the effects of nutrients and density on gender and size of *Woodwardia radicans* gametophytes

Gender refers to four categories on the basis of the presence of gametangia i.e. asexual, female, male or bisexual. Size refers to gametophyte area. The effects of growth chamber (2 units) and time (10, 14, 25 weeks since sowing; nested within chamber) were also included. Gender was considered as an effect in the models for size. Significant values are in bold.

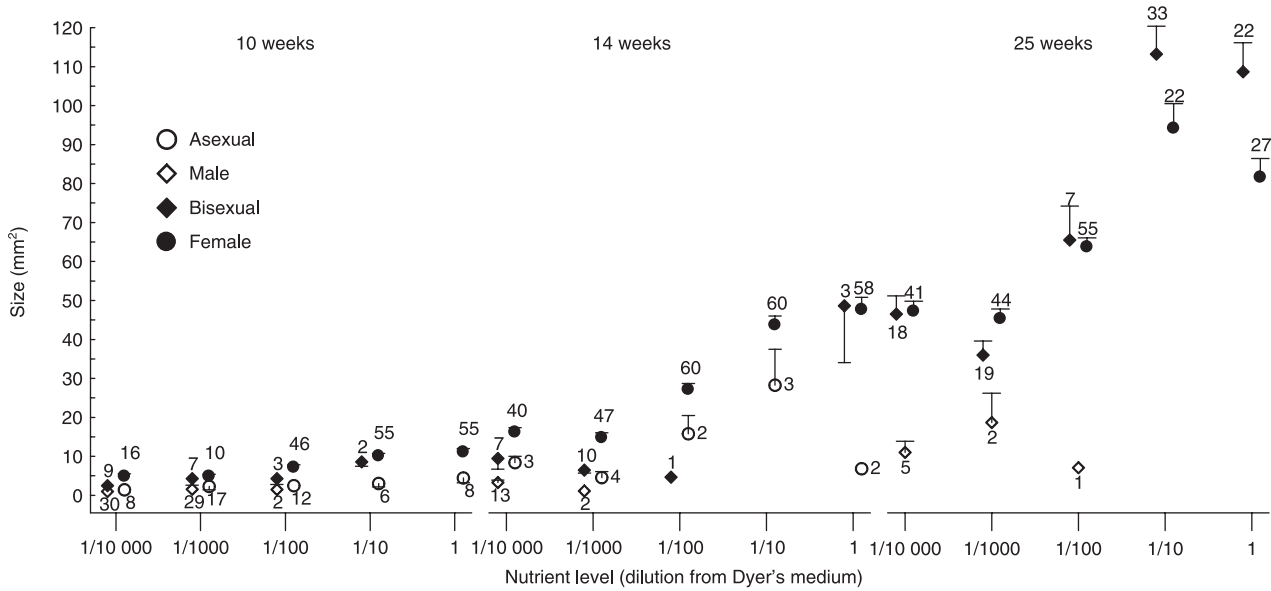


Fig. 2. Gametophyte size (mean \pm SE) at the three harvest times in each of the five nutrient levels. To improve clarity, symbols have been slightly shifted laterally and error bars that are shorter than the symbol width have been omitted. Figures close to the symbols indicate sample size.

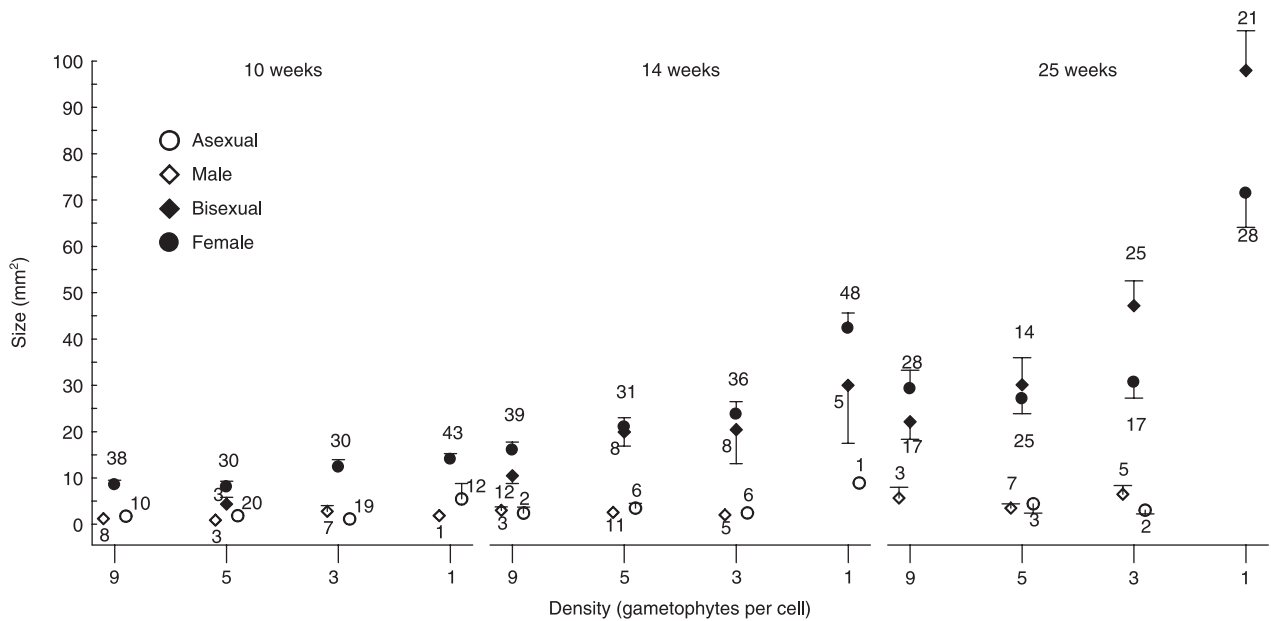


Fig. 3. Gametophyte size (mean \pm SE) at the three harvest times in each of the four density levels. To improve clarity, symbols for the asexuals and the males have been slightly shifted laterally and error bars that are shorter than the symbol width have been omitted. Figures close to the symbols indicate sample size.

treatments (Fig. 1b). Both gametophyte density and time had significant effects on gender (Table 1). At the three sample times, no or almost no isolated gametophyte became male, whereas at higher densities (\geq three gametophytes per cell) there were low frequencies ($< 20\%$) of males (Fig. 1b). Females were dominant irrespective of density level and sample time (the only exception being bisexual predominance within 3 gametophytes per cell after 25 weeks). The frequency of bisexuals increased over time.

Size was significantly affected by all main effects except chamber and by the interactions density \times time and gender \times time (Table 1). The size ranking of genders was similar to that in the nutrient experiment: male $<$ bisexual $<$ female after 10 and 14 weeks, and female $<$ bisexual after 25 weeks in the richer growing levels (\leq five gametophytes per cell) (Fig. 3). There was a trend towards increased size of females and bisexuals with decreasing density level and over time within levels. The final size of females and bisexuals in the one

Table 2. Number of gametangia per gametophyte (mean \pm SE) and its Pearson correlation (r) with gametophyte size at the three harvest times in nutrient and density experiments

Gender	Nutrients			Density		
	10 weeks	14 weeks	25 weeks	10 weeks	14 weeks	25 weeks
Gametangia						
Female						
Archegonia	6.5 \pm 0.3 $r = 0.672$ (182)	17.2 \pm 0.7 $r = 0.630$ (266)	52.5 \pm 2.2 $r = 0.612$ (189)	8.7 \pm 0.5 $r = 0.591$ (141)	18.2 \pm 0.9 $r = 0.571$ (154)	36.5 \pm 2.2 $r = 0.411$ (98)
Male						
Antheridia	10.8 \pm 1.4 NS (61)	41.8 \pm 14.5 NS (15)	124.4 \pm 24.1 NS (8)	9.0 \pm 2.5 NS (19)	13.3 \pm 4.0 NS (19)	23.7 \pm 5.3 $r = 0.842$ (15)
Bisexual						
Archegonia	2.8 \pm 0.5 $r = 0.394$	9.0 \pm 1.9 $r = 0.883$	58.8 \pm 4.3 $r = 0.827$	4.0 \pm 2.5 not tested	14.0 \pm 2.1 $r = 0.453$	36.1 \pm 3.3 $r = 0.453$
Antheridia	6.1 \pm 1.7 NS	12.0 \pm 2.9 NS	45.4 \pm 6.3 NS	9.3 \pm 4.1 not tested	7.8 \pm 2.3 NS	30.0 \pm 5.4 $r = 0.478$
Archegonia + antheridia	8.9 \pm 1.7 NS (21)	21.1 \pm 3.8 $r = 0.567$ (21)	104.2 \pm 8.0 $r = 0.564$ (99)	13.3 \pm 2.4 not tested (3)	21.7 \pm 2.8 $r = 0.428$ (33)	75.4 \pm 11.1 $r = 0.573$ (77)

Values in brackets are sample sizes. In the density experiment at 14 weeks, correlations for bisexuals were not tested because of the small sample size (three gametophytes). NS, not significant.

gametophyte per cell level of the density experiment were similar to those reached in the undiluted nutrient level (note that conditions in these two levels were identical). The size of males slightly increased over time but means were $< 7 \text{ mm}^2$ in all density levels.

NUMBER OF GAMETANGIA AND GAMETOPHYTE SIZE

In female gametophytes, the number of archegonia significantly increased with gametophyte size in both experiments and across the three harvest times (Table 2). In male gametophytes, antheridia number was not related to gametophyte size except after 25 weeks in the density experiment (Table 2). In bisexual gametophytes, archegonia and total gametangia significantly increased with gametophyte size across all experiments and harvest times (Table 2). Antheridia number increased with gametophyte size only after 25 weeks in the density experiment (Table 2).

In the cases in which both antheridia and archegonia increased with gametophyte size, an ANCOVA showed no differences in slope between regression lines, i.e. a non significant sex \times size interaction term (bisexuals: $F_1 = 0.03$, $P = 0.873$; male vs. female gametophytes: $F_1 = 1.50$, $P = 0.223$). The effect of sex was not significant either (bisexuals: $F_1 = 0.90$, $P = 0.343$; male vs. female gametophytes: $F_1 = 0.28$, $P = 0.600$).

Discussion

ONTOGENETIC SEQUENCES IN GAMETOPHYTES AND ESD

Although we did not follow individual gametophytes through time, our temporal sampling suggests that sex changes in

W. radicans fitted the model of sequential hermaphroditism. We propose the following ontogenetic sequence. Under harsh growth conditions (high density or low nutrient), growth prospects were bad and gametophytes matured sexually at a small size, turning into males. Only when further growth was possible could some of those males turn into females or bisexuals. Under good growth conditions (low density or high nutrient), gametophytes matured sexually at a relatively large size and turned into females. Our interpretation of bisexuals as deriving mostly from males is based on the intermediate size of the bisexuals in our experiments. However, a transition from vegetative to female to bisexual was also present, especially in the treatment levels with better growing conditions where bisexuals were the largest individuals after 25 weeks (Figs 2 and 3).

The ontogenetic sequence for *W. radicans* found in the present study departed from the simple transition from female to bisexual described by Quintanilla *et al.* (2005) for single individuals growing in Dyer medium or for other *Woodwardia* species by Klekowski (1969b). This supports the view of Masuyama (1975), who considered a rigid view of ontogenetic sequences too simplistic. Our study, as well as others using a range of growth conditions (Masuyama 1975; Rubin & Paolillo 1983; Korpelainen 1994), suggests that ontogenetic sequences can differ depending on environmental conditions. We anticipate that incorporation of a broader range of growth conditions than those traditionally used to culture gametophytes will reveal a diversity of intra-specific ontogenetic sequences. The study of such intra-specific diversity would allow a more complete assessment of the fit of ontogenetic sequences to ESD theory in ferns.

Ontogenetic sequences involving beginning as male and subsequent addition of, or change to, female sexual expression,

as gametophytes grow larger, fit comfortably into the ESD theory. A larger size for female expression in *W. radicans* and fern gametophytes in general can be explained in two, non-mutually exclusive ways. Firstly, female reproduction is more costly because successful sporophytes develop only on large gametophytes (Sakamaki & Ino 1999). Secondly, an architectural constraint allows production of archegonia only on large gametophytes. Archegonia are embedded in the thickened tissue of the gametophyte centre (see Study System) and such a tissue develops only after a given size has been achieved by heart-shaped gametophytes. By contrast, antheridia develop on the surface of tissue that is one cell-layer thick and can therefore be formed by young spatulate gametophytes.

On the other hand, ontogenetic sequences ending with a bisexual stage after a female stage, such as sequences B and C described by Klekowski (1969a), are more difficult to accommodate into the ESD framework. Traditionally, a final bisexual stage is considered to derive from female gametophytes and has been explained as a reproductive insurance mechanism after a period of femaleness and outbreeding (Klekowski 1969a; Lloyd 1974). However, an interpretation of ontogenetic change in sex from an outcrossing perspective faces several problems. Firstly, sequence D is difficult to fit into that framework (Klekowski 1969a). Secondly, sequences are variable with environmental conditions and this leads to complex mixtures of genders within populations, thus rendering them difficult to interpret in terms of outcrossing (Lloyd 1974; Chiou *et al.* 1998). Thirdly, other traits such as relative position of sexual organs on the gametophyte and presence of antheridiogen also affect outcrossing (Lloyd 1974). Fourthly, isozyme assessment of inbreeding (Soltis & Soltis 1992; Haufler 2002) has revealed that (i) most ferns are extreme outcrossers and (ii) mixed mating systems are very rare. All this evidence argues against an interpretation of the hermaphroditic stage of gametophytes as a reproductive insurance mechanism. Certainly, reproductive insurance does not seem to be important in *W. radicans* because allozyme variation in this species indicates a high outcrossing rate (Quintanilla *et al.* 2007a).

A full explanation of late bisexuals in gametophyte ontogeny is still lacking. We suggest two avenues for further research: (i) resource depletion in large gametophytes after arquegonia production could lead to late production of antheridia (Lloyd 1980); and (ii) late growth of small gametophytes reaching the female or hermaphroditic status could make it advantageous for females to produce antheridia and achieve fitness through the male function, in line with the proposal of Brunet & Charlesworth (1995).

SEXUAL EXPRESSION IN GAMETOPHYTES: BEYOND ANTHERIDIEN

Quintanilla *et al.* (2007b) found that antheridiogen induces frequencies of males ca. 70% in *W. radicans*. In the present study, we have shown that a high percentage of small, male gametophytes in *W. radicans* can be due purely to environmental stress, such as low nutrient availability, without any need to

invoke antheridiogens. Size of the different sexual types (asexuals, males, females, and bisexuals) in this study was also similar to those reported in Quintanilla *et al.* (2007b). Although an effect of antheridiogen cannot be ruled out in our density experiment, it is remarkable that the frequency of males was lower than in the nutrient experiment (Fig. 1) or than in our previous study on antheridiogen (Quintanilla *et al.* 2007b).

In addition to antheridiogen and stress-related differences in growth rate, variation in gametophyte size can derive from differences in spore size (Verma & Selivan 2001) or germination time (Rubin & Paolillo 1983). Schedlbauer (1976) showed that both factors are interrelated: bigger spores germinated earlier and produced faster growing gametophytes. Thus, even in a homogenous environment, differences in spore size could generate a hierarchy of gametophyte sizes and differences in gender expression.

The relative role of spore size, environmental stress and antheridiogen in determining sexual expression in nature remains an open question for ferns. Hamilton & Lloyd (1991) found strong size hierarchies and strong biases towards maleness in natural populations of gametophytes, which they interpreted as effects of antheridiogens. However, the other two factors were not assessed. Few studies of gametophyte populations have been carried out in natural conditions, due to the difficulty of species identification (Ranker & Houston 2002). Studies on the natural variation in gametophyte density, microhabitat quality, spore size and germination conditions could shed light on this question.

Such field studies are also needed to gather information pertaining to local mate and local resource competition in ferns. Local resource competition is expected to be low because most gametophytes only produce a single sporophyte (Miller 1968). Haig & Westoby (1988) assumed that sperm dispersal is limited in fern gametophytes but the extent of dispersal in fern sperm remains, as far as we know, largely unexplored. Lloyd & Bawa (1984) argued that ESD was very rare in angiosperms due, in part, to the influence of local mate or resource competition. Tentatively, the high local mate competition in ferns would saturate the male fitness gain curve and favour large female gametophytes but further work is needed on this topic.

ESD AND GAMETOPHYTE FITNESS

The 'size advantage model' predicts that the rate of increase in fitness should differ between genders (Warner 1975). Our analysis of the relationship between the number of gametangia and gametophyte size supports this hypothesis. While number of antheridia did not increase with gametophyte size, number of archegonia did. Quantification of gametophyte fitness in cultures is hindered by the difficulties of triggering the formation of fecund gametophytes in those conditions (Quintanilla *et al.* 2005). In our study, we assumed that reproductive success is directly related to number of gametangia. This seems obvious for antheridia but not for archegonia, because in homosporous ferns usually only one arquegonium per

gametophyte develops into a sporophyte (Haig & Westoby 1988). However, such an assumption has been made before (Haig & Westoby 1988) and it has been shown that sporophyte formation increases with the size of the female or bisexual gametophytes (Sakamaki & Ino 1999).

Most previous studies of ESD in plants have dealt with the 'patchy environment' or the 'size advantage' model, but not both, although a simultaneous consideration of these models would be fruitful. Kinoshita & Harada (1990) have theoretically argued that the size for sex change should not be constant if the relationship between size and reproductive success changes in space or time. Evidence for variation in threshold sizes for the sex switch exists for angiosperms (Bierzychudek 1984b). Our findings indicated variation in the threshold size for female expression along a nutrient and density gradient. Ten weeks after sowing, male gametophytes did not show differences in size across treatment levels but females became progressively smaller with decreasing nutrient or increasing density (Figs 2 and 3). A similar decrease in female gametophyte size across a stress gradient has also been reported for *Dryopteris filix-mas* (Korpelainen 1995). This decrease in average female size with increasing stress qualitatively agrees with models of optimal norms of reaction for age and size to maturity when mortality rate does not depend on growth rate (Stearns 1992). Male gametophyte size did not show apparent variations across the gradients tried in the present study, but data from other studies (Rubin & Paolillo 1983; Quintanilla *et al.* 2007b) indicate similar responses to stress to those found here for female gametophytes.

In conclusion, we have shown that stress induced by limiting nutrient supply or by crowding affected sexual expression in *W. radicans* gametophytes in a way compatible with ESD. In addition, size-related patterns of sexual expression were consistent with the size-advantage model, because female function benefited more from a larger size than male function. The sex switch threshold size was variable and decreased with increasing stress, as predicted by age and size to maturity models. Advances in the study of natural gametophyte populations and fitness estimation of different sex types could well turn fern gametophytes into model systems for the study of ESD. Future research should monitor individual ontogenetic sequences under contrasting environmental conditions, paying attention to both gametophyte size and number of gametangia, in addition to gender. The use of homosporous ferns lacking antheridiogen would allow the study of ESD without confounding factors. In particular, the relative role of spore size, germination time and environmental stress need to be disentangled. It is also necessary to assess to what extent the size advantage model can account for ESD alone, or needs to be combined with the patchy environment model. Finally, more accurate individual estimates of fitness need to be developed to test the predictions of the size advantage model.

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