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DO ANTHERIDIOGENS ACT VIA GAMETOPHYTE SIZE? A STUDY OF *WOODWARDIA RADICANS* (BLECHNACEAE)[1]

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For many plants, sex is not fixed by genotype but determined by environmental conditions during development. In homosporous pteridophytes, sex is environmentally determined by the presence or absence of antheridiogens, maleness-inducing pheromones. It has been proposed that antheridiogens primarily reduce growth rate, with small gametophyte size responsible for maleness. To test this hypothesis, the effects of antheridiogen and intergametophytic competition on gender expression and gametophyte size were studied in a culture experiment with *Woodwardia radicans*. We found that (1) antheridiogen inhibited growth of gametophytes; and (2) slow growth favored maleness, whereas fast growth favored femaleness, irrespective of the presence or absence of antheridiogen. Both conclusions are consistent with the hypothesis that, in *W. radicans*, antheridiogen effect is mediated by size. They also agree with the "size-advantage" hypothesis in which energetic limitations associated with relatively small individual size impose a less severe limitation for male reproductive success than for female reproductive success. The results are also discussed with regard to a genetic sex-determining pathway that has recently been identified.

Key words: antheridiogen; Blechnaceae; gametophyte; sex expression; size-advantage hypothesis; *Woodwardia radicans*.

Mechanisms of sex determination in plants have attracted much attention within plant biology (Tanurdzic and Banks, 2004). Environmental sex determination is particularly interesting for physiological, ecological, and evolutionary reasons. Environmental sex determination is a form of phenotypic plasticity, by which individuals produce either female, male, or both sex organs depending largely upon environmental circumstances (Bull, 1981; Leimar et al., 2004). Evolutionary theory predicts selection for environmental sex determination when a factor that varies across the environment differentially influences female and male performance (Charnov and Bull, 1977). Environmental sex determination is widespread in many animal (e.g., Bull, 1981) and plant taxa (reviewed by Freeman et al., 1980; Korpelainen, 1998), but only in homosporous pteridophytes does it become dominant. Homosporous pteridophytes produce a single kind of spore that develops into potentially bisexual gametophytes. However, in many species actual gender is conditional on the presence or absence in the environment of antheridiogen pheromones. All antheridiogens characterized so far are gibberellin-related compounds (Yamane, 1998). They are secreted into the medium by archegoniate (female or hermaphrodite) gametophytes and induce precocious antheridium formation in nearby gametophytes (reviewed by Voeller, 1964; Schneller et al., 1990). Interestingly, in addition to becoming male, responding gametophytes are smaller than antheridiogen-secreting gametophytes (e.g., Haufler and Ranker, 1985; Korpelainen, 1994).

The causal relationships between antheridiogen, gametophyte size, and maleness remain controversial. Näf (1956) suggested that in gametophytes exposed to antheridiogen, potential vegetative growth is diverted to antheridium production. Alternatively, Korpelainen (1994) proposed that antheridiogens actually reduce gametophyte growth, and small size favors maleness. The Näf and Korpelainen hypotheses rely on different life-history arguments. The Näf hypothesis entails that antheridiogen modifies the allocation priorities of gametophytes (reproduction vs. growth). This involves a trade-off that has not been studied in ferns, as far as we know. The Korpelainen hypothesis entails that antheridiogens, like other environmental factors, act via gametophyte size. The Korpelainen hypothesis receives indirect support from manipulative experiments, which showed that maleness in gametophytes is promoted by stressing environmental conditions, such as poor light level and quality (e.g., Guillon and Fievet, 2003), low nutrient availability (Korpelainen, 1994), or high density (Huang et al., 2004). Furthermore, a theoretical basis for the relationship between stressing conditions, reduced growth rate and size, and maleness comes from an evolutionary model proposed by Haig and Westoby (1988). Based on a higher cost of female reproduction and other premises, Haig and Westoby (1988) predicted female sex expression under rich growing conditions and male expression under poor conditions.

We studied the relationships between antheridiogen, stress, gametophyte size, and maleness in *Woodwardia radicans*. In a previous study, isolated gametophytes in nutrient rich medium became female, while in paired gametophytes archegoniate individuals induced maleness in adjacent asexuals, suggesting the activity of an antheridiogen (Quintanilla et al., 2005). This species thus fits the archegonia-first strategy predicted in the sex expression model of Haig and Westoby (1988). However, size was not considered in that study. In the present paper we assess the effects of antheridiogen as a single factor or in combination with intergametophytic competition on gender expression and gametophyte size.

MATERIALS AND METHODS

Studied species—*Woodwardia radicans* (L.) Sm. is an evergreen fern found in riparian forests of the northern Iberian Peninsula, Macaronesia, and some Mediterranean localities. Leaves are extremely large (>2 m long) and arranged in crowns. Sporophytes reproduce asexually via adventitious buds formed on the rachis. In the northern Iberian Peninsula, sporangium dehiscence

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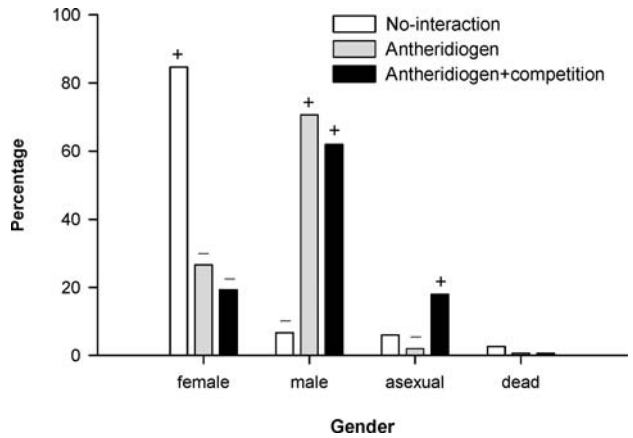

Fig. 1. Relative frequencies of female, male, asexual, and dead gametophytes of *Woodwardia radicans* in each of the three experimental treatments. $N = 150$ gametophytes/treatment. The symbols + and − above the bars indicate significant departures from the expected frequencies.

occurs around the spring equinox, when temperatures are suitable for spore germination (Quintanilla et al., 2000). Under rich growing conditions, gametophytes reach exceptionally large sizes (>20 mm wide) and are covered throughout by glandular hairs. Archegonia invariably develop below the meristem notch on both gametophyte surfaces (L. G. Quintanilla, personal observation). In bisexuals, antheridia are produced on proliferating, ameristic lobes. In males, the position of antheridia is variable. *Woodwardia radicans* has the generic base number of chromosomes, $2n = 68$ (Löve et al., 1977) and is isozymically diploid (Quintanilla et al., 2007).

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

Experimental treatments—Spores were sown on mineral agar (see Dyer, 1979, p. 282) in two plastic petri dishes (Appendix S1, see Supplemental Data accompanying online version of this article). One dish was then incubated in a growth chamber (20°C, PAR 35 $\mu mol \cdot m^{-2} \cdot s^{-1}$, 16-h photoperiod). The other dish was stored for 15 wk in the dark at 5°C, conditions that maintain spore viability but prevent germination in *W. radicans* (Quintanilla et al., 2002). Subsequently, this dish was also transferred to the growth chamber. In this way we obtained two gametophyte stocks with a 15-wk delay in germination and growth. These stocks with or without storage will hereafter be referred to as "younger" or "older" gametophytes, respectively.

Five weeks after sowing, we transplanted older gametophytes to transparent plastic trays divided into 25 square cells with 20-mm sides. Into each cell we transplanted one gametophyte on 3 mL of mineral agar. We established 12 trays, including 300 gametophytes in total (12 trays × 25 cells × 1 gametophyte). After 5 wk incubation, we transplanted younger gametophytes (still asexual and ameristic at that time) to 25-cell trays in three experimental treatments as follows. (1) "No-interaction": one isolated younger gametophyte per cell with 3 mL of fresh culture medium. (2) "Antheridiogen + competition": one younger gametophyte into a cell with an older gametophyte. The distance between both gametophytes was 10 mm. At the start of this treatment, older gametophytes had grown for 15 wk in the same culture medium (Appendix S1, see Supplemental Data with online version of this article) and were archegoniate. (3) "Antheridiogen": as in (2), but the older gametophyte was removed just before transplanting the younger gametophyte. The no-interaction and antheridiogen treatments differ not only in pheromone effects but also in nutrient levels due to uptake by the removed gametophyte (Appendix S1). However, we will consider this latter effect to be negligible given that nutrient availabilities up to 10-fold lower than that used in the current experiment do not reduce growth rate (de Soto, 2005).

For each treatment, we established six trays, giving a total of 150 younger gametophytes. These trays were incubated for 12 additional wk, and then younger gametophytes were collected and scored for gender (asexual, female, male, or bisexual), meristem presence/absence, and area. Given that gametophyte wings have ridges that hinder the determination of these variables, we softened gametophytes in boiling water for 5 min prior to mounting them. We determined gametophyte gender by observing mature gamentagia under a compound microscope. We classified filamentous and spatulate gametophytes as ameristic and gametophytes possessing either a rudimentary or fully developed notch meristem as meristic. We obtained gametophyte images with a high-resolution scanner (94.5 pixel/mm) and determined their area with the program ImageTool (version 3.0, UTHSCSA, http://ddsdx.uthscsa.edu/dig/itdesc.html). Although growth rate was not directly measured, the similarity in size of the younger gametophytes at the transplant time makes inferences on growth rate in different treatments straightforward.

Statistical analyses—The relationship between gametophyte gender (four categories: asexual, female, male, or dead) and treatment was analyzed using a chi-squared test. Significant departures from expected frequencies were tested by analyzing the standardized residuals, as proposed by Haberman (1973). Gametophyte sizes were analyzed by fixed-factor analysis of variance. Two factors were considered: treatment (2 df) and gender (asexual, female, or male, 2 df). Subsequent pairwise comparisons were made using a Ryan-Einot-Gabriel-Welsch F test ($P < 0.05$). All statistical analyses were performed using SPSS (2004).

RESULTS

Experimental treatments significantly affected gender expression of *W. radicans* ($\chi^2 = 193.101$, 6 df, $P < 0.001$). In the no-interaction treatment, most gametophytes became female, whereas in the other two treatments two thirds became male (Fig. 1). The proportion of gametophytes remaining asexual was higher in the treatment including competition than in the treatment with only antheridiogen. None of the gametophytes became bisexual after the treatments. In all treatments, 100% of the females and 100, 89, and 87% of males (no-interaction, antheridiogen, and antheridiogen + competition treatments, respectively) had a notch meristem. Asexuals were predominantly ameristic in the antheridiogen (0% meristic) and antheridiogen + competition (15% meristic) treatments, whereas in the no-interaction treatment 56% of asexual gametophytes were meristic.

The effects of treatment and gender on gametophyte area were significant (Table 1). Gametophyte areas after the three treatments decreased in the order: no-interaction > antheridiogen > antheridiogen + competition (totals in Fig. 2). The treatment × gender interaction was also significant. Thus, pairwise comparisons were performed considering all combinations of treatment and gender, rather than considering each factor separately (see Zar, 1999). In the no-interaction and antheridiogen treatments (Fig. 2), gametophyte sizes were similar, with females (means ~50 mm^2) bigger than males (~18 mm^2) and these bigger than asexuals (<5 mm^2). In the antheridiogen + competition treatment, gametophyte sizes were smaller, with females similar to males of the other two treatments, and males in the size range of asexuals.

DISCUSSION

Sex determination by antheridiogen—In agreement with previous research (Quintanilla et al., 2005), we found a marked antheridiogen effect on gender expression in *W. radicans*. In

TABLE 1. ANOVA with dependent variable gametophyte area and two factors, treatment and gametophyte gender.

Source	df	MS	*F*	*P*
Treatment	2	4592.802	9.903	***
Gender	2	23 830.172	51.385	***
Treatment × gender	4	1962.706	4.232	**
Error	435	463.761		

Note: MS = mean square; $^{**}P < 0.01$; $^{***}P < 0.001$.

the presence of exudates from archegoniate gametophytes (antheridiogen treatment), most gametophytes became male, whereas in its absence (no-interaction treatment), gametophytes predominantly became female. When an archegoniate gametophyte was kept near the responding gametophyte, thereby increasing antheridiogen dosage and reducing resource availability (antheridiogen + competition treatment), the proportion of gametophytes remaining asexual increased, but the male : female ratio was similar to that of the antheridiogen single-effect treatment.

The effects of antheridiogens on gametophyte gender are fully compatible with the current knowledge on the genetics of sex determination in ferns, derived from the work on *Ceratopteris richardii* (Banks, 1997; Tanurdzic and Banks, 2004). Gender expression ultimately depends on two genes, *FEM1* and *TRA*, which promote the differentiation of male (antheridia) and female (meristem and archegonia) traits, respectively; if *FEM1* is active, *TRA* is not, and vice versa (Tanurdzic and Banks, 2004). Antheridiogen leads, via other regulator genes, to the activation of *FEM1* and repression of *TRA* (Strain et al., 2001). In agreement, we found that antheridiogen, alone or with competition, not only induced antheridia but also inhibited archegonia. However, unlike in *C. richardii*, antheridiogen did not repress meristem establishment because most males were meristic irrespective of treatment.

Antheridiogen and gametophyte size—Antheridiogen reduced growth rate, and the size of the responding gametophytes was smaller when the signalling gametophyte was kept in the vicinity than when it was removed. This result indicates that antheridiogen effects on size are dose-mediated, as found in other species (e.g., Stevens and Werth, 1999). Competition for light and nutrients caused by the signalling gametophytes may also have contributed to growth reduction.

Our results are well explained by the Korpelainen (1994) model. First, growth reduction was a direct effect of antheridiogen. This conclusion is based on the fact that in the antheridiogen treatment, as in the other two treatments, asexuals were smaller than males (Fig. 2). If the resource cost of antheridia production caused the slow growth (Näf hypothesis), then bigger asexuals than males would be expected. Second, there was a clear relationship between size and sexual expression, with males being smaller than females in all treatments. In other words, slow growth favored maleness, whereas fast growth favored femaleness, irrespective of the presence or absence of antheridiogen. These first and second conclusions can be straightforwardly combined in the Korpelainen model: antheridiogen induces growth reduction, which in turn favors maleness. By contrast, Chiou and Farrar (1997) concluded that this model is not valid for polypodiaceous species and suggested that growth reduction and antheridia production are induced by two different compounds.

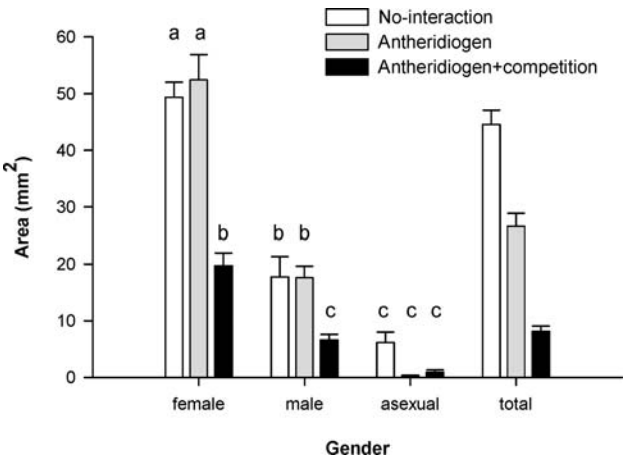

Fig. 2. Area (mean + 1 SE) of female, male, and asexual gametophytes of *Woodwardia radicans* in each of the three experimental treatments. Means with the same letter are not significantly different ($P < 0.05$, Ryan-Einot-Gabriel-Welsch *F* test). The total category averages areas of females, males, and asexuals.

However, Fernández et al. (1999) found evidence that in *Blechnum spicant* (blechnaceous, like *W. radicans*) an apolar gibberellin A_9-like compound is responsible for both growth inhibition and maleness induction. Furthermore, the explanation by Chiou and Farrar (1997) is not consistent with our result that slow growing individuals also became male in the absence of exudates, as noted earlier.

Adaptive implications of antheridiogen and size-dependent gender—There are two interpretations regarding the ultimate causes of antheridiogen systems (reviewed by Korpelainen, 1998). Most authors consider that antheridiogens benefit both the signalling and responding gametophytes (e.g., Willson, 1981; Haig and Westoby, 1988; Hamilton and Lloyd, 1991). Antheridiogens could increase the probability of cross-fertilization between adjacent gametophytes by facilitating dioecy. In agreement with this interpretation, isozymic analysis of sporophyte populations indicates that *W. radicans*, like most diploid ferns (Soltis and Soltis, 1990), is an outcrosser (Quintanilla et al., 2007). Reduction of intragametophytic selfing due to antheridiogens may confer a strong fitness advantage, given that zygotes produced by this breeding system are homozygous at all loci and, consequently, all deleterious recessive alleles are expressed. Klekowski (1969) found evidence that *W. radicans* carries genetic load of deleterious recessives. This result is consistent with cross-fertilization in *W. radicans* because outcrossing reduces homozygosity and hence the effectiveness of selection against recessive detrimental alleles (Barrett and Charlesworth, 1991). The other interpretation of antheridiogens, suggested by Willson (1981), is noncooperative. She proposed that antheridiogens could be allelopaths secreted by females to stunt the growth of unrelated individuals. The detrimental vs. beneficial effects of antheridiogen on responding gametophytes have never been tested directly. In any case, as pointed out by Korpelainen (1994), antheridiogen action via growth inhibition is consistent with cooperative and noncooperative interpretations of this pheromone, given that both are based on size-specific differences in allocation to maleness and femaleness.

We found that *W. radicans* needs to reach a threshold size for reproduction and that afterwards it follows the gender allocation rule "if small be male, if large be female." The evolutionary significance of the protandrous strategy of *W. radicans* under antheridiogen effects or other stressing factors such as competition is consistent with the predictions of the "size-advantage" hypothesis (Ghiselin, 1969; Charnov, 1982; Lloyd and Bawa, 1984) as argued theoretically by Haig and Westoby (1988) and empirically by Korpelainen (1998). The female gametophyte allocates energy to young sporophyte growth (Sakamaki and Ino, 1999). Thus, relative to female function (production of ovocell and sporophyte), male function demands a lower energetic investment for sperm production and sporophyte sire. Smaller (slow-growing) gametophytes are presumed to have smaller energetic reserves and thus less severe limitations for male reproductive success than for female reproductive success. We have explored the relationship between size and sexual expression of *W. radicans* on a nutrient and density gradient (de Soto, 2005), and the results will be reported elsewhere. In short, environmental stress induced maleness via gametophyte size, as we propose for antheridiogen. The signal transduction pathway between slow growth and antheridium production could be common to antheridiogen and other environmental factors. Ultimately, the model of antheridiogen action through gametophyte size can be combined with the genetic model discussed earlier: slow growth would be the signal that activates the transduction pathway leading to masculinization.

Conclusion—This study provides experimental evidence of antheridiogen effects through gametophyte size in *W. radicans*. Antheridiogen reduced growth rate and slow-growing individuals became male. This simple model of antheridiogen mechanism is consistent with experimental findings for other sex-determining environmental factors (e.g., light, nutrients) and with evolutionary theory established for homosporous pteridophytes and even flowering plants. Future studies might demonstrate the operation of this indirect mechanism in field conditions, consider the validity of the model for other species, and determine the effects of gametophyte size on male and female components of fitness. Such studies will undoubtedly increase our understanding of the adaptive significance of antheridiogens.

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