Antheridiogen concentration and spore size predict gametophyte size in *Ceratopteris richardii*

Mike Ganger and Tiffany Sturey

Abstract: In many plants females invest more in reproduction than males. In organisms that exhibit environmental sex determination, individuals in low-quality environments or who are slow growing are expected to develop into males. The gametophytes of *Ceratopteris richardii* Brongn., a homosporous fern, may develop as males or hermaphrodites. Hermaphrodites secrete a pheromone called antheridiogen that induces undifferentiated spores to develop as males. Given that induction is not 100% in the presence of antheridiogen, it is hypothesized that resources may alter *C. richardii* gender decisions. An experiment was undertaken to determine (*i*) whether spore size predicts gender, (*ii*) whether spore size predicts gametophyte size, (*iii*) whether antheridiogen negatively affects the growth of *C. richardii*, and (*iv*) whether wild-type *C. richardii* and *him1* mutants (genetic mutants disposed to male development regardless of antheridiogen presence) behave similarly in their response to antheridiogen. Spore size was not predictive of gender but was positively related to both male and hermaphrodite gametophyte size. Antheridiogen was found to slow the growth of male and hermaphrodite gametophytes of the wild type and male gametophytes of the *him1* mutant. These results are supportive of the idea that gender may be determined indirectly through antheridiogen's effect on gametophyte growth.

Key words: gametophyte, antheridiogen, gender determination, fern.

Résumé : Chez plusieurs plantes les femelles investissent plus dans la reproduction que les mâles. Chez les organismes montrant une détermination sexuelle environnementale, on s'attend à ce que les individus vivant dans des milieux de piètre qualité ou se développant lentement prennent la forme mâle. Le gamétophyte du *Ceratopteris richardii* Brongn., une fougère homosporale, peut prendre les formes mâles ou hermaphrodites. Les hermaphrodites secrètent une phéromone dite anthéridiogène induisant des spores non différenciées avec développement mâle. Sachant que l'induction ne se fait pas à 100 % en présence de l'agent anthéridiogène, les auteurs proposent l'hypothèse que les ressources peuvent altérer le choix du genre chez le *C. richardii*. Ils ont effectué une expérience pour déterminer si (*i*) la dimension des spores prédit le genre, (*ii*) la dimension des spores prédit la grosseur du gamétophyte, (*iii*) l'agent anthéridiogène affecte négativement la croissance du *C. richardii*, et (*iv*) le type sauvage du *C. richardii* et les mutants *him1* (mutants génétiques prédisposés au développement mâle indépendamment de la présence de l'agent anthéridogène) se comportent de la même façon en réaction à l'agent anthéridiogène. La dimension des spores ne permet pas de prédire le genre, mais montre une corrélation positive avec la dimension des gamétophytes mâles aussi bien qu'hermaphrodites. On a constaté que l'agent anthéridiogène ralentit la croissance des gamétophytes mâles et hermaphrodites du type sauvage et des gamétophytes mâles chez le mutant *him1*. Ces résultats supportent l'idée que le genre puisse être déterminé indirectement par l'effet de l'agent anthéridiogène sur la croissance du gamétophyte.

Mots-clés : gamétophyte, agent anthéridiogène, détermination du genre, fougère.

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Introduction

Gender in organisms may be determined genetically, environmentally, or through the interaction of the two (Lloyd and Bawa 1984; Sarkissian et al. 2001). In organisms where the environment plays a significant role in this decision, the factors with the greatest influence are often resources or some analog of habitat quality, with individuals that are slow growing or in poor-quality habitats tending to develop as males (Meagher 1988). This is the consequence of male function generally being energetically less expensive than female function (Schlessman 1988). This "fundamental asymmetry" is a well-established principle and has been documented in the angiosperms where females are responsible for costs associated with seeds and fruits (Ashman 1994). It has also been suggested to occur in the seedless vascular plants where females support the developing embryo and sporophyte (Sakamaki and Ino 1999). If gender is determined environmentally, such decisions would be made early in offspring development (Charnov and Bull 1977) and would be expected to be strongly influenced by resources such that faster growing individuals bias toward becoming female and slower growing

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individuals bias toward becoming male (Meagher 1988; DeSoto et al. 2008).

The environment may act on sex determination postconception and may also act through the female as maternal effects (Nager et al. 1999). The gender decisions of offspring may be affected where females differentially provision their offspring with resources (Mousseau and Fox 1998). In plants, larger seeds and spores would be expected to contain more resources than smaller seeds and spores and could lead to increased growth rates, larger sized offspring, and, where possible, an increased likelihood of the offspring being female.

Ceratopteris richardii Brongn. is a homosporous fern that serves as a model system for understanding plant physiology and development (Hickok et al. 1995). The gender-determining system in *C. richardii* is well studied and based on a pheromone called antheridiogen (A_{CE} in *C. richardii*), which is secreted by hermaphroditic gametophytes (Banks 1997*a*). In the absence of A_{CE}, spores bias toward hermaphrodite development and contain antheridia, archegonia, and a notch meristem (Banks 1997*a*). The lack of A_{CE} is thought to lead to expression of both female and male genes (Eberle and Banks 1996; Banks 1997*b*; Juarez and Banks 1998; Strain et al. 2001). In the presence of A_{CE}, spores bias toward male development through a process called induction (Banks 1997*a*), which is thought to be a consequence of the signal transduction system initiated by A_{CE} that leads to the suppression of female genes (Eberle and Banks 1996; Banks 1997*b*; Juarez and Banks 1998; Strain et al. 2001). This signal transduction system is reactive to A_{CE} within a narrow window of development, approximately 3–6 days from sowing, and A_{CE} exposure outside of this window has no effect (Banks et al. 1993).

Several mutants of *C. richardii* have been developed to understand the genetic component of gender determination (Eberle et al. 1995; Banks 1997*a*). One such mutant, *him1* (highly male), develops as a male regardless of the presence of A_{CE}. The genetic mechanism by which male development is assured in the *him1* mutant is not completely understood, although two distinct models of gene interaction, both of which result in the suppression of female genes, have been suggested (Eberle and Banks 1996).

Not all spores grown in the presence of A_{CE} develop as males and not all spores grown in the absence of A_{CE} develop as hermaphrodites (Warne and Hickok 1991). Because of this, the potential role of nutrients in modifying this hormonally determined gender system has been suggested (Haig and Westoby 1988; Korpelainen 1994; Quintanilla et al. 2007; Ayrapetov and Ganger 2009). A_{CE} may act directly through the signal transduction system to suppress female genes or, alternatively, A_{CE} may slow down growth and development. This slowing coupled with assessment of resource levels by the gametophyte may be responsible for induction (Korpelainen 1994).

The objectives of this experiment were to determine (*i*) whether spore size predicts gender, (*ii*) whether spore size predicts gametophyte size, (*iii*) whether A_{CE} negatively affects the growth of *C. richardii* gametophytes, and (*iv*) whether wild-type *C. richardii* and *him1* mutants behave similarly in their response to A_{CE}.

Materials and methods

Hermaphrodites secrete A_{CE} and it is possible to extract it from the growth medium. Wild-type spores were obtained from the Carolina Biological Supply Company (Burlington, N.C., USA). Each vial of spores was introduced onto 20 Petri dishes (100 mm × 15 mm) containing C-Fern growth medium (Hickok and Warne 2004). After 3 weeks of growth under 24 hour grow lights (24 W/m^2) at 24–28 °C, approximately 60% of spores developed into hermaphrodites. An aqueous filtrate was obtained by freezing and thawing the Petri dishes twice and passing the liquid through cheesecloth. Aqueous filtrate from 40 Petri dishes was used to make 20 Petri dishes of C-Fern media, yielding an A_{CE} concentration of 2×. A portion of the 2× A_{CE} stock solution was then serially diluted to establish 1× and 0.5× A_{CE} concentrations.

To test whether A_{CE} negatively affects the growth of gametophytes, 80 Petri dishes (60 mm × 15 mm) each were established with 0×, 0.5×, 1×, and 2× concentrations of A_{CE}. A single spore was added to each dish. Two strains of *C. richardii* were used in this experiment: *RNWT1*(a wild-type strain) and *him1*. This resulted in 40 Petri dishes being assigned for each A_{CE} treatment for each strain. Replication was high since a pilot experiment had shown germination rates to be approximately 50% across all treatments. Because the initial spore size could potentially affect the ultimate gametophyte size, an attempt was made to quantify spore size. This was done by digitizing the spore and measuring its largest circumference using UTHSCSA Image Tool for Windows image analysis software (Wilcox et al. 2002). This measurement is hereinafter referred to as "spore size".

Gametophytes were allowed to develop for 3 weeks under 24 hour grow lights (24 W/m^2) at 24–28 °C. The prothallus of *C. richardii* gametophytes divides in two dimensions, resulting in gametophytes that typically are one-cell layer thick (Banks 1999). Male and hermaphroditic gametophytes from each treatment were manipulated to be flat, photographed, and their area (hereinafter referred to as "gametophyte size") determined using UTHSCSA Image Tool for Windows image analysis software (Wilcox et al. 2002). This same experimental setup was replicated on two separate occasions, hereinafter referred to as the first and second experiments.

A logistic regression (Sokal and Rohlf 1995) was performed using SYSTAT (Wilkinson 2002) to determine if spore size predicted gametophyte gender. Only wild-type spores were used in this analysis and each antheridiogen treatment was analyzed separately.

A two-factor analysis of covariance (ANCOVA) was performed using SYSTAT (Wilkinson 2002) to determine if either A_{CE} concentration or *C. richardii* strain (*RNWT1* or *him1*) affected male gametophyte size. A blocking factor was used to partition the variation between the two experiments (Sokal and Rohlf 1995). Spore size served as a covariate. The interaction terms with spore size served to test the homogeneity of slopes assumption of the ANCOVA. With a significant effect of treatment, post-hoc *t* tests using a Bonferroni adjustment (Sokal and Rohlf 1995) were used to determine significant differences between treatments.

A single-factor ANCOVA was performed using SYSTAT (Wilkinson 2002) to determine if A_{CE} affected the size of hermaphroditic gametophytes. Spore size was included in the analysis as a covariate. The interaction between spore size and A_{CE} treatment served as a test of the homogeneity of slopes assumption. With a significant effect of A_{CE} treatment,

post-hoc tests using a Bonferroni adjustment (Sokal and Rohlf 1995) were used to determine differences between A_{CE} treatments.

Results

Germination across all treatments averaged 49% in the first experiment and 55% in the second experiment. In the first experiment, a percentage of germinated wild-type spores developed into hermaphrodites in all treatments: 92% in the 0× A_{CE} control, 73% in the 0.5× A_{CE} treatment, 35% in the 1× A_{CE} treatment, and 40% in the 2× A_{CE} treatment. In the second experiment, 89% of wild-type spores that germinated developed into hermaphrodites in the 0× A_{CE} control. Many of the *him1* mutant gametophytes in the 0× control exhibited a hermaphrodite morphology: 47% in the first experiment and 27% in the second experiment. These hermaphroditic *him1* gametophytes were not included in the analyses of male gametophyte sizes.

Overall, wild-type spore size varied between 8755 and 21 041 μm^2 (mean = 14 676 μm^2, SD = 2281 μm^2), and the sizes of wild-type and *him1* spores were not significantly different ($F_{1,312}$ = 2.486, P = 0.116). Log-linear analyses showed that spore size was not a predictor of gametophyte gender in any of the following treatments: 0× A_{CE} ($\chi^2_{1,0.05}$ = 0.759, P = 0.384), 0.5× A_{CE} ($\chi^2_{1,0.05}$ = 0.633, P = 0.426), 1× A_{CE} ($\chi^2_{1,0.05}$ = 0.461, P = 0.497), or 2× A_{CE} ($\chi^2_{1,0.05}$ = 0.148, P = 0.701).

Male gametophyte size varied between 17 236 and 199 076 μm^2 (mean = 81 386 μm^2, SD = 38 014 μm^2). Wild-type hermaphrodite sizes varied between 28 394 and 2 006 711 μm^2 (mean = 367 724 μm^2, SD = 391 655 μm^2). Hermaphrodites occurred in all treatment groups in the first experiment, but only in the 0× A_{CE} control in the second experiment.

For the male size ANCOVA, slopes were homogeneous. The interaction terms, including spore size, were all P > 0.05; therefore, the final analysis did not include these terms. The blocking factor was not significant ($F_{1,231}$ = 1.644, P = 0.201; Table 1). There was an overall, albeit very weak, relationship between spore size and gametophyte size ($F_{1,231}$ = 8.543, P = 0.0019, adjusted r^2 = 0.039; Fig. 1A). Sizes of male gametophytes of the *him1* mutant and the wild type did not differ ($F_{1,231}$ = 0.225, P = 0.635; Table 1). Male gametophytes were significantly smaller, on average, as the concentration of antheridiogen increased ($F_{3,231}$ = 11.453, P < 0.001; Fig. 2A); P-values for the post-hoc contrasts between A_{CE} treatments were all <0.008, the Bonferroni adjusted critical P-value. The 0× A_{CE} control mean was not significantly different from the 0.5× A_{CE} or 1× A_{CE} treatment means (P > 0.008 for both contrasts; Fig. 2A). The average size of males in the 0× A_{CE} control was significantly larger than that in the 2× A_{CE} treatment.

For the hermaphrodite size ANCOVA the residuals were heteroscedastic and, therefore, gametophyte size was log-transformed before the ANCOVA was performed. Slopes were homogeneous and, therefore, the spore size× A_{CE} treatment interaction was dropped from the analysis. Since hermaphrodites occurred in only the control treatment in the second experiment, data from these gametophytes were not included in the analysis. Spore size was positively related to the size of hermaphrodites ($F_{1,40}$ = 5.425, P = 0.025, adjusted r^2 = 0.134; Fig. 1B). A_{CE} had a negative effect on the gametophyte size of hermaphrodites ($F_{3,40}$ = 3.858, P = 0.016; Fig. 2B). Post-hoc tests revealed that the 0.5× and 1× A_{CE} treatments had significantly smaller gametophytes than the control treatment (P < 0.008, the Bonferonni adjusted critical P-value). No other post-hoc contrasts were significant (Fig. 2B).

Table 1. Results of analysis of covariance on male gametophyte size.

Source	MS	df	F	P
Block	1.359	1	1.195	0.275
Strain	3.264	1	0.029	0.866
A_{CE}	1.537	3	13.515	<0.001
Strain× A_{CE}	2.347	3	2.063	0.106
Spore size	1.124	1	9.878	<0.005
Error	1.137	228		

Note: Block indicates the blocking factor, strain compares wild type to *him1* mutant, and A_{CE} refers to the antheridiogen treatments.

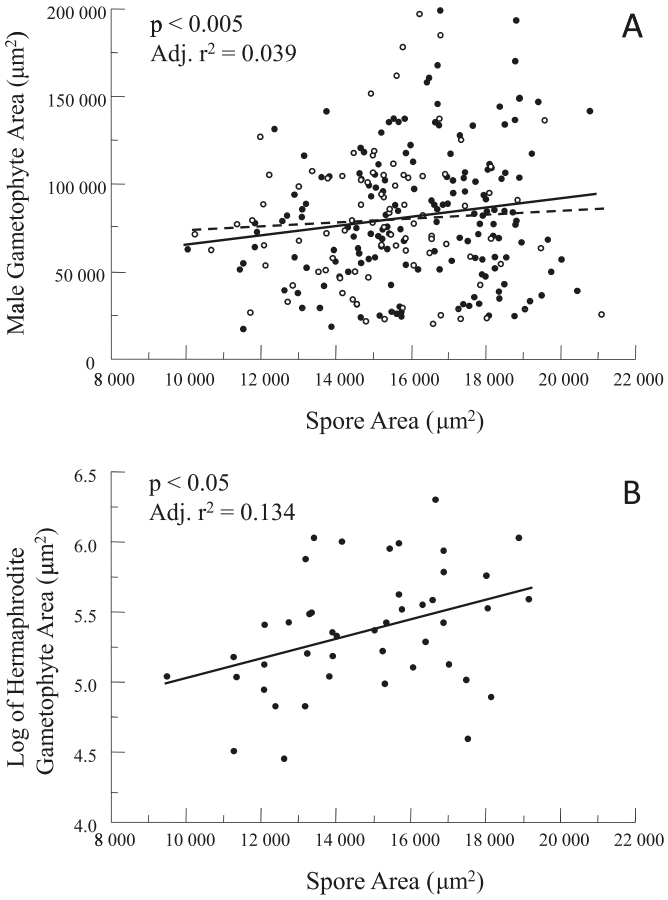
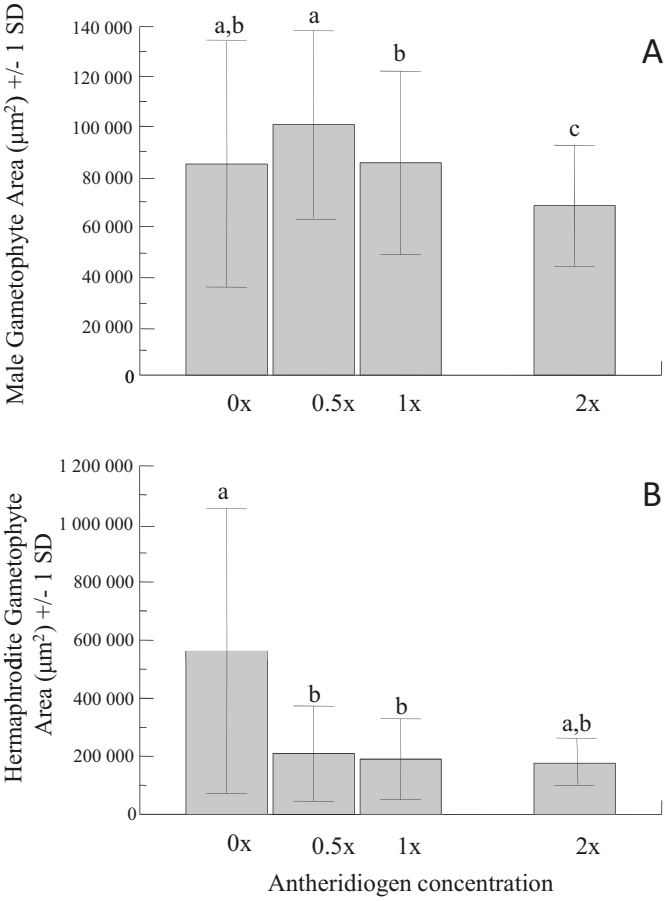


Fig. 1. (A) The size of male gametophytes 3 weeks post sowing is plotted against spore size. Both regression lines are presented: solid, *him1* strain; broken, wild type. Statistics are presented for both strains combined. (B) Log of hermaphrodite gametophyte size 3 weeks post sowing is plotted against spore size.

Discussion

In angiosperms, the decisions made by the maternal plant

Fig. 2. (A) Average male gametophyte size ± 1SD is plotted by treatment. Where lower case letters are the same, the average size of male gametophytes does not differ significantly. (B) Average hermaphrodite gametophyte size ± 1SD is plotted by treatment. Where lower case letters are the same, the average size of hermaphrodite gametophytes does not differ significantly.



that determine seed size have far-reaching effects on the offspring (Roach and Wulff 1987). These include, but are not limited to, growth rate, leaf number, and leaf size. Just as seed size is likely to be a predictor of seed resource levels, spore size is likely to be a predictor of overall spore resource levels that were provisioned by the sporophyte. Spore size in *C. richardii* was predictive of both male and hermaphrodite gametophyte size. Though the relationship between spore size and male gametophyte size was weak, the male gametophytes here were 4 weeks old and close to senescence. The relationship between spore size and the gametophyte size of younger males might be expected to be much stronger. The relationship between spore size and hermaphrodite gametophyte size was stronger. These relationships suggest a maternal effect on subsequent gametophyte growth through spore size. These differences in spore size could manifest as differences in germination rates, with larger spores germinating more quickly than smaller spores (Sayers and Hamilton 1995) and (or) as differences in growth rates overall. It is not known whether these differences in gametophyte size translate into differences in survivorship and fecundity, although a case could certainly be made. Although spores of different sizes grow at different rates, spore size did not appear to affect the gametophyte's gender. Maternal allocation decisions that were made in the production of spores do not appear to affect the offspring's gender.

In many homosporous ferns, including *C. richardii*, A_{CE} has been shown experimentally to affect the gender decision of undifferentiated spores (Haig and Westoby 1988). A_{CE} here also affects the growth of male gametophytes in a dosage-dependent manner. Quintanilla et al. (2007) and Stevens and Werth (1999) reported dosage-dependent activity of antheridiogens in *Woodwardia radicans* (L.) J. Sm. and *Onoclea sensibilis* L., respectively. This secondary effect of slowed growth appears to be independent of the primary effect of gender determination because *him1* mutants were affected similarly to the wild-type strain, despite the fact that *him1* mutants are programmed to be male.

Given A_{CE}'s negative effect on growth, the control treatment might be expected to have larger males than the A_{CE} treatments. However, this was not the case. This result may be attributable to the vast majority of spores in the wild-type control (92% of germinating spores in the first experiment and 89% in the second experiment) that developed into hermaphrodites. This left only a few males in this treatment and these males, by definition, were acting contrary to expectations, i.e., they developed into males in the absence of A_{CE}. Similarly, 47% of *him1* spores in the control of the first experiment and 27% in the control of the second experiment developed a hermaphrodite-like thallus. Although the size of the spore does not explain why some spores developed into males rather than hermaphrodites in the control, it is possible that other differences existed among these spores that in turn correlated with slower growth.

A_{CE} affected the growth of hermaphrodite gametophytes as well. Although these gametophytes "ignored" A_{CE}'s primary message and developed as hermaphrodites, these same hermaphrodite gametophytes were affected by A_{CE}'s secondary message. They experienced slower growth than hermaphrodite gametophytes in the control. One explanation is that A_{CE} affects their growth prior to the decision to become hermaphrodites. With the development of the hermaphrodite, A_{CE} is subsequently ignored (Banks 1999). This would explain why A_{CE}'s effect on hermaphrodite size is not dosage-dependent; gametophytes are only receptive to A_{CE} prior to the decision to become hermaphrodites.

Haig and Westoby (1988) have suggested that the adaptive significance of A_{CE} is that it reduces competition between gametophytes largely because the males are expected to use fewer resources than the hermaphrodites. Also, males can achieve fitness through fertilization with a hermaphrodite. That same hermaphrodite could self fertilize, but the resulting sporophyte would be homozygous across all loci (Haig and Westoby 1988; Chasan 1992). Therefore, the presence of the male nearby is said to increase the likelihood of higher quality offspring (Haig and Westoby 1988). This is an unsatisfying hypothesis, since the presence of multiple hermaphrodites would fulfill the same requirement of outcrossing. Furthermore, the more A_{CE}-secreting hermaphrodites there are, the slower male growth would become, thereby lessening the chance that the male would participate in fertilization at all. The results of this experiment suggest a suppressive effect of A_{CE} on gametophyte growth in general, regardless of gender.

The effect of A_{CE} on gender is well established, and the

results here do not disprove the hypothesis that antheridiogen directly determines gender in *C. richardii*. However, the results support the idea that gender may be determined indirectly through effects on gametophyte growth.

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