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PHEROMONAL INTERACTIONS AMONG GAMETOPHYTES OF *OSMUNDASTRUM CINNAMOMEUM* AND THE ORIGINS OF ANTHERIDIAGEN SYSTEMS IN LEPTOSPORANGIATE FERNS

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Antheridiogen systems, whereby notch-bearing, archegoniate gametophytes induce maleness in ameristic neighbors, have been detected in many core leptosporangiate ferns. Previous studies have failed to detect an antheridiogen system in Osmundales, which is sister to all other extant leptosporangiates; hence, antheridiogen systems are thought to have evolved after their divergence. Detailed studies of morphological development and patterns of gender expression in Osmundales and other basal leptosporangiate clades are needed to clarify how antheridiogen systems evolved. Here, we tracked the development and gender expression of gametophytes of *Osmundastrum cinnamomeum* grown in isolation and multispore populations exposed to basal media (control), gibberellic acid (GA₃), and an older generation of gametophytes. A notch-producing apical meristem invariably preceded antheridia and archegonia production in *O. cinnamomeum*. Exposure to either GA₃ or an older generation of gametophytes delayed growth rates and prolonged asexual and male status, whereas the multispore control possessed significantly greater proportions of females relative to isolates. Our observations confirm the existence of a putative antheridiogen system in Osmundales and a mechanism by which male-first expression is bypassed by a subset of the population. The evolution of fully developed antheridiogen systems in core leptosporangiate families may have involved the decoupling of the formation of a notch meristem from the production of antheridia in an ancestor with an Osmundales-type pattern of gender expression.

Keywords: antheridiogen, archegoniogen, phytohormone, gender, *Osmunda cinnamomeum*, *Osmundastrum*.

Introduction

Intergametophytic mating in ferns via pheromonal communication is adaptive for avoiding inbreeding and maintaining genetic variation. Antheridiogens are pheromones that promote gametophytic outcrossing and the outcome of gametophytic reproductive competition within—and in some cases between—species of leptosporangiate ferns (Lloyd 1974; Haufler and Gastony 1978; Willson 1981; Haufler and Ranker 1985; Schneller et al. 1990). These pheromones are produced by meristematic notch-bearing (i.e., cordate) gametophytes. Antheridiogens that have been tested for their effects on spore germination induce germination in darkness and precocious maleness in notchless gametophytes (Endo et al. 1972; Raghavan 1989 and citations therein). Antheridiogens have been detected in Schizaeales and in many core leptosporangiate orders and families (Voeller 1964b; Nester and Coolbaugh 1986; Raghavan 1989; MacMillan 2002) but have not been detected in Gleicheniales and Hymenophyllales, which are sister groups to Schizaeales, or in Osmundales, the sister group to all other extant leptosporangiate fern families (Pryer et al. 2004; Smith et al. 2006). All chemically characterized antheridiogens are structurally similar to and generally regarded as gibberellins

(GAs; Raghavan 1989; MacMillan 2002). Indeed, gibberellic acid (GA₃) can be substituted for the native antheridiogen in Schizaeales (Voeller 1964b; Voeller and Weinberg 1969; Näf et al. 1975; Takeno 1979). Endogenous GAs induce notch formation and thallus expansion and increase reproductive effort in leptosporangiate fern gametophytes (Greer et al. 2009). Similarly, GAs induce male gender expression and cell expansion in the apices of spermatophytes (Tanurdzic and Banks 2004). GAs induce cell expansion in differentiating cells near the gametophyte apex and in older thallus cells that divide to produce antheridia, but the planes of division differ between the two regions (Kazmierczak 2003). Thus, antheridiogen systems not only provide a means for exploring the evolution of the physiological controls of development in leptosporangiate ferns but may also provide insights into the physiological controls and mating systems possessed by the most recent common ancestor of monilophytes and spermatophytes.

As with any study of evolutionary process, efforts to elucidate the evolution of antheridiogen systems require accurate information regarding phylogenetic relationships and character states in basal and derived clades. This is particularly true for Osmundales, given its phylogenetic position among leptosporangiate ferns extant and extinct. A recent molecular phylogeny of Osmundaceae found *Osmunda* to be a paraphyletic genus and *Osmunda cinnamomea* to be sister to all other extant members of this family (Metzgar et al. 2008). Consequently, the authors recommended moving *Osmunda cinnamomea* to a new genus, *Osmundastrum*, and recognizing four genera,

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Osmundastrum, *Leptopteris*, *Osmunda*, and *Todea* (Metzgar et al. 2008; Christenhusz et al. 2011), which we follow here.

Our current knowledge of morphological development and gender expression in Osmundales is incomplete and enigmatic, reflecting genotypic variance in allometry and interactions between each genotype and its environment (i.e., phenotypic plasticity). Nayar and Kaur (1971) reported a male-to-hermaphrodite sequence of gender expression for the three extant genera of Osmundales (*Leptopteris*, *Osmunda*, and *Todea*), which can complicate detection of an antheridiogen system. For example, Voeller (1964a, 1964b) did not detect male induction by exogenous GA₃ in *Osmunda claytoniana* or *Osmundastrum cinnamomeum*; however, the sequence and duration of gender expression and its relationship to growth rate and notch development were not reported, all of which are essential for assaying an antheridiogen system, particularly in a species with a male-to-hermaphrodite gender sequence. On the basis of their literature review and results from their own experiments, Greer et al. (2009) hypothesized that gametophytes of Osmundales release a GA-based compound that prolongs asexual and male stages in less developed neighbors—that is, a putative antheridiogen system. This hypothesis has the appeal of explaining why past bioassays for an antheridiogen system in Osmundales may have missed its presence. Alternatively, Klekowski (1973) observed an increase in the frequency of female gametophytes of *Osmunda regalis*, from 0.2% among isolates to 21.3% among pairings. Although Klekowski did not offer an explanation for these observations, they may be explained by the presence of a female-inducing pheromonal system—that is, an archegoniogen in which asexual gametophytes can detect neighbors that are developmentally more advanced (e.g., male, hermaphrodite, or merely possessing an apical notch) and respond by skipping the default male stage and becoming female. A potential mechanism for an archegoniogen was observed by Greer et al. (2009) in which size, notch development, and rates of reproductive maturation decreased in multispore populations treated with Apogee, which blocks GA synthesis. They concluded that endogenous GA is required for apical notch formation and production of both antheridia and archegonia. Hence, exogenous GA-based exudates could conceivably be modified to promote precocious or prolonged maleness or femaleness.

The objectives of the investigations reported here were to (1) elucidate the default patterns of morphological development and gender expression of gametophytes of *Osmundastrum cinnamomeum* isolates, (2) identify the effects of exogenous GAs (GA₃) on growth and gender expression, and (3) determine whether this species possesses antheridiogen and archegoniogen systems.

Material and Methods

Spore Collection and Culture Conditions

Spores from six *Osmundastrum cinnamomeum* were collected in May 2009 in Kent County, Michigan, and stored at 3°C. The green spores were surface cleaned through repeated rinses and centrifugation in autoclaved deionized water (DI). This method was used in lieu of surface sterilization techniques that used sodium hypochlorite or antibacterials (nys-

tatin and streptomycin) that were shown to reduce spore germination and cause abnormal development in a previous study (Greer et al. 2009). No contamination was observed in the subsequent gametophyte populations grown on agar medium.

In all three studies, spores of *O. cinnamomeum* were sown on nutrient-enriched C-Fern agar (Carolina Biological Supply) and adjusted to pH 6.0. The subsequent gametophyte populations were maintained in growth chambers under 24-h full-spectrum fluorescent illumination at $141.2 \pm 35.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $20^\circ \pm 3^\circ\text{C}$. Spores were 1 mo old before sowing for all studies except the bioassay for a gender-influencing pheromone, which was conducted 5 mo after the others.

Phenology of Gender Expression in Isolates

The phenology of gender expression by *O. cinnamomeum* in the absence of intergametophytic interactions was investigated by growing gametophytes in isolation. Spores were germinated in a centrifuge tube in sterile deionized water for 1 wk in the conditions reported above, at which time gametophytes (five cells or fewer) were isolated, and each was sown in a 5-mL cell within a sterile polystyrene 24-well culture plate (Corning CLS3527). A total of 146 replicate isolates were prepared. In addition to its lid, each tray was wrapped in plastic film to prevent desiccation. Twenty isolates were harvested weekly for 5 wk, beginning 3 wk after sowing. Each gametophyte was mounted in a 90% deionized water/10% ethanol solution; scored as asexual, male, female, or hermaphrodite; and digitally photographed. Gender was determined by the presence of antheridia, archegonia, or both by means of a compound microscope at the time of harvest. Gametophytes scored as males or females possessed only antheridia or archegonia, respectively. In contrast, gametophytes scored as hermaphrodites possessed both antheridia and archegonia; however, functionality of the antheridia and archegonia was not determined. Photographs were taken with a compound microscope; however, a dissection microscope was used when the gametophytes became too large to be fully viewed under the lowest magnification of the compound scope. Gametophyte size (i.e., thallus area) and the ratio of notch depth to thallus length (NDL ratio) were analyzed from the digital photographs using SigmaScan Pro 5.0 (Fox and Urich 1993). NDL ratio was used to assess gametophyte developmental status—specifically, the formation of a notch meristem and cordate morphology. For example, a comparatively small NDL ratio between gametophytes of similar age and size would be the product of delayed initiation of notch formation (i.e., a reduced rate of anticlinal divisions in the meristem) or a slower rate of notch development once initiated. All measurements were standardized to account for differences in magnification during image capture.

Bioassay for Effects of Exogenous GAs (GA₃) on Growth and Gender Expression

To test for a GA₃-based pheromone system, *O. cinnamomeum* spores were sown on nutrient-enriched agar containing 0 M, 100 μM , or 1 mM GA₃ in 100-mm petri plates. Spores were sown at approximately three spores per square centime-

ter to minimize physical competitive interactions while creating circumstances that promote pheromonal interactions. Each dosage was replicated in 10 plates. Five gametophytes were harvested from each of the 10 replicate plates per GA₃ dosage at 5 and 8 wk after sowing, for a total of 50 gametophytes per dosage and age group. Data collection methods followed those used for the isolate study. Twenty of 50 digital photographs were chosen randomly for size and NDL ratio analysis. The petri plates were maintained in covered germination trays containing ~1 cm of deionized water to maintain humidity. Each petri plate was rotated within each tray and the trays were rotated within the growth chamber to randomize their positions on a weekly basis.

Bioassay for a Gender-Influencing Pheromone

Our bioassay for a gender-influencing pheromone used multispore populations sown at a density of approximately three spores per square centimeter, replicated in ten 100-mm petri plates. Each plate contained a sterile glass slide placed flat on the surface of the agar near the center of the plate, which prevented the settlement of spores and created a region without gametophytes that would later be sown with a second generation of spores.

To establish the pattern of gender expression in multispore populations at the experimental density, five gametophytes from each of the 10 plates were harvested (for a total of 50 gametophytes) and were scored for gender at 8 wk after sowing. This first generation also served as a control. To assay for a gender-influencing pheromone, the spore-blocking slide was removed after the 8-wk harvest of the first generation, and a second generation of spores was sown at approximately the same spore density. Fifty gametophytes from the second generation were harvested from the central area of each of the 10 replicate plates where no first-generation gametophytes occurred and were scored for gender, size, and NDL ratio at 5 and 8 wk after sowing. Data collection methods followed those used for the isolate study. Culture and randomization methods followed those described above for the GA₃ bioassay. This study was conducted ~6 mo after the two studies described above.

Data Analyses

One-way parametric ANOVA or Kruskal-Wallis ANOVA was used, depending on the ability to meet the assumptions of the former, to compare gametophyte size and NDL ratio between isolates and each GA₃ dosage. Differences among treatment groups were tested using Bonferroni and Tamhane's T2 multiple comparisons, following significant ANOVA and Kruskal-Wallis tests, respectively.

Differences in gender ratios within each relevant harvest age class were compared using χ^2 tests of independence between the following treatment pairings: (1) isolates versus the first generation from the pheromone bioassay to test for the presence of gender-promoting pheromones; (2) pairwise comparisons among 0 M, 100 μ M, and 1 mM GA₃ treatment groups and between these treatment groups and isolates to test for a GA₃-based pheromone; (3) 0 M GA₃ (control) versus first multispore generation of the pheromone bioassay to

determine whether gender was influenced by spore age, as the latter was conducted 6 mo after the former. Pairwise comparisons between the 100 μ M and 1 mM GA₃ treatments at 8 wk and the second multispore generation from the pheromone bioassay were used to assess the magnitude of pheromonal effects. Comparisons between other treatment combinations were not possible because of their specific gender ratios. Gender categories for each test were grouped on an as-needed basis to meet the assumptions of Pearson's χ^2 tests of independence; see each gender test in "Results" for further details. *P* values were adjusted using the Bonferroni-Holmes correction for multiple-comparison error rates.

Results

Phenology of Gender Expression in Isolates

All isolated gametophytes observed in this experiment possessed a cordate morphology with apical notches that became conspicuous as early as 14 d after spore sowing. Gametangia were observed only after the formation of an apical notch; neither antheridia nor archegonia was present in preceding stages. Antheridia were first observed in 4-wk-old cordate isolates (fig. 1), occurring along the edge and among the rhizoids at the basal end of the thallus. Archegonia were first observed in 5-wk-old hermaphroditic gametophytes occurring laterally along each side of the apical region of the central cushion, but most isolates (75%) were male at 5 wk. Females were first observed in 7-wk-olds and comprised 5% of the isolates. This proportion grew to 14.3% at 8 wk of age. At 8 wk of age, the majority of archegoniate individuals (85.7%) were hermaphroditic. Given the late arrival of females in this study, we conclude that all hermaphrodites developed from a prior male status; hence, the majority of *Osmundastrum cinnamomeum* isolates expressed a default male-to-hermaphrodite gender sequence.

Fig. 1 Gender ratios of asexual, male, female, and hermaphroditic *Osmundastrum cinnamomeum* gametophytes grown in isolation on enriched C-Fern medium from 3 to 8 wk old. *N* = 20.

*Bioassay for GA Effects on Growth
and Gender Expression*

Gametophytes grown in multispore populations exposed to 0 M, 100 μ M, or 1 mM GA₃ were significantly smaller than isolates at 5 wk (table 1) and 8 wk (table 1; fig. 2) after sowing. A minimum reduction in size of 31% observed among gametophytes exposed to GA₃ relative to isolates can be attributed to growth in multispore populations. When comparing among multispore populations, increasing GA₃ dosage had a curvilinear effect at 5 wk after sowing, and all treatments were significantly different in size (fig. 2). The lowest GA₃ dosage had a stimulatory effect on size relative to the other GA₃ dosages, as gametophytes exposed to 100 μ M GA₃ were slightly larger than those exposed to 0 M GA₃ (control) and 1 mM GA₃. Nevertheless, gametophytes exposed to the highest GA₃ dosage (1 mM GA₃) were significantly smaller than those exposed to 0 M GA₃. Eight weeks after sowing, the curvilinear effect on size was no longer present; however, the isolates maintained the largest area of all GA₃ treatments. Gametophytes exposed to GA₃ (0 M control and 100 μ M) were statistically indistinguishable in size, but both treatment groups were significantly larger than the 1 mM GA₃ treatment group (table 1; fig. 2).

Differences in NDL ratio between isolates and gametophytes exposed to 0 M GA₃ (control) were not significant in the 5-wk-old harvest; hence, reduced growth rate in multispore populations did not reduce attainment of a notch-forming meristem. Dosage-dependent effects on NDL ratio were observed among multispore GA₃ treatments and isolates. Gametophytes exposed to the lowest GA₃ dosage (100 μ M GA₃) possessed significantly larger NDL ratios than gametophytes in isolation, in the control group, and in the high-dosage group (1 mM GA₃), indicating a stimulation in the rate of anticlinal divisions in their meristems (table 1; fig. 3). In contrast, NDL ratio among gametophytes exposed to the highest GA₃ dosage (1 mM GA₃) was

~72% smaller relative to isolates, signifying a reduced rate of anticlinal cell division in the apical meristems of populations exposed to 1 mM GA₃. Notwithstanding the curvilinear effect described above, differences in NDL ratio among isolates and gametophytes exposed to GA₃ were negligible at 8 wk old. This may be the result of GA₃ degradation by the eighth week or a shift in the sensitivities to—or effects elicited by—GA₃ in gametophytes with well-developed notch meristems.

Five weeks after sowing, the reduced growth rate observed in multispore populations relative to isolates was also associated with a delay in reproductive maturation and progression in gender expression. All multispore populations exposed to GA₃ had greater proportions of asexuals relative to isolates ($\chi^2 > 5.9$, $P < 0.03$; table 2; fig. 4). Although multispore populations exposed to 0 M GA₃ possessed significantly more asexuals relative to isolates (94% and 10%, respectively; $\chi^2 = 48.0$, $P < 0.001$; asexual vs. gametangia-bearing gender categories), females were observed only in the 0 M GA₃ treatment group (2%) and were entirely absent among isolates or higher-dosage GA₃ treatment groups. Increasing GA₃ concentrations had a curvilinear effect on reproductive maturity in multispore populations: asexuals made up 94% of the gametophytes exposed to 0 M GA₃, 40% exposed to 100 μ M GA₃, and 98% exposed to 1 mM GA₃. The low GA₃ dosage (100 μ M) had a stimulatory effect on male expression (60% male) compared with the 0 M (4%) and high GA₃ (2%) treatments, with significantly fewer asexuals observed with the 100 μ M GA₃ dosage ($\chi^2 = 32.9$, $P < 0.001$; asexual vs. gametangia-bearing gender categories); however, differences in this gender ratio between the control and high GA₃ were not significant (fig. 4). A similar pattern in size and NDL ratio was observed in the multispore populations at 5 wk old; hence, attainment of reproductive maturity was closely associated with attainment of a notch-forming meristem, and both were accelerated with the control dosage (0 M GA₃). Hermaphrodites were observed only among isolates at 5 wk old. Thus, gametophytes grew

Table 1

ANOVA for Thallus Area and Morphological Metrics in 5- and 8-Wk-Old *Osmundastrum cinnamomeum*
Gametophytes Grown as Isolates and in Multispore Populations Exposed to 0 M, 100 μ M,
or 1 mM Gibberellic Acid (GA₃)—Enriched C-Fern Medium

	Age (wk)	Treatment	N	Mean rank	χ^2	P				
Kruskal-Wallis ANOVA:										
Thallus area	5	Isolates	20	64.17	50.856	<.001				
		Multispore (0 M GA ₃)	20	30.90						
		100 μ M GA ₃	20	49.90						
		1 mM GA ₃	20	15.50						
Thallus area	8	Isolates	20	63.45	53.832	<.001				
		Multispore	20	43.05						
		100 μ M GA ₃	20	45.00						
		1 mM GA ₃	20	10.50						
				Groups			df	Mean squares	F	P
		Parametric ANOVA:								
NDL ratio	5	Between	3	.595	72.591	<.001				
		Within	74	.008						
NDL ratio	8	Between	3	.002	.250	.861				
		Within	76	.008						

Note. NDL ratio = ratio of notch depth to thallus length.

Fig. 2 Mean area (± 1 SD) of *Osmundastrum cinnamomeum* gametophytes grown as isolates and in multispore populations on 0 M, 100 μ M, or 1 mM gibberellic acid (GA₃)-enriched C-Fern medium at 5 and 8 wk old. Treatments with the same letter are not statistically different from one another on the basis of Tamhane's multiple comparisons for Kruskal-Wallis ANOVA. $N = 20$.

more rapidly and developed gametangia more rapidly in isolation than in multispore populations, regardless of GA₃ dosage.

The delays in reproductive maturation and progression in gender expression associated with growth in multispore populations and exposure to GA₃ observed among 5-wk-old gametophytes persisted into the eighth week after sowing (fig. 5). At 8 wk old, multispore populations exposed to GA₃ possessed fewer archegoniate gametophytes and had categorically different gender groups compared with isolates except for the control, which possessed comparable archegoniate gender categories but had a greater proportion of females (42%) than isolates (14.3%; $\chi^2 = 5.076$, $P = 0.048$; female vs. hermaphrodite gender categories; table 3; fig. 5). A large portion of gametophytes in the control bypassed the default male-first gender sequence, resulting in a greater frequency of females in the multispore control relative to isolates. A further delay in development occurred in multispore populations when they were exposed to GA₃. Gametophytes exposed to a low GA₃ (100 μ M) dosage were 14% male and had a corresponding decrease in females relative to the 0 M GA₃ dosage; however, differences in hermaphrodites between low GA₃ and 0 M GA₃ were marginally significant ($\chi^2 = 5.657$, $P = 0.051$; hermaphrodite vs. other gender categories). The delay in reproductive maturation and progression in gender expression was greatest in populations exposed to the highest dosage of GA₃ (1 mM), being entirely asexual and male (12% and 88%, respectively) relative to the control, which was completely archegonium-bearing individuals (fig. 5). Furthermore, the high GA₃ dosage was the only treatment to possess asexuals at 8 wk after sowing. Thus, the delay in gender development due to GA₃ exposure had long-lasting consequences for the gender composition of the population.

Bioassay for a Gender-Influencing Pheromone

This experiment was conducted 6 mo after spore collection and the isolate and GA₃ studies; therefore, gender ratios were compared among 8-wk-old gametophytes between the first generation of the pheromone bioassay and 0 M GA₃ multispore populations to assess the effects of spore age on gender expression. The female-to-hermaphrodite ratio was significantly higher in the 0 M GA₃ control (0.72:1) than in the pheromone bioassay (0.28:1; $\chi^2 = 4.596$, $P = 0.032$; fig. 5); however, both groups were 100% archegonium bearing at 8 wk old. Thus, increased spore age was associated with a decreased rate of omission of the default male-first pattern of gender expression observed in isolates and in the 0 M GA₃ multispore control.

Five-week-old gametophytes in the second generation of the pheromone bioassay were 100% asexual. Exposure to the first generation delayed gender development beyond that of the GA₃ treatments of the same age, including the highest dosage (1 mM GA₃), each of which possessed a 2% minimum of male gender.

In the pheromone bioassay, gametophytes grown in the presence of an older generation of gametophytes had delayed reproductive maturation and progression of gender expression. Eight weeks after sowing, all gametophytes in the first generation were archegonium bearing, whereas the second generation was composed of only asexual (72%) and male (28%) gametophytes (fig. 5). Similarly, an absence of archegonium-bearing individuals was observed in multispore populations exposed to the highest GA₃ dosage (1 mM); however, the proportion of asexual gametophytes was significantly greater in the second

Fig. 3 Mean ratio of notch depth to thallus length (± 1 SD) of *Osmundastrum cinnamomeum* gametophytes grown as isolates and in multispore populations on 0 M, 100 μ M, or 1 mM gibberellic acid (GA₃)-enriched C-Fern medium at 5 and 8 wk old. Treatments with the same letter are not statistically different from one another on the basis of Bonferroni multiple comparisons for parametric ANOVA. $N = 20$.

Table 2

Pearson's χ^2 Test of Independence for Gender Ratios in 5-Wk-Old *Osmundastrum cinnamomeum* Gametophytes Grown as Isolates and in Multispore Populations Exposed to 0 M, 100 μ M, or 1 mM Gibberellic Acid (GA₃)–Enriched C-Fern Medium

	Variable	χ^2	Corrected <i>P</i>
Isolates vs. 0 M GA ₃	Asexual vs. gametangia bearing	48.000	<.001
0 M vs. 100 μ M GA ₃	Asexual vs. gametangia bearing	32.972	<.001
0 M vs. 1 mM GA ₃	Asexual vs. gametangia bearing	1.042	.307
Isolates vs. 100 μ M GA ₃	Asexual vs. other	5.966	.030

Note. *N* = 50 for all treatments, except *N* = 20 isolates.

generation (72%) compared with the 1 mM GA₃ dosage (12%; $\chi^2 = 36.946$, $P < 0.001$; male vs. asexual gender categories). Thus, the absence of archegonia and the high proportion of asexuals in the second generation of *O. cinnamomeum* gametophytes suggest that gender development was delayed or that male status was prolonged due to growth in the presence of the older first generation.

Discussion

The three studies reported here establish that gametophytes of *Osmundastrum cinnamomeum* form an apical notch preceding reproductive maturation and possess a default male-to-hermaphrodite sequence of gender expression that is responsive to complex intergametophytic pheromonal interactions. In isolation, gametophytes grew more rapidly and developed gametangia at a younger age than gametophytes in multispore populations. The existence of a putative GA-based antheridiogen system is supported by two lines of evidence: (1) a substantial delay in development occurred in populations exposed to exudates from a previous generation, prolonging asexual and male status; (2) exposure of multispore populations to GA₃ reduced growth rates, delayed sexual maturation, and prolonged male expression relative to isolates and multispore control populations. Populations exposed to GA₃ exhibited curvilinear dosage-level responses. A subset of the gametophytes in multispore control populations bypassed the default male-first gender expression, increasing the frequency of females relative to that among isolates of the same age.

Implications of Notch-Required Gender Expression in *O. cinnamomeum*

Antheridia and archegonia were produced in *O. cinnamomeum* only after formation of an apical notch, regardless of culture conditions. Therefore, prolonged asexuality is a consequence of a delay in the formation of a notch-forming, pluricellular apical meristem, whether in response to intergametophytic interactions or to exposure to GA₃. Likewise, prolonged maleness is a consequence of the same delay in the developmental progression, whether in response to exudates from a previous generation or to exogenous GA₃.

Apical notch meristem development and gender expression in fern gametophytes is certain to be controlled by a complex array of phytohormonal interactions. A high cytokinin-to-GA (CK:GA) ratio created by a reduction in endogenous GA

(Greer et al. 2009) or by exogenous kinetin (Greer et al., forthcoming) increases the rate of notch formation and prolonged asexuality and subsequent maleness in *Osmunda regalis*. Given the alternative methods used in these studies, it is likely that relative hormone levels may play a greater role than absolute concentrations. Our observations that exogenous GA₃—and hence a low CK:GA ratio—delayed notch formation and prolonged maleness in *O. cinnamomeum* are consistent with this model. A low CK:GA ratio should decrease rates of cell division in the apical meristem and delay the default male-to-hermaphrodite ontogeny.

Notch-required gender expression is intriguing in light of the fact that development is thought to correspond with the loss of antheridiogen sensitivity in species possessing an antheridiogen system (Döpp 1950; Näf et al. 1975; Raghavan 1989). Notch-

Fig. 4 Gender ratios at 5 wk old of asexual, male, female, and hermaphroditic *Osmundastrum cinnamomeum* gametophytes grown as isolates and in multispore populations on 0 M, 100 μ M, or 1 mM gibberellic acid (GA₃)–enriched C-Fern medium. All gametophytes in pheromone bioassays were asexual at 5 wk (not shown). Treatments with the same letter have statistically indistinguishable proportions of asexuals on the basis of χ^2 tests of independence. The letters A and B indicate comparisons between isolates and the control (0 M GA₃). The letters D and E indicate comparisons between the control and 100 μ M GA₃. The letter G indicates comparisons between the control and 1 mM GA₃. The letters J and K indicate comparisons between isolates and 100 μ M GA₃. See “Results” for specifics of gender comparisons. *N* = 50 for all treatments, except *N* = 20 for isolates.

Fig. 5 Gender ratios at 8 wk old of asexual, male, female, and hermaphroditic *Osmundastrum cinnamomeum* gametophytes grown as isolates; in multispore populations exposed to 0 M, 100 μ M, or 1 mM gibberellic acid (GA₃); and in pheromone-producing and pheromone-receiving generations grown on enriched C-Fern medium. Treatments with the same letter have statistically indistinguishable proportions of asexuals, males, females, and hermaphrodites on the basis of χ^2 tests of independence. The letters A and B indicate comparisons between isolates and the control (0 M GA₃). The letters D and E indicate comparisons between the control and 100 μ M GA₃. The letters M and N indicate comparisons between the control and the first-generation pheromone bioassay. The letters P and Q indicate comparisons between 100 μ M GA₃ and the second-generation pheromone bioassay. The letters S and T indicate comparisons between 1 mM GA₃ and the second-generation pheromone bioassay. The letter X indicates comparisons between isolates and the first-generation pheromone bioassay. See "Results" for specifics of gender comparisons. $N = 50$ for all treatments, except $N = 20$ for isolates.

required gender expression would limit the window of time in which a gametophyte could respond to antheridiogen. Despite the advantage of a prenotch response to antheridiogen, notch-required sexual maturation might reflect a constraint in *O. cinnamomeum*. Subsequently, the mere loss of the default male gender and notch requirement would establish a GA-based antheridiogen system in the ancestor of Schizaeales core leptosporangiates.

Adaptive Significance of a GA-Based Putative Antheridiogen System in *O. cinnamomeum*

The delay in gender expression following exposure to GA₃ resulted in long-lasting consequences to the gender composition

of the population. The possession of a putative antheridiogen system in Osmundales is evidenced by a default male-to-hermaphrodite gender sequence observed in isolate populations and prolonged asexual and male expression in multispore populations exposed to exogenous GA₃ or growth media supporting an older generation of gametophytes. The male-to-hermaphrodite sequence is likely to be the ancestral condition for leptosporangiate ferns, having been observed in many genera of Osmundales (Campbell 1911; Klekowski and Lloyd 1968; von Aderkas and Cutter 1983a; Huang et al. 2004) and Marattiales (Campbell 1911). Hence, the putative antheridiogen system operating in *O. cinnamomeum* prolongs but does not hasten default male expression. It would be advantageous over non-pheromone-controlled expression because the archegonium-bearing source gametophyte would increase its fitness by (1) prolonging and inducing maleness in neighbors, thereby increasing the chances of fertilization, and (2) reducing reproductive competition by neighboring archegoniote gametophytes (Greer et al. 2009). One can predict that increasing concentrations of the putative GA-based antheridiogens should spread out the attainment of sexual maturation and the subsequent progression of gender expression, such that intergametophytic matings become much more likely and the density of competing females and sporophytic offspring is reduced.

A curvilinear response to GA₃ was observed in NDL ratio. Low GA₃ increased the rate of anticlinal cell division in the notch meristem relative to the control, which subsequently increased male expression; however, the high GA₃ dosage suppressed this activity. Therefore, we attribute the activity in the control (0 M GA₃) to additional factors influencing gender, such as another pheromone.

Significance of the Increase in Females in Multispore Populations

A plausible hypothesis to explain the prevalence of females in the multispore control populations relative to isolates is the existence of an archegoniogen system, wherein gametophytes of *O. cinnamomeum* are able to perceive the presence of advanced neighbors (i.e., bearing antheridia and a notch) and block their default production of antheridia. Given that gametangium production occurs only after the formation of an apical notch in this species, response to an archegoniogen (i.e., omission of the default male-first expression by hasten-

Table 3

Pearson's χ^2 Test of Independence for Gender Ratios in 8-Wk-Old *Osmundastrum cinnamomeum* Gametophytes Grown as Isolates; in Multispore Populations Exposed to 0 M, 100 μ M, or 1 mM Gibberellic Acid (GA₃); and in Pheromone-Producing and Pheromone-Receiving Multispore Generations Grown on Nutrient-Enriched C-Fern Medium

	Variable	χ^2	Corrected P
Isolates vs. 0 M GA ₃	Female vs. hermaphrodite	5.076	.048
0 M vs. 100 μ M GA ₃	Hermaphrodite vs. other	5.657	.051
0 M GA ₃ vs. first generation	Female vs. hermaphrodite	4.596	.032
100 μ M GA ₃ vs. second generation	Antheridia bearing vs. other	45.776	<.001
1 mM GA ₃ vs. second generation	Male vs. asexual	36.946	<.001
Isolates vs. first generation	Female vs. hermaphrodite	.556	.456

Note. $N = 50$ for all treatments, except $N = 20$ isolates.

ing femaleness or blocking maleness) would occur in the developmental window after the formation of an apical notch but before the default initiation of antheridia. The mechanistic nature of an archegoniogen remains unknown and may be an artifact of agar medium or culture conditions. *Onoclea* gametophytes were shown to have a slightly larger proportion of females in agar than in soil (Rubin and Paolillo 1983). Alternatively, petri and isolate culture plates may differentially trap gases such as ethylene and oxygen. Indeed, ethylene plays a direct role in promoting female expression in cucumber flowers (Yamasaki et al. 2000). If gaseous in nature, the archegoniogen is unlikely to have ecological significance. Given that both culture plates and petri plates are likely to trap gases such as ethylene and oxygen, the increase in female expression observed in the control is likely to be caused by agar-mediated biochemical interactions.

The evolution of an archegoniogen system would have fitness benefits for a species with a male-to-hermaphrodite gender sequence. Female-first gender expression would confer increased fitness through the avoidance of otherwise-assured male-mate competition and hastened sporophyte production as well as increased competitive abilities via resource preemption. Klekowski (1973) reported a pattern of gender expression consistent with an archegoniogen system in a study of genetic load in the congener *O. regalis*. Among isolates, 94% (of 459) were hermaphroditic, 0.3% were female, and 5.7% were male, whereas among pairings established prior to gametangium formation, 78.7% were hermaphroditic, 21.3% were female, and 0.003% were male. Consistent with the prediction of female fitness gain described above, 91.4% of *O. regalis* gametophyte pairings in this study possessed one hermaphrodite and one female, and the female possessed the sporophytic offspring in all cases because of the unidirectionality of the sperm source and the high level of genetic load that was observed. Sustained growth rate in response to an archegoniogen system is expected to accelerate attainment of femaleness. Consistent with this prediction, females were observed by Huang et al. (2004) in less dense neighborhoods of multispore populations of *O. cinnamomeum* and in multispore populations of *Todea barbara*, where females were more frequent when conditions were most favorable to growth (von Aderkas and Cutter 1983b). An additional, non-mutually-exclusive possibility exists, that *O. cinnamomeum* and *O. regalis* are polymorphic in default gender expression, wherein genotypes that produce archegonia prior to antheridia are maintained at a low frequency, as may be expected in a species with a prevailing male-to-hermaphroditic gender

expression. Further studies are needed to establish the mechanistic basis and ecological relevance of the archegoniogen system as well as the existence of a gender-based polymorphism.

Insights into the Evolution of GA-Based Antheridiogens

The quantitative genetic model of gender expression proposed by Banks (1999) for *Ceratopteris* may provide insights into the controls of gender expression in Osmundales and, hence, the evolution of an antheridiogen systems. In Banks's model, perception and response to antheridiogen is controlled by the quantitative trait locus HERMAPHRODITE (HER) and two mutually antagonistic quantitative trait loci, FEMINIZING (FEM), which promotes maleness, and TRANSFORMER (TRA), which promotes femaleness. Fully developed antheridiogen systems, known only in Schizaeaceae and core leptosporangiate families that form cordate gametophytes, induce maleness in those lacking an apical notch by activating HER, which represses TRA and subsequently MAN1, and activates FEM to promote antheridia production (Banks 1999). In contrast, production of antheridia in *O. cinnamomeum* requires the presence of an apical notch. Applying the HER-TRA-FEM model to explain our observations, we hypothesize that HER activation is linked to the formation of an apical notch in *Osmundastrum*, perhaps by endogenous GAs, resulting in its default male-first expression. Given that antheridia production always followed formation of an apical notch and preceded archegonium formation in our study of *O. cinnamomeum*, HER, if present, is not receptive to endogenous or exogenous signals (GA₃ or native antheridiogen) prior to attainment of an apical notch. Thus, the evolution of antheridiogen systems in more derived families may have required HER to become inducible only by an exogenous signal and only prior to notch formation.

Investigation into the presence of antheridiogen systems in basal leptosporangiate clades, particularly Gleicheniales and Hymenophyllales, as well as species within the eusporangiate Marattiales may provide further insights into the evolution of the phytohormonal controls of morphological development and gender expression and the evolution of antheridiogen systems of leptosporangiate ferns.

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