Pheromonal Interactions among Gametophytes of *Osmundastrum Cinnamomeum* and the Origins of Antheridiogen Systems in Leptosporangiate Ferns

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

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Antheridiogen systems, whereby notch-bearing, archegoniate gametophytes induce maleness in amercistic 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 multisporal 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 multisporal 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 cinnamomea, 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; Haufier and Gastony 1978; Willson 1981; Haufier 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; Endo et al. 1972; Raghavan 1989; Endo et al. 1972; Raghavan 1989; Naf 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 spermophytes (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 spermophytes.

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,
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 variation 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 cinnamonum; 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 as 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 feminality.

The objectives of the investigations reported here were to (1) elucidate the default patterns of morphological development and gender expression of gametophytes of Osmundastrum cinnamonum 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 cinnamonum 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 (nystatin 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. cinnamonum 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 ± 35.5 μmol m−2 s−1 and 20° ± 3°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. cinnamonum 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 CLS5327). 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. cinnamonum 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 centi-
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 multispor 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 multispor 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 $\chi^2$ 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 μM, 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 multispor 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 μM and 1 mM GA₃ treatments at 8 wk and the second multispor 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 $\chi^2$ 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 were 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](image-url) 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 μM, or 1 mM GA3 were significantly smaller than isolates at 5 wk (table 1) and 8 wk (table 2; 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 μM 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 μM) 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 among 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 μM 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 (χ2 > 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; χ2 = 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 μM GA3, and 98% exposed to 1 mM GA3. The low GA3 dosage (100 μM) 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 μM GA3 dosage (χ2 = 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 μM, or 1 mM Gibberellic Acid (GA3)–Enriched C-Fern Medium

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Treatment</th>
<th>N</th>
<th>Mean rank</th>
<th>χ²</th>
<th>P</th>
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<tr>
<td>Thallus area 5</td>
<td>Isolates</td>
<td>20</td>
<td>64.17</td>
<td>50.856</td>
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<tr>
<td></td>
<td>Multispore (0 M GA3)</td>
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<td>30.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 μM GA3</td>
<td>20</td>
<td>49.90</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>1 mM GA3</td>
<td>20</td>
<td>15.50</td>
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<td></td>
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<tr>
<td>Thallus area 8</td>
<td>Isolates</td>
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<td>63.45</td>
<td>53.832</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
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<td>20</td>
<td>43.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 μM GA3</td>
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<td></td>
<td>1 mM GA3</td>
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<td>10.50</td>
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<tr>
<td>Groups</td>
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<td>Mean squares</td>
<td>F</td>
<td>P</td>
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<td>NDL ratio 5</td>
<td>Between</td>
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<td>72.591</td>
<td>&lt;.001</td>
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<td>Within</td>
<td>74</td>
<td>.008</td>
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<td></td>
<td>Within</td>
<td>76</td>
<td>.008</td>
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</table>

Note. NDL ratio = ratio of notch depth to thallus length.
more rapidly and developed gametangia more rapidly in isolation than in multisporic populations, regardless of GA3 dosage. The delays in reproductive maturation and progression in gender expression associated with growth in multisporic populations and exposure to GA3 occurred in the eighth week after sowing (fig. 5). At 8 wk old, multisporic 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. hermaphroditic 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 multisporic control relative to isolates. A further delay in development occurred in multisporic populations when they were exposed to GA3. Gametophytes exposed to a low GA3 (100 μM) 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 (\( \chi^2 = 5.657, P = 0.051 \); hermaphroditic vs. other gender categories). The delay in reproductive maturation and progression in gender expression was greatest in populations exposed to the highest GA3 dosage (1 mM), 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 multisporic populations exposed to the highest GA3 dosage (1 mM); however, the proportion of asexual gametophytes was significantly greater in the second

**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 multisporic populations to assess the effects of spore age on gender expression. The female-to-hermaphroditic 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 multisporic 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.

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Populations exposed to GA3 exhibited curvilinear growth rates, delayed sexual maturation, and prolonged 
sequence of the same delay in the developmental progression, among isolates of the same age.

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 multisporp 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 multisporp populations to GA3 reduced growth rates, delayed sexual maturation, and prolonged male expression relative to isolates and multisporp control populations. Populations exposed to GA3 exhibited curvilinear dosage-level responses. A subset of the gametophytes in multisporp control populations bypassed the default male-first gender expression, increasing the frequency of females relative to that among isolates of the same age.

### 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 multisporp 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 multisporp populations to GA3 reduced growth rates, delayed sexual maturation, and prolonged male expression relative to isolates and multisporp control populations. Populations exposed to GA3 exhibited curvilinear dosage-level responses. A subset of the gametophytes in multisporp 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 *Osmundastrum 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 (Dopp 1950; Naf et al. 1975; Raghavan 1989). Notch-

![Fig. 4 Gender ratios at 5 wk old of asexual, male, female, and hermaphrodite *Osmundastrum cinnamomeum* gametophytes grown as isolates and in multisporp populations on 0 M, 100 μM, 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 $\chi^2$ tests of independence. The letters A and B indicate comparisons between isolates and the control (0 M GA3). The letters C and E indicate comparisons between the control and 100 μM GA3. The letter G indicates comparisons between the control and 1 mM GA3. The letters J and K indicate comparisons between isolates and 100 μM GA3. See “Results” for specifics of gender comparisons. N = 50 for all treatments, except N = 20 for isolates.](image-url)
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 μM, 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 asexuels, males, females, and hermaphrodites on the basis of χ² 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 μM 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 μM 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 Marattiaceae (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 archegoniate 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

<table>
<thead>
<tr>
<th>Pearson’s χ² Test of Independence for Gender Ratios in 8-Wk-Old Osmundastrum cinnamomeum Gametophytes Grown as Isolates; in Multispore Populations Exposed to 0 M, 100 μM, or 1 mM Gibberellic Acid (GA3); and in Pheromone-Producing and Pheromone-Receiving Multispore Generations Grown on Nutrient-Enriched C-Fern Medium</th>
<th>Variable</th>
<th>χ²</th>
<th>Corrected P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolates vs. 0 M GA3</td>
<td>Female vs. hermaphrodite</td>
<td>5.076</td>
<td>.048</td>
</tr>
<tr>
<td>0 M vs. 100 μM GA3</td>
<td>Hermaphrodite vs. other</td>
<td>5.657</td>
<td>.051</td>
</tr>
<tr>
<td>0 M GA3 vs. first generation</td>
<td>Female vs. hermaphrodite</td>
<td>4.596</td>
<td>.032</td>
</tr>
<tr>
<td>100 μM GA3 vs. second generation</td>
<td>Antheridia bearing vs. other</td>
<td>45.776</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>1 mM GA3 vs. second generation</td>
<td>Male vs. asexual</td>
<td>36.946</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Isolates vs. first generation</td>
<td>Female vs. hermaphrodite</td>
<td>3.536</td>
<td>.456</td>
</tr>
</tbody>
</table>

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 Adernas 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 Bank'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 Schizaeaceous 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 (GA_3_ 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.

**Acknowledgment**

This article is dedicated to the memory of Eric A. Andres.

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